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Effect of elevated atmospheric CO on phosphorus cycling in a short rotation poplar plantation

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Abstract

Global climate change and the rising concentrations of atmospheric CO_2 have increased concerns about the future of planet Earth. Numerous studies have been carried out to investigate and clarify the response of elevated atmospheric CO_2 on different plant species. The forests of the world play significant role in the global carbon budget. Previous studies of elevated CO_2 were mostly focused on single plant species grown in controlled environments and for relatively short periods and often in small root volumes. These systems do not allow investigation of the fate of phosphorus under changing environmental conditions. In this study, a P budget has been estimated to investigate the effect of elevated CO_2 on P cycling. The experimental site used is in central Italy (EUROFACE), where three *Populus* species were grown under elevated atmospheric CO_2 conditions, using FACE (Free Air CO_2 Enrichment). Phosphorus fractionation and organic phosphorus were determined under ambient (350 ppm) and elevated (550 ppm) atmospheric CO_2 conditions in three *Populus* species; *P. alba*, *P. nigra* and *P. x euramericana* in two fertilization treatments. P fractionation was carried out by determining water, NaOH extractable, HCl extractable and HNO_3 digestible P fractions. Within the 0-60 cm soil depth investigated, growth of poplar under elevated CO_2 increases P stores in the soil. The values of total P in 0-60 cm layer for the three species ranged between 130-175 mg kg^{-1} in ambient and 205-230 mg kg^{-1} in FACE. Total P content decreased down the soil profile especially at the soil depth of more than 50 cm. Higher organic P was found under FACE (22-29 mg kg^{-1}) as compared to ambient (15-25 mg kg^{-1}). In all treatments organic P was highest at 50-60 cm soil depth. There was no significant difference between species and between different fertilisation treatments in the amount of inorganic P determined. The organic matter content was 4-8% in the soils of experimental site, but was less under FACE. In leaves, roots and wood samples, P was determined and an estimate of total biomass P was calculated. General trends show that most of the species contained more phosphorus under FACE in leaf and root samples, while in the wood samples the opposite was found. Using a multi element analysis, an attempt was made to estimate the pools of P taken up by the trees. Ca/Sr ratios have often been used for this purpose. In this work Ca/Sr ratios did not change under FACE nor were species and treatments effects shown. Similarly no clear insight was gained from the other element ratios about the source of P utilized by the trees. By constructing a P budget, it was shown that P stores in the soil clearly increased under FACE, and this was mainly in the HCl extractable fraction. As no parallel decrease was seen in any other soil P pools, this suggests that P is being moved into the 0-60 cm soil layer. The possible mechanisms of this movement are discussed.

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List of Abbreviations

ADP	Adenine Diphosphate
AM	Vesicular-arbuscular mycorrhiza
ATP	Adenosine Triphosphate
DOC	Dissolved organic matter
EM	Ecto-mycorrhiza
EFMA	European Fertilizer Manufacturers Association
EUROFACE	European Free Air Carbon dioxide Enrichment on Poplar Plantation (2^{nd} Rotation)
FACE	Free Air Carbon dioxide Enrichment
HPLC	High Performance Liquid Chromatography
ICP	Inductively Coupled Plasma Spectrometry
IPCC	Intergovernmental Panel for Climate Change
IRGA	Infra-red Gas Analyser
NPP	Net Primary Production
NEP	Net Ecosystem Production
OTC	Open Top Chambers
PAR	Photosynthetic Active Radiation
POPFACE	European Free Air Carbon dioxide Enrichment on Poplar Plantation (1^{st} Rotation)
SOM	Soil Organic Matter

Framework of study

The activities of mankind have made tremendous changes in the environment globally over the last two centuries. One of the main causes of these changes is the use of natural non-renewable resources such as fossil fuels. One of the most significant effects of anthropogenic activity is that on the composition of the earth's atmosphere, seen as an increase in the atmospheric content of carbon dioxide (CO_2) and other trace gases (Vitousek *et al.*, 1997). The increase in CO_2, an essential requirement for photosynthesis in all plants, is expected to have considerable impacts on the future climate. Except for human land use, no type of global change has been documented to be more substantial and rapid than the increase in atmospheric CO_2 concentration. From the beginning of the industrial age (ca. 1850) until today, atmospheric CO_2 concentration has risen from approximately 280 to 382 ppm (Nösberger and Long, 2006), a 30% rise in just the last 150 years. The rate of increase of CO_2 concentration has been about 1.5 ppm per year over the past two decades, and commonly oceans and land are taking up about half of the atmospheric emissions (IPCC-2001). This rise is continuing at the rate of 1% every year (Scarascia-Mugnozza *et al.*, 2001), and CO_2 concentration is predicted to reach 550 ppm by the middle of 21st century (Schimel *et al.*, 1995). This increase may cause temperature changes, directly and indirectly influencing atmospheric, terrestrial and aquatic, abiotic and biotic processes within a complex web of interactions, and is leading to a major climate change (IPCC, 1996). Increased CO_2 represents the most important human enhancement to the greenhouse effect. The consensus of the climate research community is that it has already had a detectable influence on the earth's climate (Levitus *et al.*, 2001), and will further drive substantial climate change during the 21st century. In contrast to other green house gases, CO_2 is a plant fertilizer rather than a pollutant. The initial effect of an elevated atmospheric CO_2 concentration is a photosynthetic stimulation resulting in faster growth and higher production (Long and Drake, 1992).

P cycling includes inputs of phosphorus through rainfall, dry deposition, weathering and loss via leaching. Atmospheric inputs of mineral elements into rain forests may constitute an important input of plant nutrients, especially for soils of low inherent fertility (Proctor, 1987; Bruijnzeel, 1991). Such atmospheric inputs are divided into wet deposition (input of mineral elements dissolved in rainwater) and dry

deposition (input from deposited aerosol particles or as dust). The separation of dry versus wet deposition is fraught with technical difficulties (Lindberg *et al.*, 1986), but for many tropical forest studies a simple combined measure of the two has been obtained by sampling in a forest clearing or sometimes above the canopy. Irrespective of the source, hydrological studies have shown that a significant proportion of the atmospherically derived phosphorus appears to be retained by moist tropical forests, rather than being leached out of the system (Bruijnzeel, 1991). Bruijnzeel (1991) suggests that this general pattern of phosphorus accumulation in the forest/soil ecosystem is real and that it may rise as a consequence of P fixation onto iron and aluminium oxides.

In addition to substantial inputs of phosphorus occurring as a consequence of wet and dry deposition, substantial enrichment of rainwater phosphorus concentrations occurs during the passage of rainwater through tropical forest canopies (Vitousek and Sanford 1986; Proctor 1987; Veneklass, 1990; McDowell, 1998). Again, exact values of the enrichment through fall are subject to considerable uncertainties as a consequence of methodological problems. Marschner (1995) and Richards (1996) consider "canopy leaching" to provide the main source of nutrient additions to rainfall as it passes through canopy.

From the basin wide studies in South America, phosphorus weathering rates of 0.3-1.0 mmol P m^{-2} have been reported (Lewis *et al.*, 1987: Gardner, 1990). The degree to which such weathering of parent material may supply nutrients for plant growth has been considered by Burnham (1989) and Bruijnzeel (1989). They point out that for already highly weathered soils, the active zone of rock weathering occurs a considerable distance below the zone where active root uptake of any nutrient released by the weathering process is likely. Nevertheless, there are some cases where moist tropical forest roots can penetrate the underlying weathered rock (Ballie and Mamit, 1983), and this would certainly be expected for montane forests.

Vitousek and Sanford (1986) grouped lowland forests according to the underlying soil fertility and showed that forests growing on moderately fertile soils tend to have foliar N, P, K, Ca and Mg concentrations higher than do those growing on the common Oxisols or Ultisols soil types of moderate to low fertility. Forest on the later tend to have foliar concentrations not very different from forests growing on the very low-fertility spodosol or psamment soil types (Sanchez, 1976). The relationship between above-ground carbon and above-ground phosphorus density is very strong

(Vitousek and Sanford, 1986: Hughes *et al.*, 1999). Foliar phosphorus concentrations typically decline with canopy depth in tropical rain forests (Lloyd *et al.*, 1995) and so it is not straightforward to relate bulked canopy values to physiological measurements made on individual leaves. Along with faster growth rates and higher phosphorus and nitrogen requirements for the species from the higher nutrient soil (Veenendaal *et al.*, 1996), a picture emerges of species adapted to higher nutrient soils being successful by virtue of high potential growth rates and as ability to rapidly acquire nutrients. Tropical forest foliage typically accounts for less than 15% of the above-ground P pool (Hase and Fölster, 1982; Uhl and Jordan, 1984).

The phosphorus content in coarse and fine root content is less than that in above-ground biomass and is also affected by soil fertility level (Lloyd *et al.*, 2001). The available information on root P content is less than that on above-ground biomass. Nevertheless, the available data suggest that the effects of soil fertility on root P concentrations are similar to leaves and above-ground woody tissue (Golley *et al.*, 1975; Uhl and Jordan, 1984).

Nutrient availability exerts a major control over the response of plants and ecosystems to rising atmospheric CO_2 content (Bowes 1993; Lloyd & Farquhar 1996; BassiriRad, Gutschick & Lussenhop 2001). Phosphorus is a particularly important limiting nutrient because its supply rate directly controls the CO_2 responsiveness of photosynthesis (Conroy *et al.* 1986; Lewis *et al.* 1994; Stocklin, Schweizer & Korner 1998). For example, legumes in a calcareous grassland do not respond to elevated CO_2 content unless P is added (Stocklin *et al.* 1998) and ecosystems become more P limited with increasing atmospheric CO_2 content above current levels (Vance *et al.* 2003). A critical uncertainty is the degree to which P limitations were significant in past CO_2 atmospheres. At present, the atmospheric CO_2 content is 35% greater than the average CO_2 content of the Holocene epoch and is more than double the CO_2 content of 180 μmol mol^{-1} that predominated in the Late Pleistocene 20 000 years ago (Petit *et al.* 1999). Over an evolutionary timescale of many thousands of years, low CO_2 content was the norm. In the past 500 000 years, over 96% of the time corresponded to an atmospheric CO_2 content below 280 μmol mol^{-1} and for two-thirds of the time, atmospheric CO_2 content was less than 240 μmol mol^{-1} (Petit *et al.* 1999; Sage & Coleman 2001). Because of this predominance of low CO_2 content in recent evolutionary time, the proper baseline control for CO_2 enrichment studies generally should be 180–280 μmol mol^{-1}, not the already elevated content of the current era.

Plants may adapt to this low CO_2 content, and these adaptations may constrain the ability of species to respond to rising atmospheric CO_2 content (Sage & Cowling 1999; Sage & Coleman 2001). The work present in this thesis is a step towards predicting the future of phosphorus (P) cycling and its possible effects on forest ecosystems under increasing CO_2.

Hypothesis

In this work the following general hypothesis was tested "*increased biomass inputs influence phosphorus availability in soils. Increased growth of trees under elevated CO_2 increase P demand and will decrease soil P pools.*"

Objectives

The work has following objectives;

(a) *Determine effects of increased biomass inputs on the soil P pools.*

(b) *Determine total ecosystem P pools.*

(c) *Estimate the effects of elevated CO_2 on P cycling.*

Structure of thesis

Chapter 1 is a general introduction of the study with literature review on phosphorus cycling, phosphorus in soils and different plant materials. This chapter also contain a review of the FACE technology and history of CO_2 enrichment.

Chapter 2 gives information about the EUROFACE experimental site and the results obtained so far.

Chapter 3 describes all the materials and methods used to perform different experiments during the research.

Chapter 4 describes the results of different phosphorus fractions in soils under ambient and elevated atmospheric CO_2 conditions and some other experiments related to soil analysis.

Chapter 5 presents results and the effects of elevated atmospheric CO_2 on phosphorus in different plant materials.

Chapter 6 gives details of the Ca and Sr in soils and plant materials of the same site and about previous history of phosphorous at the experimental site.

Chapter 7 summarises the results of the study, discusses the overall effects of elevated CO_2 on P-cycling and suggests directions for future research in this area.

Chapter 8 gives concluding comments on the research.

Appendix 1 discusses the experiments investigating the role of mycorrhizal hyphae in P cycling.

Chapter 1

1 General introduction

1.1. Phosphorus in soils

Phosphorus is one of the most important essential mineral nutrients for plant growth. Phosphorus makes up about 0.12% of the earth's crust. It is present in almost all soils and rocks, in water and in plant and animal remains. The world's supply of P comes from mineral deposits, a non-renewable natural resource. The most common minerals containing phosphorus are of the apatite group [Ca_{10} (PO_4, CO_3) (F, OH) $_{2-3}$], obtained from sedimentary rocks (Cathcart, 1980). The igneous rocks also contain phosphates. The solubility of the various inorganic phosphorus compounds directly affects the availability of phosphorus for plant growth. The solubility is influenced by the soil pH. Soil phosphorus is mostly available for plant use at pH values of 6 to 7. When pH is less than 6, plant available phosphorus becomes increasingly tied up in aluminium phosphates. As soils become more acidic (pH below 5), phosphorus is fixed in iron phosphates. When pH values exceed 7.3, phosphorus is increasingly made unavailable by fixation in calcium phosphates (Oldham, 2003).

Soil phosphorus exists in inorganic and organic forms. Each form is a continuum of several compounds, existing in equilibrium with each other and ranging from solution P to very stable unavailable compounds. Soil P is usually classified into 3 categories (Larsen, 1967). The first (1) is P in soil solution which is considered to be direct source of P for plants. The second (2) is labile P and it is in fairly rapid equilibrium with fraction 1. The fraction 1 and 2 are assumed to characterize the supply of P to the roots. The third (3) is nonlabile P which is not readily exchangeable. In soil solution available phosphorus usually exists in the form of orthophosphate ions i.e. $H_2PO_4^-$ and HPO_4^{2-}. Absorption of $H_2PO_4^-$ by plant roots is greater at low pH values, whereas HPO_4^{2-} is taken up at higher values of pH. In contrast to nitrate nitrogen, phosphate anions are either adsorbed by the soil or are precipitated as products with low solubility.

The chemistry of soil P transformations giving rise to this situation is complex (Sanyal and DeDatta, 1991). But even for the simplest understanding of soil phosphorus, it is necessary to consider labile and nonlabile pools of phosphorus in both the organic and inorganic forms as well as the significant fluxes in microbial pool

(Brookes *et al.*, 1984; Singh *et al.*, 1989; Lodge *et al.*, 1994; Gijsman *et al.*, 1996). Labile pool is the material which is labile to displacement or change, while, nonlabile is considered to be in a stable state for the time scales from years to centuries (Lloyd *et al.*, 2001). Inorganic phosphorus occurs at fairly low concentrations in the soil solution whilst a large proportion of it is more or less strongly held by diverse soil minerals (Hinsinger, 2001). In both physical and chemical sense, soils are strongly heterogeneous media and elements such as phosphorus do not really partition into such simple compartmented states (Barrow, 1999). Likewise when attempts are made to fractionate P into pools of varying stability, the exact nature of the different P pools within the soil that these chemically isolated fractions represent is also not entirely clear (Gijsman *et al.*, 1996).

The labile component of inorganic phosphorus is generally taken to comprise aluminium bonded phosphates, iron bonded phosphates and calcium bonded phosphates. For highly acid and highly weathered tropical soils iron and aluminium phosphates tend to dominate and thus adsorption capacity for P is usually quite high (Sanchez, 1976). Crystalline clay minerals are also able to specifically adsorb P through a ligand-exchange reaction with the (OH)H groups co-ordinated with the Al ion on the edge of the crystal (Muljadi *et al.*, 1966).

In most soils 50-75% P is inorganic. Organic P compounds range from readily available unrecompensed plant residues and microbes within the soil to stable compounds that have become part of soil organic matter. In forest soils the proportion of organic P may rise to 80-90% of the total P (Zech *et al.*, 1987), including 7-8% located in mycelial hyphae (Baath and Soderstörm 1979). Organic phosphorus in soil can be associated either with soil organic matter (humus) or recently added organic debris coming from plants or animals.

Organically bound phosphorus generally accounts for 20-80% of the total P in forest soils (Tiessen *et al.*, 1994a; Newberry *et al.*, 1997). This organic P represents a wide range of compounds, reflecting the diverse biological origins of soil organic matter (Magid *et al.*, 1995). Labile forms of organic P include phospholipids and nucleic acids which are of primarily bacterial origin. Inositol phosphates often constitute the bulk of nonlabile organic P pool, forming sparingly soluble salts with ions such as iron, aluminium and calcium. They can also form strong complexes with proteins and can be

strongly adsorbed by clay minerals, typically constituting about 50% of organic P (McLaren and Cameron, 1996). In most soils the P content of the surface horizon is more than that of the subsoil. This is as most of inorganic and organic P is initially stored in the top soil and migrates to lower horizons.

Organic phosphorus is considered to play a key role as a source of P for plants in tropical soils (Sanchez *et al.*, 1976). These organic molecules cannot be used directly by plants. They have to be broken down by soil microbes to release inorganic phosphate ions which can be taken up by plant roots or enter into the same reactions as other phosphate ions originating in inorganic P pools (Johnston & Steen, 2000). Biological processes in the soil tend to control the mineralization and immobilization of organic P. In this context it is important to note that, in contrast to nitrogen, phosphorus is to a large degree mineralized independent of carbon (McGill and Cole, 1981). This is a result of the production of phosphatases by plant roots, mycorrhizae, and microbes. These specifically hydrolyze phosphate ester linkages on soil organic compounds, releasing phosphorus and making it available for plant uptake (Lloyd *et al.*, 2001). According to Gijsman *et al.*, (1996), data of Ognalaga *et al.*, (1994) also suggest that organic P can be stabilized into nonlabile forms independently of organic carbon. A similar conclusion was also reached by McGill and Cole (1981). This means that there is much more chance for variation in C/P ratios of the labile soil organic pool than is the case for C/N ratios. This has important implications for the response of P-limited systems to increases in atmospheric carbon dioxide concentrations (Lloyd *et al.*, 2001).

1.2. Phosphorus in plants:

In plants, phosphorus occurs in concentrations between 0.1-0.4%, lower than those typically found for N and K (Tisdale *et al.*, 1993). In its prime importance in plant nutrition, phosphorus plays important roles in photosynthesis and oxidation-reduction reactions. The most essential function of P in plants is in energy storage and transfer. Energy obtained from photosynthesis and metabolism of carbohydrates is utilized in the synthesis of the energy-rich phosphate compounds (ADP and ATP) for subsequent use in growth and reproductive processes (Tisdale, *et al.*, 1993). Phosphorus is an important structural component of nucleic acids, coenzymes, nucleotides, phospho-proteins, phospholipids and sugar phosphates (Schachtman *et al.*, 1998). A good supply of phosphorus is associated with increased root growth, early maturity of grain crops and

greater straw strength. The quality of certain fruits, forage, vegetables and grain crops is also improved and disease resistance increased with the satisfactory P nutrition (Tisdale, *et al.*, 1993). Furthermore, inorganic phosphorus controls some key enzymes reactions. For example, in fruit tissue of tomato, inorganic P released from the vacuoles into the cytoplasm can stimulate phosphofructokinase activity, an enzyme which can initiate burst correlated with fruit ripening (Woodrow and Rowan, 1979). Delayed fruit ripening in phosphorus-deficient tomato plants (Pandita and Andrew, 1967) may be related to this function of inorganic phosphorus. The phosphorus requirement for optimal growth is in the range of 0.3-0.5% of the plant dry matter during the vegetative stage of growth (Marschner, 1995). Phosphorus deficiency is associated with a reduction in leaf expansion and leaf surface area (Freeden *et al.*, 1989) and number of leaves (Lynch *et al.*, 1991). Moreover, due to P deficiency flower initiation is delayed (Rossiter, 1978), the number of flowers decreased (Bould and Parfitt, 1973) and seed formation restricted in particular (Barry and Miller, 1989). Premature senescence of leaves may also limit seed yield in the phosphorus-deficient plants (Batten *et al.*, 1986).

Phosphorus is taken up by plant roots from a number of forms. The form in which Pi (Inorganic P) exists in solution changes according to pH. The pKs for the dissociation of H_3PO_4 into $H_2PO_4^-$ and then into HPO_4^{2-} are 2.1 and 7.2, respectively. Therefore, below pH 6.0, most Pi will be present as the monovalent $H_2PO_4^-$ species, whereas H_3PO_4 and HPO_4^{2-} will be present in minor proportions (Schachtman *et al.*, 1998). Most studies on the pH dependence of Pi uptake in higher plants have found that uptake rates are highest between pH 5.0 and 6.0, where $H_2PO_4^-$ dominates (Ullrich-Eberius *et al.*, 1984: Furihata *et al.*, 1992), which suggests that Pi is more easily taken up as a monovalent ion.

Calculations indicate that root interception is of minor importance in meeting plant demand for most mineral nutrients due to very small volume of soil displaced (Marschner, 1995). Thus mass transport and / or diffusion, meet plant demand for most mineral nutrients. Due to the low solubility of P containing minerals the concentrations of P in the soil solution is low, less than 1% of total soil P. As a consequence mass flow is unable to meet plant demand of P and diffusion is more important to replenish P levels around the plant root (Marschner, 1995). The diffusion rate of P is controlled by a number of factors, for example soil water content (Bhadoria *et al.*, 1991), with rates of diffusion decreasing as soil water content decreases. A number of studies have shown that P uptake is more impaired than that of any other mineral elements in dry soils

(Marschner, 1995). As plant demands often exceed rates of diffusion, zones of depletion form in the rhizosphere which may be several mm wide (Jungk and Claassen, 1989). Although the zones of low P concentration will increase the driving force for diffusion, they also result in lower soil solution concentrations of P in contact with the roots. Plant can increase P acquisition by increasing the volume of the soil explored for example with root hairs. Jungk and Claassen, (1989) could also show that root hairs were more effective than the root cylinder in absorbing P per unit area.

There is a general perception that Pi uptake by plants occurs as a direct consequence of uptake from the soil by root cells. However, in more than 90% of land plants, symbiotic associations are formed with mycorrhizal fungi (Schachtman *et al.*, 1998). In these plants the fungal hyphae play an important role in the acquisition of P for the plant (Bolan, 1991; Smith and Read, 1997; Hinsinger, 2001). Mechanism of P acquisition by mycorrhizas is generally similar to those found in nonmycorrhizal roots and other fungi (Thomson *et al.*, 1990; Smith and Read, 1997). Mycorrhizae can be divided into two main categories: ectomycorrhizae and endomycorrhizae, of which vesicular arbuscular mycorrizae are the most widespread in the plant kingdom (Smith and Read, 1997). The mycorrhizal symbiosis is founded on the mutualistic exchange of C from the plant in return for P and other mineral nutrients from the fungus. Influx of P in roots colonized by mycorrhizal fungi can be 3-5 times higher than in nonmycorrhizal roots (Smith and Read, 1997).

1.3. Elevated atmospheric carbon dioxide

The ability to predict the likely effects of atmospheric change on natural ecosystem is one of the major challenges of ecology at present (Schlesinger, 1999; Lukac, 2002). Attempts to predict future vegetation responses to elevated CO_2 have resulted in a diverse range of experimental designs and facilities created to test the reaction of objects from a single photo-synthesising cell to a whole ecosystem (Lukac, 2002). As well as scale, researchers varied the length of exposure of plants to elevated CO_2 from only few days' fumigation to assess immediate physiological responses, to continuous measurements on plant grown under elevated CO_2 conditions for several growing seasons (Ashenden & McLeod, 1993). This section explains various techniques used to simulate future atmospheric levels of CO_2.

1.4. CO_2 Enrichment methods

As soon as the scientific community realised that elevated atmospheric CO_2 might have profound consequences for ecosystem, the effect of elevated CO_2 on plant came under close scrutiny. As is always the case with emerging fields of research, the first to be examined were the simplest ecosystems: those based on agricultural crops (Lemon, 1983). Numerous studies were carried out on agricultural plants, followed by examination of wild herbaceous species and communities. Literature is reviewed by among others, Bazzaz (1990) and Allen *et al.* (1992). However, it is estimated that trees account for up to 70% of terrestrial carbon dioxide fixation (Melillo *et al.*, 1993) and therefore might play an important role in carbon sequestration. Subsequently, studies aimed at improving our understanding of possible effect on tree species emerged (Ceulemans and Mousseau, 1994; Norby *et al.*, 1999), even though, in comparison to crop species, the response of trees to CO_2 enrichment is slightly less well understood. This might be due to a large buffering effect of the woody storage compartments (Janssens *et al.*, 2000) or due to the obvious complications arising from the size of the ecosystems studied and from simulating the development of natural canopy in time. As already mentioned, numerous techniques have been devised to study the effects of elevated CO_2 has on plants, the most important of which are briefly explained in the following four sections. Since the subject of this research is poplar plantation, emphasis has been put on studies dealing with woody species.

1.4.1. Controlled environment chambers

In the early stages CO_2 experiments were conducted in greenhouses and closed chambers due to existing infrastructure and simplicity. Initially juvenile plants were grown in pots in greenhouses with altered atmospheric conditions to simulate ambient and future levels of CO_2 (Enoch and Kimbell, 1986). The tree species studies were limited to examining seedlings of very young trees (Saxe *et al.*, 1998). The invention of phytotron technique allowed researchers to examine tree species in chambers where all environmental conditions were controlled (Ceulemans and Mousseau, 1994). A phytotron allows the exposure of plants to a wide combination of factors, CO_2 concentration being only one of them (Bazzaz, 1990).

A number of closed chamber techniques have been used like installation of controlled plastic chambers inside a greenhouse (Radoglou and Jarvis, 1990) or field

applications of chambers with different environmental variables other than CO_2 concentration kept at ambient levels (Overdiek, 1993). A technique called 'branch bag method' can be considered a modification of closed chamber technique. This method involves the enclosing of selected branches of mature tree in plastic bags and exposing them to elevated CO_2 concentrations (Barton and Jarvis, 1999; Hogg *et al.*, 2000) and assumes the validity of branch autonomy theory (Sprugel *et al.*, 1991). Common to all these close-space facilities are some limiting factors hindering their usage for tree research, notably limited space and inadequate light conditions.

1.4.2. Open top chambers (OTCs)

Open top chambers are considered to be an improvement over closed chambers, and have been extensively used for studies involving CO_2 enrichment. These chambers permit enclosure of part of an ecosystem and exposure of plants to different environmental conditions. In contrast to closed chambers, environmental conditions other than CO_2 concentration in OTCs more closely approximate natural conditions, and larger plants can be studied. The OTCs were originally developed for pollution studies (Olczyk *et al.*, 1980; Wang *et al.*, 1986). They have been used for examining a variety of plant communities (Allen *et al.*, 1992; Ashenden *et al.*, 1992; Owensby *et al.*, 1993). The open top chambers differ in dimensions within a diameter range of 0.5 -3 m and heights of 1.3-6 m. The plants in OTCs are generally grown in pots, open bottom root boxes, or free-rooted in the soil.

Although this method has been extensively used and developed, it still suffers from lots of chamber effects that are ascribed to controlled environment chambers. These include change in temperature, microclimate and limited dimensions. It has to be mentioned that, similarly to the closed chamber technique, application of OTC imposes a considerable size constraint on the tree being studied. During the decade (1990-2000), 36% of all studies on poplar used OTCs (Gielen and Ceulemans, 2001).

1.4.3. Natural springs

The possibility of using natural CO_2 springs to study long term effects of elevated CO_2 on plants was shown in 1990's (Miglietta *et al.*, 1993). These springs have existed for decades or centuries and offer a unique opportunity to study acclimation of natural ecosystem to elevated CO_2 conditions, albeit in a rather uncontrolled manner (Newton *et al.*, 1996). Experimental use of these sites has highlighted their variability

and their spread across the world (CO_2 springs and their use in biological research workshop, San Miniatao, 1993). Naturally occurring CO_2 vents have been used to study perennial grasslands (Cook *et al.*, 1998), Mediterranean macchia (Tognetti *et al.*, 1998; Stylinski *et al.*, 2000) or carbon and nitrogen pools in the soil (Ross *et al.*, 2000). The drawbacks that have to be taken into account when studying plants exposed to elevated CO_2 by a natural vent include lack of proper control plots and great spatial and temporal variability of CO_2 enrichment. Only 3.5 % poplar studies on the response of elevated CO_2 in the last ten years have been carried out using these natural springs (Gielen and Ceulemans, 2001).

1.4.4. FACE technology

In the last 15 years, the free air CO_2 enrichment (FACE) technique has been developed to treat entire ecosystem (Hendrey *et al.*, 1993). Using this technique many of the chamber effects that were ascribed to OTCs have been overcome (McLeod and Long 1999; Zak *et al.*, 1993). This technique was originally designed for agricultural crops and was used for a wide range of ecosystems. Table 1.1 lists some of the experiments utilising FACE technology of CO_2 enrichment which are carried out around the world.

FACE experiments are almost unanimously considered to provide the best opportunity to expose parts of plant to elevated atmospheric CO_2 conditions (Lukac, 2002). In its original design, a FACE system consists of sets of horizontally or vertically positioned blowers releasing either ambient or CO_2 enriched air into the atmosphere. The blowers are usually arranged in some sort of circular shape enclosing the experimental area into which air mixed with CO_2 is blown. This allows for observation of the plant reaction to elevated CO_2 with minimal artefacts due to the operation of CO_2 enrichment equipment. Although, the use of blowers requires large air feeding pipes to be installed and to circulate large volumes of air significant infrastructure must be installed (Hendry *et al.*, 1993). This implies construction of control plots where everything, apart from CO_2 enrichment, is operated in the FACE (Miglietta *et al.*, 2001a).

Table1.1: FACE experiments currently implemented in the world
(Source: http://cdiac.esd.ornl.gov/programs/FACE/whereisface.html)

Location	Ecosystem	CO_2 level (ppm)	Plot Ø (m)
Bangor (UK)	beech, birch & alder	570	8
Brauschweig (D)	agronomic crops	550	20
Bulls (NZ)	pasture grassland	475	12
Caribou/Poker Creeks (USA)	bog	560	1
Cedar creek (USA)	C3 & C4 grasses (BioCON)	550	20
Clermont-Ferrand (F)	grassland (MEGARICH)	600	1-2
Cumbria (UK)	bog (BERI)	560	1
Dublin (IRL)	grassland (MEGARICH)	600	1-2
Durham (USA)	loblolly pine forest (FACTS-1)	570	30
Eschikon (CH)	grassland	600	18
Giesson (D)	potatoes (CHIP) & grassland	560	8
Godollo (H)	grassland (MEGARICH)	600	1-2
Hofstetten (CH)	mature temperate forest	600	-
Jasper Ridge (USA)	grassland	2 x amb.	2
Kopparasmyren (S)	bog (BERI)	560	1
LesChaux-des-Breuleu (CH)	Bog (BERI)	560	1
Maricopa (USA)	agronomic crops	570	23-25
Mekrijarvi (FI)	bog (BERI)	560	1
Munich (D)	grassland (MEGARICH)	600	1-2
Nevada desert (USA)	majave desert scrub community(NDFF)	550	23
New Delhi (IND)	brassica spp. & rice (oryza) cultivars	560	-
Oak Ridge (USA)	sweetgum plantation	570	25
Pontville (AU)	native grassland	550	1.5
Rapolando (I)	grassland (MEGARICH)	600	1-2
Rapolando (I)	potatoes (CHIP) & agronomic crops	560 & 600	22&30
Rhinelander (USA)	aspen forests (FACTS-II)	570	30
Sardinilla (P)	tropical rain Forest	550	30
Shizukuishi (JPN)	rice and paddy fields	570	12
Sky Oaks (USA)	chaparral	550	16
Stillberg – Davos (CH)	treeline ecotone	550	1.3
Urbana-Champaign (USA)	soybean	550	20
Viterbo (I)	**poplar plantation (EUROFACE)**	**550**	**20**
Wageningen (NL)	bog (BERI)	560	1
Yabulu (AU)	tropical savana + planted eucalyptus & acacia seedlings	450 500	15

To further improve the technique, a method releasing pure CO_2 into the air has been devised (Okada *et al.*, 2001). This design offered some advantages over the conventional system, notably dispensing with the need for large infrastructure and with construction of ambient air releasing pipes in the control plots. This system is used at EUROFACE site and is explained in more detail in the next chapter.

Chapter 2

2 Experimental site (EUROFACE)

The work presented in this thesis was carried out as a part of the project 'A European Free Air Carbon dioxide Enrichment Experiment on poplar plantation' in Viterbo, Italy. The main objective of this project is to investigate the possible effects of elevated atmospheric CO_2 on *Populus* species. The project participants designed and built a large scale FACE experiment which started operation in June 1999. The first phase of this project from June 1999 to November 2001 was named POPFACE. The second phase mainly from which this study is presented is called EUROFACE. The following sections will give information about the technology used for CO_2 enrichment and the field performance of the system.

2.1 Site plan

The experimental site is located in central Italy, near the city of Tuscania, Viterbo province. The site is at grid references 42°37′04″N, 11°80′87″E and 150 m above the sea level. The climate of the site is typically Mediterranean, with warm and dry summer, mild and humid autumns and winter. The mean annual air temperature is 14°C while mean annual precipitation is about 800 mm. Three *Populus* species were grown *(P. alba, P. nigra* and *P. x euramericana)* under ambient and elevated atmospheric CO_2 conditions. Table 2.1 lists some general characters and differences between these species. The *P. x euramericana* (I-24) cuttings were planted during the spring of 1999 over 9 ha of a former agricultural field at a commercial spacing of 2x1 m. Within this plantation, 6 experimental plots were equally spaced in order to avoid enrichment pollution of 'control' plots with air blown in from FACE plots (Fig. 2.1). The whole plantation was drip irrigated with 6 to 10 mm of water per day only during the growing season starting from early April to early November.

In each experimental plot cuttings of *Populus* species were planted at the same time as the rest of plantation. The *Populus alba* (clone 2AS-11), *Populus nigra* (Clone Jean Pourtet) and *Populus x euramericana* (*Populus deltoides* x *Populus nigra*, clone I-214) were planted in all 20 x 20 m plots at 1 x 1 m spacing to facilitate early canopy closure.

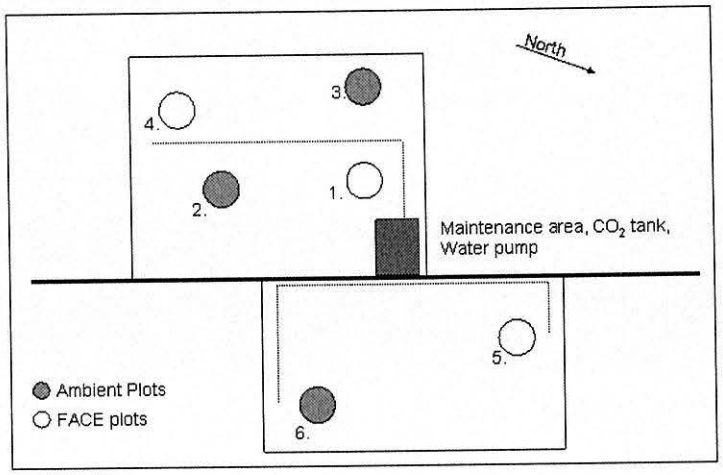


Figure 2.1: Layout of EUROFACE plantation

Each plot was divided into 3 sections, each of which was planted out with trees of a single species. Figure 2.2 illustrates the planting scheme for plot 1 and 2. In the remaining two pairs of plots, i.e. 3-4 and 5-6, the planting scheme was rotated to avoid possible bias due to uneven growth of different species.

Table 2.1: General characteristics of poplar species planted in EUROFACE

Species & Genotype	Sex & Origin	Rooting capacity	Branching Habit	Apical control	Bud-burst	Bud set
P. alba L. 2AS11	M Italy	Medium	medium	good	End of March	End of Oct.
P. nigra L. Jean Pourtet	M France	Very good	Very high	good	End of March	Start of Oct.
P. x euramericana I-214	F Italy	Very good	Low	V. good	End of March	Mid. of Sept.

Source: Calfapietra *et al.*, 2001.

Within each plot, a circle of 20 metres in diameter surrounded the experimental trees, enclosing 314 individuals on which the measurements were carried out. Trees located on the border of each section, either next to trees of a different species or on the perimeter of the plot, were, excluded from the measurements. Three plots (1, 4 and 5) were with FACE infrastructure and three (2, 3 and 6) were used as ambient CO_2

controls. The octagonal-shaped FACE plots were providing atmospheric CO_2 enrichment. Thus, the experimental set-up provided a block design consisting of three replicates per treatments. All results reported in this work were averaged for each *Populus* species and for each plot prior to performing ANOVA (see section 3.8, page 31). It is important to consider, that this approach, although illustrating a sound basis for testing the effects of elevated CO_2, obscures possible interactions between plot and CO_2 treatment.

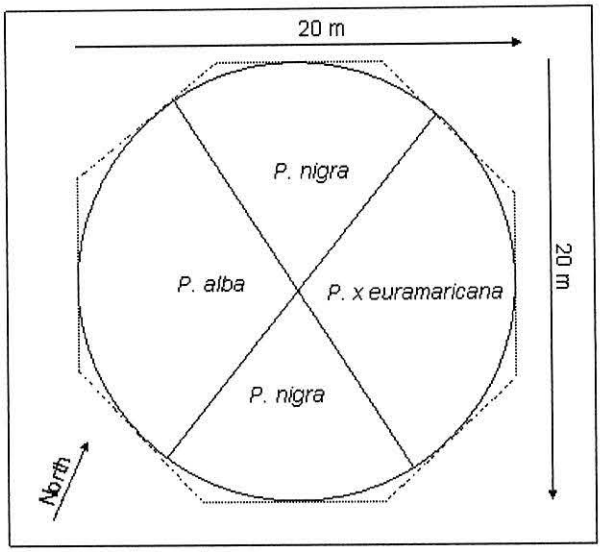


Figure 2.2: Layout of experimental plot

Each FACE plot covers an area of circa 350 m^2. The size of ring was designed to provide an internal area with equal distribution of CO_2 with a diameter of 16 m. Each plot had eight masts supporting layers of horizontally positioned polyethylene pipes (diameter 25 mm) releasing pure CO_2. The pipes were suspended at various heights to facilitate even vertical distribution of CO_2 within the canopy and the masts allowed for easy and rapid vertical movement of the pipes to account for fast growth of the poplars. At the beginning of the experiment, immediately after planting, an initial single layer of pipes was located at approximately 50 cm above ground. Later into the experiment a second layer of pipes was added, reaching a maximum height of 12 m. In each layer, a side of the octagon consisted of two paired pipes carrying 350 to 500 jets respectively. The jets were of a mean diameter 0.3 mm, drilled manually for the initial season, using laser technology for the subsequent seasons to improve performance and accuracy of CO_2 release. The directional control of CO_2 fumigation was provided by 16 on/off

solenoid valves and an automated pressure regulator controlling the amount of released CO_2. The system was run by a programmable microprocessor unit based in the centre of each FACE ring. An algorithm used by this unit adjusted the amount of released CO_2 every second, according to the measurements of wind speed, wind direction (30 sec averages) and CO_2 concentration. Detailed description and analysis of the design of POPFACE fumigation system is given by Miglietta *et al.* (2001b).

In the winter of 2001, after three years of growth, all trees were cut to the base of the stem (5-8 cm above the ground), resulting in stools with many new resprouting shoots in the spring of 2002. From 1999 until 2001 all plants were unfertilized. In 2002 a fertilizer treatment was added to one half of the plot. In the fertilized treatments (half of each plot), a total amount corresponding to 212 kg N per ha during the first year and 290 kg N per ha during the second and third year was supplied. Hydraulic pumps, installed outside each plot, were used to distribute the fertilizer (first year Navarson 20-6-6 ($N\text{-}P_2O_5\text{-}K_2O$); second and third year ammonium nitrate 34-0-0 ($N\text{-}P_2O_5\text{-}K_2O$)); dissolved in 200 L tanks, through the drip-irrigation system (Liberloo *et al.*, 2006). Fertilization was provided once per week throughout the growing season, in constant amounts during the first year, and in amounts proportional to the growth rates during the second and third year. Each half plot was further divided into three triangular sectors for each of the different species, thus yielding six sectors per plot. Plantation management included continuous drip irrigation, mechanical herb removal, and a limited application of BT toxin based insecticides (Liberloo *et al.*, 2006).

2.2 Soil description

A detailed survey was carried out, in November 1998, to characterize the soil properties of the experimental site, before planting trees and starting CO_2 atmospheric enrichment, and also to assess spatial variability in order to appropriately locate experimental blocks and plots. The soil was classified as Pachic Xerumbrept-silt loam agricultural soil belonging to order Inceptisols and suborder Umbrepts except plot 5 which was Ochrepts, more than 1 meter deep, originated from geological substrate derived from sedimentary material of volcanic origin and marine deposits (Hoosbeek *et al.*, 2004). The average stone content (%; fraction >2mm) of the top layer (0-20 cm) was 1.93 percent and pH was found 5.04. The average value for bulk density was 1.34 g cm^{-3} with a C/N ratio of 8.0 (Scarascia-Mugnozza *et al.*, unpublished material).

Table 2.2: Mean values and standard deviations of the top layer (0-20 cm) soil characteristics of the POPFACE site (From Scarascia-Mugnozza *et al.* 2006).

Soil texture	Silt loam
Stone content (%; fraction > 2mm)	1.93 (0.97)
pH(KCl)	5.04 (0.22)
total C (%)	0.98 (0.20)
total N (%)	0.12 (0.02)
C/N ratio	8.00 (1.01)
bulk density (g/cm^3)	1.34 (0.09)

2.3 Fertilization

Before the trees were planted and the CO_2 enrichment started, a detailed soil survey to characterize soil properties of the experimental site, was carried out, in November 1998. This survey also assessed soil spatial variability in order to appropriately locate experimental blocks and plots. Maximizing the distances between the plots, six areas 48 × 48 m^2 were identified in the 9 ha field for soil sampling and samples were taken at 3 depth intervals, 0-10, 10-20 and 20-30 cm. Sixteen sampling points were located in each area, for a total of 96 soil samples collected in the field, along the soil vertical profile. Also, some deeper, complete pedological descriptions were added to the soil analyses. Soil was then classified as heavy loam agricultural soil, more than 1 meter deep, originated from a geological substrate derived form sedimentary material of volcanic origin and marine deposits (Hoosbeek *et al.* 2004).

2.3.1 Nitrogen fertilization

In the first 3-year rotation cycle no fertilization was applied to the plantation and to the plots because chemical analyses showed a fairly availability of nitrogen for the poplar trees because of previous ample supply of fertilizers to the former agricultural crops. In the second rotation cycle a fertilization treatment was added to one half of each experimental plot because soil analyses showed the occurrence of limiting conditions of nitrogen availability in the soil. The total amount of nitrogen supplied was 212 Kg ha^{-1} y^{-1} in 2002 and 290 Kg ha^{-1} y^{-1} during 2003 and 2004. The nitrogen was

supplied in constant weekly amounts with a 4:1 NH_4^+:NO_3^- ratio in 2002 whereas was supplied in weekly amounts proportional to the growth rate with a 1:1 NH_4^+:NO_3^- ratio in 2003 and 2004. Each experimental plot was equipped with a plastic 200 l tank where the fertilizer is dissolved and a hydraulic pump (Ferti-injector Amiad, IMAGO srl, Italy) connected to the irrigation system.

2.4 Performance of CO_2 enrichment

The CO_2 enrichment became operational on 29th June 1999 and remained in operation till the end of growing season i.e. 5th of December 1999. The performance of FACE system was evaluated by recording of 1 minute average CO_2 concentration by IRGA (Infra-red Gas Analyser) located at the centre of each FACE plot. In the first growing season (POPFACE) this target was achieved for all three FACE replicates with seasonal means at 545, 544.8 and 541.7 ppm for plots 1, 4 and 5 respectively which represents a 91% rate of CO_2 concentration being within ±20% of 550 ppm target.

The CO_2 concentration within the FACE plots was 554 ± 1.6 ppm during the first year after coppice (2002), 535.9 ± 20.4 μmol mol^{-1} during the second year after coppice (2003) (F. Miglietta, CNR-IATA, Florence, Italy, unpublished results). The elevated CO_2-concentrations, measured at 1-min intervals, were within 20% deviation from the pre-set target concentration for 89.4, 72.2 and 70 percent of the time during the three years of the second rotation (Liberloo *et al.*, 2006). A general benchmark for the successful performance of a FACE system is if thel minute average of CO_2 concentration is measured every second is within ±20% variation of a target for more than 80% of the time (Miglietta *et al.*, 2001b).

The major exceptions from overall satisfactory performance were days with very high wind conditions, during which the daily mean CO_2 concentrations dropped significantly. This can be explained by the fact, that an automatic cut-off of CO_2 supply was imposed for wind in excess of 10 ms^{-1}, as gas flow through the pressure regulator became insufficient. However, it should be noted that the wind climate of Tuscania had a positive effect on the system performance as the air CO_2 mixing was enhanced by windy conditions. On average, the wind rotated from north-east in the morning to south-west in the afternoon with a slight dominance of wind south-westerly direction. This fact must be taken into consideration, because the plants on south-west side of the FACE plots were exposed to marginally higher concentrations of CO_2 than the plants growing on the north-east side.

Overall performance of the fumigation system was satisfactory during all POPFACE and EUROFACE growing seasons. The final average heights of the plants were measured 9.97 m for *P. alba*, 9.44 m for *P. nigra* and 10.11 m *for P. x euramericna,* and 2 layers of horizontal pipes were used to supply sufficient amount of CO_2 in last growing season.

2.5 Background results

This FACE infrastructure has made it possible to conduct one of the first European experiments on climate change at the scale of planted forest ecosystem. None of the three study clones exhibited any consistent photosynthetic down regulation during the first 3 year rotation cycle (Bernacchi *et al.*, 2003). Measurement of stomatal conductance in this experiment showed that stomatal conductance (g_s) was generally decreased by elevated CO_2, varying between 16% and 35%, but this reduction was often not statistically significant (Bernacchi *et al.*, 2003; Tricker *et al.*, 2005). Nevertheless, the reduction of stomatal conductance was maintained, even after 5 years of exposure to elevated CO_2 (Tricker *et al.*, 2005).

Plant N is one of the limiting factors when the long term responses of plants to elevated CO_2 are analysed (Scarascia-Mugnozza *et al.*, 2006). The N concentration in leaves was investigated in 2003 (second year of the second rotation cycle), assuming that this parameter is strongly related to the nutrient status of the whole tree (Kozlowski and Pallardy, 1997). Leaf N concentration on a mass basis increased under fertilization 16-22%, depending on species and CO_2 treatment. However, the foliar N concentration was found to slightly decrease under elevated CO_2, although not significantly. Differences between the treatments disappeared for all species by the end of the growing season and values of N concentration showed similar values in all treatments around 2% (Scarascia-Mugnozza *et al.*, 2006). Moreover, the limiting levels of PAR by the end of the growing season could have inhibited N accumulation in the leaves, as it was also observed along the vertical profile of the canopy (Gielen *et al.*, 2003).

Elevated CO_2 caused roots to develop into the soil and the analysis of vertical distribution of roots showed increased allocation of biomass into deeper soil horizon (20-40 cm) (Lukac *et al.*, 2003). The increase in root biomass at deeper soil layers under elevated CO_2 conditions for *P. alba* and *P. nigra* was from 23% to 36% and from 20% to 39% respectively, but not for *P. x euramericana,* suggesting a genotype-specific response. Mycorrhizal symbiosis can produce profound effects on the availability of N

and other nutrients to plants; and, conversely, an increased supply of C under elevated CO_2 can be beneficial for fungal symbionts. The response of arbuscular mycorrizal colonization to elevated CO_2 was found to vary among *Populus* species (Lukac *et al.*, 2003). In both, *P. alba* and *P. nigra*, hyphal presence inside fine roots increased (by +29% and +36% respectively), but little effect was observed on *P. x euramericana* (+2%). A similar species-specific effect was observed for ectomycorrhizae as EM colonization significantly increased in *P.alba* (+78%).

Responses of poplar agro-forestry systems to atmospheric CO_2 enrichment can be grouped into the effect of elevated CO_2 before and after canopy closure. Initially, elevated CO_2 stimulated growth of *Populus,* although not to the same extent for all three studied species (Calfapietra *et al.*, 2003a). Finally, the production of biomass (stem, branches, and coarse roots) after 3 year of growth was stimulated under elevated CO_2 by 24%, averaged across the species (Calfapietra *et al.*, 2003b). In the second rotation, after coppicing, an increase in biomass production was observed under elevated CO_2 (Liberloo *et al.*, 2005); and this increase in biomass as a response to elevated CO_2 was caused by an initial stimulation of absolute and relative growth rates, which disappeared after the first growing season following coppicing. Fertilization did not influence aboveground growth, although some responses to elevated CO_2 were more pronounced in fertilized trees.

The exposure of all three genotypes to elevated CO_2 resulted in larger trees with greater root system (Lukac *et al.*, 2003). However, *Populus* genotypes utilized in this research did not increase root production by the same magnitude under elevated CO_2. The smallest increase in standing root biomass induced by elevated CO_2 occurred in P. *alba* (+47%). *P. nigra* and *P. x euramericana* responded to elevated CO_2 with +76% and +71% respectively. Ingrowth core measurements showed a corresponding increase in fine root production. Elevated CO_2 enhanced fine root growth of *P. alba* by 56%, *P. nigra* by 97% and *P. x euramericana* by 73%. Elevated CO_2 increased not only the amount on fine roots produced, but also the rate of root turnover in all three genotypes, on average from 1.5 to 2.0. This increase was largest for *P. alba*, which speeded up the root turnover under elevated CO_2 by 45%, while *P. nigra* and P. *x euramericana* showed a 27% increment (Scarascia-Mugnozza *et al.*, 2006).

In all the species elevated CO_2 induced a small, and not significant, increase in the annual litter production, from 3% to 6% (Scarascia-Mugnozza *et al.*, 2006). The atmospheric CO_2 enrichment also affected litter decay rates, with two different

mechanisms: (a) by altering litter quality, the decomposition rate of litter slowed down on average by 7% after 8 months of incubation, (b) when incubated in the field under elevated CO_2, litter decomposition accelerated, especially in the initial stage of the process, possibly as a consequence of increased soil biological activity and soil C input into the rhizosphere environment under elevated CO_2 compared to control CO_2. Also these reasons were genotype specific and *P. nigra* was the most affected by the treatment (Cotrufo *et al.*, 2005).

If based on observed increment in above and belowground biomass under elevated CO_2, was expected a greater increase in soil C_{new} and C_{total} under elevated CO_2 than under control CO_2 (Hoosbeek *et al.*, 2004). C_{new} is the amount of C taken up by the soil during the experiment and was estimated by C_3/C_4 stable isotope method (Van Kessel *et al.*, 2000). The old C pool (C_{old}) was defined as C_{total} minus C_{new}, while the respired C was calculated as the difference between C_{old} at the beginning of the experiment. C_{total} ($C_{old} + C_{new}$) increased 12% and 3%, 484 g m^{-2} and 107 g m^{-2} under the control and elevated CO_2 respectively. During the same time span, 704 g m^{-2} and 926 g m^{-2} C_{new} were formed in the soil under control and elevated CO_2 respectively. The old C pool lost relatively more C under elevated CO_2, resulting in a loss of C by respiration of respectively 220 g m^{-2} and 819 g m^{-2} under control CO_2 and elevated CO_2 (Scarascia-Mugnozza *et al.*, 2006). This is opposite of the original hypothesis. The priming effect was defined as the stimulation of SOM decomposition caused by the addition of labile substrates (Dalenberg and Jager, 1989). This priming effect induced by elevated CO_2 may have also caused increased respiration rates. However soil C data for second rotation showed that priming effect was a temporary effect. Due to the change of land use at the beginning of the POPFACE project, total C content kept increasing but, as opposed to the first rotation, the increase in the soil C_{total} under elevated CO_2 during the second rotation was larger than control CO_2 (Scarascia-Mugnozza *et al.*, 2006).

Growth and anatomical wood properties in secondary sprouts in three poplar clones showed no uniform response pattern to elevated CO_2 or N-fertilization (Luo *et al.*, 2005). In cross-sections of young poplar stems, tension wood accounted 2-10% of the total area and was not affected by elevated CO_2. In *P. nigra*, N-fertilization caused an about 2 fold increase in tension wood, but not in other clones.

Chapter 3

2 General methods

This chapter covers different methods used to conduct the research presented in this thesis.

3.1 Sample collection

Soil samples were collected at the EUROFACE site. This site has six experimental plots, three were FACE (Free Air Carbon-dioxide Enrichment) three were under ambient CO_2 conditions. The FACE plots were supplied with elevated CO_2 at 200 ppm higher than ambient. Three *Populus* species ; *P. alba*, *P. nigra* and *P. x euramericana* in two fertilisation treatments were grown under ambient and elevated atmospheric CO_2 conditions (see site description in chapter 2).

Soil samples were collected in August 2003. The samples were taken at depths 0-10, 10-20, 20-40 and 50-60 cm from each plot, species and fertilizer treatment. In addition soil samples were taken at the same depths at the edges of the plantation without trees. The samples were taken at least 2 m from the edge of the tree plantation at the nearest north or south point from each plot. The soil samples were oven dried at 70°C for 72 hours, after which they were ground and passed through a 2 mm sieve and stored in polythene bags for further analyses. These soil samples were used to analyse organic, inorganic P fractions and the organic matter content.

For microbial P a set of soil samples were collected in June 2005 as described above. The soils were immediately cooled to 4°C and transported to Bangor in a cool box. The samples were then stored at 4°C.

3.2. Inorganic phosphorus

Inorganic P fractions were determined using a modified Hedley method (Hedley *et al.*, 1982) (Fig. 3.1). Water soluble P was extracted by placing 0.5 g soil in 10 cm^3 of distilled water and was shaken for 16 hrs. After shaking, the samples were centrifuged at 1500 g for 10 minutes at 5°C and the supernatant was transferred into a new centrifuge tube. From the residue second fraction was extracted using 0.1 M NaOH. Thirty cm^3 of 0.1 M NaOH was added to the residual soil from the water extraction and again shaken for 16 hrs. After this period the samples were centrifuged as described

above. The supernatant was removed and the residual further extracted in 30 cm^3 of 1 M HCl, shaken and centrifuged as described above. The HCl supernatant was removed and the final pellet washed into Teflon vessels, oven dried to remove moisture and then digested in 2 cm^3 of concentrated HNO_3. The samples were digested in an oven at 130°C for 16 hrs to collect the final extract. The extract was obtained by filtering through a 90 mm filter (Schleicher & Schuell GmbH) and diluted to a final volume of 25 cm^3 using distilled water. Before analysis pH of the HCl extracts was adjusted by adding equal volumes of 1 M NaOH.

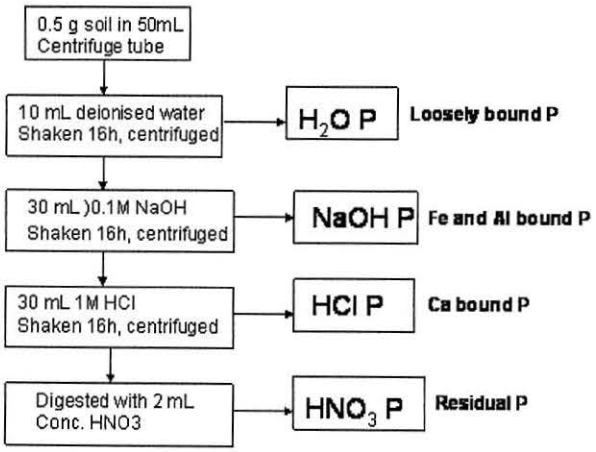


Figure 3.1: Sequential P fractionation scheme, modified from Hedley *et al.*, (1982).

3.2.1 Ames reagent

P was determined using Ames reagent as prepared below.

Reagent A (AMES)

3 g of ammonium molybdate was dissolved in 62 cm^3 of distilled water, and 0.0727 g of potassium antimony tartrate was dissolved in 25 cm^3 of distilled water. Finally 250 cm^3 of 5N H_2SO_4 was made by diluting 37 cm^3 of concentrated H_2SO_4. These 3 solutions were mixed and made up to a final volume of 500 cm^3.

Reagent B (AMES)

To make reagent B, 50 cm^3 of reagent A were mixed with 0.264 g of ascorbic acid.

3.2.2 Standard preparation

Standards were made by dissolving 0.435 g of K_2HPO_4 in 250 cm^3 of distilled water to make a 0.01 M stock solution. Aliquots of 12.5, 25, 50, 100 and 150 µl were diluted in 25 cm^3 of distilled water, 0.1 M NaOH, 1 M HCl or 5% HNO_3 to give 5, 10, 20, 40 and 60 µM standards in the same matrices as the extracts.

3.2.3 P determination

For determination of P, 40 µl of Ames reagent B were mixed with 200 µl of extract in a 96 well micro plate (Fisher) and left for 30 minutes for colour development. The absorbance was measured at 850 nm in VERSA max. micro plate reader (Molecular Devices). All measurements were made in triplicate. The mean of the absorbance readings was then used to convert to mg P kg^{-1}soil (Dwt).

3.3 Organic phosphorus

Organic P was determined using the method of Walker and Adams (1958). Soil organic P was determined by comparing the concentration of ignited and non-ignited soil samples. For the ignited soils, 0.5 g of oven dried soil were placed in porcelain crucible and put in muffle furnace at 450°C for 16 hrs. After cooling, the samples were transferred into a 50 cm^3 centrifuge tube and extracted with 30 cm^3 of 0.1M NaOH by shaking for 16 hrs. The extracts were centrifuged at 1500 g for 10 minutes at 5°C and the supernatant transferred into new clean centrifuge tube. The non-ignited soils were extracted in 0.1M NaOH as described above. The extracts were later analysed for P as described in section 2.3.3. Organic P was calculated as the difference between the P concentrations of ignited and non-ignited samples.

3.4 Microbial phosphorus

Microbial P was determined using chloroform fumigation (Jenkinson and Powlson, 1976). Three replicates of 10g for each soil sample were taken. For chloroform fumigation one replicate of soil was placed in a glass culture tube (Jenkinson and Powlson, 1976). Twenty eight samples were placed in a desicator together with 30-40 cm^3 of chloroform, and placed under vacuum using a vacuum pump. The chloroform was allowed to boil for approximately 1 min after which the vacuum pump was switched off. The desicator was then covered with a dark cloth and

left in a fume cupboard for 5 days. Soil samples were removed after five days and were extracted on the same day with 0.5 M $NaHCO_3$ (Brooks *et al.*, 1982; Hedley and Stewart, 1982) at pH 8.5, using 2g of fumigated soil and 40 cm^3 0.5 M $NaHCO_3$. The samples were shaken for 30 minutes and then centrifuged for 5 minutes at 1500 g. After centrifugation the samples were acidified using 2.5M H_2SO_4 and a p-nitro phenol indicator. 5 drops of p-nitro phenol indicator were added to 5cm^3 of extract and then 2.5 M H_2SO_4 was slowly added until the solution became colourless. The non-fumigated second replicate was extracted using the same procedure. The P concentration was determined as described in section 3.2.3.

The third sample was used to determine the water content of the soil for correction to soil dry weight. The samples were dried in an oven at 105°C for 16 hrs, and moisture content was calculated as the difference between fresh and dry weight. The difference between fumigated and non-fumigated P concentrations was taken as microbial P after the moisture correction.

3.5 Method assessment

While developing the Ames P analysis method to find different fractions of P we observed that no colour was developed in HCl. This suggests that method was pH sensitive or sensitive to HCl. As the sequential P extraction results in extracts in different matrices, the effect of the matrix on colour development was determined.

To test the influence of pH 500 cm^3 of dist. H_2O was adjusted to pH 0.66, 1.0, 2.0, 4.87, 6.7 and 10 by using either 1 M HCl or 1 M NaOH. These solutions were then used to prepare a series of standards containing a range of P concentrations (10, 20 and 60 μm). A 10 μM stock solution was taken and 25, 50 and 150 μL aliquots pipetted into different volumetric flasks and made to volume by adding pH adjusted water. This resulted in 18 standards of 6 different pH values.

These were analysed at 850nm wavelength, with similar procedure illustrated above, to find the readings at different pH levels and concentrations. We studied the P concentration at different time intervals; the first reading was taken after 5minutes and the next after 10 minutes, followed by series of readings after every 10 minutes. After 5^{th} reading after 40 minutes the time interval was increased to 60 minutes, the readings were taken till 400 minutes. Data collected was used to draw the graph in Figure 2.2. The results of this investigation showed clear interactions between the pH values of the matrix and the time allowed for colour development.

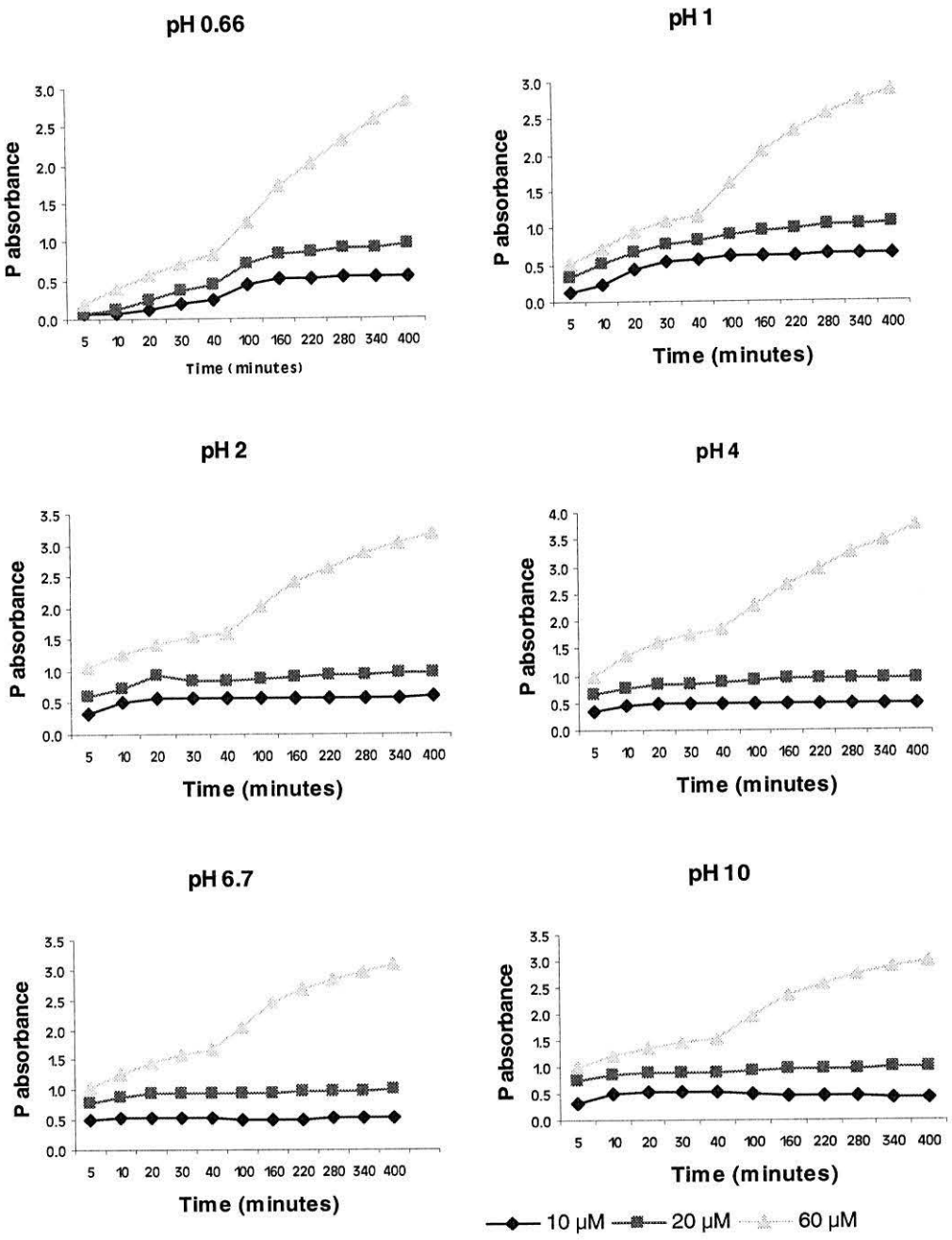


Figure 3.2: Phosphorus absorbance at different pH levels and different time intervals measured in different standards to find the best pH and time for the reading.

The analysis showed at pH values between 2 and 10, colour development continued over the whole of 400 minute period investigated. The increase over time in colour development was greatest at 60 µM P concentration, but could still be seen at the lower P values. At pH values greater than 2, at P concentrations of 10 and 20 µM,

colour development was stable after a minimum of 30 minutes. This time for full colour development was shorter at pH 6.7 and 10. However, at the 50 μM P concentration colour development continued over the 400 minute period.

Table 3.1: The mean P absorbance values at different pH levels and concentrations

	P-concentrations (μM)		
pH	**10**	**20**	**60**
0.66	0.35	0.59	1.40
1	0.52	0.83	1.69
2	0.55	0.86	2.09
4.8	0.47	0.87	2.36
6.7	0.53	0.94	2.10
10	0.47	0.92	2.01

Based on these results a colour development time of 30 minutes was used in all further analysis. Table 3.1 shows the effect of pH on colour development after 30 minutes. This shows that between pH 2 and pH 10 there is a little effect of pH on colour development, but a strong effect below pH 2. Due to the continuing colour development over time at P concentrations greater than 20 μM, all assays contained less than 20 μM P. Sample solutions were diluted to avoid the effect of P concentration on colour development.

3.6 Phosphorus in plant materials

The plant materials were collected from EUROFACE experimental site Viterbo, Italy. The leaf and root samples were collected in May, 2003. For each species in each plot, 10 of the youngest fully expanded leaves were collected from 3-4 trees within reach of the canopy access tower to make a composite sample. The leaves were than air dried and then finally oven dried at 60°C for 72 hrs.

The root samples were taken using an 8 cm diameter soil corer to depths of 40 cm. For each species in each plot and treatment, 2 cores per segment were taken to form a composite sample. The cores were cut into soil depths of 0-20 and 20-40 cm, and the roots were removed from the core and washed. The root samples were also first air

dried and then oven dried at 60°C for 72 hrs.

The wood samples for *P. alba* and *P. x euramericana* were taken from the base of the stem in 2003 by the University of Göttingen, Germany. Samples for *P. nigra* were collected in June 2005. For each species in each plot and treatment, wood samples per segment were taken to form a composite sample. In both Bangor and Göttingen, the wood samples were air dried and ground before further analysis.

3.6.1 Digestion of plant material

For leaf, root and wood samples, 200 mg of each material was digested in Teflon vessels in 2 cm^3 of concentrated HNO_3. After closing the lids tightly, the samples were put in the oven at 130°C for 16 hrs. The Teflon vessels were then allowed to cool, and the digest was washed into 25 cm^3 volumetric flask using distilled water, and made up to a final volume of 25 cm^3. The samples were filtered using Schwartz band ash free filter paper, and diluted using 5% HNO_3 for further analysis. In Göttingen a 50 mg dry wood was ground by a mill (Retsch, Haan, Germany). Subsequently 2 ml of 65% HNO_3 was added to the fine powder and the mixture was incubated for 12 hours at room temperature. After filtration of the mixture, the solution was added distilled water to 25 cm^3.

3.6.2 Phosphorus analysis

Phosphorus was analysed using two methods. Phosphorus was analysed using the Ames method as described in 3.2.1, with the following modifications. Eighty µl of sample were added to 80 µL of Ames reagent A and 30 µL 10% ascorbic acid. The samples were left for 10 minutes for colour development, and the absorbance read at 850 nm. These readings were then converted to mg P kg^{-1} Dwt from standard curves. The standards were made with 5% HNO_3.

In addition, wood samples were analysed for P by using induction coupled plasma spectroscopy (ICP). The samples were prepared as described in 3.6.1, and measured against standards made with 5% HNO_3. In Bangor a Jobin Yvon JY138 Ultrace Sequential Spectrometer and in Göttingen inductively coupled plasma-atomic emission spectrometer (ICP-AES; Spectro Analytical Instruments, Kleve, Germany) were used to analyse P concentrations.

3.7 Elemental ratios in soil and plant materials

The calcium and strontium were analysed from the same soil, leaf and root samples as used before for phosphorus analysis by atomic absorption spectrophotometery. In addition for soil samples only, Ca, Sr, P, Ba, Mg, K, Mn, Na, Fe and Al were also analysed by inductively ICP (Jobin Yvon JY138 Ultrace Sequential Spectrometer), only for soil samples, to observe the elemental ratios. In the following the method used for this analysis is described and the results are presented in chapter 6.

3.7.1 Elements in soil

The calcium (Ca) and strontium (Sr) in soils was extracted with the same extraction scheme as described before is section 3.2 as proposed by Hedley *et al.*, (1982). The Ca and Sr were measured only in 0-10 cm depth soil samples of non-fertilized treatment plots. The different extractions were later analysed on Varian flame atomic absorption spectrophotometer. Different set of standards in the extraction medium were made to analyse the calcium and strontium. The same extracts were diluted for the measurements of calcium. The same extracts except H_2O extract were also analysed by ICP (Jobin Yvon JY138 Ultrace Sequential Spectrometer) for calcium (Ca), strontium (Sr), barium (Ba), phosphorus (P), potassium (K), aluminium (Al), manganese (Mn), Magnesium (Mg), iron (Fe) and sodium (Na).

3.7.2 Elements in plant materials

Calcium and Strontium were measured from the HNO_3 digestible extracts already prepared for phosphorus analysis as mentioned in section 3.6. The extracts were analysed on a Varian flame AAS. The standards were made with 5% HNO_3. The extracts were again diluted for the measurements of calcium.

3.8 Statistical Analysis

Sigma stat 3.0 was used for all the statistical analysis. The main experimental factors were CO_2 treatment, poplar species, soil depths and fertilization. Interactions among experimental factors that were specifically investigated were: CO_2 treatment x poplar species, CO_2 treatment x soil depths and CO_2 treatment x fertilization, to quantify the range of different responses to changing environmental factors. Data that were not normally distributed or did not show equal variance were transformed by using

a $\log_{10}$ transformation. The significance was tested by Holm-Sidak method for all pair wise multiple comparison procedures.

Chapter 4

4 Phosphorus in soils

4.1 Introduction

The chemistry of phosphorus (P) in soils is complex. Inorganic P can react with Ca, Fe and Al to form discrete phosphates and organic P can be found in different forms with varying resistance to microbial degradation (Zhang & Kovar, 2000). This chapter describes investigation of the availability of P in soils under poplar grown at ambient and elevated atmospheric CO_2 levels.

The geochemical and biological processes regulate the availability of phosphorus in soils (Cross & Schlesinger 1995). At the global scale and over the long term, geochemical processes link the movement and distribution of phosphorus between two large pools which are terrestrial soils and ocean sediments (Richey, 1983; Schlesinger, 1991; Ramirez and Rose, 1992). In most natural ecosystems, geochemical processes may also determine the long term distribution of phosphorus in soils, but in the short term, biological processes influence phosphorus distribution because most of the plant available phosphorus is derived from soil organic matter (Ballard, 1980; Wood *et al.*, 1984; Smeck, 1985; Tate and Salcedo, 1988; Walbridge, 1991). The proportion of total phosphorus held in various forms changes as soil develops and the weathering of primary minerals supplies phosphate to the plant available pool in the soil (Walker and Sayers, 1976). In a mature, undisturbed, the Hubbard Brook Experimental Forest, in New Hampshire, USA, about 8 times as much phosphorus cycles in the intrasystem P cycle compared to the annual release of P from rock weathering (Schlesinger, 1991).

The biological portion of the phosphorus cycle is controlled primarily by bacterial and fungal decomposition, immobilization, and mineralization, and secondarily via plant uptake (Wood *et al.*, 1984; Jurinak *et al.*, 1986; Walbridge, 1991; Bolan, 1991). Biological processes regulate the movement and distribution of labile forms of phosphorus, and organic P cycling is important to the availability of soil P (Stewart and Tiessen, 1987). Rates of plant litter decomposition depend on substrate quality (including C/P ratios), soil moisture, and temperature (McGill and Cole 1981; Harrison, 1982b). Microbial mineralization and immobilization of phosphorus depend on phosphorus availability (Harrison, 1982b). Walbridge and Vitousek (1987) found that phosphorus mineralization rates from phosphorus rich soils were twice as high as

those from phosphorus deficient bog soils.

Over time, both biological and geochemical processes transform inorganic phosphorus into stable forms of organic and inorganic phosphorus in soil (Tiessen *et al.*, 1984; Sharpley *et al.*, 1987). In desert ecosystem geochemical processes dominate P cycling when calcium carbonate is abundant in soils (Lajtha and Schlesinger, 1988). Ligand exchange between P and carbonate minerals limits P availability in desert soils (Ardisols), and precipitation of phosphate with calcium establishes the upper limit for the availability of P (Lajtha & Bloomer 1988). Soils that dominate humid temperate and tropical regions (Ultisols and Oxisols) are highly weathered, acidic, and dominated by large quantities of sesquioxides. These soils easily adsorb and geochemically fix phosphorus, in many cases leading to phosphorus limitations (Johnson and Cole, 1980; Sanchez *et al.*, 1982; Sollins *et al.*, 1988). Walker and Syers (1976) suggested that the proportion of phosphorus in labile, non-labile, non-occluded, and occluded fractions should vary between soil taxa along a gradient of soil weathering intensity. In ecosystems with young, slightly weathered soils, most of the phosphorus should be found in primary minerals, such as hydroxyapatite. In ecosystem with a moderate weathering regime, most of the phosphorus should be found in organic compounds or adsorbed with secondary clay minerals. And, finally, in ecosystems with highly weathered soils, most of the phosphorus should be in the non-labile, occluded, or stable organic forms.

Lake sediments originate as seston, containing biotic and abiotic particulate matter present in the water column (Hutchinson, 1967). Phosphorus is often the limiting nutrient for algal growth in lakes (Hutchinson, 1973) and may limit marine productivity (Smith, 1984). Phosphorus may enter an aquatic system in the particulate form or dissolved P may become associated with particles as they settle out of the water column (pathways reviewed by Bloesch *et al.*, 1988). Sedimentation is the major P sink for the epilimnia (well-mixed surface water) of lakes, transporting P to the hypolimnion (deep water) and ultimately the sediments (Imboden, 1974., Stabel 1984).

Few studies have been carried out on how plant productivity affects P availability in soil. Previous investigation have been concerned with the effects of P fertilization and elevated CO_2 interactions on plant growth. The potential response of terrestrial ecosystem to elevated CO_2 may be constrained by the availability and cycling of nutrients (Johnson *et al.*, 2004). The most of the studies were conducted in greenhouses or open top chambers experiments and relate to nitrogen cycling. Johnson

et al., (2001) in a 3 year study looked at the effects of elevated CO_2 on soil N and P, soil pCO_2, and calculated CO_2 efflux in a fire-regenerated Florida scrub oak ecosystem. In the case of P, buried resin bags and anion exchange membranes were used to analyse ortho-P. The resin lysimeter used in this study consisted of a 5.5 cm long, 4 cm-i.d. PVC pipe within which a resin bag was sandwiched between layers of washed silica sand. After removing them they were extracted with 1 M KCl and analyzed for ortho-P by automated calorimetric analysis. While the membranes were extracted with 0.5 M NaCl and the extracts were analysed for orthophosphate by using Alpkem RFA300 colorimeter. The results suggested a negative effect of CO_2 on P availability during the first year. However, this effect decreased over time. P availability declined overall, probably in response to P uptake. In the same experiment elevated CO_2 had no effect on any measured soil property except extractable phosphorus (P), which was lower with elevated CO_2 after five years (Johnson *et al.*, 2003).

At the sweetgum FACE site at Oak Ridge similar experiments on nutrient cycling of phosphorus and other nutrients under elevated CO_2 in sweetgum plantation have been carried out. In 1999, they used Plant Root Simulator probes (PRS^{TM}), which consisted anion and cation exchange membranes embedded in plastic stakes (Western Ag Innovations, Inc., Saskatoon, Canada). The stakes were pounded in the soil and allowed to remain in contact with it for 1 month. After removal they were extracted with 1 M HCl and extract was analysed for ortho-P. The results of ortho-P showed no significant differences between ambient and elevated P concentrations. In 2001, they changed the technique to the WECSA Soil Access system (Warrington Ecological Analysis, Ft. Collins, CO). The system employs a mixed-bed cation/anion exchange resin capsule (Unibest PST-1; Yang and Skogley 1992; Dobermann *et al.* 1994) fitted to the end of a permanently-installed PVC pipe. The capsules were allowed to remain in contact with the soil for a 1 month period after which they were extracted with 2 M KCl and measured ortho-P measured in them. In general, the effects of elevated CO_2 on nutrient cycling in this study were not large (Johnson *et al.*, 2004).

In a FACE experiment in northern Japan rice (*Oriza sativa* L.) grown under with FACE (ambient + 250 mumol mol^{-1}) was compared to rice grown in ambient CO_2, the increase rate in dry matter was lower after panicle initiation. The nitrogen (N), phosphorus (P), potassium (K), and magnesium (Mg) concentrations of rice were significantly 21, 6, 14, and 9% lower, respectively, in rice grown under FACE than the concentrations of these elements in ambient grown rice at panicle initiation, and at

harvest the N and K concentrations were significantly lower under FACE (Yamakawa *et al.*, 2003).

Using spring wheat (*Triticum aestivum* L. cv. Minaret) (Fangmeier *et al.*, 1997) field exposure systems (OTC's) were used to enrich the atmosphere in CO_2 and/or ozone. Nutrient element concentrations and grain quality were assessed in spring wheat grown under elevated CO_2 concentrations and contrasting levels of tropospheric ozone at different nitrogen supply rates at several European sites. At five of the sites, nutrient element concentrations (macronutrients: nitrogen, phosphorus, potassium, magnesium, and micronutrients: manganese, zinc, iron) in different parts of plants from the various treatments were analysed. Under elevated CO_2, nearly all nutrient elements tended to decrease in concentration in all the plant parts (Fangmeier *et al.*, 2002). Carbon dioxide enrichment proved to affect nutrient concentrations in a complex manner. In green leaves, all elements (with exception of phosphorus and iron) decreased (Fangmeier *et al.*, 1999). The lack of a pronounced reduction of P concentrations in green leaves supports earlier suggestions that the P demand of leaves increased under CO_2 enrichment is met by greater uptake or redistribution (Conroy *et al.*, 1992). The results of a glasshouse experiment, on interactive effects of elevated CO_2 on P availability and legume presence on calcareous grassland, showed phosphorus addition pooled for all treatment combinations resulted in an almost 10%, 16% and 14% increase in phytomass in three years of experiment respectively (Stöcklin & Körner, 1999).

Since the recognition that nutrient availability is a major control on crop productivity, many methods have been devised to provide indices of soil nutrient status (Daubeny, 1845 in Nelson *et al.*, 1953). Chemical extraction techniques developed to evaluate soil P fertility have been studied in earnest for >60 years. Nelson *et al.* (1953) reviewed the early studies and an updated discussion of methods for evaluating "available P" can be found in Tiessen and Moir (1993). Multiple or sequential extraction procedures have evolved over several decades. Their use is based on the premise that since it is not possible to extract plant-available P quantitatively, a logical approach is to partition P into several reasonably well-defined chemical pools whose ability to contribute P to vegetation can be evaluated through experiments (e.g., Bray & Kurtz, 1945; Chauhan *et al.*, 1981; Hedley *et al.*, 1982a). Two paradigms that evolved in concert with multiple or sequential P-extraction procedures are frequently referenced in the recent literature. Walker and Syers (1976) developed a model describing the change in four soil P fractions during pedogenesis in New Zealand. Hedley *et al.*, (1982a,

1982b) and Tiessen *et al.*, (1984) developed a conceptual model linked to a sequential fractionation procedure that separates inorganic P (Pi) and organic P (Po) into several labile and non-labile pools and delineates major pathways by which these pools are linked through short and long-term chemical and biological processes (Johnson *et al.*, 2003).

Tiessen and Moir (1993) argued that the Hedley sequential fractionation is the only available method that provides an assessment of available Po. In its early application to agricultural soils, the Hedley fractionation procedure was used to quantify labile and non-labile pools of Po and track transformations involving P from those organic pools (Hedley *et al.* 1982a, 1982b). This method has since been applied to a large number of agricultural and non-agricultural soils covering many biomes and most soil orders (Tiessen *et al.*, 1984; Sharpley *et al.*, 1985, 1987). Judging from the recent studies of P in forest soils, it has become the method of choice among forest scientists during the past decade. Cross and Schlesinger (1995) summarized data from many applications of this fractionation procedure to soils from what they judged to be natural ecosystems. Several authors have explored the availability to plants and microbes of the fractions measured by the Hedley-based methods. Over the past decade, this technique has become the method of choice in the ecology community, and as such, it seems useful to try to relate the values produced by this method to measures of species composition and productivity, but this has yet to be accomplished (Johnson *et al.*, 2003).

4.2 Results

In the following section, data on the influence of the three genotypes of *Populus* grown under elevated atmospheric CO_2 and different fertilization regimes on different phosphorus pools in soil are presented. This section is divided into three subsections that cover the data obtained for inorganic, organic and microbial phosphorus.

4.2.1 Inorganic phosphorus fractions

The inorganic P was measured by a modified sequential extraction scheme from Hedley *et al.* (1982). The results are presented for each species for soil samples taken at 4 soil depths.

4.2.1.1 Water extractable phosphorus

The water extractable P fraction in soils under *P. alba* is shown in figure 4.1 (A-F). Under ambient CO_2 concentrations in the non-fertilized treatment (Fig. 4.1 A) the amount of water extractable P in the soil decreased with soil depth. A similar trend was found in the elevated CO_2 treatment this was however less strong. This trend was not found to be significant. There was a general increase in the water extractable P under FACE compared to ambient CO_2, this however was not statistically significant. Under ambient CO_2, fertilization increased (Fig. 4.1 B) the levels of water extractable P at 0-10 cm soil depth. In contrast to the non-fertilized treatment, in the fertilized treatment lower levels of water extractable P were found in the FACE treatment compared to the ambient treatment at all soil depths. However, the differences were not statistically significant. Under FACE the levels of water extractable P were also lower in the fertilized treatment compared to the non-fertilized treatment at all soil depths.

Similar trends were found in soils under *P. nigra* (Fig 4.1.C and D), but were much less consistent. There was no clear decrease in water extractable P with increasing soil depth, and in the non-fertilized treatment under FACE higher levels of water extractable P were only found at 10-20 and 30-40 cm soil depth. Again this increase was not statistically significant. However, in the fertilized treatment water extractable P was less under elevated CO_2 compared to ambient CO_2 at all soil depths except 0-10 cm, but again the difference was not significant.

For *P. x euramericana* (Fig 4.1 E and F) similar trends in the levels of water extractable P to both *P. alba* and *P. nigra* were found. There was no clear change in the levels with soil depth, but in the non-fertilized treatment higher levels were found under the FACE treatment, except for at 50-60 cm levels. The opposite trend was found in the fertilized treatment, here higher levels were found under ambient CO_2 compared to the FACE treatment. The analysis of variance showed no significant differences among different species or fertilizer treatments.

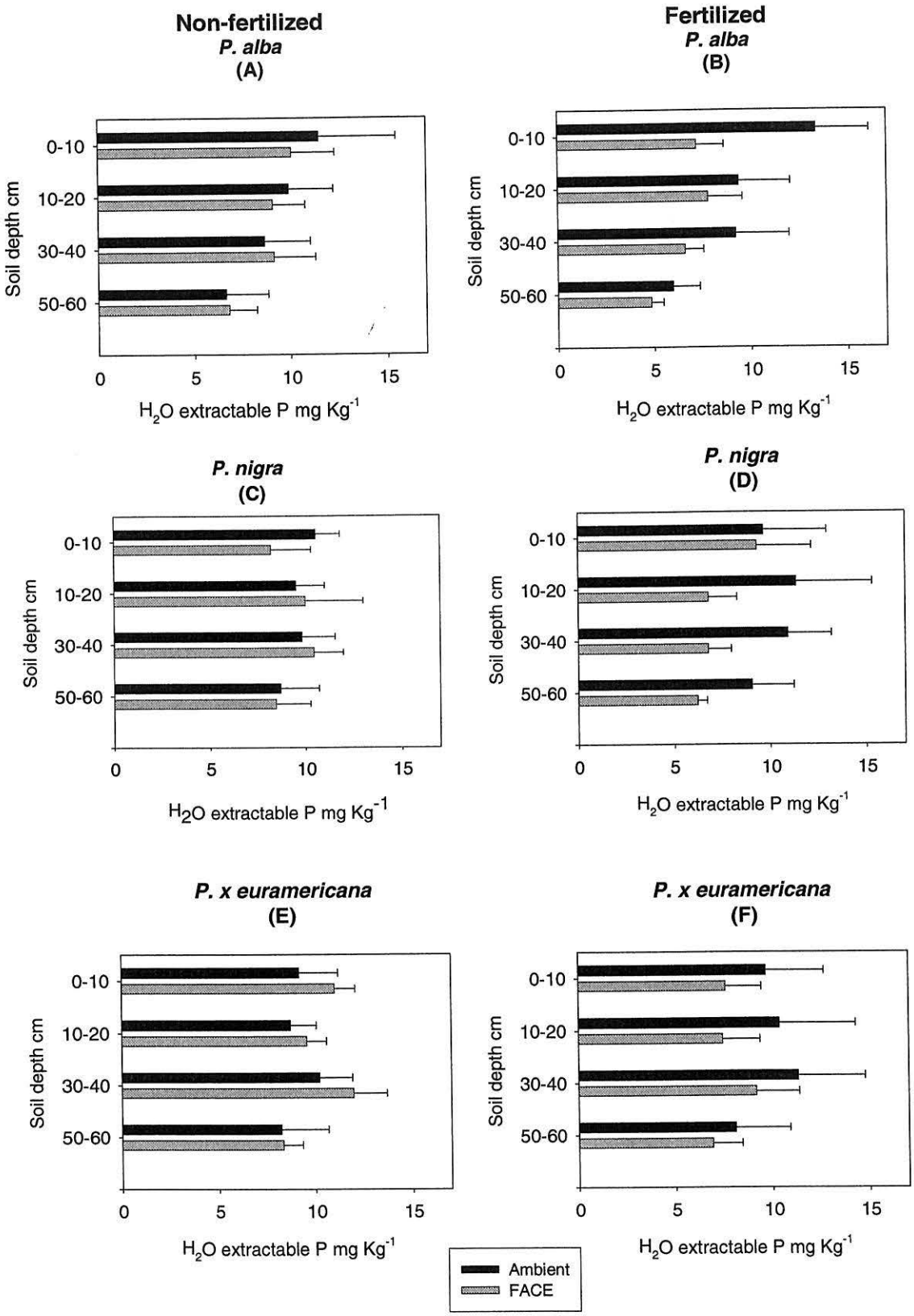


Figure 4.1: The concentration of water extractable P at different soil depths in 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown are means ± SE.

NaOH extractable phosphorus

The second P fraction was extracted by using 0.1M NaOH and has been suggested to be P extracted from Fe and Al containing minerals; however from the brown colour of the NaOH extracts also clearly extracted soil organic matter. In general the amounts of P extracted in NaOH exceeded those extracted in water by a factor of four. For soils under *P. alba* in the non-fertilized treatment significant (P=0.019) effect of FACE was found (Fig 4.2 A). The P levels at 50-60 cm were reduced to about 40% of those of the other soil layers. Also the 50-60 cm soil depth was found to be statistically different from all other soil layers. In the fertilized treatment similar to the water extractable P, higher levels of NaOH extractable P were found in the ambient compared to the FACE treatment at all soil depths. These differences were not significant. Again similar to the non-fertilized treatment the levels of NaOH extractable P were lower at 50-60 cm soil depth compared to the other soil layers.

For *P. nigra* (Fig 4.2 C and D) similar trends were found to those for *P. alba*. In non-fertilized treatment pooled data showed significant difference between ambient and FACE (P=0.010) and 50-60 cm depth was also found to be statistically different (P=0.009) from the 0-10 cm depth. The lowest levels of NaOH extractable P were found at 50-60 cm soil depth, and in the fertilizer treatment lower levels were found in the FACE treatment compared to the ambient treatment. There was however, no significant difference between ambient and FACE in fertilized plots.

These patterns were also found for *P. x euramericana* (Fig 4.2 E and F). However, in the non-fertilized treatment the levels of NaOH extractable P under FACE treatment exceeded those of the ambient treatment by 2 times. The results showed that there is a highly significant difference (P<0.001) between ambient and FACE plots in the non-fertilized treatments. However, no significant differences were found in fertilized plots.

4.2.1.3 HCl extractable phosphorus

The concentrations of P extracted with 1M HCl were 2 times higher than those extracted with NaOH. For all *Populus* species (Fig 4.3 A-F) much higher levels of HCl extractable P were found in the FACE compared to ambient treatments for both the non-

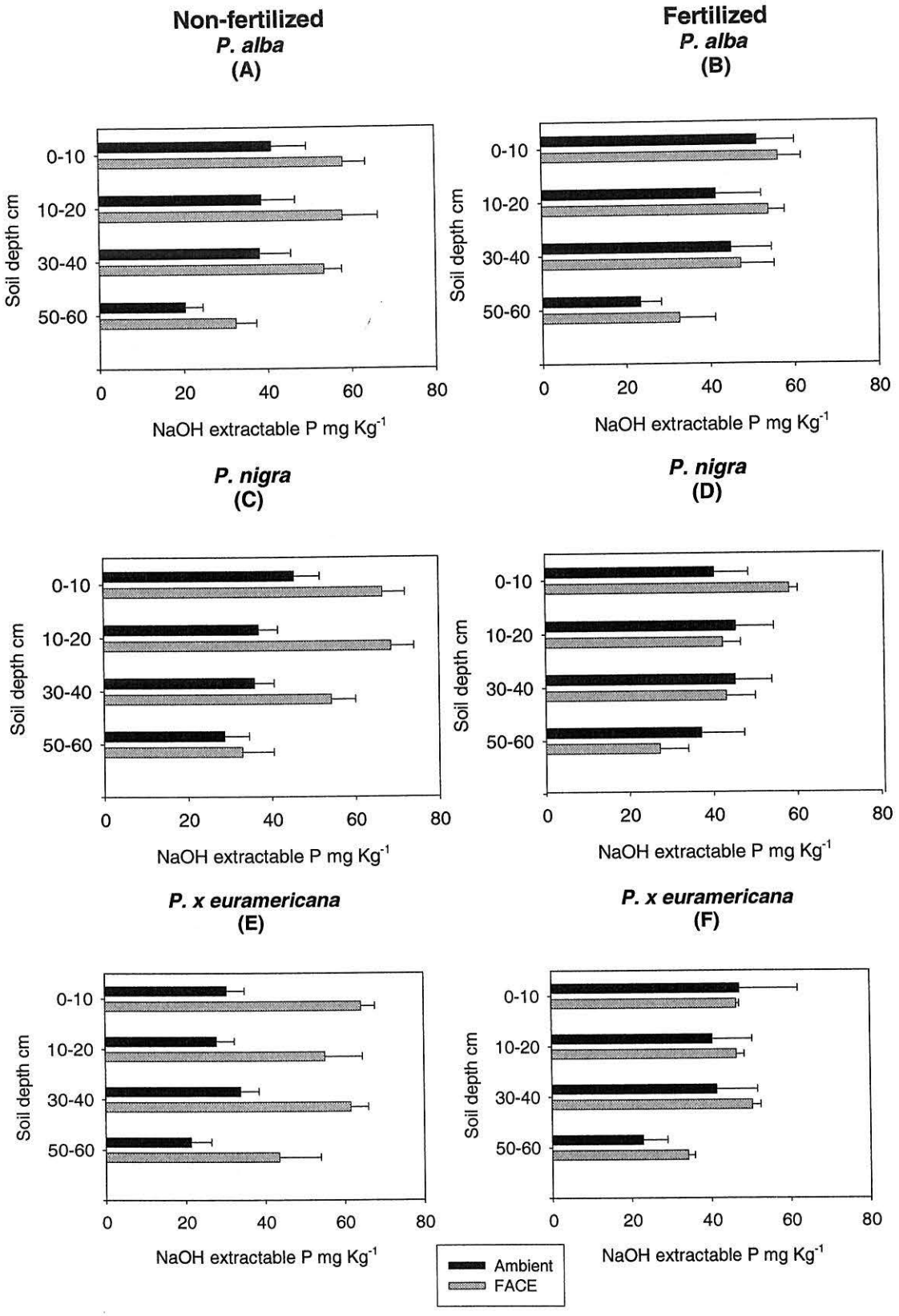


Figure 4.2: The concentration of NaOH extractable P at different soil depths in 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown are means ± SE.

fertilized and fertilized plots. The differences between the FACE and ambient treatments were greatest at 50-60 cm soil depth and greater in the non-fertilized compared to the fertilized treatment in all the species. The high standard errors in FACE results are mainly due to the lower P concentrations measured in plot 3 of ambient treatment and plot 4 of FACE treatment. This concentration in these plots was recorded most of the time 50% less than the other plots under study. This reduced P level in plot 3 and 4 were found for all the species. Analysis of variance also confirmed that there are highly significant differences between ambient and FACE ($P<0.001$) in both fertilizer treatments when the data were pooled. However, no significant differences were found between different species and depths.

4.2.1.4 HNO_3 extractable phosphorus

The final P fraction was extracted by digesting the residual soil in Teflon vessels using concentrated HNO_3. The levels of P extracted were again in general in a similar range to those extracted in NaOH.

For *P. alba* there were no significant trends in the non-fertilized treatment (Fig 4.4 A). However in fertilized treatment (Fig 4.4 B) showed statistically significant difference ($P=0.019$) between ambient and FACE. In both the fertilization treatments more HNO_3 extractable P was found in the FACE treatment. A similar trend was shown in the non-fertilized treatment in soils under *P. nigra* (Fig 4.5 C), whereas in the fertilized treatment (Fig 4.5 D) this increase was not consistent. The analysis of variance showed no significant differences between different treatments and soil depths. In contrast the non-fertilized treatment (Fig 4.5 E) of *P. x euramericana* at all soil depths, except 30-40 cm, showed higher HNO_3 extractable P levels under ambient CO_2. However in the fertilized treatment (Fig 4.5 F) *P. x euramericana* was found to have more P under FACE except 0-10 cm depth. Statistical analysis again showed no significant differences among different depths and treatments.

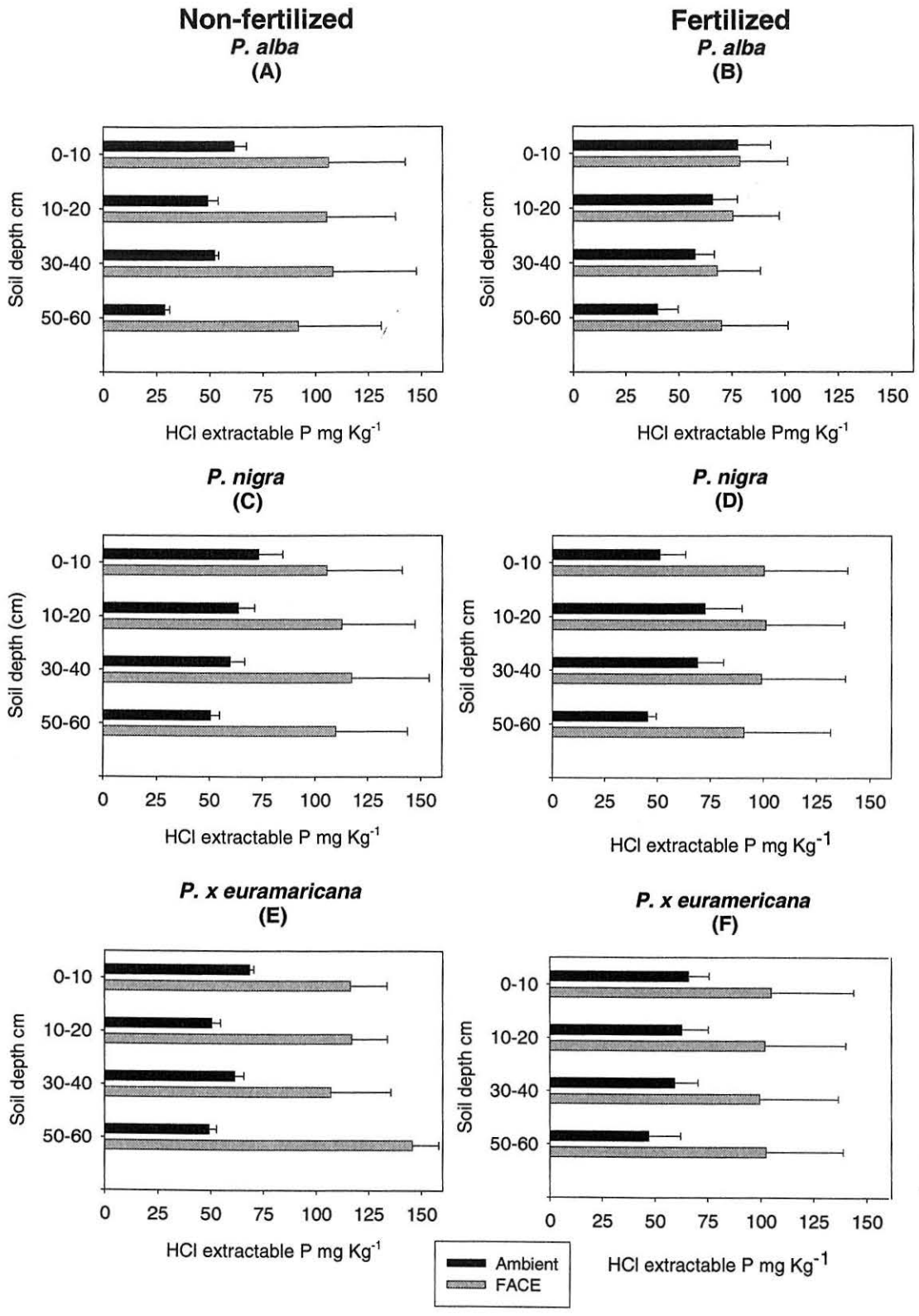


Figure 4.3: The concentration of HCl extractable P at different soil depths in 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown are means ± SE.

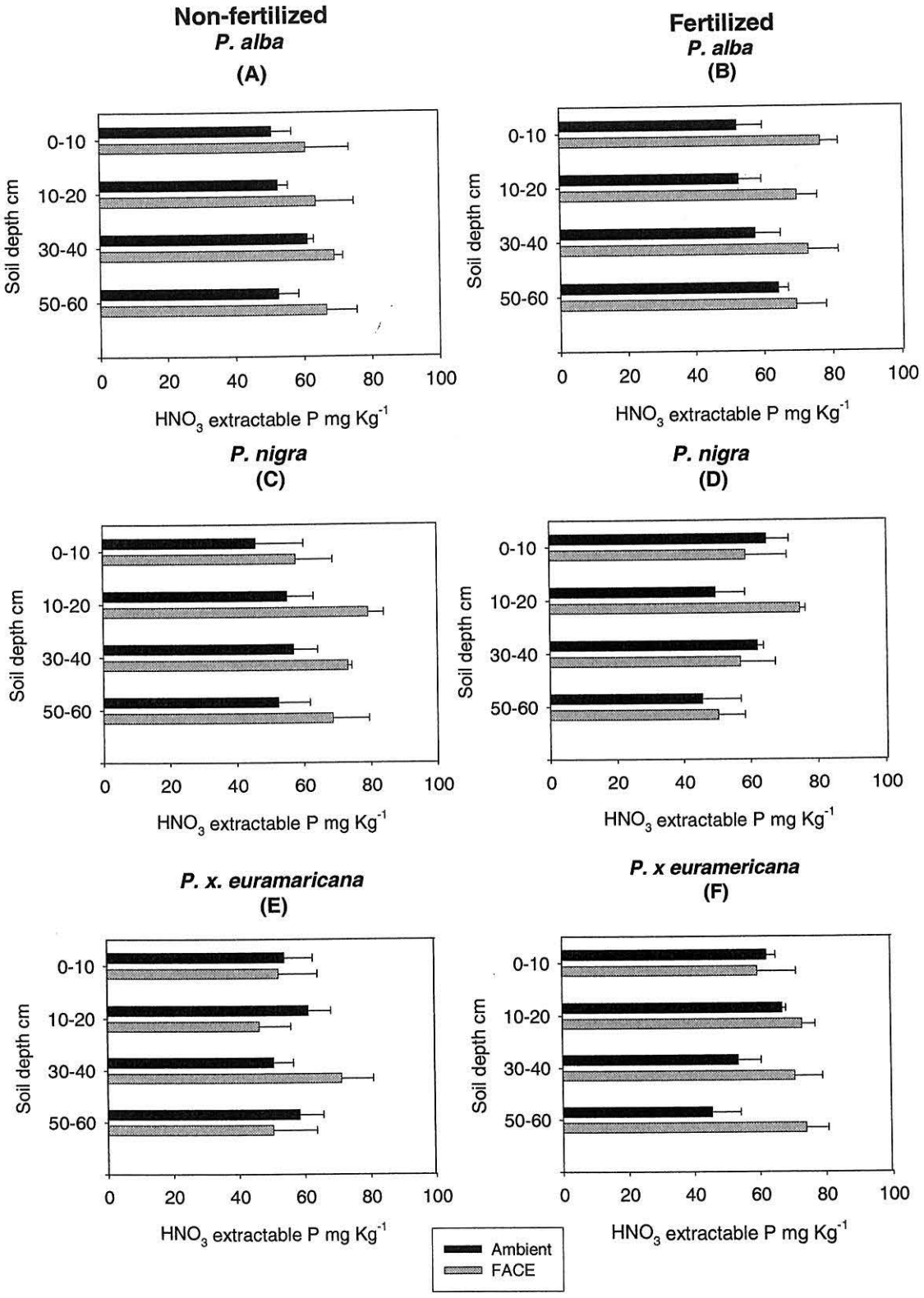


Figure 4.4: The concentration HNO_3 digestible P at different soil depths in 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown are means ± SE.

4.2.2 Organic phosphorus

Organic phosphorus was measured by using a loss on ignition method (Walker and Adams, 1958). The ignited and non ignited samples were extracted by using 0.1M NaOH. For *P. alba* (Fig 4.5A and B) at all soil depth in both fertilization treatments, except at 50-60 cm soil depth in the non-fertilized treatment, higher levels of organic P were shown under FACE treatment. Statistically significant difference was found between ambient and FACE in both non-fertilized treatment (P=0.043). There was no statistically significant difference found in fertilized plots. The results for *P. nigra* (Fig 2.6 C and D) showed similar trends to those found in *P. alba* there was more organic P in both fertilization treatments under elevated CO_2 at all the depths and also the amount of organic P was highest at 50-60 cm depth. The statistical analyses revealed that there was no significant difference between ambient and FACE in the non-fertilized and fertilized in *P. nigra*. The depths were also showing no significant differences. In *P. x euramearicana* (Fig 2.6 E and F) again more organic P was shown under FACE at all the soil depths for fertilized and non-fertilized treatments. Statistical analysis also showed that there was a highly significant difference between ambient and FACE (P=0.045) for non-fertilized. The 50-60 cm soil depth showed higher organic P values in both fertilizer treatments, but this difference was found not to be statistically significant. When the data were pooled, again no significant difference between species and soil depths was shown.

In *P. alba* the organic P was measured between 14-29 mg kg^{-1} in all ambient plots with no fertilizer treatment and average value was 18 mg kg^{-1} at all depths. While this value was 21-28 mg kg^{-1} in FACE plots with an average value of 23 mg kg^{-1}. In fertilized samples the amount of organic P was 14-22 mg kg^{-1} with an average value 17 mg kg^{-1} in ambient and similar values in FACE were found 18-26 mg kg^{-1} and the average value was 22 mg kg^{-1}. In *P. nigra* the average values in no fertilizer ambient plots were amounted to 19 mg $kg^{-1}$and 24 mg kg^{-1} in FACE plots, while in fertilized plots these values were 18 mg kg^{-1} in ambient and 26 mg kg^{-1} in FACE. The *P. x euramericana* species analysed for organic P had 17 mg kg^{-1} in no fertilizer ambient and 27 mg kg^{-1} in FACE plots. The fertilized samples showed 18 mg kg^{-1} in ambient and 25 mg kg^{-1} average organic P at all depths. Higher organic P was found under FACE (22-29 mg kg^{-1} in FACE 15-24 mgkg^{-1} in ambient), and in all treatments organic P was highest at 50-60 cm soil depth. When all the data was pooled, statistical analysis also confirmed that a there is highly significant difference between ambient and FACE

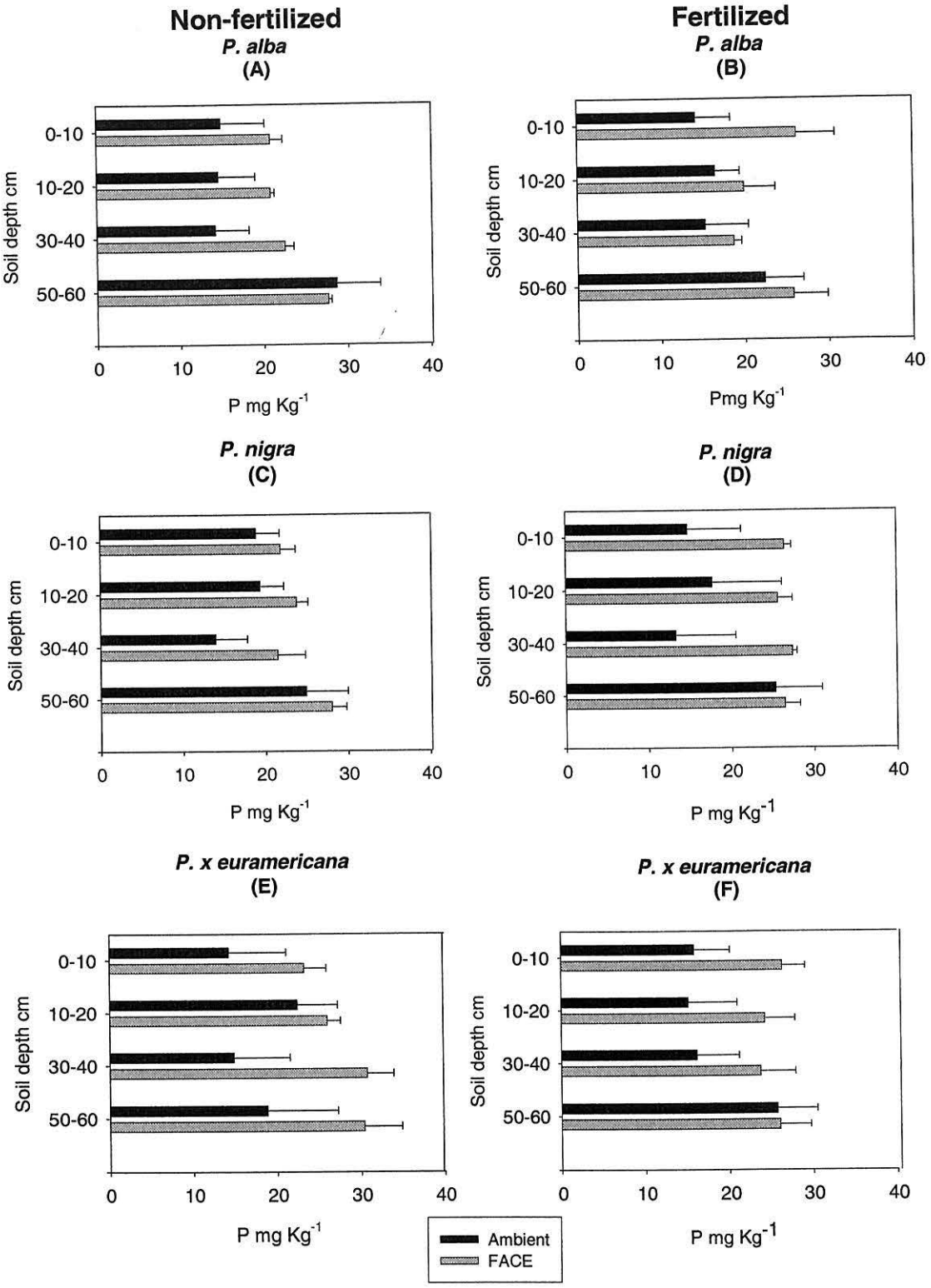


Figure 4.5: The concentration of organic P at different soil depths in 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown are means ± SE.

(P<0.001) and 50-60 cm depth is also significantly different (P=0.007) from all other depths in all species.

4.2.3 Microbial phosphorus

Microbial P was measured by using the chloroform fumigation technique as proposed by Jenkinson and Powlson 1976, following by extraction with 0.5M $NaHCO_3$. The difference between fumigated and non fumigated samples after moisture corrections was taken as microbial P. In the following section the results for microbial P for all the species are presented.

The microbial P concentrations in ambient non-fertilized treatment was found to be very low, the average values obtained for *P. alba* were 7.1 (3.1se), 1.1 (0.4se), 3.9 (1.3se) and 3.3 (1.5se) mg kg^{-1}at 0-10, 10-20, 30-40 & 50-60 cm depths respectively. The corresponding values of *P. nigra* and *P. x euramericana* were also found very low as shown in table 4.2. The results in fertilized ambient samples were also found to be very low.

Table 4.1: Microbial phosphorus mg kg^{-1}in non-fertilized ambient plots

Ring	***Populus alba***				***Populus nigra***				***P. x euramericana***			
Ambient (-F)	0-10	10-20	30-40	50-60 cm	0-10	10-20	30-40	50-60 cm	0-10	10-20	30-40	50-60 cm
2	0.8	0.7	6.9	6.6	1.2	2.7	0.6	1.3	0.7	0.2	1.7	0.5
3	14	2.1	3.6	3.3	3.7	1.3	1.9	0.8	2.9	0.3	8.4	3.5
6	6.5	0.4	1.3	0.1	1.0	0.5	0.01	0.1	0.3	0.5	0.1	13.2
Mean	**7.1**	**1.1**	**3.9**	**3.3**	**1.9**	**1.5**	**0.9**	**0.7**	**1.3**	**0.3**	**3.4**	**5.7**
SE	*3.1*	*0.4*	*1.3*	*1.5*	*0.7*	*0.5*	*0.5*	*0.3*	*0.7*	*0.1*	*2.1*	*3.1*

Table 4.2: Microbial phosphorus mg kg^{-1}in fertilized ambient plots

Ring	***Populus alba***				***Populus nigra***				***P. x euramericana***			
Ambient (+F)	0-10	10-20	30-40	50-60 cm	0-10	10-20	30-40	50-60 cm	0-10	10-20	30-40	50-60 cm
2	8.5	0.3	0.01	2.0	1.1	0.2	3.1	0.9	4.6	0.1	3.7	2.3
3	9.3	1.9	7.0	0.3	4.8	3.0	1.8	3.3	16.1	4.3	0.8	1.0
6	0.3	0.1	2.5	0.7	0.1	1.3	1.4	0.1	0.2	8.8	1.0	1.3
Mean	**6.0**	**0.8**	**3.2**	**1.0**	**2.0**	**1.5**	**2.1**	**1.4**	**7.0**	**4.4**	**1.8**	**1.5**
SE	*2.3*	*0.5*	*1.7*	*0.4*	*1.2*	*0.7*	*0.4*	*0.8*	*3.9*	*2.1*	*0.8*	*0.3*

The results of non-fertilized treatment, FACE samples showed a more or less similar picture apart from *P. nigra* species which was found to have comparatively high microbial P values only in plot 4 at all depths. The fertilizer treated FACE rings also showed similar values, but again more microbial P was found in plot 4 for all the species and at all depths. The overall picture showed that there is very little or no microbial P present in most of the experimental sites.

Table 4.3: Microbial phosphorus mg kg^{-1} in non-fertilized FACE plots

Ring	***Populus alba***				***Populus nigra***				***P. x euramericana***			
FACE (-F)	0-10	10-20	30-40	50-60 cm	0-10	10-20	30-40	50-60 cm	0-10	10-20	30-40	50-60 cm
1	3.3	0.8	0.4	6.2	4.6	10.7	0.4	1.6	17.7	2.3	1.1	0.2
4	0.6	7.2	3.3	6.1	128.2	67.1	10.9	48.5	0.6	2.0	13.0	3.2
5	1.7	0.6	0.1	6.2	0.2	8.6	9.2	0.1	0.2	1.3	0.05	0.3
Mean	**1.9**	**2.9**	**1.2**	**6.2**	**44.3**	**28.8**	**6.8**	**16.7**	**6.2**	**1.8**	**4.7**	**1.2**
SE	*0.6*	*1.8*	*0.8*	*0.04*	*34.3*	*15.7*	*2.7*	*13.0*	*4.7*	*0.3*	*3.4*	*0.8*

Table 4.4: Microbial phosphorus mg kg^{-1} in fertilized FACE plots

Ring	***Populus alba***				***Populus nigra***				***P. x euramericana***			
FACE (+F)	0-10	10-20	30-40	50-60 cm	0-10	10-20	30-40	50-60 cm	0-10	10-20	30-40	50-60 cm
1	4.1	0.1	19	7.5	1.2	2.5	2.3	0.2	0.2	0.6	0.5	0.4
4	64.6	28.2	27.6	61.6	88	5.7	123	13.1	189	10.2	14.4	27.8
5	17.7	12.9	5.1	0.1	0.1	2.4	1.0	16	8.9	0.05	0.5	1.0
Mean	**28.8**	**13.7**	**17.2**	**23.1**	**29.8**	**3.5**	**42.2**	**43.1**	**66.1**	**3.6**	**5.1**	**9.7**
SE	*1.5*	*6.6*	*5.4*	*15.8*	*23.8*	*0.9*	*33.1*	*28.8*	*50.3*	*2.7*	*3.8*	*7.4*

4.2.4 Soil organic matter content

Soil organic matter (SOM) percentage was measured by loss on ignition method as proposed by Storer (1984). The results are presented in figure 4.6 for all three species under study. The results clearly show that there is more organic matter in ambient plots than FACE plots. Generally all the species show a similar percentage of organic matter at all soil depths. It should be noted that the organic matter content is similar throughout the soil profile under ambient and FACE plots. The uniform distribution may be the result of the site being an agricultural field before the start of the experiment and ploughed before the trees were planted.

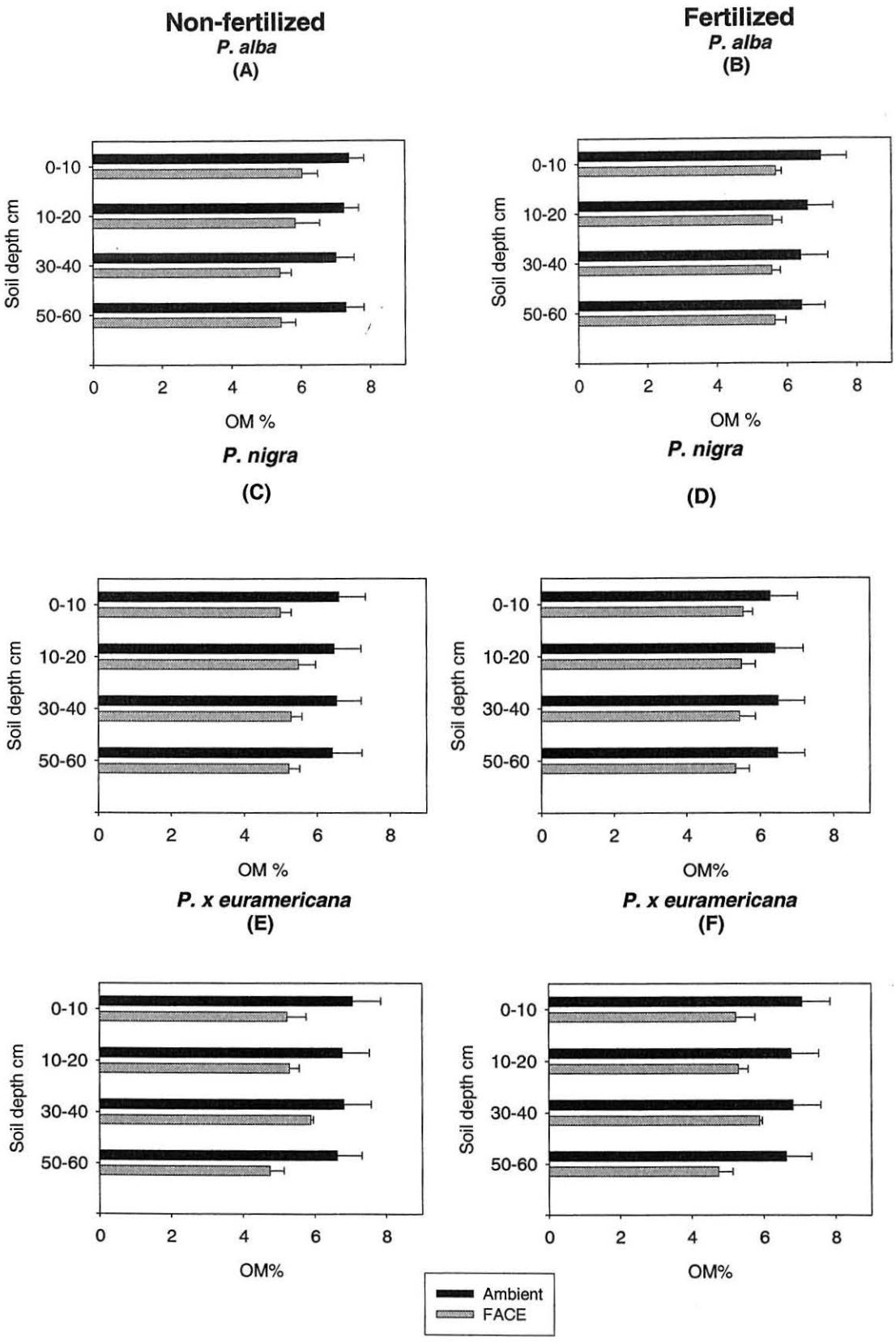


Figure 4.6: The percentage of organic matter at different soil depths in 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown are means ± SE.

In both treatments the organic matter was found around 5% in FACE plots and around 7% in ambient plots. The percentage is found higher because of the ignition method used as it also burns clay content of soil with organic matter.

4.2.6 Phosphorus fractions at the edges of experimental plots

Soil samples collected from the edges close to ambient and FACE plots were also analysed to determine phosphorus concentrations at different soil depths present before the plantation was established. The results are presented in Figure 4.7 (A-E) for all the fractions. The samples were taken outside the experimental plots and no treatment was applied to them.

The phosphorus content measured for inorganic fractions except the H_2O extractable, at all the edges of ambient and FACE plots, showed a consistent trend of decreasing P-content down the profile. When the ambient and FACE data were pooled statistically significant difference (P=0.044) was found for depth only in the HNO_3 extract. The differences among the depths and species were not significantly different in H_2O, NaOH and HCl extracts. The highest P content was measured in the HCl fraction. The organic fraction was found to have a different trend; the highest levels of organic P were found at 50-60 cm depth in both ambient and FACE edges. The analysis of variance also showed significant difference (P=0.016) between ambient and FACE edges but no significant differences were found between different depths. In general the trends of edges were similar to the trends found inside the ambient plots.

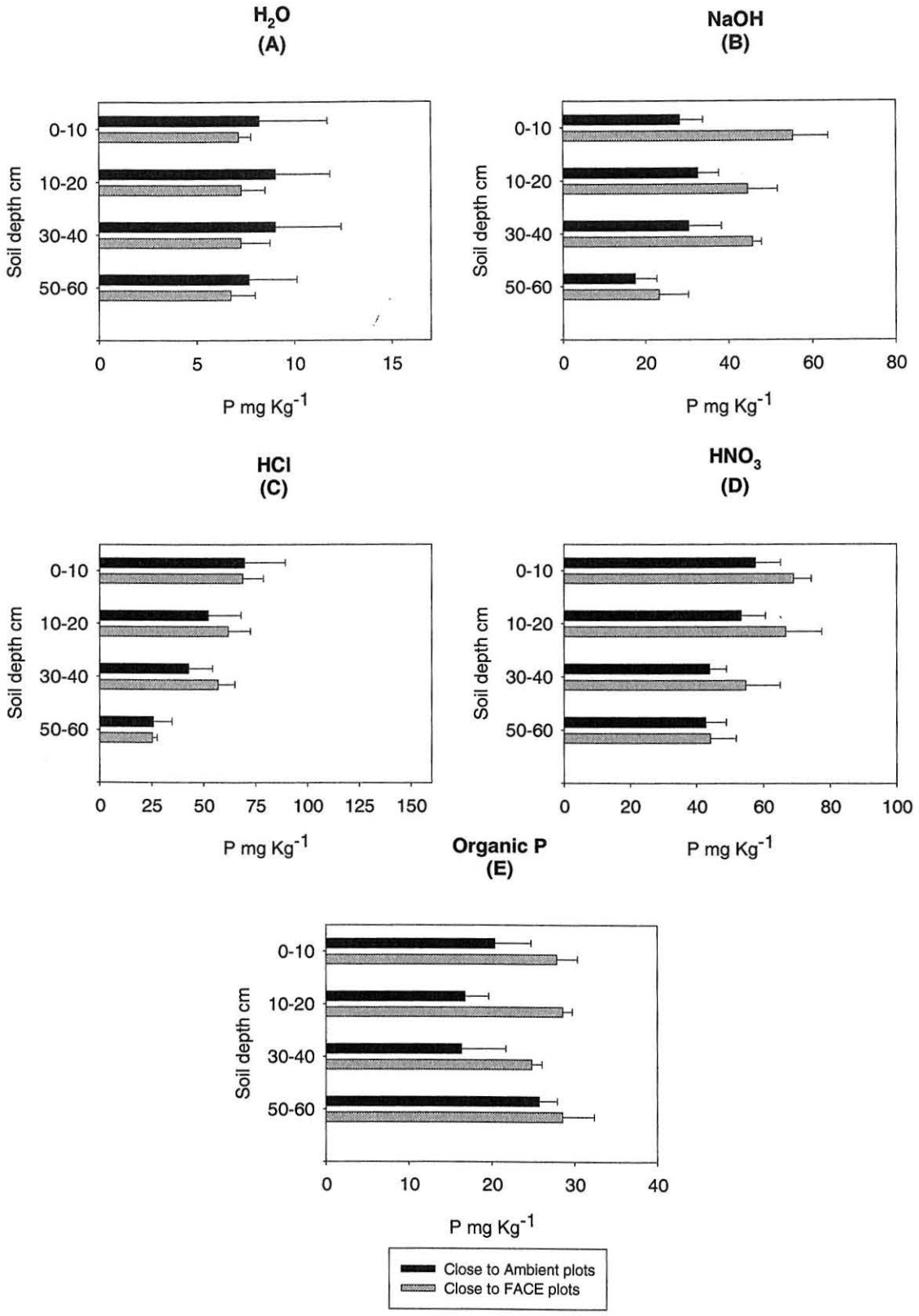


Figure 4.7: The concentration of phosphorus at different soil depths close to ambient and elevated atmospheric CO_2 plots. Data shown are means ± SE of different P fractions.

4.2.7 Total phosphorus in soils

In *P. alba* the organic P was measured between 14-29 mg kg^{-1} in all ambient rings with no fertilizer treatment and average value was 18 mg kg^{-1} at all depths. While this value was 21-28 mg kg^{-1} in FACE rings with an average value of 23 mg kg^{-1}. In fertilized samples the amount of organic P was 14-22 mg kg^{-1} with an average value 17 mg kg^{-1} in ambient and similar values in FACE were found 18-26 mg kg^{-1} and the average value was 22 mg kg^{-1}. In *P. nigra* the average values in no fertilizer ambient rings were measured 19 mg $kg^{-1}$and 24 mg kg^{-1} in FACE plots. While in fertilized rings these values were 18 mg kg^{-1} in ambient and 26 mg kg^{-1} in FACE. The *P. x euramericana* species analysed for organic P had 17 mg kg^{-1} in no fertilizer ambient and 27 mg kg^{-1} in FACE plots. The fertilized samples showed 1.8 mg kg^{-1} in ambient and 2.5 mg kg^{-1} average organic P at all depths. Higher organic P was found under FACE (22-29 mg kg^{-1} in FACE 15-25 mg kg^{-1} in ambient), in all treatments organic P was highest at 50-60 cm soil depth. The statistical analysis also confirms the results that a there is significant difference between ambient and FACE readings and 50-60 cm depth is also different from all other depths in all species.

Total extractable P increased under FACE (130-175 mg kg^{-1} in ambient, 205-230 mg kg^{-1} in FACE) for both treatments and all the species. This increase was primarily due to an increase in the HCl extractable fractions. In *P. alba* the average value of total inorganic P found at all depths in ambient non-fertilized treatment was 146 mg kg^{-1}, this value in FACE plots was 227 mg kg^{-1}. The average values of fertilized soils of the same species showed 167 mg kg^{-1} in ambient and 199 mg kg^{-1} in FACE plots. The difference in fertilized samples was not significant. The *P. nigra* showed similar results with 160 mg kg^{-1} in ambient and 246 mg kg^{-1} in non-fertilized FACE plots, while the fertilized treatment had 167 mg kg^{-1} in ambient and 208 mg kg^{-1} in FACE plots. This trend continued in the *P. x euramericana* species, the average total inorganic P values were 151 mg kg^{-1} in ambient and 243 mg kg^{-1} in FACE, for the non-fertilized treatment. The fertilized samples also showed similar picture as they showed 164 mg kg^{-1} in ambient and 224 mg kg^{-1} in FACE plots. The overall trend of low inorganic P at 50-60 cm depth was constant in all treatments and species but for P. x euramericana in FACE plots.

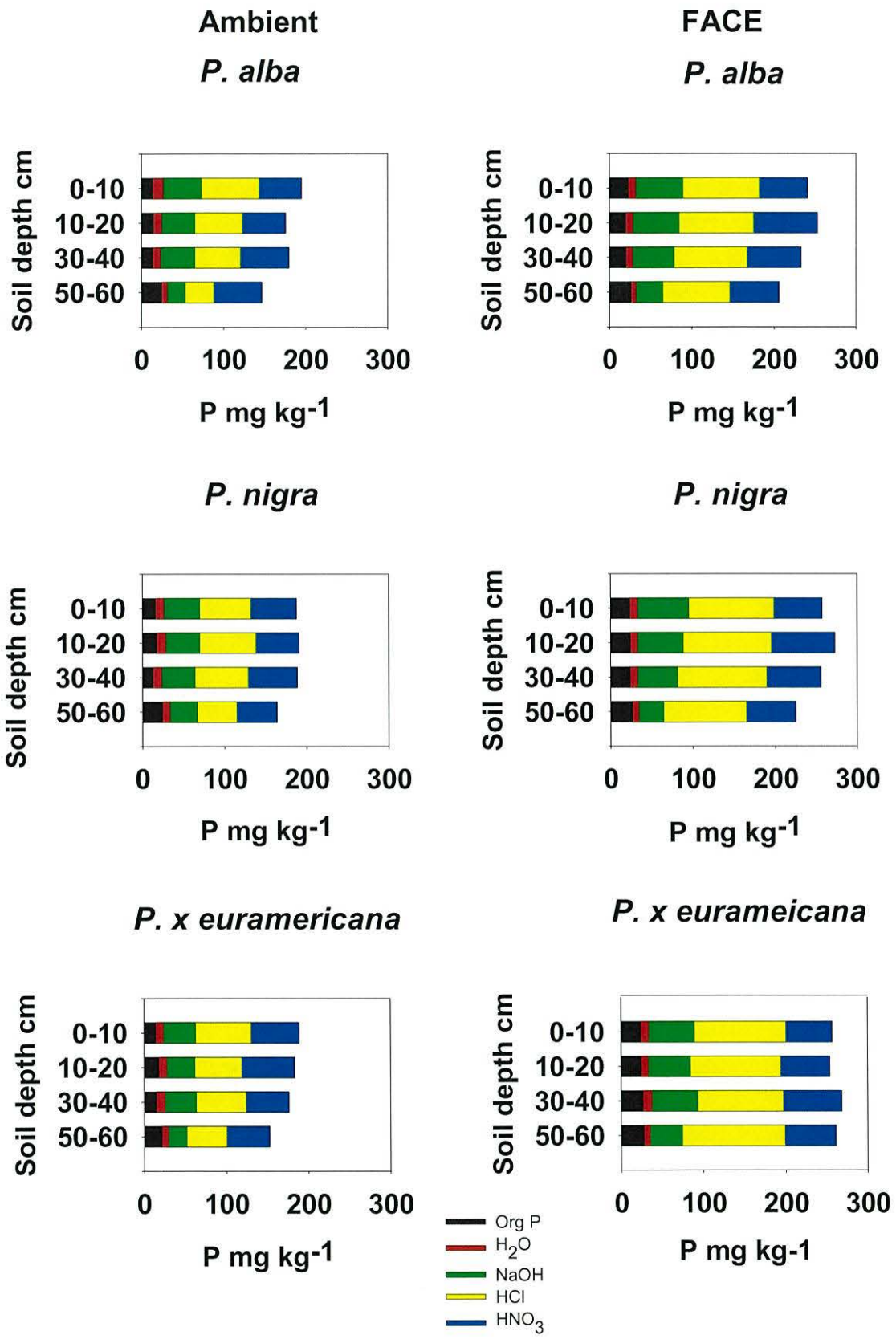


Figure 4.8: P fractions in soils under 3 poplar species grown under elevated (FACE) and ambient CO_2 concentrations. The data presented are average values for each species obtained from non-fertilized and fertilized samples from all plots.

When the data for all species was combined to form a mean value for the ambient and FACE treatment (Fig. 4.9). The increase in P stocks under FACE could clearly be seen at all soil depths. Under ambient the mean sum of all fractions was 159 mg kg^{-1}, compared to 225 mg kg^{-1} under FACE.

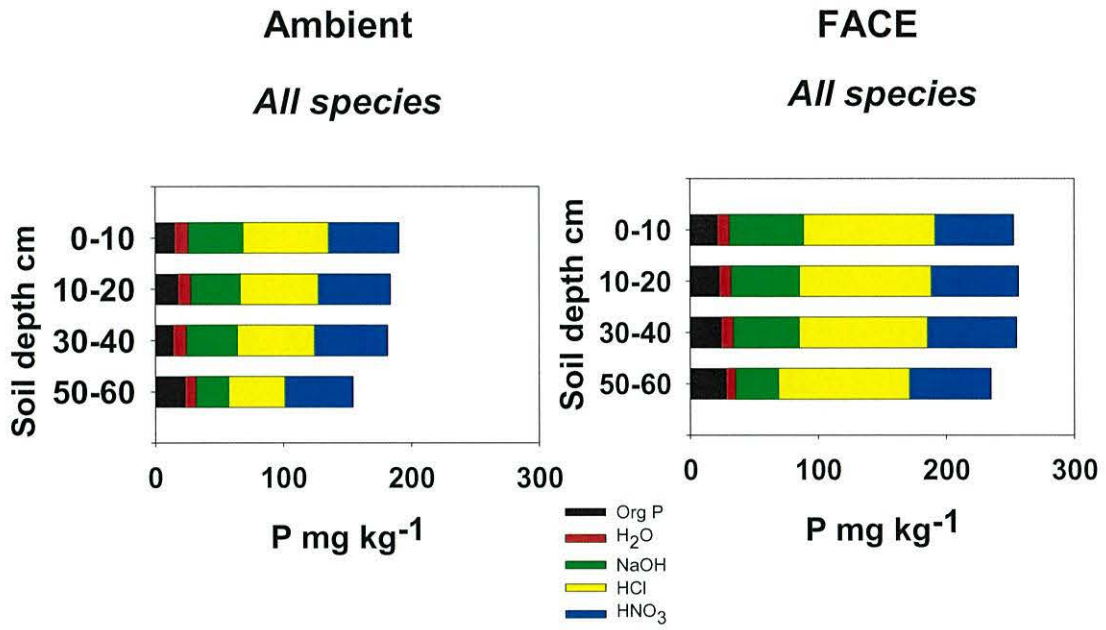


Figure 4.9: P fractions in whole field for all the species grown under elevated (FACE) and ambient CO_2 concentrations. The data presented are average values for all the species obtained from non-fertilized and fertilized samples from all plots.

4.3 Discussion

In soils phosphorus is present in organic and inorganic P pools of differing availability. The organic P pool is replenished through cycling of biomass. The relative size of inorganic pool is controlled by weathering. "The major factors that determine the chemical mobility, bioavailability, as well as the speciation of soil P are, the pH, the concentrations of anions that compete with P ions for ligand exchange reactions and the concentrations of metals (Ca, Fe and Al) that can coprecipitate with P ions. The chemical conditions of the rhizosphere are known to considerably differ from those of the bulk soil, as a consequence of a range of processes that are induced either directly by the activity of plant roots or by the activity of rhizosphere microflora." (Hinsinger, 2001).

The Hedley method used in this research has been modified. To determine loosely bound P Hedley originally used anion exchange resin (Dowex 1x 8-50, > 500 μm bead size) contained in a polyester mesh bag (Estel mono PE, 400μm), while we used deionised water for the same Pi fraction. This fraction, loosely bound P, estimates a P fraction from which plants normally draw their P supply (Amer *et al.*, 1955; Bowman *et al.*, 1978). Of the original Hedley method, we also skipped the second fraction which is extracted by $NaHCO_3$ (0.5 M, pH 8.5), which extracts additional Pi that is available to plants and also extracts the more labile forms of soil P_o (Bowman and Cole, 1978). We extracted the second fraction with 0.1 M NaOH solution that extracts some of the labile P_o (Anderson, 1964); it also partially dissolves iron and aluminium phosphates (Chang and Jackson, 1958) and also desorbs Pi from the surfaces of sesquioxides (McLaughlin *et al.*, 1977; Parfitt, 1978). Subsequent to alkaline extraction, 1M HCl was used to extract the next P fraction, which dissolves acid soluble Pi, probably in the form of calcium phosphate and some Pi which is occluded within sesquioxides and released on the partial dissolution of these oxides. The remainder of the occluded P appear in the residual P fraction with the most stable organic phosphates (Hedley *et al.*, 1982). We used concentrated HNO_3 acid for the digestion of this final extract.

Previous studies have produced conflicting results on the effect of elevated CO_2 on phosphorus. Elevated CO_2 has been found to cause increases (e.g., Norby *et al.*, 1986), decreases (e.g., Johnson *et al.*, 1995, 2000*a*), or no effect (e.g., Johnson *et al.* 1995, 2000*a*) in soil extractable P and exchangeable Ca^{2+}, K^{+}, and Mg^{2+} in various greenhouse studies and open top chamber studies. Norby *et al.* (1986) found an increase in soil extractable P with elevated CO_2 in a green house study with white oak (*Quercus alba* L.) and speculated that elevated CO_2 increased phophatase activity. On the other hand Johnson *et al.* (1995) found reduced soil-extractable P under elevated CO_2 in a greenhouse study of ponderosa pine growing in a poor soil, but no effects of elevated CO_2 were found, on either P uptake or soil-extractable P, when the plants were grown on a richer soil.

In this work the influence of elevated CO_2 on soil P fractions has been investigated at the EUROFACE experimental site. At this site elevated atmospheric CO_2 increases biomass inputs into the soil, mainly via root litter inputs (Gielen *et al.*, 2005). At the FACE ORNL the effects of CO_2 conditions on nutrient cycling in sweetgum plantation was also investigated. In Sweetgum no significant effects of

elevated CO_2 on ortho-P availability were shown (Johnson et *al.* 2004). In contrast we found a totally different picture which is shown in the Fig. 4.8 and 4.9. The data show a FACE effect on both organic and inorganic phosphorus pools in soils. The increase in total extractable P fractions under FACE and the significant differences are primarily due to an increase in the putative Ca bound P fraction. This increase was seen in the total organic, NaOH and HCl extractable fraction. However, it should be noted that NaOH also extracts for the organic P pool. However, there was no movement of P between the fractions determined, to account for the increase in the HCl fraction. The unplanted soil edges of the plantation were used to gain an indication of the effects of tree planting and the FACE treatment. The P concentration in all fractions was similar between the edge of the plantation and ambient plots, but a clear increase was shown in the total organic, NaOH and HCl fractions. The increase under FACE was particularly prominent in the deeper soil depths.

The FACE treatment stimulated growth of *Populus*, although not to the same extent in all three species studied (Calfapietra *et al.*, 2001). The increase in aboveground biomass under elevated CO_2 was positive in first three year of experiment, while in 2^{nd} rotation cycle, after coppicing, this response to FACE disappeared. An ontogenetic decline in growth in the FACE treatment, together with strong competition inside dense plantation, may have caused this decrease. Fertilization did not influence aboveground growth, although some FACE responses were more pronounced in fertilized trees (Scarascia-Mugnozza *et al.*, 2006). Root productivity and turnover rate constitute crucial component of ecosystem and carbon cycling (Aber *et al.*, 1985) and its response to elevated CO_2 might be of a great importance when considering the ecosystems potential for C sequestration. Root systems are often reported, in terms of size, as the biggest beneficiaries of elevated atmospheric CO_2 among plant parts. This is a fact that fine root growth and maintenance costs in trees may represent as much as 67-70% of total net primary production (Fogel, 1985). Exposure of all three *Populus* genotypes to elevated atmospheric CO_2 resulted in bigger trees with greater root system, which is in accordance with generally accepted hypothesis. Elevated CO_2 has the same effect on all three genotypes, bigger root systems are produced and maintained (Lukac *et al.*, 2003). At the EUROFACE site, elevated CO_2 both increased the rates of the root turnover and resulted in an increase in fine root biomass in the deeper soil layers (Lukac *et al.*, 2003). Death of fine roots and associated mycorrhizas will result in return of root P to the soil, and may be a factor in the change in P pools.

The results of organic P were also found high in FACE than the ambient plots, but the amount of organic matter was lower in FACE plots than the ambient plots in all the species. The FACE experiments in aggrading forests and plantations have demonstrated significant increases in net primary production (NPP) and C storage in forest vegetation (Norby *et al.*, 2001; Calfapietra *et al.*, 2003a; Norby *et al.*, 2005). The extra C sequestered is stored in forest vegetation, also in forest floor litter and in the soil (Hoosbeek *et al.*, 2006). The fate of this additional C allocated belowground remains unclear (Jastrow *et al.*, 2005; Litcher *et al.*, 2005). Enhanced carbon transfer to root system may result mainly in enhanced root respiration or, otherwise, in an increase of dry matter, mycorrhizal activity and subsequent transfer of carbon to soil P pools (Hoosbeek *et al.*, 2006). In the soil in 2003 both the organic matter contents as shown in this work, and the C contents (Hoosbeek *et al.*, 2003) were lower under FACE. This organic matter loss has been suggested to be due to priming effect. The increased turnover of soil organic matter might have also resulted in a redistribution of P between the P fractions.

These results can be compared to results from other sites. Cross and Schlesinger, (1995) reviewed the results of Hedley fractionation method used in different soil types for determining P. The results reported for Inceptisols were similar to our findings. The phosphorus status of soils measured under sweetgum (*Liquidambar styraciflau* L.) plantation at Oak Ridge National Environmental Research Park in Roane County, Tennessee, USA were found very low, i.e. 3.0±1.0 to 9.63±0.63 $\mu g\ g^{-1}$ in FACE and 4.72±0.23 to 11.41± 1.81 $\mu g\ g^{-1}$ in ambient plots, before the start of elevated CO_2 treatment (Johnson *et al.*, 2003). It was measured in the soil profile from 0-90 cm depth and it was decreasing down the profile. Another study performed in northern Chihuahuan desert, New Mexico, USA, showed much higher P content measured in HCl extracted fractions in both grassland and shrubland soils. The largest fraction in both vegetation types was $CaCO_3$ bonded HCl extractable P, which comprised 41.4% of the total phosphorus pool in the grassland and approximately half of the total phosphorus pool in the shrubland (58.1%). The sum of organic fractions made up only a small portion of the total phosphorus pool, comprising 13.3% in the grassland and 12% in the shrubland (Cross and Schlesinger, 2001). These results were similar to our findings with largest HCl fraction and low organic fraction. Cassagne *et al.* (2000), reported the total phosphorus content in the Inceptisols of Eastern Pyrenees (France), ranged from 700 to 1100 $\mu g\ g^{-1}$. This was about 3 times higher than our findings and

might have been due to different vegetation type as most of the Pyrenees are covered by mountain pine forest.

The direct determination of phosphate minerals in soils is very difficult. P is present as a minor constituent in soils, usually ranging from 0.02-0.5%, with an average of 0.5% (Lindsay and Vlek, 1977). The total P determined in this study as a sum of all the fractions are lower than those P generally found in soils in a total P analyses. This could be due to the fractionation method used, which did not extracted all the P from soil, specially the final digestion was done by using HNO_3 acid rather than mixture of $HClO_3$ and concentrated H_2SO_4 acid used in Hedley fractionation scheme. To determine this, the total P was also measured by an independent organisation (Institüt fur Düngenmittel und Saatgut, LUFA, Germany), using a pressure microwave digestion method. In essence, 1 g of soil sample was digested with a mixture of 7 ml HNO_3 and 2 ml HF. This yielded an average total soil P value of 366 g P m^{-2}, comparable with our measurement of 152 g P m^{-2} using the sequential extraction method.

4.4 Conclusions

The results clearly show that there is more organic and inorganic P under elevated atmospheric CO_2 conditions. The elevated CO_2 is making an impact on P quantity in the soil profile. The elevated CO_2 is clearly making changes in the soil phosphorus pools positively.

Chapter 5

5 Phosphorus in plant materials

5.1 Introduction

There is a growing concern among the scientific community that environmental conditions are being modified globally and the atmospheric CO_2 concentration is expected to reach 700 ppm by the end of 21st century (IPCC, 2001). The worldwide increase in atmospheric concentration of CO_2 constitutes part of evidence of this global change. This change might have profound effects on terrestrial ecosystem and it is an imposing task to unravel the complexity of ecosystems responses. One of the reason for difficulties encountered might be the fact that the key components of the ecosystem response may reside out of sight, the below ground systems of roots, soil and associated organisms (Norby and Jackson, 2000). Little information is available on the interactions between CO_2 enrichment and phosphorus availability and nutrition. This chapter will give us some knowledge about the effect of elevated CO_2 on phosphorus in plant materials. We will discuss about phosphorus content found in leaves, roots and wood samples taken at the EUROFACE experimental site.

Many studies have shown that elevated CO_2 can lead to increased production of biomass per unit uptake and therefore cause reduced tissue nutrient concentrations (Zak *et al.*, 1993; Mcguire *et al.*, 1995; Benston and Bazzaz 1996; Johnson *et al.*, 1997, 2003; Curtis *et al.*, 2000; Medlyn *et al.*, 2000; Finzi *et al.*, 2002). These findings have led to speculation that nutrient concentrations in litterfall and root detritus could be reduced, causing reduced decomposition and N mineralization (Strain, 1985).

Zak *et al.*, 1993, published the results of their experiment conducted at University of Michigan Biological Station (UMBS) on the effect of elevated atmospheric CO_2 on plant productions, soil micro-organisms, and the cycling of C and N in plant-soil system. The experiment was carried out in OTC's by planting *Populus grandidentata* Michx., in a poor nutrient soil for one growing season at ambient and twice ambient atmospheric CO_2 concentrations. They reported significant increase in photosynthesis, total and belowground biomass, labile C and microbial biomass C in the rhizosphere, N availability at the bulk soil, under elevated atmospheric CO_2.

Mcguire *et al.*, 1995 reviewed experimental studies to evaluate how the N cycle influence the response of forest net primary production (NPP) to elevated atmospheric

CO_2. Their survey also concluded that at the tissue level, elevated atmospheric CO_2 reduces N concentration on an average 21%, but it has a smaller effect on N concentrations in stems and fine roots. In contrast, higher soil N availability generally increases leaf N concentration.

Bazzaz and Benston (1996) also reviewed the belowground negative and positive feedbacks on CO_2 growth enhancement. They concluded that in the majority of cases, elevated CO_2 leads to significant increases in biomass, though the magnitude and duration of these increases is highly variable between species and environmental conditions (e.g. Ceulemans and Mousseau, 1994; Kimball and Idso, 1983; Poorter, 1993; Wullschleger *et al.*, 1995). In a study of the growth responses of six co-occurring temperate deciduous tree species after three full years of exposure to ambient and elevated CO_2 atmospheres, Bazzaz and co-workers demonstrated that variability in tree seedling/sapling growth responses to elevated CO_2 is a function of species as well as environment (Bazzaz and Miao, 1993; Bazzaz *et al.*, 1993). Thus, even within a single ecosystem there is enough variation in potential growth responses to rising atmospheric CO_2 levels that in order to discuss system-level potential responses we have to know a lot about the nature of variation within-system in species composition and environmental variation. Johnson *et al.*, (1997) reported the effects of elevated CO_2 and N fertilization on vegetation and soil nutrient content in juvenile ponderosa pine (*Pinus ponderosa* Laws.). They presented the data on nutrient uptake and soil responses in open top chamber site at the Institute of Forest Genetics in Placerville, California. Their results of three year study concluded that there is a positive effect of elevated CO_2 on plant growth and nutrient uptake. They found short term reduction in foliar P, S, B and Mg as well as N concentrations with elevated CO_2, and this was explained by simple growth dilution. Elevated CO_2 caused reductions in soil available N and P and increases in exchangeable Al^{3+}, but had no consistent or significant effects upon total C, total N, exchangeable Ca^{2+}, K^{+}, or Mg^{2+}. The reductions in available P exceed which could be accounted for by plant uptake and may have been due to increased P adsorption, increased microbial immobilization, or both. In a subsequent study Johnson *et al.*, (2000) also published their findings of field and laboratory studies on the effects of CO_2 and N fertilization on decomposition and N immobilization in ponderosa pine litter (*Pinus ponderosa* Dougl.). This experiment was performed in open top chambers. Needle mass decreased by 8 to 15% over the 26-month litter bag experiment, but there were no treatment effects of either CO_2 or N. Nitrogen concentrations were lower in the

initial samples with elevated CO_2 and low N treatment levels, but this was not statistically significant and disappeared in the 14 and 26 month collections. No statistically significant differences in N concentration or content due to treatment were found at any time. There were no statistically significant treatment effects on total ^{15}N immobilization or microbial ^{15}N immobilization in the laboratory studies on senesced needles.

Another experiment was conducted at the University of Michigan Biological Station, Pellston, Michigan, USA on gas exchange, and leaf nitrogen and growth efficiency of *Populus temuloides* in CO_2 enriched atmosphere. Curtis *et al.*, (2000) grew 6 genotypes of *Populus temuloides* at ambient (35 Pa) or elevated (70 Pa) CO_2 in soil with high and low N mineralization rate. They concluded that elevated atmospheric CO_2 had a sustained, positive effect on carbon gain capacity and above ground biomass accumulation in *P. tremuloides,* but only under conditions of high soil N availability. Carbon dioxide enrichment increased photosynthetic N-use efficiency regardless of soil N availability but in low N soil growth at elevated CO_2 resulted in lower leaf N concentration. This led to a reduction in photosynthetic capacity relative to plants at ambient CO_2. Gains in net CO_2 assimilation rate under elevated CO_2 were largely offset by increased dark respiration and fine root turnover, resulting in no net increase in growth efficiency at high CO_2. Medlyn *et al.*, (2000) also predicted the responses of forest growth to elevated temperature (T) and atmospheric CO_2 concentration. In their study, the plant-soil model G'DAY was used to simulate forest growth responses to T and CO_2 on different time scales for forests in cool and warm climates. They found that doubled CO_2 caused a large initial increase (~20%) in net primary productivity, but this did not persist in the long term. By contrast, a 2°C increase in T caused a persistent long-term increase in NPP of approximately 10-15%. In particular, the predicted long-term increase in NPP under elevated T reflected an increase in predicted N mineralization and plant N uptake, assuming that a constant fraction of mineralized N is taken up by plants.

Finzi *et al.*, (2002) also published their results of study performed at FACE experimental site in Duke Forest, Orange county, USA. They studied the N budget of pine forest under free air CO_2 enrichment. They also reported that elevated atmospheric CO_2 increases plant biomass, NPP and plant demand for N. The biomass of loblolly pine needles was significantly greater under elevated CO_2 in all years. The biomass of loblolly pine wood and coarse roots increased throughout the 4 years of this study and

was significantly higher under elevated CO_2 in years 3 and 4. The biomass of the remaining components, deciduous leaves, deciduous wood plus coarse roots, and fine roots was not significantly different between ambient and elevated CO_2 in any year of this study. At the end of the 3rd growing season under elevated CO_2, the total biomass of vegetation in the plots under elevated CO_2 was significantly higher than that under ambient CO_2. In other years, total biomass was higher in the plots under elevated CO_2, but only marginally statistically significant ($P<0.10$). The quantity (g/m^2) of N in loblolly pine needles was significantly greater in the plots under elevated CO_2 in the 2nd and 3rd years but not in the 1st and 4th years of this study. The quantity of N in deciduous leaves, deciduous wood and coarse roots, and fine roots was not significantly different between ambient and elevated CO_2. Only the increment of N in fine root biomass was significantly higher under elevated in all other components increased under elevated CO_2 but the increases were not statistically significant.

In contrast to nitrogen, leaf phosphorus is thought to increase with increasing CO_2 since more Pi is required because of altered balance between photosynthetic carbon reduction cycle and photosynthetic carbon oxidation (photo respiratory) cycle (Sharkey, 1985; Conroy, 1992; Morin *et al.*, 1992). Consequently, critical phosphorus concentrations are often found to increase under CO_2 enrichment (Conroy, 1992: Rogers *et al.*, 1993), and leaf concentrations showed no or at least less, reduction compared with nitrogen under CO_2 enrichment (Overdieck, 1993). Conroy, (1992) worked on the influence of elevated atmospheric CO_2 concentrations on plant nutrition. He suggested that the rising levels of atmospheric CO_2 are likely to increase biomass production of C_3 species in both natural and managed ecosystems because photosynthetic rates will be higher. The greatest absolute increase in productivity will occur when nitrogen and phosphorus availability in the soil is high. Low nitrogen does not preclude a growth response to high CO_2, whereas some C_3 species fail to respond to high CO_2 when phosphorus is low, possibly because insufficient phosphorus is available to maintain maximum photosynthetic activity at high CO_2. Morin *et al.*, (1992) conducted an experiment on growth kinetics, carbohydrate and leaf phosphate content of clover (*Trifolium subterranean* L.) after transfer to a high CO_2 atmosphere or to high light ambient air. Clover seeds were grown in sand and germinated in greenhouse. The seedlings were replanted in pots with perlite as the substrate and were then transferred to environment chambers. The concentration of Pi (soluble P), Pe (esterified acid-soluble P) and total P and Pi/Pe ratios (soluble pools) were determined during 1st day

and prolonged exposure of the plants to the high CO_2 atmosphere or to ambient air and high light. The Pe based on fresh weight decreased by 27% between day 1 and 13. Total soluble Pi/fresh weight decreased by 50% and total soluble P (Pi +Pe) decreased by 40% between day 1 and day 13 in high CO_2.

Rogers *et al.*, (1993) measured the influence of increasing atmospheric CO_2 on shoot growth, leaf nitrogen and phosphorus concentrations and carbohydrate composition in cotton (*Gossypium hirsutum* L.) and wheat (*Triticum aestivum* L.). The critical concentration of N in the leaves of cotton and wheat was reduced at high CO_2. Shoot dry weight of both species was generally higher at elevated CO_2, especially at high rates of available soil N and P. For both species, the critical N concentration is lower at elevated CO_2 whereas the critical P level is higher, indicating that leaf standards will need reassessing as the atmospheric CO_2 concentration rises.

Overdieck, (1993) conducted an experiment to study the CO_2 enrichment effects (300–650 μmol mol^{-1}) on mineral concentration (N, P, K, Ca, Mg, Mn, Fe, Zn), absolute total mineral contents per individual and of whole stands of four herbaceous (*Trifolium repens* L., *Trifolium pratense* L., *Lolium perenne* L., *Festuca pratensis* HUDS.) and two woody species (*Acer pseudoplatanus* L., *Fagus sylvatica* L.). The plants were grown in three different greenhouses for long term exposure to CO_2 at Osnabruck. In general, the mineral concentration of the plant tissues decreased (all six species: N>Ca>K>Mg) with the exception of P. On the average, a slight decrease of P was detected for the two clover species in the studies, whereas the two grasses maintained their P concentrations at approximately the same level. As far as this element is concerned, however, all four herbaceous species belong to the group in which the P-uptake was proportionally enhanced parallel with the carbon uptake and dry weight increase. The tree species of their studies behaved differently. The results concluded the P concentrations of plant tissues are less influenced by additional CO_2 than most other minerals.

5.2 Results

In the following section the results of phosphorus measured in the plant materials are presented. The results are presented to show the effect of elevated CO_2 on different plant materials under ambient and FACE. This section covers the amount of phosphorus analysed in leaves, root and wood samples, at the EUROFACE experimental site.

5.2.1 Phosphorus in leaves

The data presented in figure 5.1 show the amount of P in leaves for each species and each plot. In *P. alba* and *P. x euramericana* (5.1 A & C) the P content in leaves in FACE plot 1 was 16- 20% higher than the FACE plots 4 and 5. For *P. alba* this was found in both non-fertilized and fertilized plots, but only in the non-fertilized treatment for *P. x euramericana.*

The mean values for each treatment (Figure 5.2 A & B) in all species were not significantly different between the non-fertilized and fertilized treatments. When the non-fertilized and fertilized plot data were pooled, the mean values for *P. alba* were 1035 mg kg^{-1} for ambient and 1083 mg kg^{-1} for FACE treatment, and were not statistically different. This difference between ambient and FACE was similar in both *P. nigra* and *P. x euramericana* with a difference in mean P concentration in leaves between ambient and FACE of 28-60 mg kg^{-1}. There was no statistically significant FACE effect in both fertilizer treatments.

5.2.2 Phosphorus in roots

The data presented in figure 5.3 show the amount of P in roots for each species at two soil depths and each plot. The results for *P. alba* (Figure 5.2A) showed that in the non-fertilized treatment at 20-40 cm depth the values of phosphorus were 43-50% higher in ambient plot 3. While, at the same depth of the same species were 28-47% higher in plots 3 and 6 of fertilized plots. There is more phosphorous found on average in FACE plots than ambient plots with no fertilizer treatment (Figure 5.4 A & B) at both depths. The statistical analyses revealed the difference was not significant among the mean values at different levels of CO_2 (P=0.623). While the fertilized samples (Figure 5.4 C& D) showed a different picture with a higher P content in ambient plots, but again the difference was not statistically significant (P=0.772). The *P. nigra* (Figure 5.3 B) showed 53% higher value at 20-40 cm depth of plot 6 in no fertilizer treatment. The average values in non-fertilized ambient plots (Figure 5.4 A & B) were found to be higher than FACE plots at both the soil depths but analysis of variance showed no significant differences between ambient and FACE values (P=0.278).

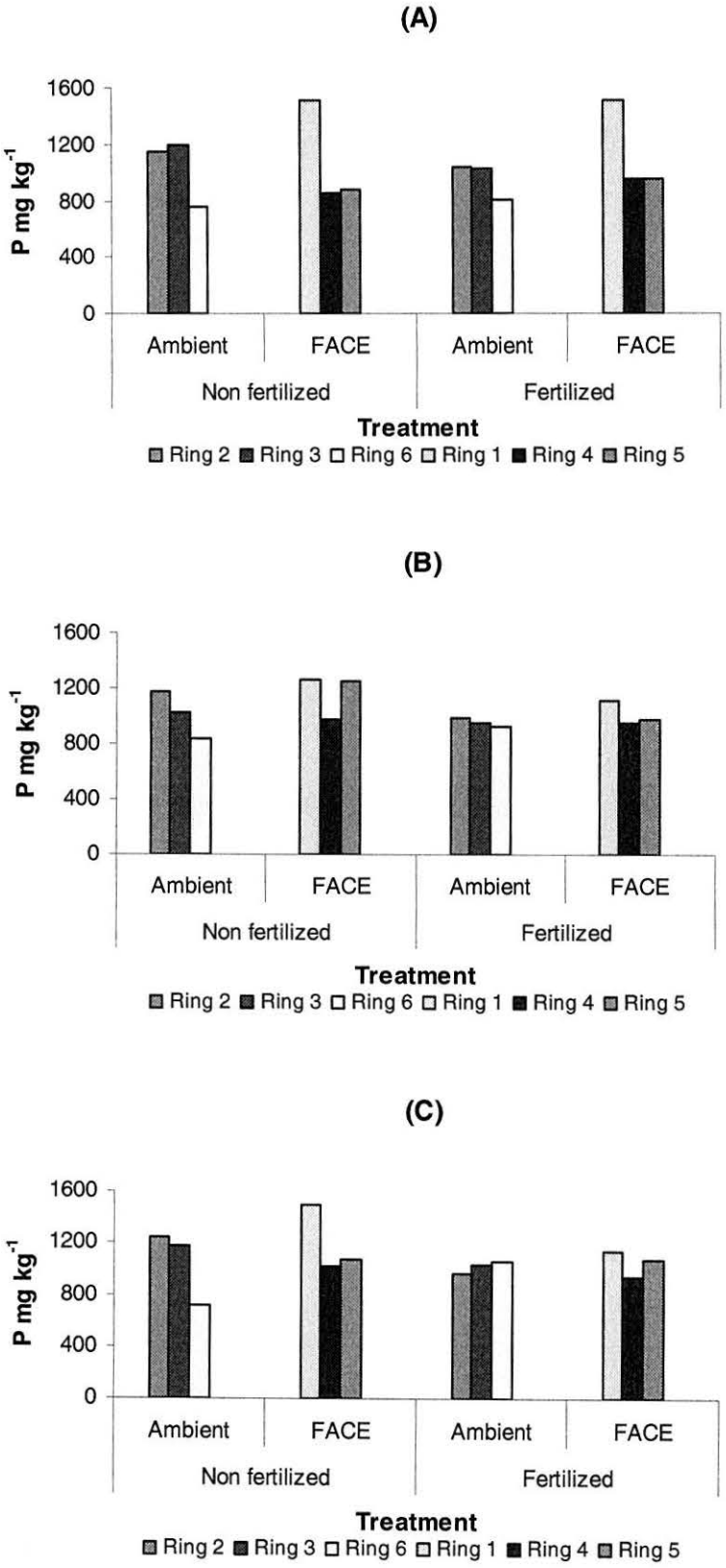


Figure 5.1: The concentration of P in leaves for 3 *Populus* species (A= *P. alba*, B= *P. nigra*, C= *P. x euramericana*) grown under control and elevated atmospheric CO_2 with and without fertilizer. Data shown is the value for each plot.

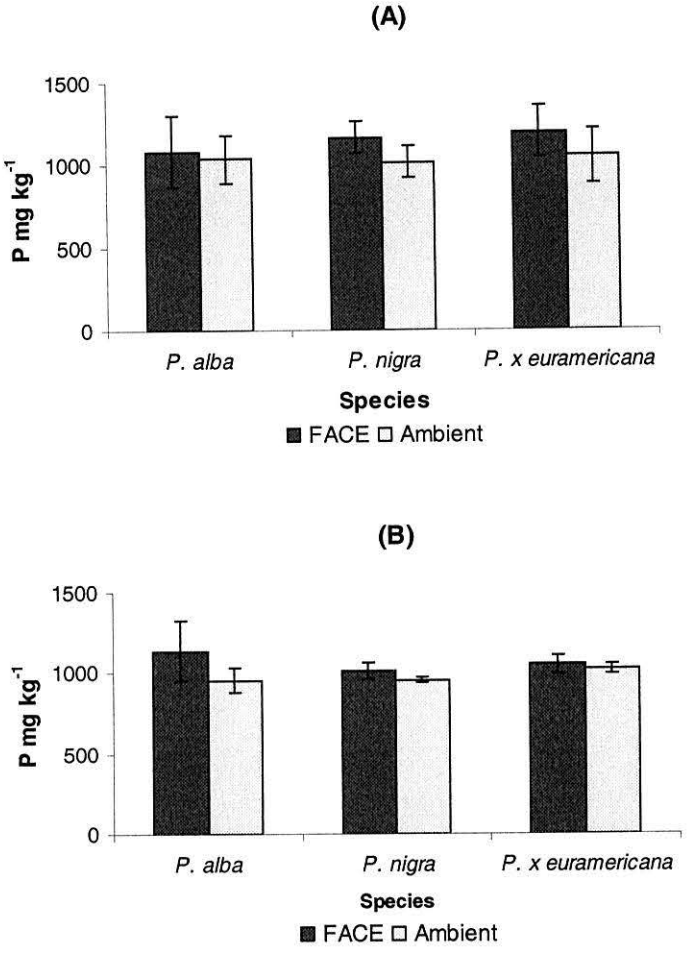


Figure 5.2: The concentration of P in leaves for 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer (A= Non-fertilized, B= Fertilized). Data shown are means ± SE.

The *P. x euramericana* (Figure 5.3 C) results revealed 15-55 % higher phosphorus in fertilized root samples at both depths in plot 6. The average values for phosphorus (Figure 5.4 A & B) in *P. x euramericana* was found higher in FACE plots at 0-20 and 20-40 cm depths for non-fertilized root samples. While in fertilized treatment (Figure 5.4 C & D), we found a different trend, as the average values at both sampling depths were found higher in ambient plots.

The difference in the mean values among the different levels of CO_2 evaluated within *P. x euramericana* was not significant (P=0.100), while the difference between the different levels of fertilizer treatment was found to be statistically significant (P=0.016) only at 0-20 cm depth. The statistical analysis of the overall mean values

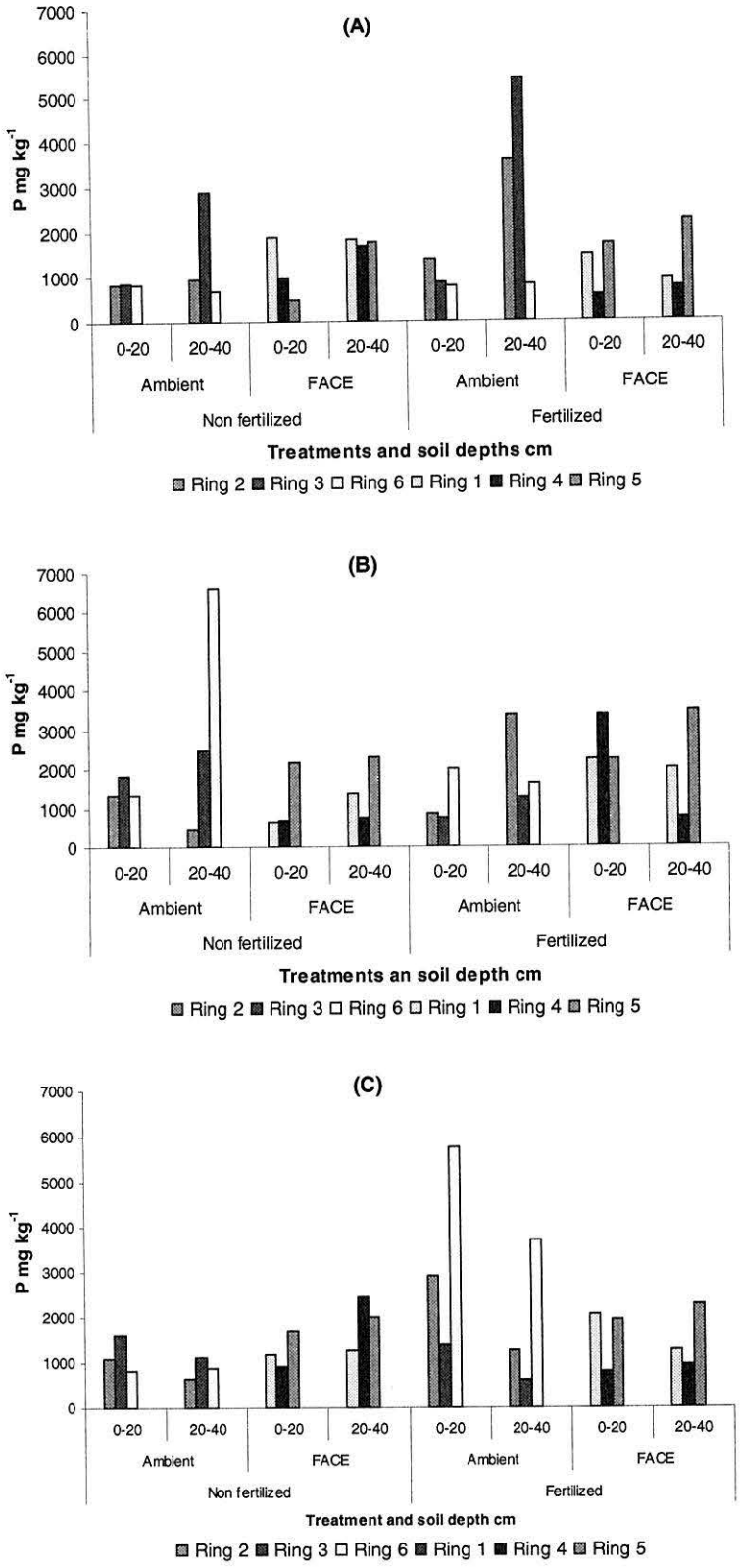
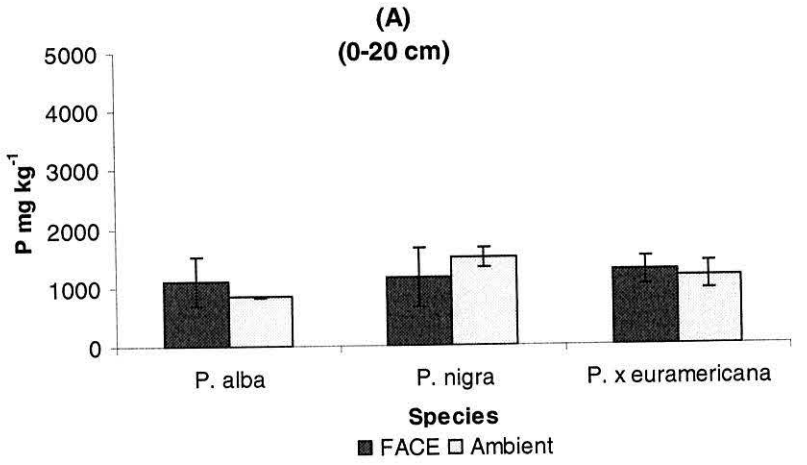
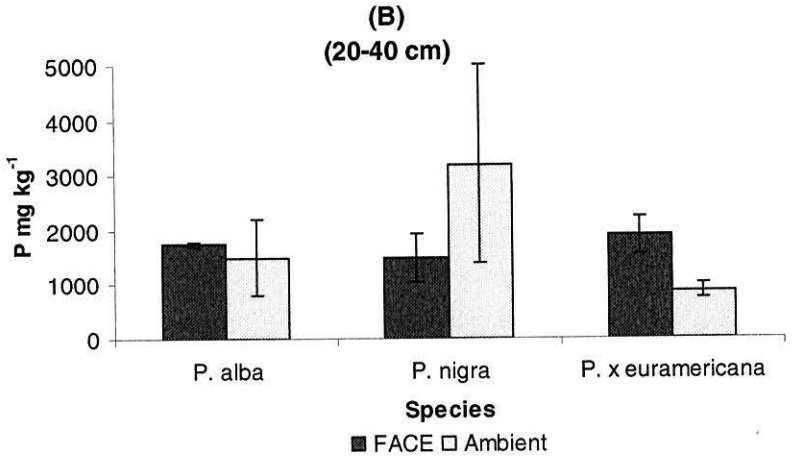
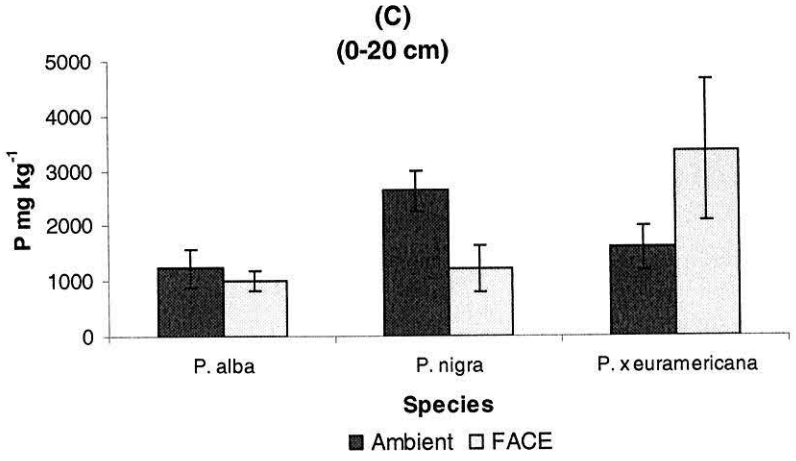


Figure 5.3: The concentration of P in roots for 3 *Populus* species (A= *P. alba*, B= *P. nigra*, C= *P. x euramericana*) grown under control and elevated atmospheric CO_2 with and without fertilizer. Data shown is the value for each plot at two depths.


(A)
(0-20 cm)
5000
4000
3000
2000
1000
0
P mg kg-1
P. alba
P. nigra
P. x euramericana
Species
FACE
Ambient


(B)
(20-40 cm)
5000
4000
3000
2000
1000
0
P mg kg-1
P. alba
P. nigra
P. x euramericana
Species
FACE
Ambient


(C)
(0-20 cm)
5000
4000
3000
2000
1000
0
P mg kg-1
P. alba
P. nigra
P. x euramericana
Species
Ambient
FACE

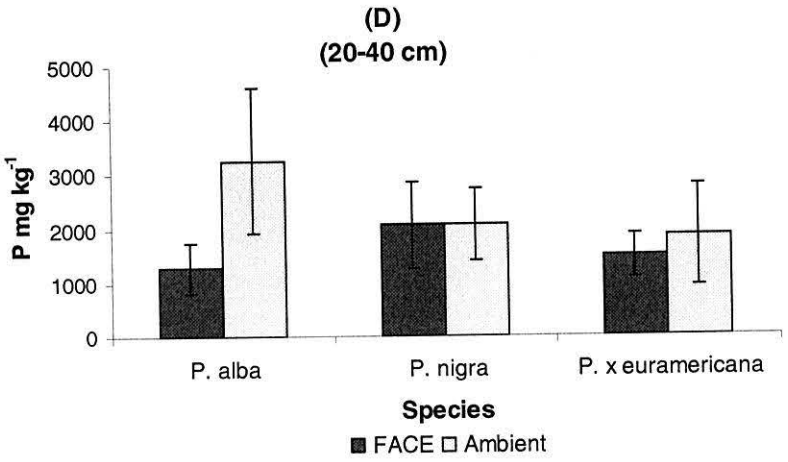


Figure 5.4: The concentration of P in roots at different depths for 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer (A & B= Non-fertilized, C & D= Fertilized). Data shown are means ± SE.

among the different CO_2 levels were not found to be significant (P=0.633). The difference among different fertilizer treatments and different species were also statistically not significant (P=0.269 & and P=0.742).

5.2.3 Phosphorus in Wood

Phosphorus in wood samples was also determined by ICP after HNO_3 acid digestion in Teflon vessels. Two species were analysed in Germany i.e. *P. alba* and *P. x euramericana*, *P. nigra* was sampled in June, 2005 and analysed for phosphorus. The following section gives the results for all species.

The results for phosphorus in wood samples are presented in figure 5.5 (A-C) for three different species. In *P. alba* (Figure 5.5 A) showed very much similar values in both fertilizer treatments for different CO_2 levels. The average values in fertilized and non-fertilized plots for the same species showed (Figure 5.6 A & B) higher P content in the ambient plots but difference was not statistically significant. However, *P. alba* was found to be statistically different from other species. The P content was lower in wood of *P. alba* than the two other species.

In *P. nigra* (Figure 5.5 B) the results were found to have highest P content than other species analysed. In non-fertilized plot 1 18-27% higher P content, while fertilized plot 5 was found to have 6-10 % more phosphorous. The average values (Figure 5.6 A) in non-fertilized plots the difference is very little but slightly higher P content is found in ambient samples, while the FACE plots (Figure 5.6 B) showed higher P

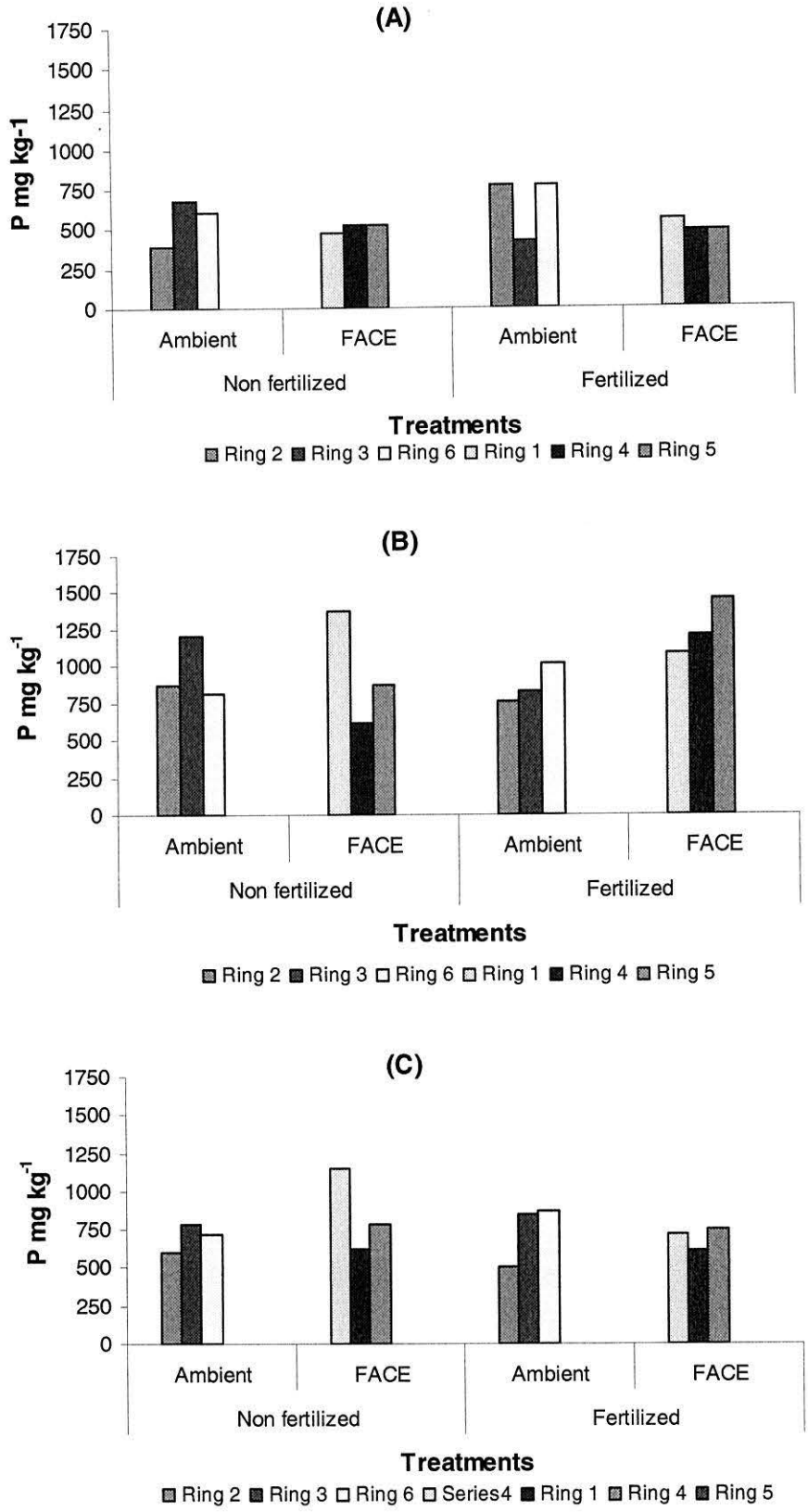


Figure 5.5: The concentration of P in wood for 3 *Populus* species (A= *P. alba*, B= *P. nigra*, C= *P. x euramericana*) grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the value for each plot.

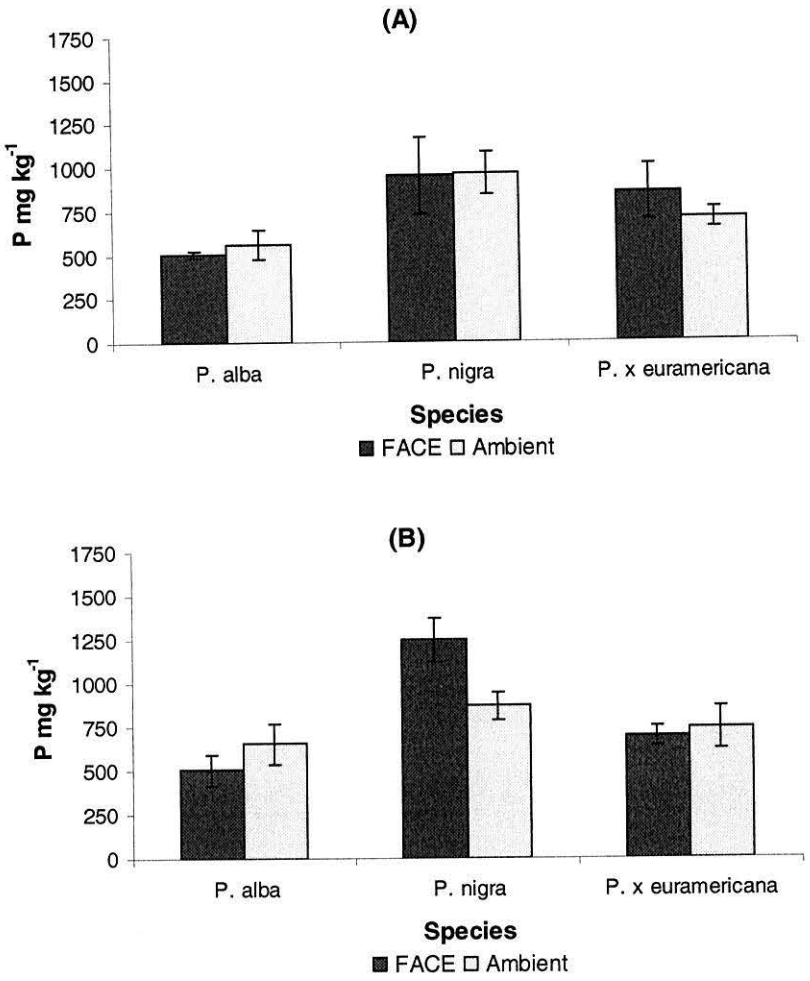


Figure 5.6: The concentration of P in wood for 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer (A= Non-fertilized, B= Fertilized). Data shown are means ± SE.

concentration for fertilizer treated plots. The analysis of variance showed no significant differences between ambient and FACE.

The *P. x euramericana* (Figure 5.5 C) results showed the 14-21 % higher phosphorus in non-fertilized wood samples in plot 1. The average values of the same species showed (Figure 5.6A & B) different trends in non-fertilized and fertilized plots. There are no significant differences among different levels of CO_2 treatment. However, the species were found to be significantly different from each other ($P<0.001$). Especially under FACE all species are found significantly different from other. While under control conditions only *P. alba* was found significantly different from *P. nigra* ($P=0.010$).

5.2.4 Mean phosphorus content in plant materials

In plant materials, the response to elevated CO_2 was not consistent as the trend was different for different species and treatments. In *P. alba* the leaf samples showed 1083 mg kg^{-1}(±213 se) in FACE plots and 1035 mg kg^{-1} (±140 se) in ambient plots of non-fertilized plots. In the *P. nigra* the values obtained were 1161 mg kg^{-1}(±95 se) in FACE and 1013 mg kg^{-1}(±96 se), again showing higher P content under FACE. In *P. x euramericana* the values were 1193 mg kg^{-1}(±153 se) and 1047 mg kg^{-1}(±164 se) respectively. The trend was more phosphorus under FACE. In fertilized samples the trend was very much similar but less difference between concentrations of FACE and ambient.

In root samples of non-fertilized samples at 0-20 cm depth the overall results for *P. alba* showed a similar picture as the results of leaves. The average values obtained in the field were 1099 mg kg^{-1}(±406 se) in all FACE plots and 845 mg kg^{-1} (±10 se) in ambient plots. In *P. nigra* the results for roots are different from the results of leaves. P content was found much higher in ambient plots. The mean values found in FACE and ambient plots were 1164 mg kg^{-1} (±507 se) and 1494 mg kg^{-1} (±166 se) respectively for the whole field. In *P. x euramericana* the values were again higher in FACE plots as the average values were 1261mg kg^{-1}(±231 se) in FACE and 1169 mg kg^{-1}(±232 se) in ambient plots of the same treatment. At 20-40 cm depth results show more or less similar trend as the 0-20 cm depth samples. The *P. nigra* again showed different picture than the remaining two species.

In fertilized plots, the response was totally different for all the species. At 0-20 cm depth the average values measured in *P. alba* for FACE plots was 1229 mg kg^{-1} (±339 se) and it was 1000 mg kg^{-1}(±180 se) in ambient plots. General trend found in this species showed more phosphorus under FACE. The *P. nigra* also showed the same trend with average values of 2619 mg kg^{-1}(±377 se) in FACE and 1211 mg kg^{-1}(±404 se) in all ambient plots. The P content was again higher in FACE plots. In the *P. x euramericana* the trend was totally different as at 0-20 cm depth FACE plots showed 1595 mg kg^{-1}(±398 se) P, while ambient plots were having 3348 mg kg^{-1}(±1289 se) P in all the samples analysed. At 20-40 cm depth in *P. alba* showed higher P content 3260 mg kg^{-1}(±1346 se) under ambient plots than FACE plots. *P. x euramericana* also showed similar results with higher P content in ambient plots. The *P. nigra* showed similar values for ambient and FACE.

In wood samples analysed for phosphorus we also found different trends in species. In *P. alba* the average value obtained in non-fertilized FACE plots was 506 mg kg^{-1}(±17 se), while this value was 562 mg kg^{-1}(±86 se) in ambient plots. P content was higher in ambient plots but the difference was not significant. In *P. nigra* we found the average values of phosphorus 950 mg kg^{-1} (±223 se) in FACE and 964 mg kg^{-1}(±121 se) in ambient plots of the same treatment. The trend was much similar to *P. alba* here, a higher P content in ambient rings and again the difference was not significant. The last species *P. x euramericana* showed a different trend with higher P content under FACE. The average values were 854 mg kg^{-1}(±157 se) in FACE and 701 mg kg^{-1}(±54 se) in ambient plots. The fertilizer treated wood samples of the experimental site showed different trend in *P. nigra* species in which FACE plots were having more phosphorus than ambient plots. The *P. alba* and *P. x euramericana* showed more P content in ambient plots. The samples of *P. nigra* also showed relatively higher phosphorus than other species used in the experiment. Generally there is a trend to slightly higher P content in leaves under FACE, and low P content in roots under FACE, and no effect on the P concentration in wood. However, the variation between the plots is high, and none of the differences shown were statistically significant.

5.3 Discussion

Studies on the effects of elevated CO_2 on nutrients other than N are fewer in number and have produced conflicting results (Johnson *et al.*, 2003). The results of this study generally are in agreement with the past research. The trend in leave samples showed higher P content under FACE but the differences were not found statistically significant in any treatment or species. As suggested by Overdick, (1993), elevated CO_2 has little or no effect on leaf P content. Rogers *et al.*, (1993) found changed (low N and high P) critical concentrations in leaf samples of wheat and cotton under elevated atmospheric CO_2. Johnson *et al.*, (2003) studied the effect of elevated CO_2 on nutrient distribution in a fire adapted Scrub Oak forest, in Merritt Island. They reported elevated CO_2 caused increased vegetation P, K, and Mg contents and increased Ca content in O horizon. In another study Johnson *et al.*, (2004) also reported results of nutrient concentration for foliage and litter fall in sweetgum plantation at Oak ridge FACE experiment. They found 1.3±0.01 mg g^{-1} of P content in ambient and 1.2±0.0 in elevated foliage samples, while it was measured 0.81±0.05 and 0.75±0.04 mg g^{-1} respectively in litterfall samples. These values showed there was no effect of elevated

CO_2 on foliage and litterfall.

The root samples in non-fertilized plots showed a different picture for *P. nigra* species, which showed reduced P content under elevated CO_2 at both sampling depths but the two other species showed the reverse trend. This might be due to species response to elevated CO_2. In fertilized plots different trends were found in different species and depths. The difference was mainly due to some high values in plot 6 under *P. x euramericana* species. These differences may be due to fertilizer effect on species. In sweetgum root P concentration was measured at ORNL 0.81±0.05 and 0.75±0.04 mg g^{-1} under ambient and elevated CO_2 respectively (Johnson *et al.*, 2004). The P content of poplar was generally higher in roots than leaves and wood samples. The elevated CO_2 was found to increase P leaching from O horizon measured by resin lysimeter in sweetgum plantation (Johnson *et al.*, 2004). The higher contents in root shown in *Populus* may be due to the contribution of mycorrhizas in the roots. Roots of all 3 species were shown to be colonized by both ectomycorrhizas and VAM (Luhae, *et al.*, 2003). Mycorrhizas are known to contain high concentration of P in their tissue (Cox *et al.*, 1980). Norby *et al.*, (1991) suggest that P uptake may increase due to higher rates of organic acid release into rhizosphere under elevated CO_2. However, in the *Populus* roots P was lower under FACE in *P. nigra* and *P. x euramericana*, and unaffected in *P. alba*. This suggests if anything responsible for dilution of P contents is due to increased root growth.

In wood samples again there was no significant FACE effect in most of the species under different treatments and differences are only in species, which confirms that there is little or no effect of elevated CO_2 on wood samples. Which is in confirmation with the past result that elevated CO_2 had no significant effect on tissue P concentration (Johnson *et al.*, 2004), and its effect is highly variable in species and environmental conditions (Benston and Bazzaz, 1996).

5.4 Conclusions

The above results clearly show that there is no consistent trend in the different plant material samples. The leaves showed the only consistent trend that there is relatively higher P content under FACE but this difference is not found statistically significant. In roots non- fertilized samples showed the same trend in two species but the trend was different in *P. nigra* showing high phosphorus in ambient plots. While root samples showed a mix trend in fertilized plots. The wood samples showed a

different trend as phosphorus was mostly found higher in ambient plots. The results were again not statistically significant. We can conclude from the results presented in this study that there is little or no effect of elevated CO_2 on the different plant materials in EUROFACE experimental site.

Chapter 6

6 Element ratios in soils and plant materials

6.1 Introduction

Calcium is one of the major elements and is essential for plant growth. It is supplied to plants by atmospheric inputs, decomposition and weathering of soil minerals, organic materials, fertilizers, and by liming materials. It is delivered to trees via uptake through fine feeder roots in the organic-rich forest floor and underlying mineral soils of forested watershed (Bullen and Bailey, 2005). There is a strong preference for Ca^{++} on the cation exchange sites of most soils and it is the predominant cation in most soils with a pH of 6.0 or higher. Calcium, an essential part of plant cell wall structure, provides for normal transport and retention of other elements as well as strength in the plant (Street and Kidder, 1997). In soil minerals the average amount of calcium is 2400 ppm (Sposito, 1989). Amounts of calcium in plants fluctuate between 5 and 50 mg g^{-1} of the dry weight according to species, organ and stage of development (Poszwa *et al.*, 2000). Calcium tends to accumulate in older plant parts (Asta, 1992; Marschner, 1995). The sensitivity of trees to natural or anthropogenically induced environmental change in part reflects the sensitivity of their fine roots to change in both root tip vitality and the availability of nutrients such as Ca (Momoshima and Bondietti, 1990). Over the past century, increased atmospheric deposition of acid anions (Mayewski *et al.*, 1986) has negatively impacted the Ca status of sensitive forest ecosystem, due to both depletion of readily available Ca on the soil exchange sites (Shortle and Bondietti, 1992; Likens *et al.*, 1996, 1998; Markewitz *et al.*, 1998; Huntington, 2000) and mobilization of monomeric aluminium (Al_i) that is toxic to fine roots (Lawrence *et al.*, 1995). Either Ca deficiency or aluminium toxicity may lead to an increased importance of the upper forest floor relative to deeper Al_i-impacted organic and mineral soils as a Ca source for the fine roots (cf., Joslin and Wolfe, 1992). Ca and isotopic approaches can reveal transitions of Ca sources for tree roots would be useful for determining the historic timing of, systemic response to and potential recovery from soil acidification (Bullen and Bailey, 2005).

Strontium is divalent alkaline earth element and its ionic radius is similar to that of calcium. The ionic and hydrated radii of Ca, 0.099 nm and 0.6 nm, respectively, are

close to those of Sr, 0.113 nm and 0.5 nm, respectively (Faure, 1986). Sr substitutes Ca in minerals including plagioclase feldspar and apatite, in sulphates such as gypsum and anhydrates and carbonates (calcite, dolomite and aragonite) (Capo *et al.*, 1998). In forest ecosystem, the strontium and calcium absorbed by trees originate from two primary sources: atmospheric inputs and weathering of primary soil minerals (Poszwa *et al.*, 2000). The alkaline earth element barium (Ba) behaves similarly to Sr during nutrient uptake, due to similar ionic radius ($r_{Ba}/r_{Sr} = 1.13$) and charge (Bullen and Bailey, 2005). Bullen *et al.*, (2005) has suggested that ratios of neighbouring alkaline earth elements (e.g., Ca/Sr and Sr/Ba ratios) as well as Sr isotope ($^{87}Sr/^{86}Sr$) ratios of tree tissue may prove useful for identifying sources of alkaline earth elements, if there is systematic variability of these ratios in plant-available pools down the soil profile. Sr isotopes are increasingly being used in biogeochemical studies of forested ecosystems (e.g., Aberg *et al.*, 1990; Miller *et al.*, 1993; Bailey *et al.*, 1996; Blum *et al.*, 2002; Kennedy *et al.*, 2002). In favourable conditions, the $^{87}Sr/^{86}Sr$ isotopic ratio of atmospheric deposition and weathering differ enough to measure the contribution of each source to plant, soil exchangeable and stream water Sr (Wickman, 1996). $^{87}Sr/^{86}Sr$ of plant available Sr in the organic soils will differ from that in the underlying mineral soil if: (1) there is a significant difference in $^{87}Sr/^{86}Sr$ of atmospherically derived and mineral weathering-derived Sr, and (2) biological cycling of Sr does not homogenize the Sr isotope signal (Bullen and Bailey., 2005). Assuming that Ca and Sr behave similarly, the results obtained for Sr have been applied to Ca (Aberg, 1995). The $^{87}Sr/^{86}Sr$ variation with soil depth of root available Sr has been used to study the contribution of specific soil horizons to Ca uptake (Dambrine *et al.*, 1997; Wickman and Jacks, 1992). Using the same assumption, relative contributions of the Ca deposited in rain and released by weathering to forest pools have been calculated (Jacks *et al.*, 1989). There are also many reports suggesting that Ca and Sr may behave differently in the soil plant system. Uptake of Ca and Sr in plants varies according to species, organs, sites and soil characteristics (Garten *et al.*, 1977; Watmough and Dillon, 2003). Sr/Ca exchange isotherms on organic substrates indicate almost no preferential adsorption on carboxylate groups at neutral pH, while a preference for Ca over Sr was obtained at low pH (Baes and Bloom, 1988). Sr/Ca ratios were found similar or slightly higher in the exchangeable phase than in soil solution extracted from a set of soils by centrifugation (Veresoglou *et al.*, 1996). Many scientists have calculated a discrimination ratio (DR) between the Sr/Ca ratio in plants and soils [(DR= (Sr/Ca) in plant / exchangeable

(Sr/Ca) in soil (Memon *et al.*, 1983)] or soil solution [DR= (Sr/Ca) in plant / (Sr/Ca) in soil solution (Veresoglou *et al.*, 1996)]. It was observed that above ground parts of plants generally accumulates Ca over Sr (DR < 1) in relation to soil and soil solution, but some species accumulate Sr over Ca (DR up to 3). In lettuce grown in pot experiments, an increase in Ca concentration of soil solution decreased the Sr concentration and Sr/Ca ratio in the plant and the discrimination ratio (Lembrechts *et al.*, 1990). Bailey *et al.* (1996) reported a roughly 8-fold variation in the Ca/Sr ratio of red spruce tissues at the Cone Pond watershed (New Hampshire). However, the roots and foliage had Ca/Sr ratios 6.2 times that of stemwood, respectively.

These observations suggest that the Sr biological cycle could differ from that of Ca, which might limit the use of Sr as a tracer of Ca (Poszwa *et al.*, 2000). In order to clarify this point, Poszwa *et al.*, (2000), compared the relationship between Ca and Sr concentrations and fluxes in atmospheric inputs, soil and soil solutions, and tree organs in three forested ecosystems. Poszwa *et al.* (2000) used the Jura experimental site 40 Km north-west of Lausanne (Switzerland). They reported that the Ca/Sr of foliage from Norway spruce was 8 times that of roots sampled from the B-horizon having soils developed on granite materials.

Blum *et al.*, (2002) studied the Ca/Sr and $^{87}Sr/^{86}Sr$ ratios in a mixed conifer/hardwood forest. The results showed higher Ca/Sr ratios in foliage than in exchangeable soil because of trees preferentially take up Ca over Sr by a factor of 2.3 to 8.5 for different forest species. They suggested that the high Ca/Sr ratios of spruce foliage relative to those of NH_4Cl-exchangeable and HF/HNO_3-acid digestible fractions of the underlying soils at Hubbard Brook result from preferential ectomycorrhizal weathering of the high Ca/Sr mineral apatite in deep mineral soils.

There is clear evidence of fractionation between the alkaline earth elements, and particularly between Ca and Sr, within spruce following uptake from soil nutrient pools (Bullen and Bailey, 2005). Bullen and Bailey, (2005) studied whether Sr isotope and alkaline earth elements ratios of spruce tissues and spatially associated soils, can be used to infer sources of Ca taken up by trees, and to determine whether changes in Sr isotope and alkaline earth element ratios in spruce stemwood cores can be used to infer changes in sources of Ca taken up by trees during their life span. This study was conducted on the base-poor Cone Pond watershed, where earlier work suggested that atmospheric deposition of inorganic acidity has caused depletion of Ca from the soil nutrient pools (Bailey *et al.*, 1996).

In this chapter we will discuss the results obtained for different elements in soil and plant materials of EUROFACE experimental site. The soil, leaves and roots were analysed by AAS for Ca and Sr. The soil samples were also analysed for Ca, Sr, Ba, Mg, Al, Fe, Na, P, K and Mn by ICP. The different element ratios are also discussed to find the history of mineral nutrients present in soils of the experimental site which might have contributed to high phosphorus measured in HCl extractable fraction (Ca bound P), discussed earlier in the chapter 4. We have also tried to find the effect of elevated atmospheric CO_2 on these elements and different elemental ratios.

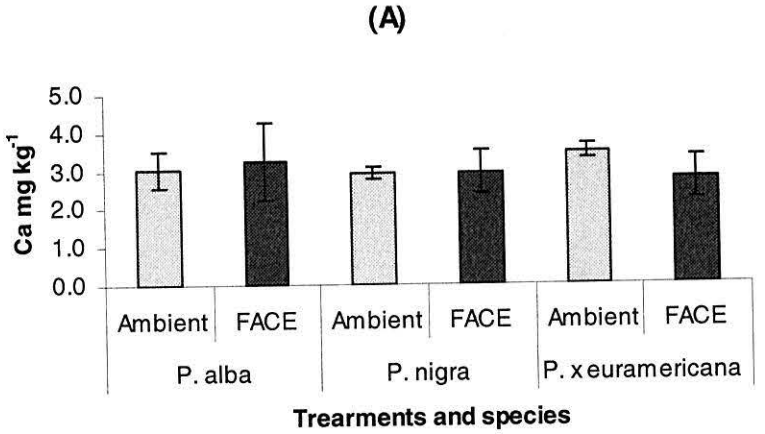
6.2 Results

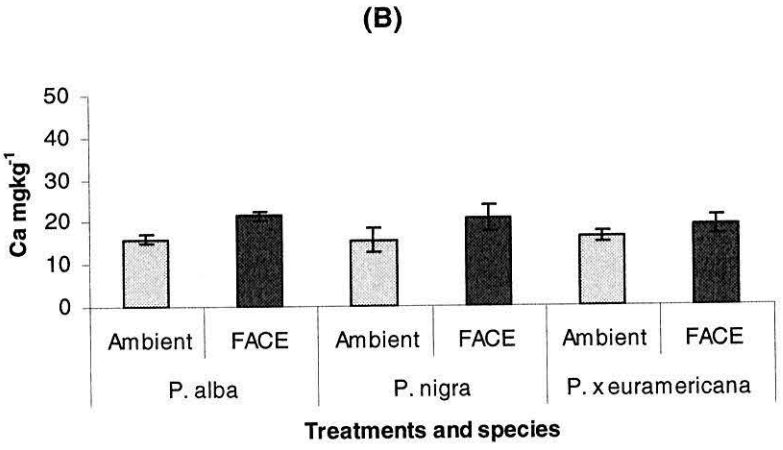
In the following section the results of calcium and strontium measured by the atomic absorption spectrophotometer in the soils and plant materials are presented. The results are presented to show the effect of elevated CO_2 on different plant materials under ambient and FACE CO_2 concentration. We have also tried to show the previous history of soil nutrient levels and the effect of weathering on nutrient concentrations. This section covers the amount of calcium and strontium analysed in soils, leaves and root samples at EUROFACE experimental site.

6.2.1 Calcium in soil

The samples analysed for calcium were from the non-fertilized plots at 0-10 cm depth. The samples were extracted as previously described in chapter 4 for the different fractions of phosphorus, followed by measurement on a Varion flame atomic absorption spectrophotometer. The figure 6.1(A-C) shows the amount of calcium analysed in the different fractions. The NaOH extract were found to have negligible levels of Ca and are not presented.

The H_2O extracts showed lowest Ca values and there was no consistent trend between different species and treatments (Figure 6.1A). Statistical analysis also confirmed that the difference in the mean values among the different levels of species and treatments is not great enough to exclude the possibility that the difference is just due to random sampling. There was no statistically significant difference (P= 0.915 & P= 0.703). The HCl extracted fraction showed a much different pattern as shown in figure 6.1(B). The figure shows that there is a continuous trend of more Ca in FACE





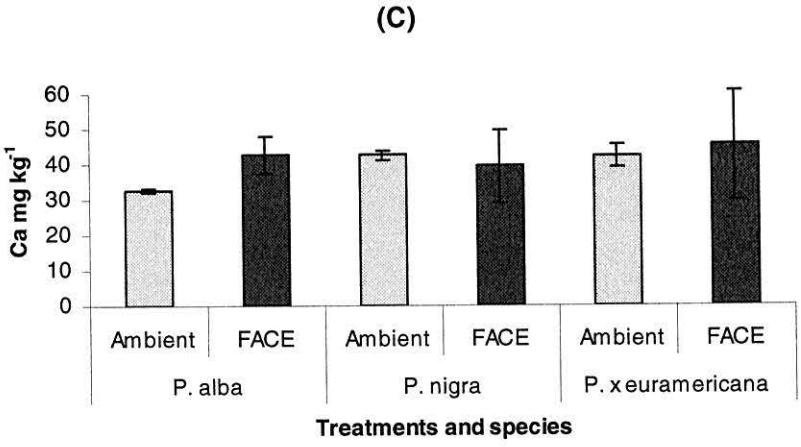


Figure 6.1: The concentration of calcium in soil (0-10 cm) for 3 *Populus* species grown under ambient and elevated atmospheric CO_2 without fertilizer in 3 different extracts (A=water, B=HCl, C=HNO_3). Data shown is the average value for each species with ±SE in different in different extraction solutions.

treatment. Statistical analysis also confirmed the results that the difference in the mean values among the different levels of treatment is greater than would be expected. There is a statistically significant difference (P=0.021). However, there was no significant difference between the different species (P=0.851). The final HNO_3 fraction (Figure 6.1 C) showed highest Ca concentrations but again no consistent trend was found between different species and treatments. The statistical analysis also revealed that there was no significant difference between the different treatments and species.

6.2.2 Strontium in soil

Strontium was also measured as described in section 6.1. The results are presented in the figure 6.2 (A-C). In the NaOH fraction Sr was below detection limits and hence is not included in the results.

The water extractable Sr results are shown in figure 6.1 (A), it can be seen from the figure that only a small amount of Sr is present and no consistent trend was found. The concentrations do not vary for different species and treatments. Statistical analysis also confirms that there are no statistically significant differences among the different treatments and species. The levels of Sr in HCl extract again showed a different pattern (figure 6.2 B). The *P. alba* species show relatively less Sr especially in FACE samples. However, the other two species showed the same trend as was found for Ca i.e. more Sr in FACE than ambient. Statistical analysis showed that there are no significant differences between species and treatments. This might be due to the lower value in *P. alba*. However the values were close to the significance level i.e. P= 0.059 & P= 0.081 for species and treatments respectively. The results for the HNO_3 extraction are presented in figure 6.2 (C), which clearly shows the trend of more Sr in FACE than ambient in all the species. The statistical analysis revealed the difference in the mean values among the different levels of treatments and species is not great enough to exclude the possibility that the difference is just due to random sampling. There are no statistically significant differences between the treatments and species.

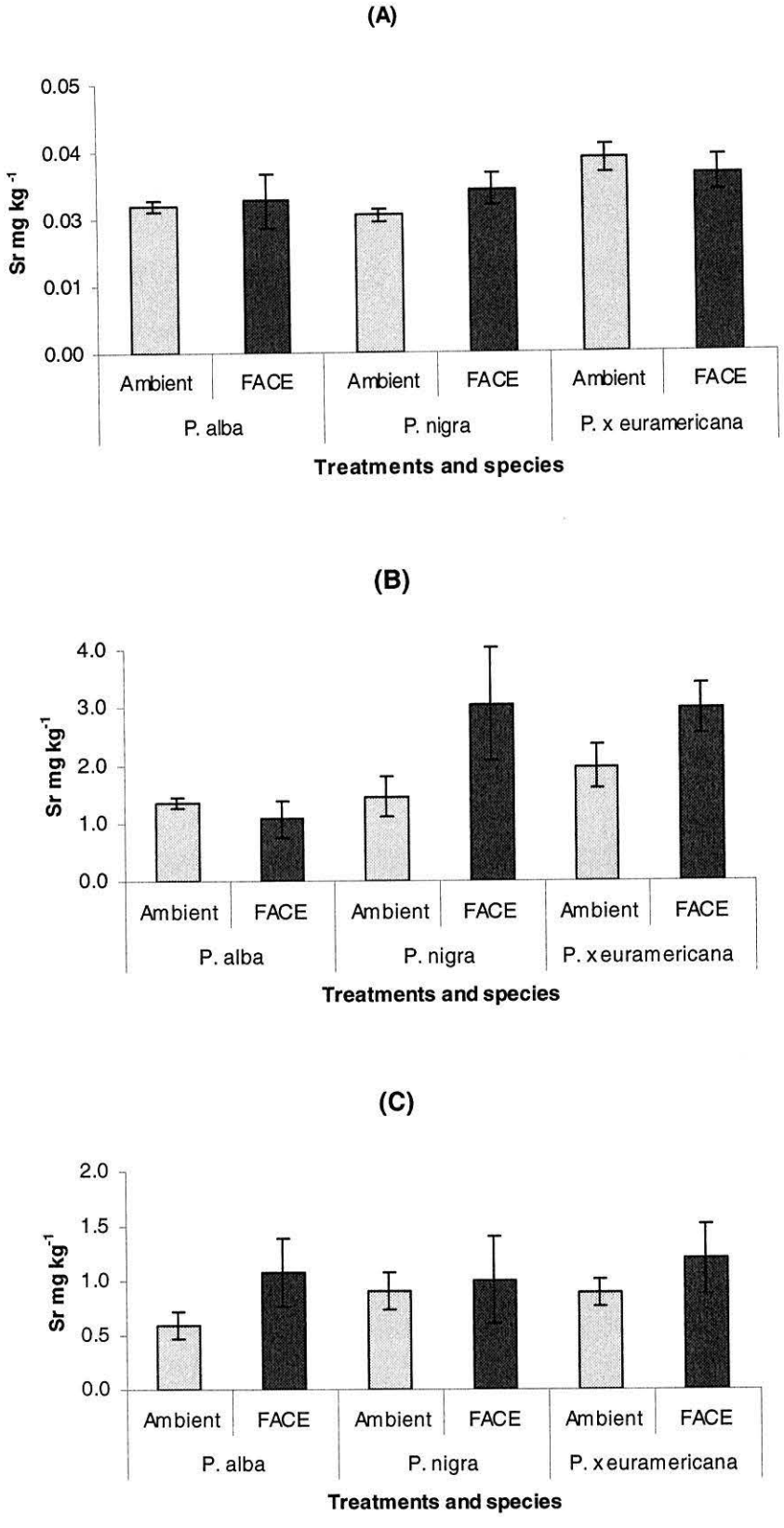


Figure 6.2: The concentration of strontium in soil (0-10 cm) for 3 *Populus* species grown under ambient and elevated atmospheric CO_2 without fertilizer in 3 different extracts (A=water, B=HCl, C=HNO_3). Data shown is the average value for each species with ±SE in different plots.

6.2.3 Calcium in leaves

Calcium in leaf samples of EUROFACE experimental were analysed after nitric acid digestion. The analysis was carried out using a Varian flame atomic absorption spectrophotometer. The results are presented in figure 6.3 (A &B).

Figure 6.3 (A) illustrates the results obtained for calcium concentrations in the non-fertilized plots. It is evident from the graph that there is no trend in different treatments. However, there are some differences in Ca concentrations for different species. Analysis of variance showed that the *P. alba* species is significantly different from the *P. nigra* and *P. x euramericana* species with *P* values 0.025 & 0.017 respectively. There was no significant difference between *P. nigra* and *P. x euramericana* species in both ambient and FACE (*P*= 0.139).

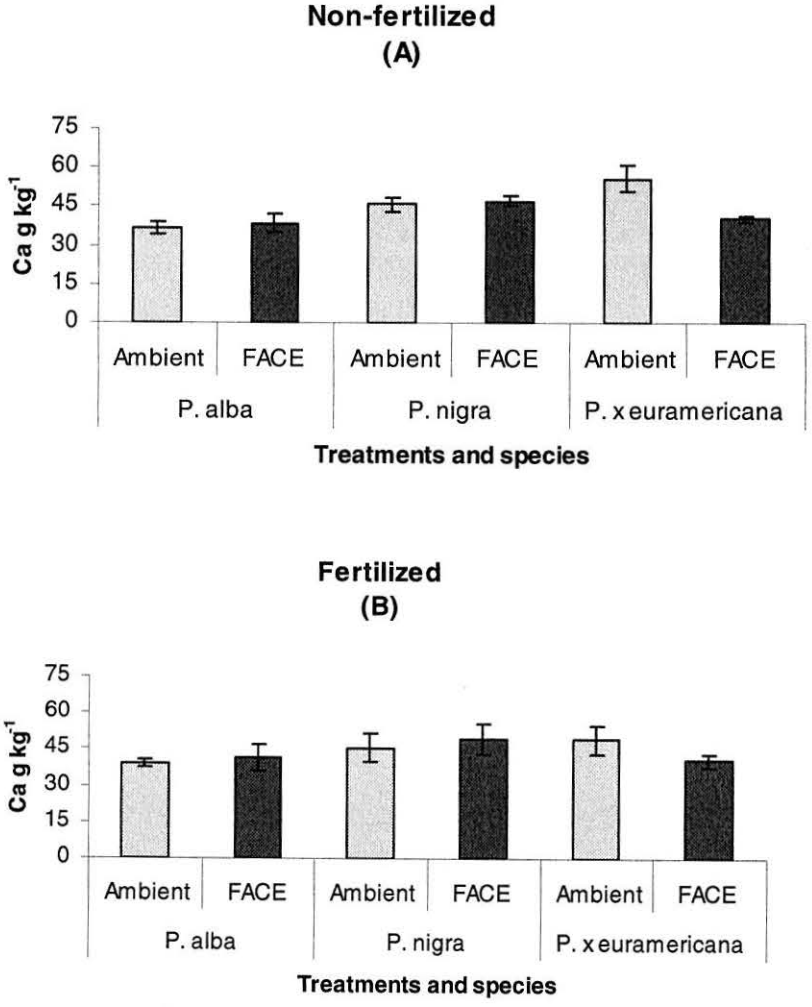


Figure 6.3: The concentration of calcium in leaves of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the average value for each species with ±SE in different plots.

Figure 6.3 (B) presents the results for calcium in fertilized plots, which again clearly shows that there is no specific trend among the results obtained for different species and treatments. Statistical analysis also confirms the results that the difference in the mean values among the different levels of treatments (P= 0.369) and species (P= 0.840) is not great enough to exclude the possibility that the difference is just due to random sampling variability.

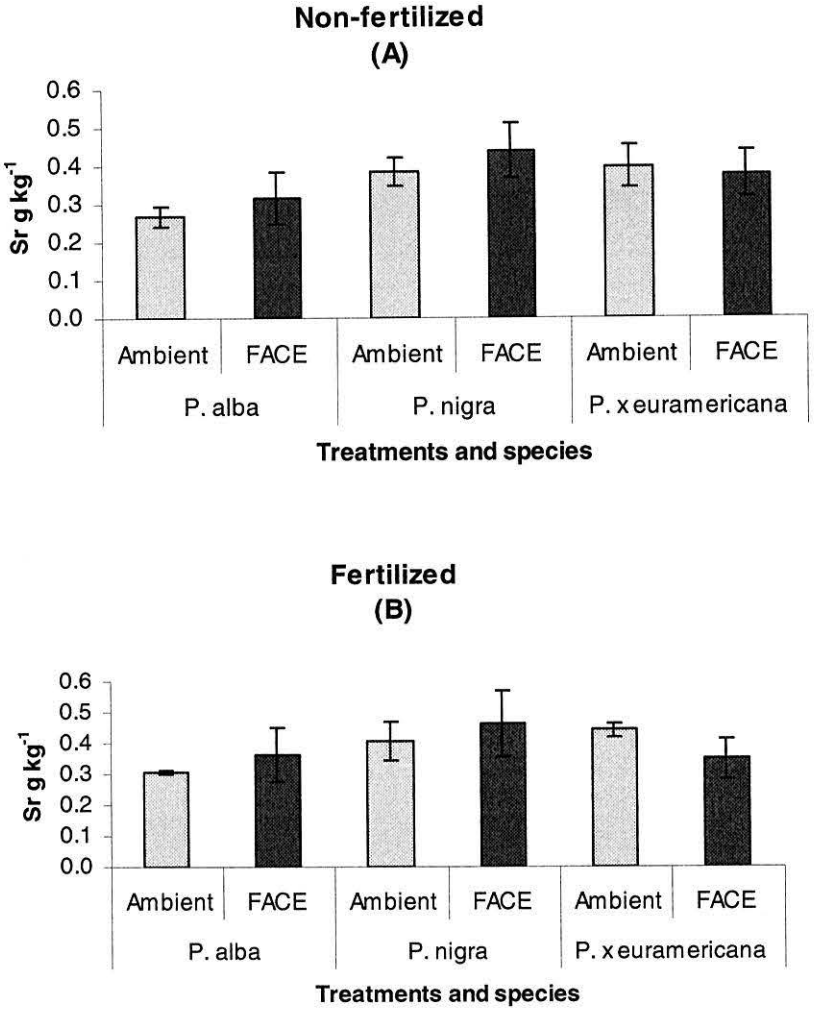


Figure 6.4: The concentration of strontium in leaves of 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the average value for each species with ±SE in different plots

6.2.4 Strontium in leaves

Strontium in leaf samples were also analysed in the nitric acid extracts by atomic absorption spectrophotometery. The results are presented in the figure 6.4 (A & B) for different species and treatments.

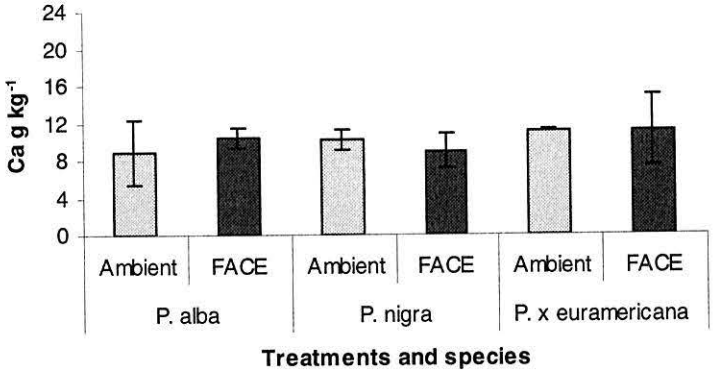
The results for non-fertilized plots (Figure 6.4 A) clearly show that there is again no consistent trend in the results for all species and treatments. In *P. alba* and *P. nigra* species more Sr was measured in FACE plots, while the trend is opposite to that in *P. x euramericana* species. Statistical analysis also confirms the results that there is no significant correlation among the different species and treatments. Figure 6.4 (B) shows the results of strontium in fertilized plots under control and FACE treatments. The results are very much similar to the non-fertilized plots. There is no consistent trend and again *P. alba* and *P. nigra* species had more Sr in FACE, while, there is more Sr in ambient for *P. x euramericana* species. Analysis of variance also showed no significant differences between different species and treatments.

6.2.5 Calcium in roots

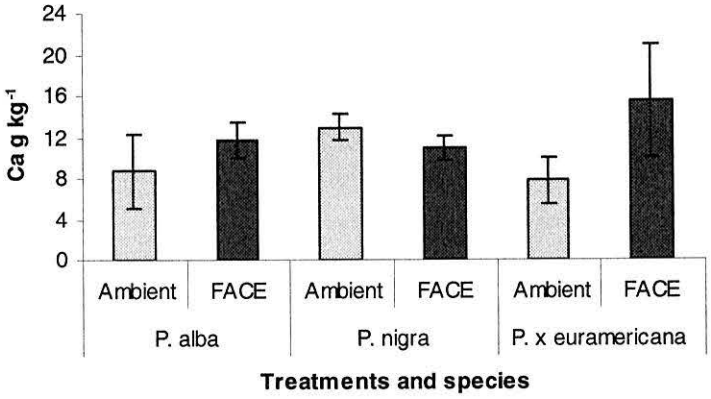
Calcium content in roots was also measured as described in sections 6.1-6.4. The results obtained for fertilized plots are presented in figure 6.5 (A & B). For non-fertilized plots at 0-20 cm depth (Figure 6.5 A) no differences were found between either treatments or species. At 20-40 cm depth (Figure 6.5 B) in *P. alba* and *P. x euramericana* more calcium was found in FACE, while the *P. nigra* species showed more calcium in ambient. The species also showed varying concentrations of calcium at 20-40 cm depth. Statistical analysis revealed that these differences are not statistically significant with *P* values 0.787 & 0.228 for species and treatments respectively.

The results for 0-20 cm depth of fertilizer treated plots are shown in figure 6.5 C and showed similar patterns to those found in the non-fertilized treatment; however average values are relatively higher than in the non-fertilized samples. Again no significant differences were found for both species and treatments. The results of 20-40 cm depth fertilized plots again showed more calcium in *P. alba* and *P. x euramericana* species under FACE, and *P. nigra* species was found to have less calcium under FACE. However, these differences were not statistically significant.

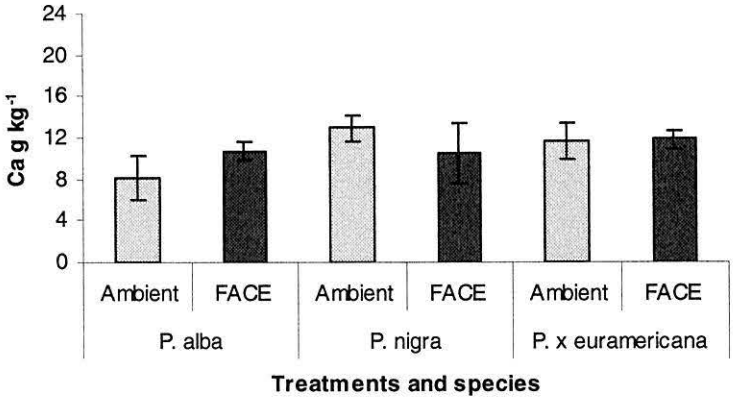
Non-fertilized
(A) 0-20 cm



(B) 20-40 cm



Fertilized
(C) 0-20 cm



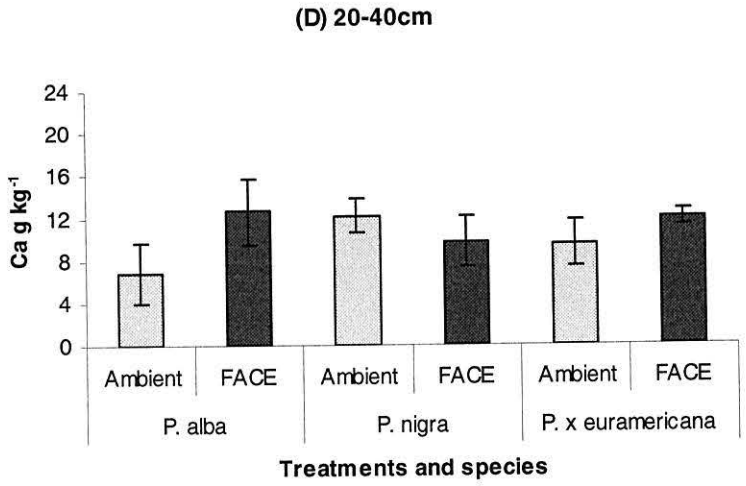


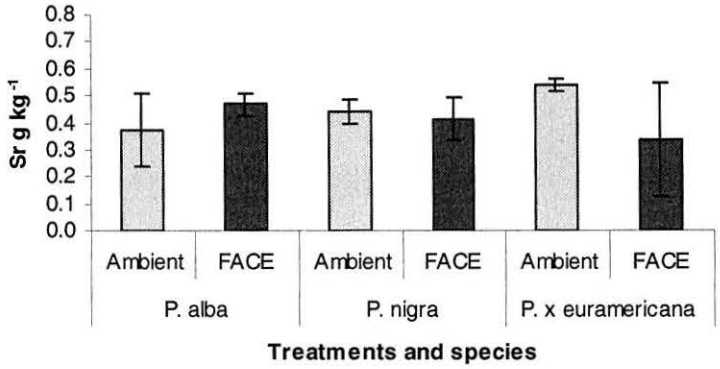
Figure 6.5: The concentration of calcium in roots of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the average value for each species at two depths with ±SE in different plots.

6.2.6 Strontium in roots

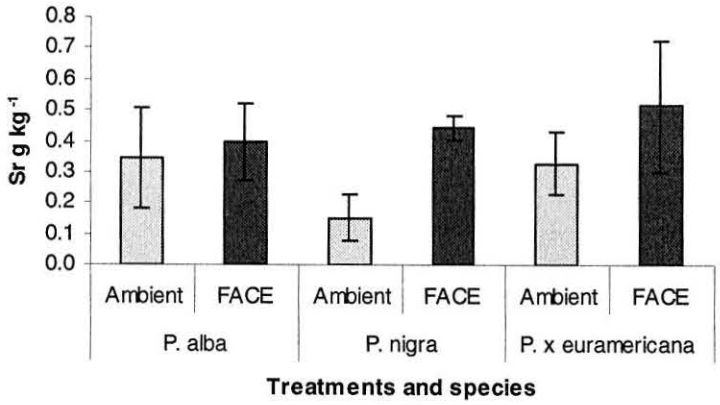
Non-fertilized plots at 0-20 cm depth (Figure 6.6 A) generally showed higher levels of Sr in ambient roots. At 20-40 cm depth there was a trend for more Sr under FACE in all the species. However, the difference was not statistically significant (P=0.094). The differences between species were also found to be un significant.

At 0-20 cm in fertilized plots also no statistical differences between treatments and species was found. At 20-40 cm soil depth in fertilized plots a trend of more Sr under FACE treatment for *P. alba* and *P. x euramericana* species was shown. The differences between treatments are statistically significant (P=0.029). However, there were no significant differences between the species.

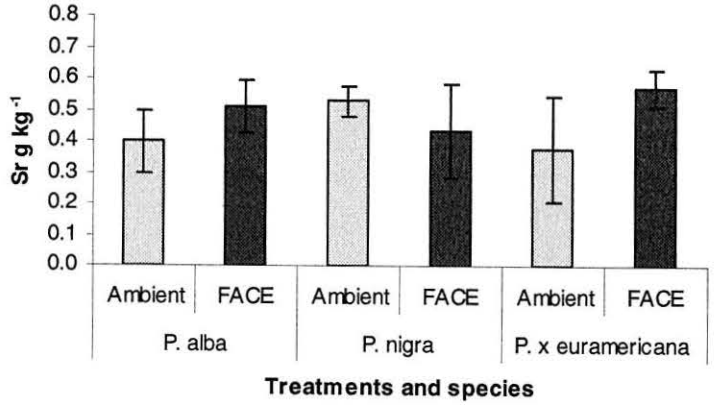
Non-fertilized
(A) 0-20 cm



(B) 20-40 cm



Fertilized
(C) 0-20 cm



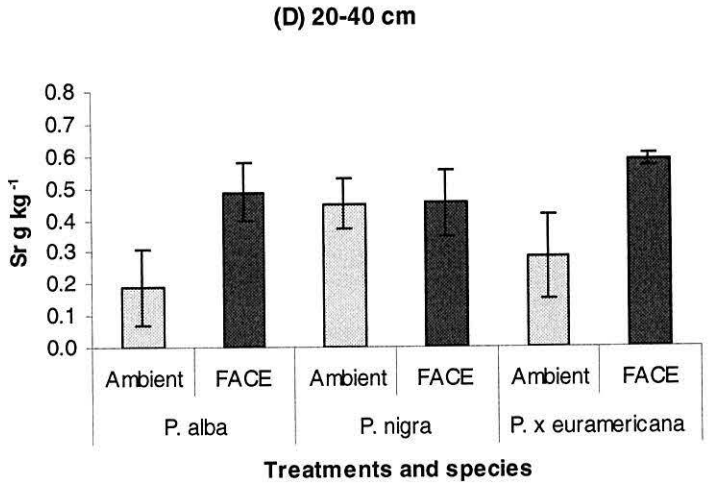


Figure 6.6: The concentration of Strontium in roots of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the average value for each species at two depths with ±SE in different plots.

6.7 Ca/Sr ratios in soil, roots and leaves

The calcium and strontium results presented earlier are used to calculate Ca/Sr ratios (Figure 6.7 A-C). The ratios of water extractable Ca/Sr (Figure 6.7 A) showed that the ratios were ranging from 63-138 for the different species. The highest ratios are observed in *P. alba* with slightly higher value in the FACE treatment. In *P. nigra and P. x euramericana* the ratio is higher in ambient plots. However, there was no significant differences between the ambient and FACE. However, the average values were higher in ambient (107.32) than FACE (98.26).

The Ca/Sr ratios in the HCl extract are presented in figure 6.7 (B). Higher ratios were found in ambient treatment in all the poplar species. The values were again highest in *P. alba*. The values ranged from 5 to12, and significant difference was found between ambient and FACE (P=0.028) treatments. However, no significant differences were found between different species.

The calcium to strontium ratios of the final extract are presented in figure 6.7(C). The average values ranged from 25 to 116 but no significant differences among the different treatments and species were found.

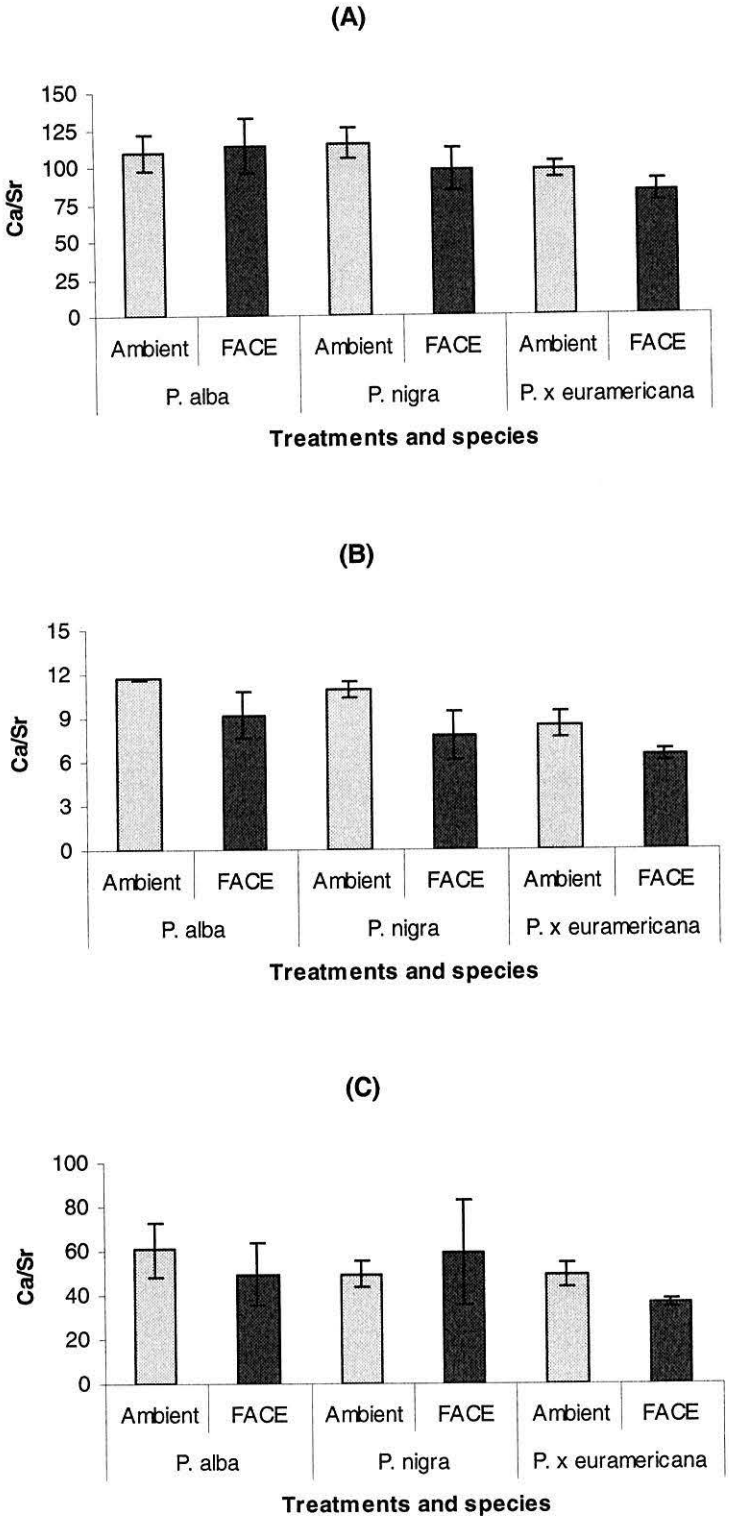


Figure 6.7: The Ca/Sr in soil of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 without fertilizer in 3 different extracts (A= water, B= HCl, C= HNO_3). Data shown is the Ca/Sr value for each species with ±SE in different extraction solutions.

The Ca/Sr ratios determined in leaves are presented in figure 6.8 A & B. In both non-fertilized (6.8 A) and fertilized (6.8 B) the Ca/Sr ratios ranged between 256 and 290, however no statistical differences were found between treatments or species.

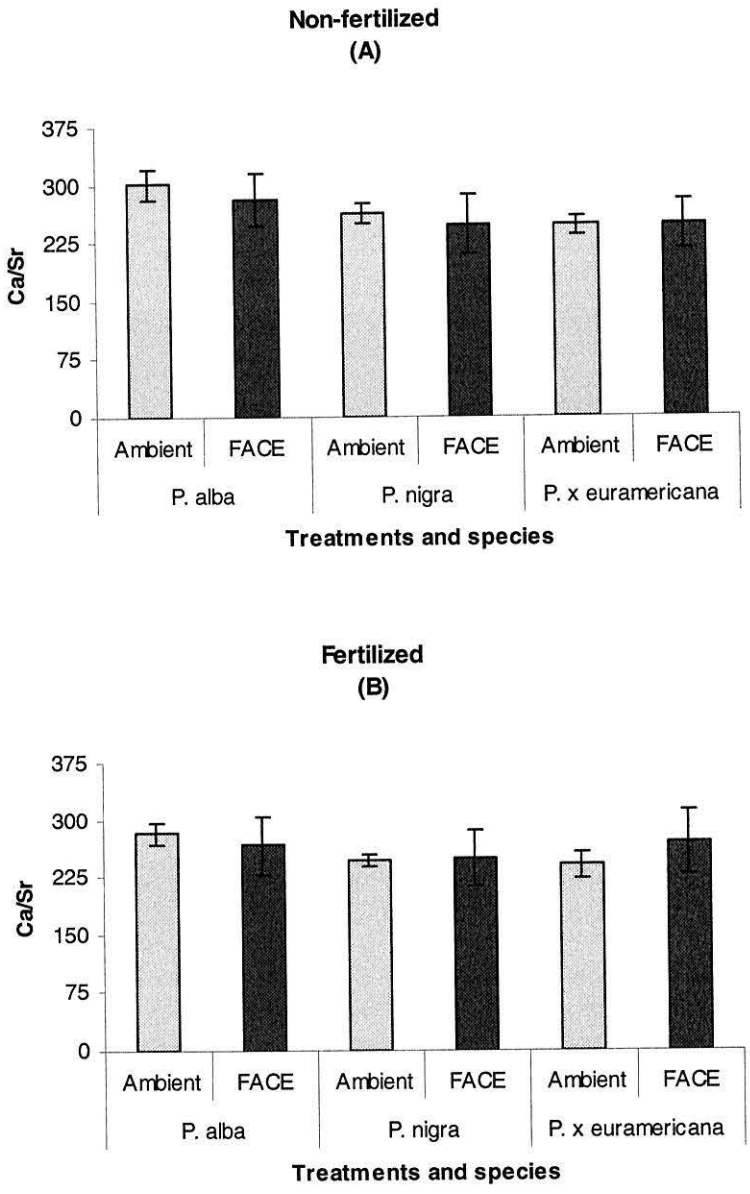
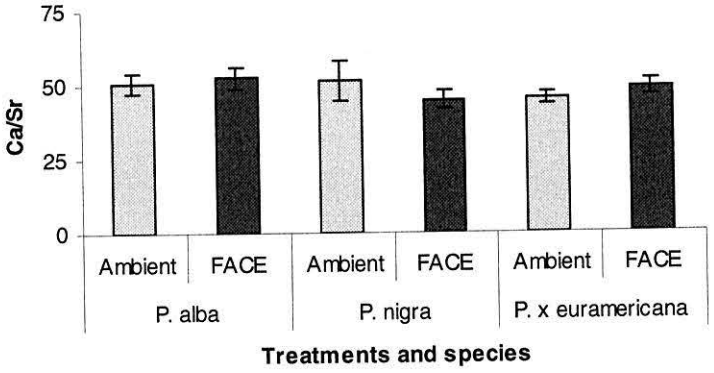


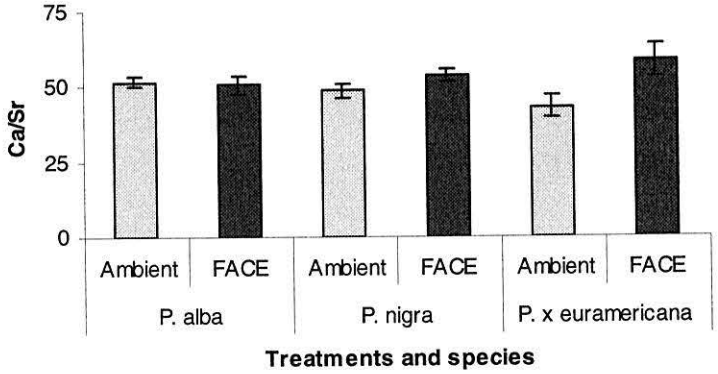
Figure 6.8: The Ca/Sr in leaves of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the Ca/Sr value for each species with ±SE in different plots.

In roots taken from 0-20 cm soil depth ratios (Figure 6.9) were again similar in all treatments and species. However, at 20-40 cm depth although there were no statistical differences between species, if the species are pooled, the increase under FACE was statistically significant (P= 0.037).

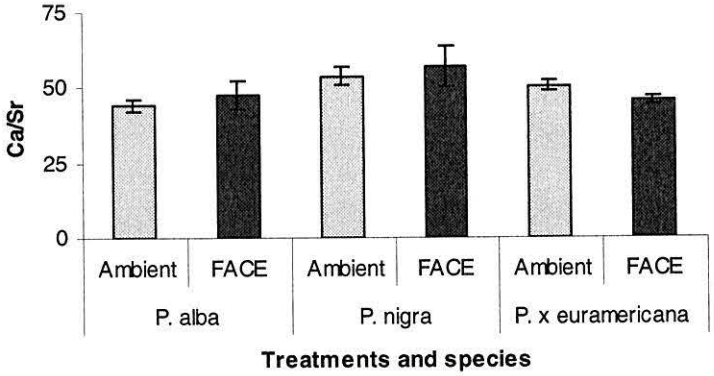
Non-fertilized
(A) 0-20 cm



(B) 20-40 cm



Fertilized
(C) 0-20 cm



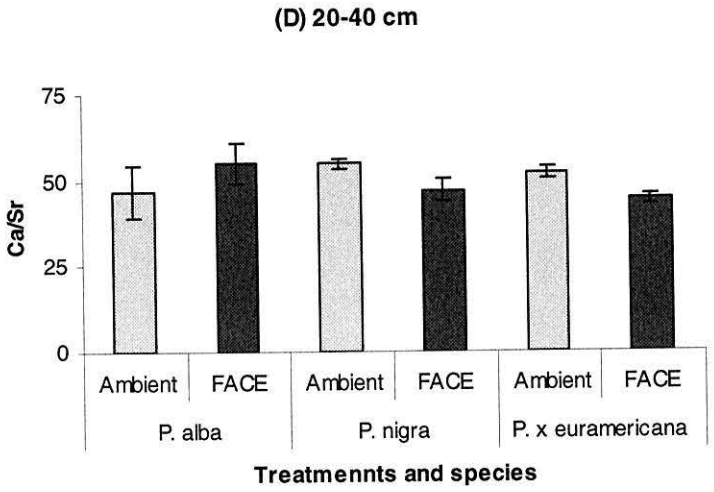


Figure 6.9: The Ca/Sr in roots of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer (A&C=0-20 cm, B&D=20-40 cm). Data shown is the Ca/Sr value for each species at two depths with ±SE in different plots.

6.8 Elements ratios measured on ICP

The soil samples of the top soil (0-10 cm) extracted with HCl and HNO_3 were also analysed on ICP for comparison of nutrient levels and to determine elemental ratios. These elements are used to determine the following elemental ratios. The results were found to be different from those measured by AAS which may be due to the different instrument used for analysis.

6.8.1 Ca/Sr in soil

The calcium and strontium results obtained from ICP were used to calculate Ca/Sr ratios (Figure 6.10). The results for Ca were found to have more calcium in FACE plots, while there was no significant FACE effect on strontium. The Ca to Sr ratios in the HCl extract are presented in figure 6.10, clearly higher ratio was shown in FACE treatment in all the poplar species. The average Ca/Sr values ranged from 82 to111 in different species and treatments. The average values were 89 in control and 104 in FACE plots. The significant differences was found between ambient and FACE (P= 0.014) treatments. The different plots were also found significantly different (P= 0.003) from each other. However, no significant differences were found between different species.

The calcium to strontium ratios of the HNO_3 extract are also presented in figure 6.10. The FACE plots again showed higher Ca/Sr ratio in HNO_3 extracts. The average values ranged from 56 to175 among the different treatments and species. The average

values in ambient plots were 78, while they were found 123 in FACE plots. The statistical analyses revealed no significant differences among the different species, treatments and plots.

6.8.2 Sr/Ba in soil

The results obtained for Sr and Ba by ICP analysis are used to measure molar Sr/Ba ratios. The results for HCl extract (Figure 6.11) clearly showed higher values in ambient plots. While, the individual result for Ba and Sr showed more Ba in FACE plots and no clear effect of FACE treatment on Sr. The average Sr/Ba values ranged between 0.17-0.31, with a mean of 0.29 in ambient and 0.19 in FACE plots. The analysis of variance also confirmed that there is a significant difference between control and FACE values (P=0.027). The statistical analysis also revealed that there is a significant difference between the different plots (P=0.003), while there were no significant differences between the 3 poplar species.

The Sr to Ba ratios of HNO_3 extract showed opposite to HCl extract results, in *P. alba* and *P. nigra* species, with higher values in FACE plots. The results of Ba and Sr showed higher concentrations in ambient plots for all the species. The average values of Sr/Ba ranged from 0.66 to 1.2, while these values were 0.76 in ambient and 0.99 in FACE plots. The statistical analysis again showed that there are significant differences between ambient and FACE treatments (P= 0.047), while the plot 3 is also different from plot 1 and 2 (P= 0.005).

6.8.3 Mg/K in soil

Magnesium and potassium measured on ICP were found to have absolute values higher in HCl extract of soil from FACE plots. In contrast, higher concentration of Mg and K were found in ambient plots in HNO_3 extracts. The Mg to K ratio was not found to have any significant trend as shown in figure 6.12. The average values of Mg/K were 0.09 to 0.11 in the HCl extract and 1.03 to 1.08 in the HNO_3 extract. The analysis of variance also confirmed that there are no significant differences among the different treatments, species and plots. The only significant difference was between plot 1 & 3 in the HCl extract (P=0.017).

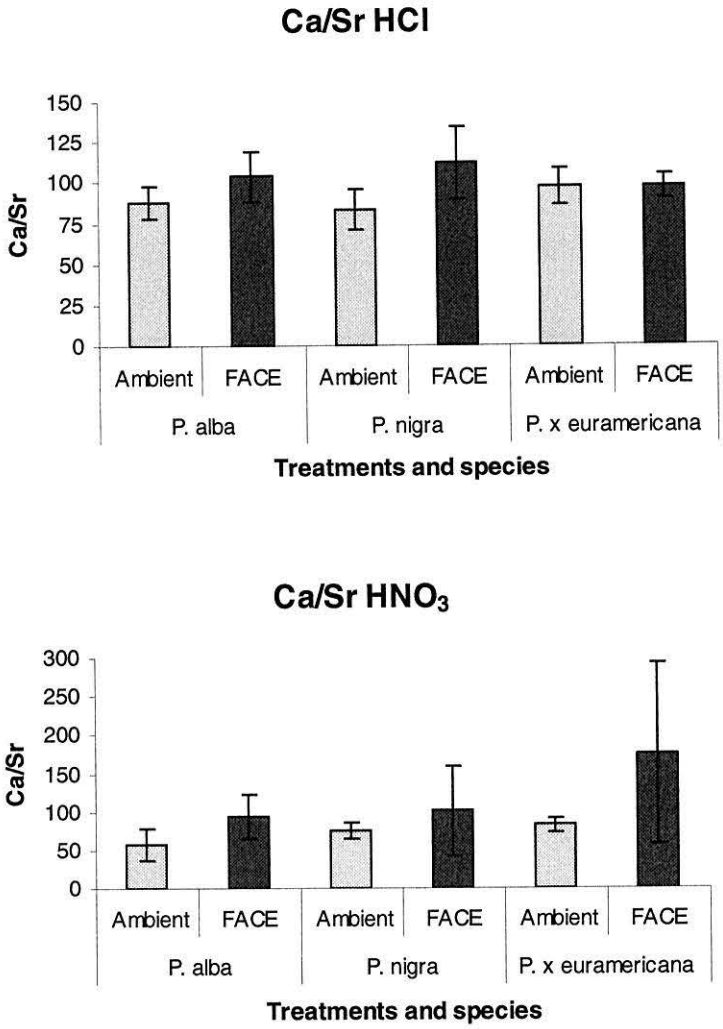


Figure 6.10: The Ca/Sr in soil of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 without fertilizer in 2 different extracts. Data shown is the Ca/Sr value for each species at 0-10 cm depth with ±SE in different extraction solutions.

6.8.4 K/Sr in soil

The potassium to strontium ratios are presented in figure 6.13. The graphs reveal that there is no specific trend in the K/Sr ratios in both the extracts. The average values in the HCl extract ranged from 431 to 540, with *P. alba* and *P .x euramericana* showing higher values in ambient plots, while *P. nigra* showed a higher value in FACE plots. The statistical analysis also confirmed that there are no significant differences among the different levels of treatments and species, while the ambient plot 3 was found significantly different from FACE plot 1 and ambient plot 2.

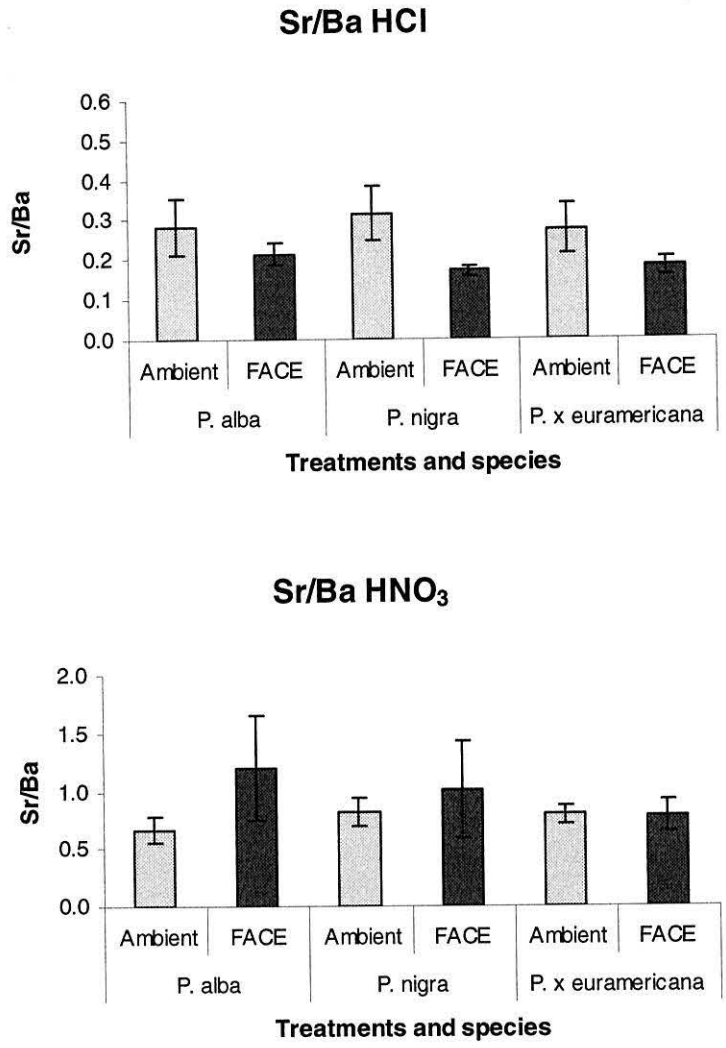


Figure 6.11: The Sr/Ba in soil of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 without fertilizer in 2 different extracts. Data shown is the Sr/Ba value for each species at 0-10 cm depth with ±SE in different extraction solutions.

6.8.5 P/Ca in soil

The phosphorus concentrations in HCl extract of soil measured by ICP again showed significantly higher P concentration in FACE plots for all the species. The average values found in ambient plots were 2.5-3.5 mg kg^{-1} but the similar values in FACE plots were 5.2-8.4 mg kg^{-1}. The results measured from Ca and P concentration measured on ICP were used to calculate Ca/P and the values are presented in figure 6.14. In HCl extract, P/Ca in soil showed higher values in FACE plots for all the species. The HNO_3 extract ratios were found to have higher values in FACE plots for *P. nigra* and *P. x euramericana*, while *P. alba* showed opposite result.

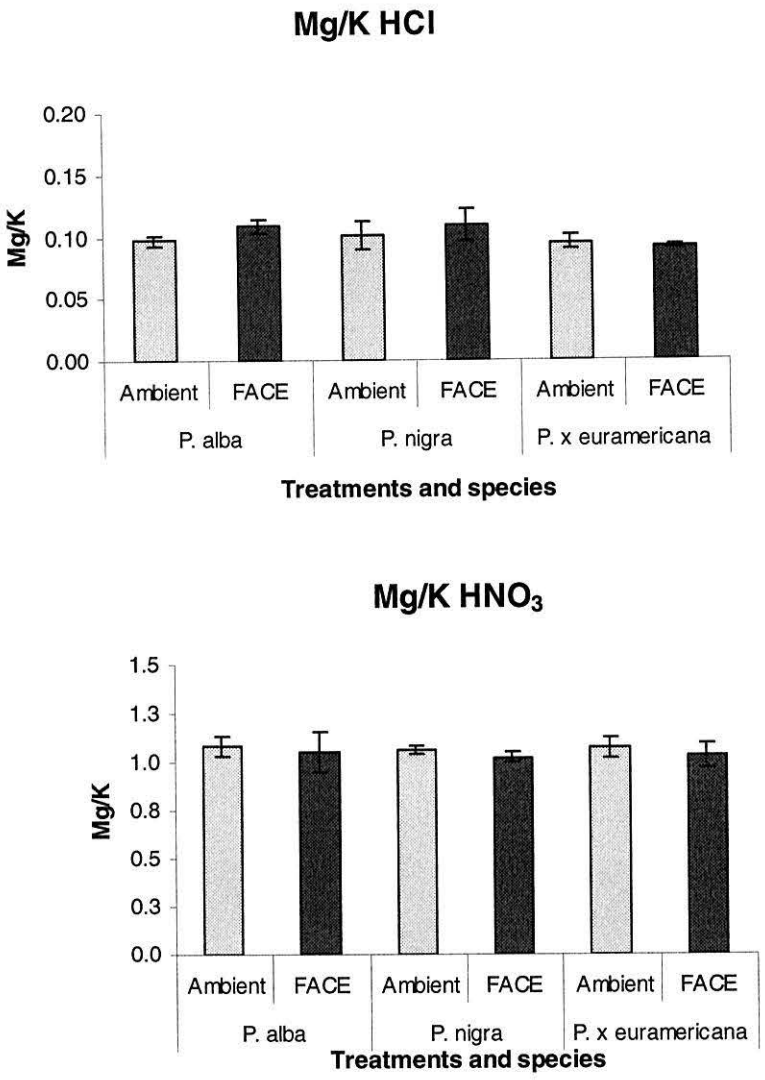


Figure 6.12: The Mg/K in soil of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 without fertilizer in 2 different extracts. Data shown is the Mg/K value for each species at 0-10 cm depth with ±SE in different extraction solutions.

The average values in HCl extract ranged between 0.10-0.25 and these values were 0.04-0.10 for HNO_3 extract. The analysis of variance revealed no significant differences between the different treatments and species in both the extracts. In HCl extract only plot 3 was found significantly different (P=0.017) from plot 2.

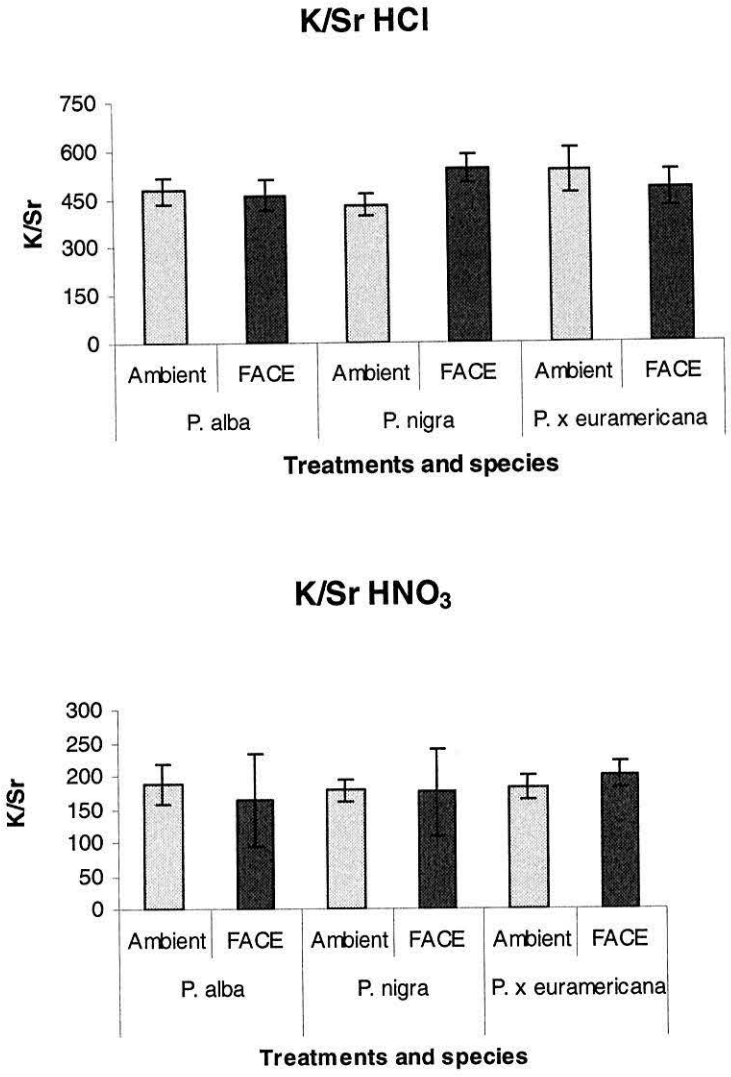


Figure 6.13: The K/Sr in soil of 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 without fertilizer in 2 different extracts. Data shown is the K/Sr value for each species at 0-10 cm depth with ±SE in different extraction solutions.

6.8.6 Ca/(Ca+Na) in soil

The values measured for Na on ICP were used to measure Ca/(Ca+Na) and the ratios in two extracts are presented in figure 6.15. The absolute Na concentrations measured in HCl extracts differed greatly between ambient and FACE (Figure 6.16). The average values calculated for ambient were 3.0 to 5.7 mg kg^{-1}, while the concentration was 21.3 to 31.6 mg kg^{-1}in FACE samples. The values calculated for HNO_3 extract did not show a big difference in *P. alba* and *P. nigra*. However in

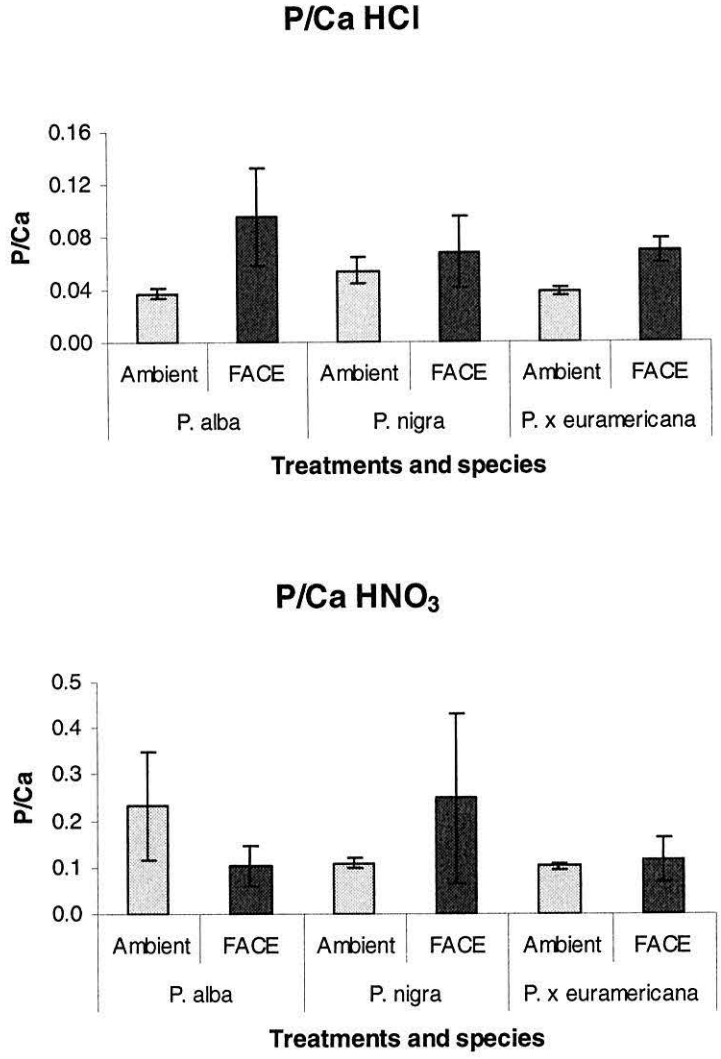


Figure 6.14: The P/Ca in soil of 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 without fertilizer in 2 different extracts. Data shown is the Ca/P value for each species at 0-10 cm depth with ±SE in different extraction solutions.

P. x euramericana a large difference was found mainly because of the value determined in FACE plot 4. The Ca to (Ca+Na) ratio (Figure 6.15) calculated for HCl extract showed higher values in ambient rings. The average values for these ratios in HCl extracted samples ranged from 0.77 to 0.96. Statistical analysis confirmed that there is a significant difference between ambient and FACE (P=0.023). While, there were no significant differences among different species and plots. The results of HNO_3 extract were also very much similar showing higher ratios in ambient plots. The average values of this extract ranged between 0.67-0.77. The analysis of variance revealed no significant difference among any of the parameters.

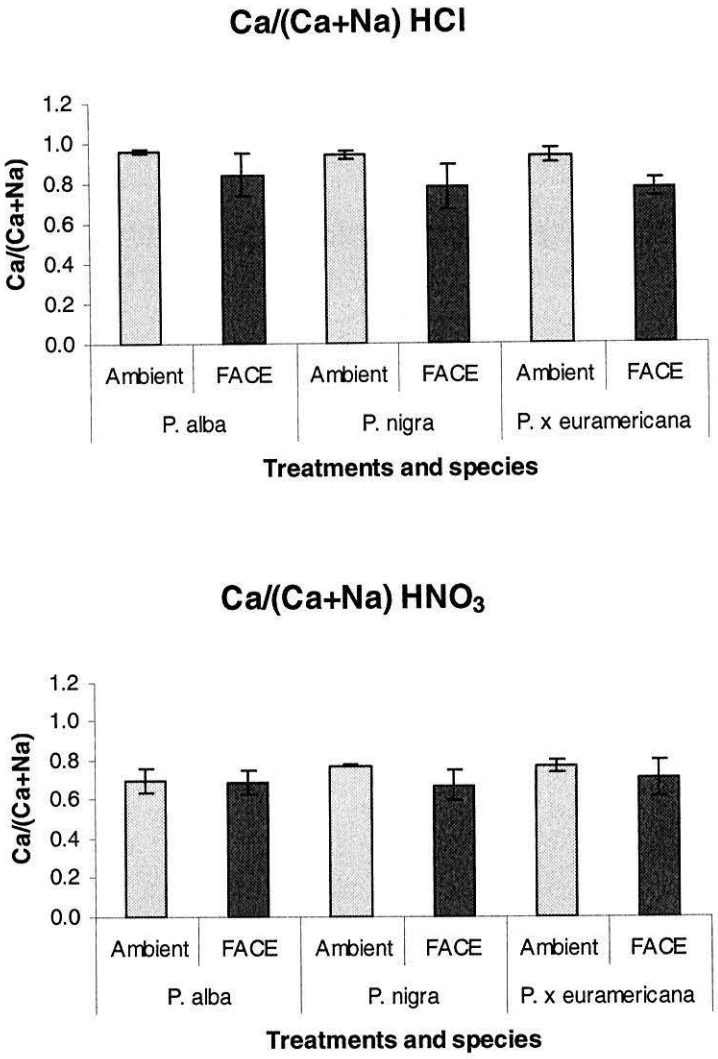


Figure 6.15: The Ca/(Ca+Na) in of 3 species of *Populus* grown under ambient and elevated atmospheric CO_2 without fertilizer in 2 different extracts. Data shown is the Ca/(Ca+Na) value for each species at 0-10cm depth with ±SE in different extraction solutions.

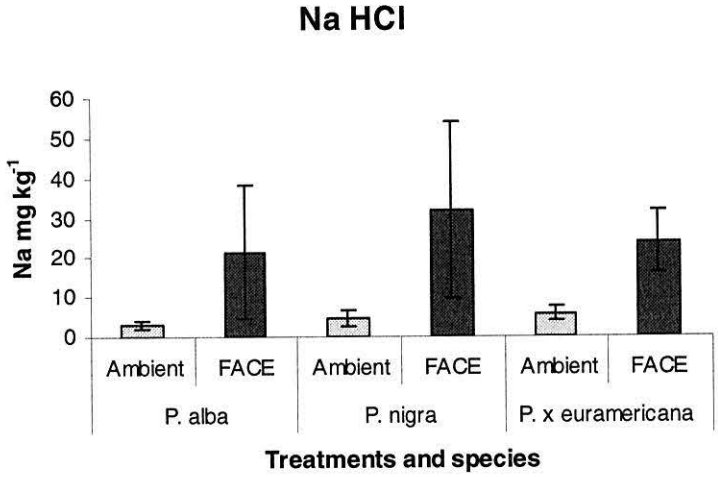


Figure 6.16: The Na in soil of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 without fertilizer in HCl extracts. Data shown is the Na value for each species at 0-10 cm depth with ±SE in different extraction solutions.

6.9 Discussion

Chemical approaches for identification of nutrient sources require an understanding of the nutrient pools that are actually available to fine roots in soils (Bullen and Bailey, 2005). The conventional approach is to consider the exchangeable ions in soils to be the most 'plant available' (Suarez, 1996), although any laboratory procedure for the determination of exchangeable inventories is strictly operational and may not truly approximate nutrient uptake by fine roots (Bullen and Bailey, 2005). Kennedy *et al.* (2002) found that plants rooted in shallow soil at a Chilean watershed did not take up isotopically labelled Sr residing on mineral soil exchange sites during the two years following application of the tracer to a forest plot. They suggested that the plants obtained alkaline earth elements predominantly from soil organic layer, rather than exchange sites of the mineral soil. However a number of research experiments have suggested that fine roots and their fungal associates are able to derive nutrients directly from minerals in the soil, utilizing chemical exudates that are more aggressive than typical salt extractants (Van Breemen *et al.* 2000; Wallander *et al.* 2002). Bullen and Bailey, (2005) suggested that a more reasonable estimate of plant-available soil pools is obtained by considering the operational exchangeable and leachable fractions of the soils together as a bulk pool.

We measured the different elements and element ratios to find the history of Ca in the soils of EUROFACE experimental site in Italy. The results of Ca, Sr and Ca/Sr in different soil fractions and in leaves and roots of poplar measured by atomic absorption

spectrophotometer are presented in Table 6.1 and 6.2. The table 6.3 gives the results of Ca, Sr and Ca/Sr ratio measured by ICP, these are for soil samples only.

Blum *et al.* (2002) tried to trace of source of Ca uptake using Ca/Sr and $^{87}Sr/^{86}Sr$ ratios. In their work they assumed no fractionation within tree species they investigated. The results presented here show strong fractionation of Ca/Sr between roots and shoots in *Populus*. This strongly suggests that Ca/Sr ratios in leaves cannot be related to the ratios in different minerals with the soil. The presented results in this work are in agreement with the study of Watmough and Dillon, (2003) which suggests that as Ca/Sr can vary greatly between different parts of a plant, Ca/Sr ratios are poor indicators of the source of Ca for trees. The values obtained in our study for leaf samples are similar to some forest species analysed by Watmough and Dillon, (2003) and Bullen and Bailey, (2005), but mostly are lower than their values. This again may show that Ca/Sr values vary in different forest plants. The Ca/Sr values in roots were also found lower than the values of Watmough and Dillon, (2003)) and Bullen and Bailey, (2005). However, the results suggest that element levels and ratios in roots may be better indicators of element sources in the soil, than element levels and ratios measured in the soil.

The Ca/Sr values of soil samples, measured on AAS, were also found much lower than the values presented by Blum *et al.* (2002). This was mainly because of higher Ca content in soils of EUROFACE experimental site. Highest Ca/Sr values were

Table 6.1: Average concentrations of Ca, Sr and Ca/ Sr ratios in different soil fractions under ambient and elevated CO_2 (FACE) measured on AAS.

Soil Fractions	Species	Ambient			FACE		
		Ca	Sr	Ca/Sr	Ca	Sr	Ca/Sr
H_2O	*P. alba*	121	2.4	109	129	2.5	114
	P. nigra	117	2.2	115	118	2.7	98
	P.x euramericana	139	3.2	97	112	2.9	83
HCl	*P. alba*	636	118	12	856	229	9.2
	P. nigra	620	127	11	827	267	7.8
	P.x euramericana	644	172	8.5	754	261	6.4
HNO_3	*P. alba*	1307	52	60	1712	94	49
	P. nigra	1702	79	49	1576	88	59
	P.x euramericana	1693	77	49	1806	104	36

Element concentrations are given in terms of mg Kg^{-1}. The Ca/Sr ratios are given in molar proportions.

Table 6.2: Average concentrations of Ca, Sr and Ca/ Sr ratios in plant material under ambient and elevated CO_2 (FACE) with (+F) or without (-F) fertilization measured on AAS.

Plant Materials	Species	Ambient			FACE		
		Ca	Sr	Ca/Sr	Ca	Sr	Ca/Sr
Leaves -F	*P. alba*	36.7	0.27	300	38.6	0.32	281
	P. nigra	46	0.39	263	47.2	0.44	247
	P.xeuramericana	55.7	0.40	246	40.6	0.37	247
Leaves +F	*P. alba*	39.3	0.31	282	41.6	0.36	267
	P. nigra	45.5	0.41	247	49.4	0.46	249
	P.xeuramericana	48.9	0.44	241	40.3	0.35	269
Roots -F (0-20 cm)	*P. alba*	8.8	0.37	50.6	10.3	0.47	52.6
	P. nigra	10.1	0.44	51.3	8.9	0.41	44.7
	P.xeuramericana	11.1	0.54	45.3	11.1	0.34	48.8
Roots -F (20-40 cm)	*P. alba*	8.7	0.35	51.9	11.7	0.40	50.7
	P. nigra	12.9	0.15	48.6	10.9	0.44	53.6
	P.xeuramericana	7.7	0.33	43.0	15.4	0.51	58.2
Roots +F (0-20 cm)	*P. alba*	8.1	0.40	44.3	10.7	0.51	47.4
	P. nigra	12.9	0.53	53.7	10.5	0.44	57.1
	P.xeuramericana	11.6	0.38	50.5	11.7	0.57	45.3
Roots +F (20-40 cm)	*P. alba*	6.8	0.19	47.1	12.6	0.49	54.9
	P. nigra	12.1	0.45	54.9	9.7	0.45	47.1
	P.xeuramericana	9.5	0.28	52.1	11.5	0.59	44.3

Element concentrations are given in terms of mg Kg^{-1}. The Ca/Sr ratios are given in molar proportions.

found in water soluble fraction, while they were lowest in HCl fraction. If the values determined by AAS and ICP are compared the values of H_2O extracted fraction with values determined for an organic horizon in Bullen and Bailey, (2005), then Ca/Sr of the water extract are similar to leachable and digestible fraction of Bullen and Bailey, (2005), but lower than values found in their exchangeable fractions. Bullen and Bailey, (2005), extracted exchangeable fraction with ammonium acetate, leachable fraction with HNO_3 digestion and finally digestible fraction with a mixture of HNO_3 and HF after ashing the soil in muffle furnace at 650° C. The Ca/Sr results of HCl and HNO_3 soil fractions in this study were compared with the mineral horizon results of Bullen and Bailey (2005), and they were found lower than most of the values. This was mainly because of the higher Ca concentrations in the mineral horizons analysed by Bullen and Bailey (2005). The results also showed that higher Ca/Sr, for both soil and plant materials, in FACE plots for all the species but the differences were not found statistically significant.

However, it could clearly be shown in the work presented here that different Ca/Sr ratios are obtained depending on the analytical method used. Determination by either AAS (Table 6.2) or ICP (Table 6.3) resulted in an almost 10 fold difference in Ca/Sr ratio in the soil fraction. This was primarily due to lower Ca and Sr values determined by ICP compared to AAS, which result in significantly different ratios. The values were again found higher in FACE plots with significant differences, these differences may have been due to the significant differences between the different plots. Bullen and Bailey (2005) also used ICP for determination of element. The absolute Ca and Sr values measured in this work were higher than those of Bullen and Bailey (2005), but the Ca/Sr molar ratios were very much similar in mineral horizons.

Table 6.3: Average concentrations of Ca, Sr and Ca/Sr ratios of two soil extracts under ambient and elevated CO_2 measured on ICP.

Soil Fractions	Species	Ambient			FACE		
		Ca	Sr	Ca/Sr	Ca	Sr	Ca/Sr
HCl	*P. alba*	66	0.76	88	85	0.84	104
	P. nigra	63	0.77	83	83	0.77	111
	P. x euramericana	66	0.69	97	76	0.79	96
HNO_3	*P. alba*	37	0.71	57	36	0.38	94
	P. nigra	54	0.75	75	41	0.42	100
	P. x euramericana	55	0.70	83	78	0.58	175

Element concentrations are given in terms of mg Kg^{-1}. Element ratios are given in molar proportions

If other ratios are compared, Sr/Ba ratios in HCl fraction were found lower than exchangeable and digestible fractions reported by Bullen and Bailey (2005). The values of Ba were found about 10 times higher than leachable and exchangeable fractions, while they were very much similar to digestible fraction. The Ca/P ratio was also found lower and the difference was again due to the higher Ca values, while P values were much similar as reported by Bullen and Bailey (2005). These differences may clearly be due to the different soil type, Aquic, Lithic, and Typic Haplorthods, analysed by Bullen and Bailey (2005), and also the extraction procedure used. However, this publication provides one of the few comparisons with the work presented here. The differences found using AAS or ICP show that the element ratios should be considered as indicative by not finite.

Clearly elevated atmospheric CO_2 results changes in the element ratios found in extracts of the soil. In most cases the biggest differences between ambient and FACE was found in the HCl extract of the soil. This was the extract where the greatest increase in soil P was shown in chapter 4. In this fraction a decrease was seen in the Ca/ (Ca+Na) and Sr/Ba ratios under FACE compared to ambient CO_2. The Ca/Sr ratio increased or decreased depending upon the analytical method used.

However, the elevated atmospheric CO_2 is clearly affecting the mineral composition of the HCl extract. The HCl extraction procedure is representative of a poorly available fraction to plant roots, which can only be assessed by exudation of protons and organic acid by plant roots and mycorrhizas (Jones, 1998). This would suggest that under FACE a secondary mineral is forming by biological action either from biomass turnover, in particular roots and mycorrhizas, or due to turnover of soil organic matter due to priming of organic matter (Hoosbeek *et al.* 2003) shown to occur at the EUROFACE site.

Chapter 7

7 Total phosphorus budget

The following chapter presents the total phosphorus budget in an attempt to gain an overall picture of P cycling under ambient and elevated atmospheric CO_2. Biomass and soil bulk density values were used to calculate P in soil measured in living biomass on a g m^{-2} basis. Finally, these results obtained for leaves, roots and stem are combined to an ecosystem P budget on P cycling to assess the overall impact of the elevated atmospheric CO_2.

7.1 Total P in soil

The total P was calculated from the amount of P obtained from inorganic, organic and microbial P. The amounts obtained were converted into g m^{-2} from the results already given in chapter 4 by using soil bulk density data (Table 2.2 p.21) for each plot of experimental site given in Hoosbeek *et al.* (2003). The results are presented in figure 7.1 (A&B) and 7.2 (A&B). The figure 7.1 shows the values obtained for total inorganic P in the soil at depth of 0-60 cm. In non-fertilized plots (figure 7.1 A) there were statistically significant higher levels of P under FACE compared to ambient (P=0.007). The values obtained for fertilized plots are presented in figure 7.1(B), which also show a similar trend of more inorganic P under FACE, but the difference is not as great as in the non-fertilized plots. In the fertilized plots the differences between ambient and FACE were also not statistically significant (P=0.107). No significant differences were also found between species and fertilizer treatments. If the values for the species are pooled in the non-fertilized treatment the total inorganic P was 114 g m^{-2} under ambient and 182 g m^{-2} under FACE, clearly showing the FACE effect. The untreated edges of the plots had a P stores of 103 g m^{-2}, slightly less than the values of the ambient plots.

The total organic P is presented in figure 7.2 (A&B). Again significantly more organic P under FACE in both fertilizer treatments (P=0.025) was shown. The microbial P is not included as the concentration was negligible in soils. The soils of EUROFACE clearly show a FACE effect on soil P in the soil profile sampled to a depth of 60 cm.

Table 7.1: The amount of phosphorus in different soil fractions for 3 species of poplar grown under ambient and FACE, with and without fertilization. The difference FACE minus ambient concentration (g P m^2).

Soil Fractions	Species	Ambient g P m^{-2}	FACE g P m^{-2}	Difference G P m^{-2}	Mean g P m^{-2}
			Non-fertilized		
H_2O	*P. alba*	6.7 (±2.3)	6.6 (±1.8)	-0.1	
	P. nigra	7.2 (±1.5)	7.2 (±2.3)	0.0	0.3
	P. x euramericana	6.9 (±1.6)	7.8 (±1.1)	0.9	
NaOH	*P. alba*	25.6 (±5.8)	37.2 (±4.4)	11.6	
	P. nigra	26.4 (±3.7)	40.1 (±3.3)	13.7	15.3
	P. x euramericana	21.3 (±3.4)	41.9 (±5.2)	20.5	
HCl	*P. alba*	35.9 (±3.4)	80.1 (±36.9)	44.3	
	P. nigra	44.9 (±7.1)	88.2 (±34.7)	43.3	45.5
	P. x euramericana	42.9 (±2.2)	91.8(±20.3)	48.9	
HNO_3	*P. alba*	41.6 (±2.4)	49.3 (±6.6)	7.7	
	P. nigra	38.4 (±5.0)	52.6 (±4.5)	14.2	7.4
	P. x euramericana	41.8 (±4.2)	42.1 (±2.5)	0.3	
OP	*P. alba*	13.9 (±4.7)	17.3 (±0.6)	3.5	
	P. nigra	14.5 (±3.9)	17.3 (±0.9)	2.8	4.5
	P. x euramericana	13.6 (±5.9)	20.9 (±1.8)	7.3	
			Fertilized		
H_2O	*P. alba*	7.0 (±2.0)	4.8 (±1.1)	-2.2	
	P. nigra	7.3 (±2.0)	5.4 (±1.4)	-1.9	-2.0
	P. x euramericana	7.7 (±2.9)	5.7 (±1.7)	-1.9	
NaOH	*P. alba*	30.3 (±6.4)	34.5 (±4.8)	4.2	
	P. nigra	30.2 (±6.7)	30.5 (±3.6)	0.3	2.9
	P. x euramericana	28.4 (±7.5)	32.7 (±1.1)	4.3	
HCl	*P. alba*	43.3 (±8.0)	52.5 (±20.9)	9.2	
	P. nigra	45.0 (±10.8)	77.8 (±38.6)	32.8	24.2
	P. x euramericana	42.6 (±10.7)	73.2 (±32.2)	30.7	
HNO_3	*P. alba*	42.3 (±1.3)	53.4 (±6.9)	11.2	
	P. nigra	40.1 (±2.5)	44.6 (±1.0)	4.6	8.8
	P. x euramericana	41.8 (±3.8)	52.3 (±6.8)	10.5	
OP	*P. alba*	13.2 (±4.1)	15.9 (±2.1)	2.8	
	P. nigra	13.7 (±6.4)	19.3 (±0.2)	5.6	4.2
	P. x euramericana	14.0 (±4.7)	18.2 (±2.4)	4.2	

The contribution of each extraction fraction on g P m^{-2} basis is shown in table 7.1. In the non-fertilized treatment in the water extracted fraction no differences were seen between ambient and FACE. In the fertilized treatment 2 g m^{-2} less P was found in the water extracted fraction under FACE. The NaOH fraction increased under FACE in the non-fertilized treatment, and the difference was greater than the increase in organic P. As discussed in chapter 4, the NaOH extraction removes both inorganic and organic P. In the fertilized treatment an increase in the NaOH extracted fraction and the organic P fraction was also seen under FACE. By far the biggest increase was seen in the HCl extracted fraction in both non-fertilized and fertilized treatments. In non-fertilized

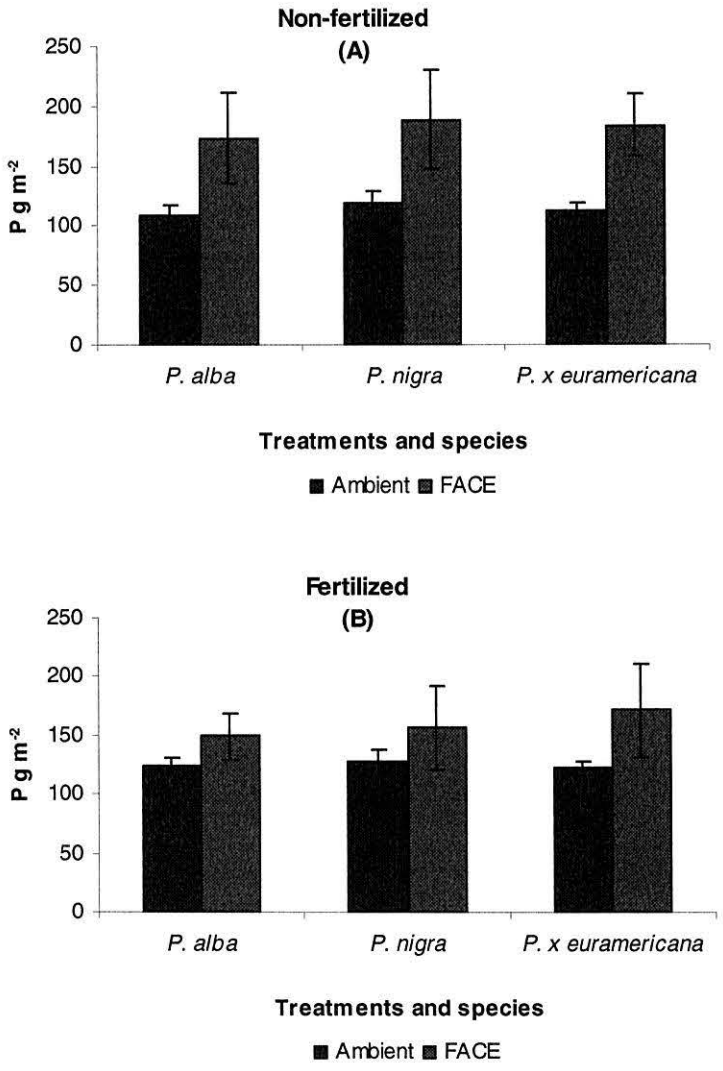


Figure 7.1: Total inorganic P in soil of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the total P value for each species to a soil depth of 60 cm ±SE.

treatment the HCl extracted P contributed 67% of the P increase in soils under FACE. While NaOH and HNO_3 fractions accounted for 22% and 11% of the total P increase. In fertilized plots the HCl extracted P accounted for 61% of the increase in soil P. The NaOH and HNO_3 were responsible for 10% and 29% of the increase respectively.

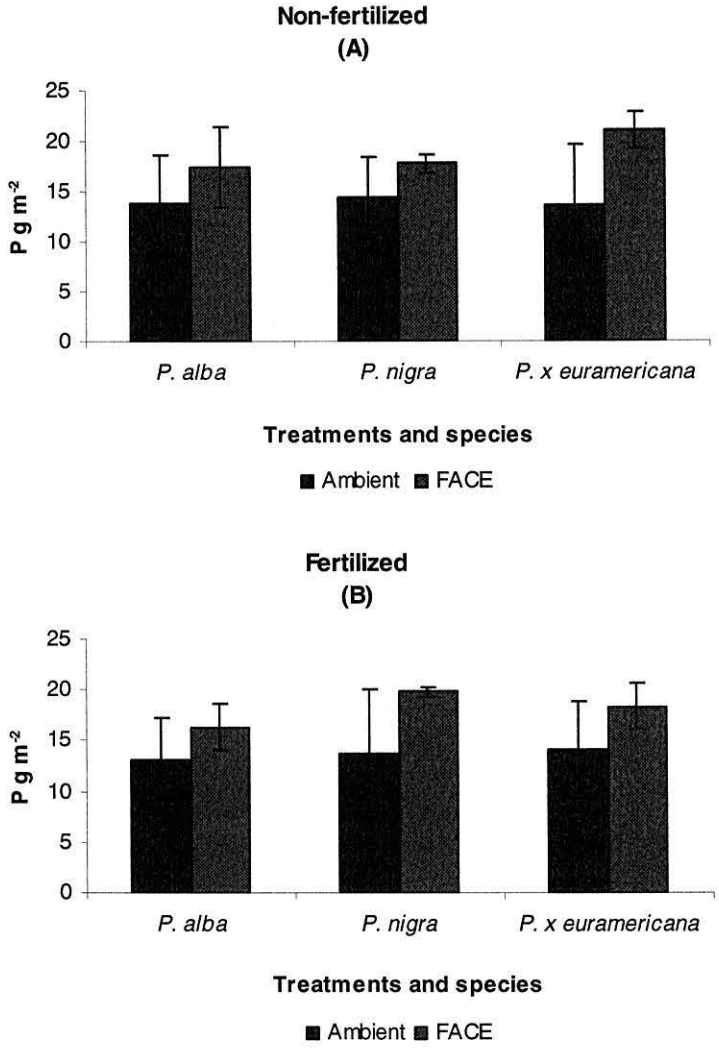


Figure 7.2: Total organic P in soil of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the total P value for each species to a soil depth of 60 cm ±SE.

7.2 Total P in living Biomass

The amount of total P in living biomass including roots, stem and leaf is presented in figure 7.3 (A&B). The values were converted into g m^{-2} by using the P concentrations in mg kg^{-1} given in chapter 5 and biomass data from EUROFACE data

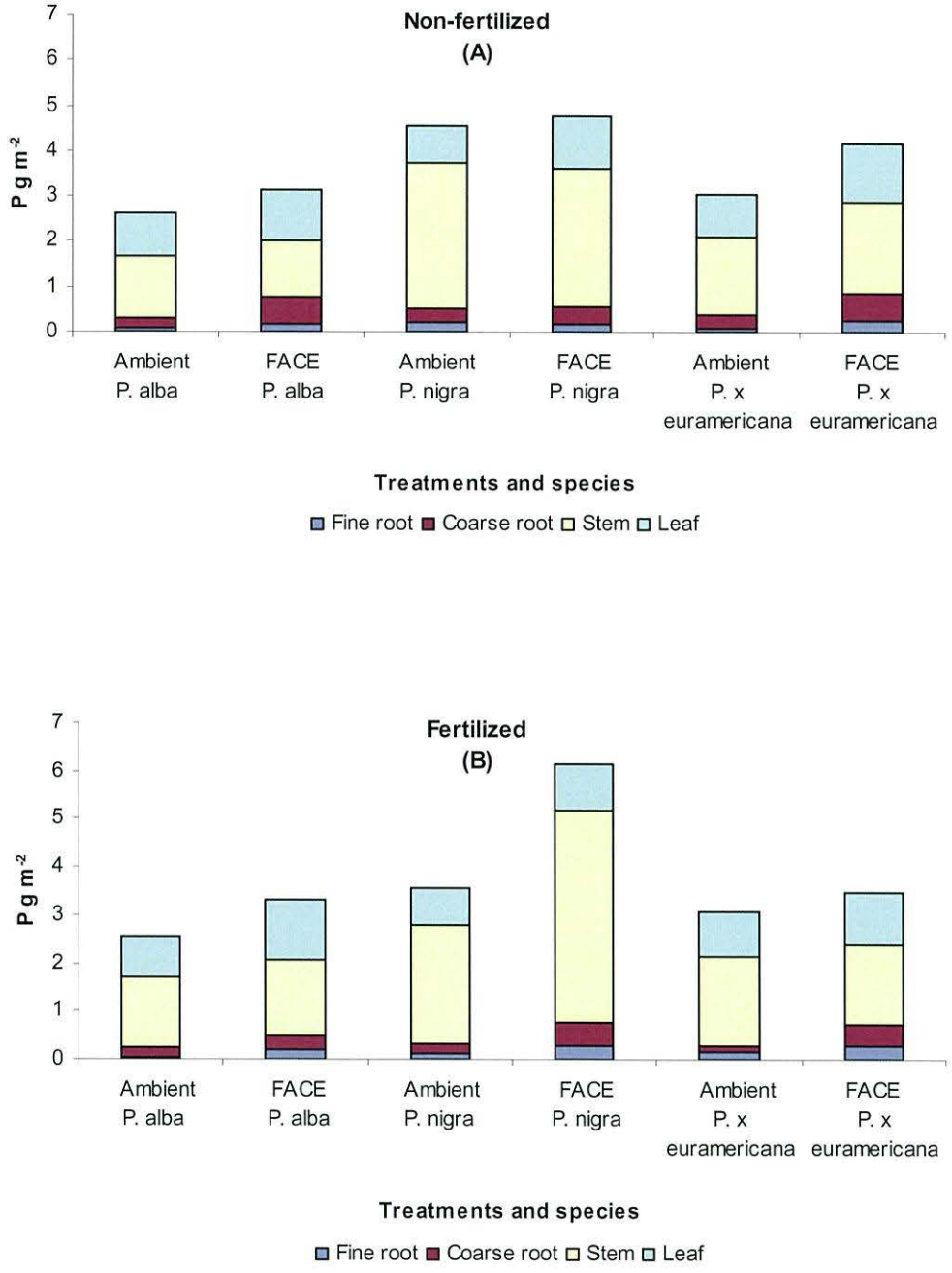


Figure 7.3: The total P in living biomass of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the total P value for each species in root, stem and leaf in different plots.

base, and from Lukac *et al.* (2003), Geilen *et al.* (2005) and Liberloo *et al.* (2006). The figure 7.3 (A) shows the P values calculated for non-fertilized plots. It is evident from the figure that generally there is more P under FACE for all the species and highest values are found in *P. nigra*. In non-fertilized plots the difference between ambient and FACE was found statistically significant only in coarse roots (P=0.009). The same pattern was shown in fertilized plots (Figure 7.3 B), but there were significant differences for leaves (P=0.024), fine roots (P=0.045), coarse roots (P<0.001) and stems (P=0.012) between ambient and FACE. Although analysis of variance of total P biomass in non-fertilized plots showed no significant difference among the different treatments, between the species significant differences were found only between *P. alba* and *P. nigra* (P=0.01) in both ambient and FACE. Total P biomass in fertilized plots showed statistically highly significant differences (P<0.001) among the treatments and species. *P. nigra* was found significantly different from *P. alba*(P=0.017) and *P. x euramericana* (P=0.025) under FACE. In contrast there were no significant differences between *P. alba* and *P. x euramericana* (P=0.050). There were no significant differences between the species in ambient plots.

7.3 Total phosphorus pool

The summed phosphorus in soil and living biomass data is presented in the figure 7.4 (A & B). The figure 7.4 (A) shows the values calculated in non-fertilized plots. For the total P stored in soils and biomass there is more total P under FACE in all the species, and the difference between ambient and FACE is statistically significant (P=0.007). The results of fertilized plots for total P are presented in figure 7.4 (B). A trend which was found very much similar to non-fertilized plots, but the difference between ambient and FACE was smaller in all the species and not statistically significant (P=0.097).

When the values for the species and fertilizer treatment are pooled an increase in P storage of 18% was found under FACE. In non-fertilized plots the increase under FACE was 23%, while it was 12% in fertilized plots.

7.4 P inputs from biomass turnover

The root biomass and turnover data of Lukac *et al.* (2003) was used to calculate the annual P return from fine root turnover in all the species grown at EUROFACE experimental site. The P return from leaves was calculated by assuming a leaf litter

turnover value of 1 per year. The calculated values are presented in figure 7.5 A & B. The figure clearly shows that the P return is greater under FACE in both the non-fertilized and fertilized treatments. In ambient plots of non-fertilized treatment (Figure 7.5 A) the average amounts of P input through leaf and fine root turnover was calculated to be 0.89 and 0.21 g P m^{-2} in ambient plots, and was 1.19 and 0.40 g P m^{-2} respectively, in FACE plots. The average extra P input in FACE plots was thus 0.30 and 0.19 g P m^{-2} through leaf and fine roots turnover. In fertilized plots (Figure 7.5 B) the

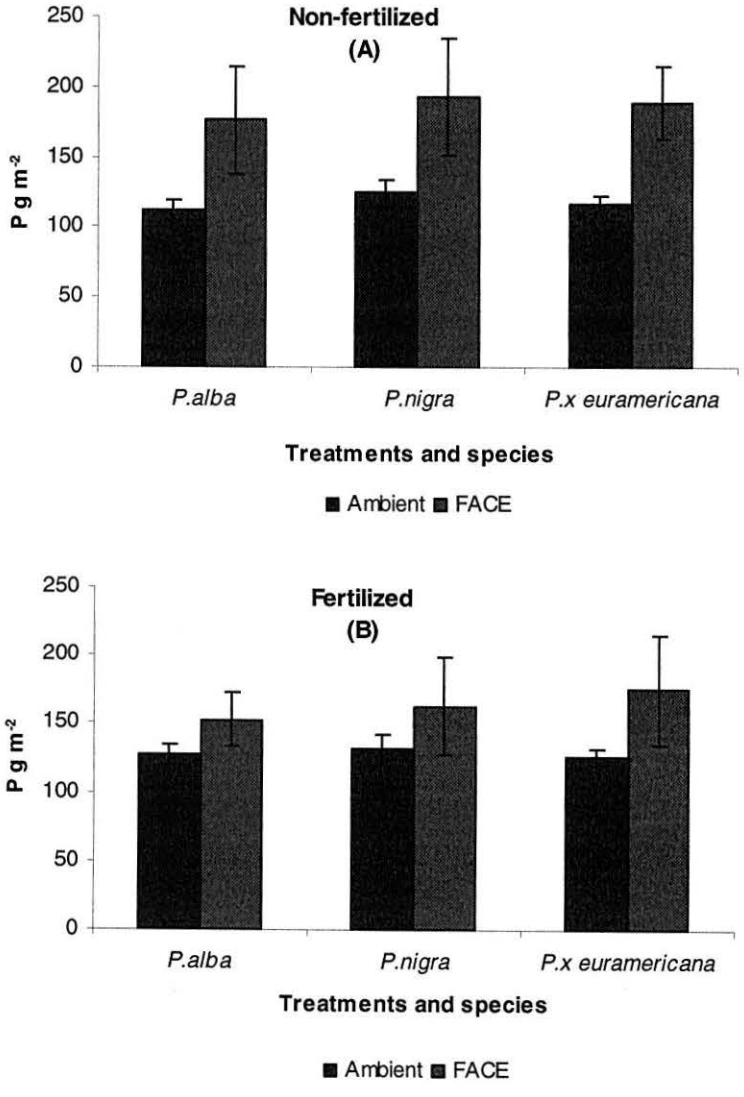


Figure 7.4: The total P storage (soil+ living biomass) of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the total P value for each species with ±SE in different plots.

average values were 0.12 and 0.35 g P m^{-2}. In total 0.49 and 0.60 g P m^{-2} was added to soil through biomass inputs in non-fertilized and fertilized plots each year respectively.

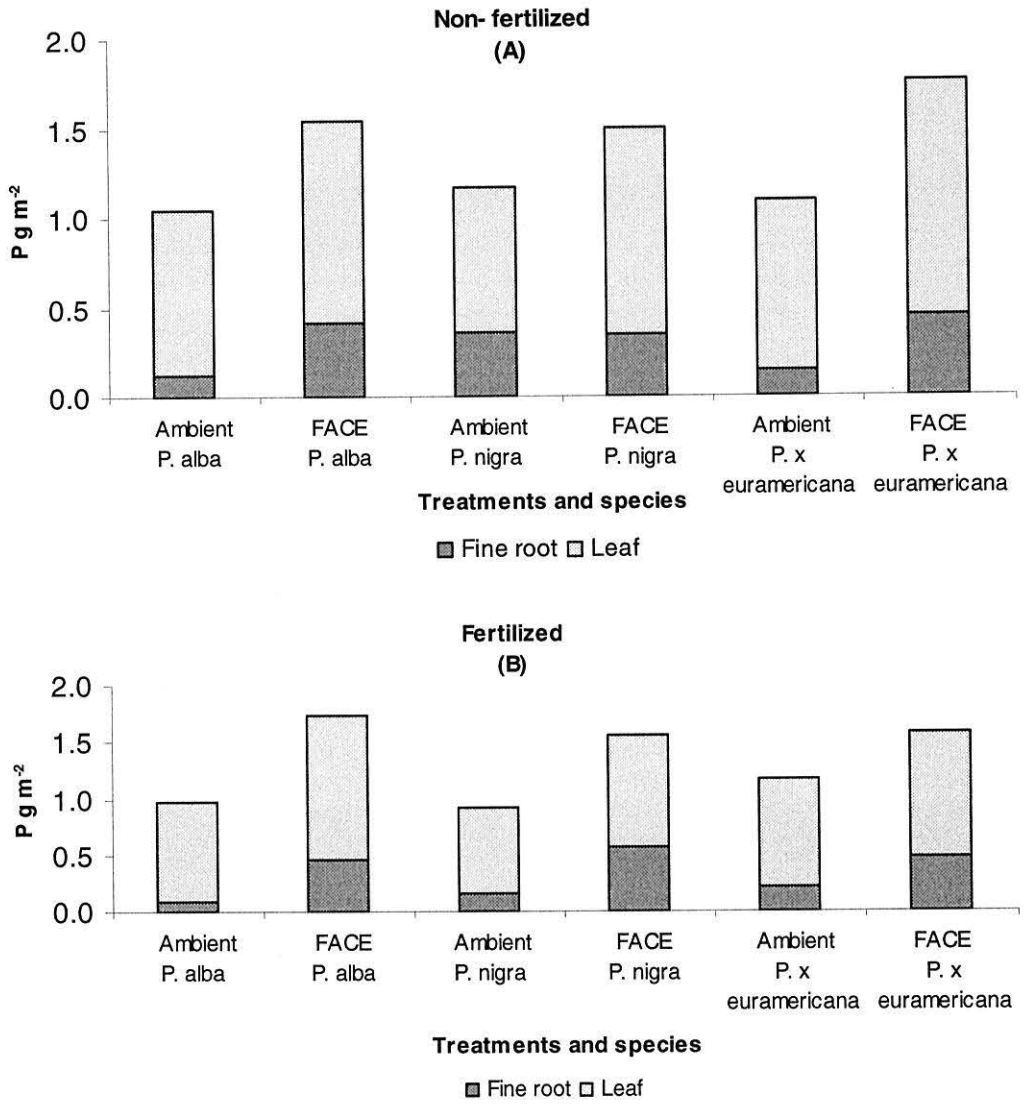


Figure 7.5: The total P return from biomass of 3 *Populus* species grown under ambient and elevated atmospheric CO_2 with and without fertilizer. Data shown is the total P value for each species in root, and leaves in different plots

7.5 Discussion

Phosphorus is limiting for crop yield on > 30% of the world's arable land and, by some estimates, world resources of inexpensive P may be depleted by 2050 (Vance *et al.* 2003). Phosphorus has also been suggested to be a major factor which will limit carbon acquisition of plants and ecosystems in response to increasing atmospheric CO_2

(Conroy *et al.* 1986; Lewis *et al.* 1994; Stocklin *et al.* 1998; Vance *et al.* 2003, Campbell and Sage, 2006). Campbell and Sage (2006) suggested P acquisition is adapted for the low atmospheric CO_2. Plants have evolved a diverse array of strategies to obtain adequate P under limiting conditions, including modifications of root architecture, carbon metabolism and membrane structure, exudation of low molecular weight organic acids, protons and enzymes, and enhanced expression of the numerous genes involved in low-P adaptation (Vance *et al.* 2003).

In contrast to the suggestion that P deficiency will limit the response of plants to elevated CO_2, in this study, the total P in soils (Table 7.2) and biomass (Table 7.3) was shown to be higher under FACE than the ambient treatment. In soils there was an increase in available P particularly in the HCl extractable soil pool which was more than 60% of the total increase in both fertilizer treatments. As discussed in chapter 4, the increase in P in the HCl extractable pool was not due to movement of P from other soil pools, but rather smaller increases were also seen in the NaOH and HNO_3 extractable pools. The increase in the HCl pool thus cannot be explained by movement of P between the pools. The increase in the HCl extractable pool rather suggests that the P pool in the 0-60 cm soil layer has increased under FACE. This can only be the result of P input into the 0-60 cm soil layer from either atmospheric inputs or from deeper soils layers. Lukac *et al.*, (2003) could show an increase in rooting depth of fine roots of all the poplar species under FACE. This suggest that the increase in soil P in the 0-60 cm layer could be a result of uptake of P in lower soil layers and movement to its subsequent higher layers either via root and mycorrhizal turnover. Plants can affect the concentration of P in the soil solution directly by the activity of plant roots or by the activity of rhizosphere microflora (Hinsinger, 2001). The release of root exudates, such as organic ligands can alter the concentration of P in the soil solution (Hinsinger, 2001). Delucia *et al.* (1997) could show an increase in oxalate in the soil under elevated CO_2, hence both increased rooting depth and increased P uptake may have resulted in retranslocation of P pools within the soil profile. If P were moved from lower soil layers to the 0 -60 cm layer the principle method of return to the soil should be via fine root and mycorrhizal turnover. However, the amount of P returned to the soil via root turnover is not sufficient to account for the increase in soil P pools. The difference in the amount of P returned via fine root turnover was 0.19 g m^{-2}, giving a value of ca. 1.1 g m^{-2} for the 6 year enrichment period. Similarly, P return via leaf litter is also insufficient to account for in the increase in soil inorganic P pools. The amount of P

from leaf fall turnover was calculated to be between 0.26-0.30 g P m^{-2} per year assuming leaf litter turnover of 1 per year. As the experiment ran for 6 years, the total P input by litter fall can be estimated to be approximately 1.6-1.8 g P m^{-2}. This however, cannot explain the increase of available P in HCl extractable fraction in soil as this flux is too small when compared to the values obtained for the soil. However, if the rate of return via leaf and root biomass is compared to the increase in organic P under FACE then the P return via biomass is sufficient to account for ca. 50 % of the difference between ambient and FACE.

Elevated CO_2 commonly increases the rate of surface soil CO_2 efflux as a result of increased autotrophic and heterotrophic soil respiration (Hungate *et al.* 1997; Andrews *et al.* 1999; Norby *et al.* 2002; King *et al.* 2004). During the first rotation of the POP/EUROFACE total soil C content decreased under FACE, but more new C was incorporated than under ambient CO_2, indicating a decrease in old soil organic matter (Hoosbeek *et al.*, 2006),. The decrease in old soil organic matter may be due to a priming effect, defined as the stimulation of soil OM decomposition caused by the addition of labile substrates (Hoosbeek *et al.*, 2006). Thus, more soil organic matter was mineralized under FACE, this could be seen in the lower % soil organic matter seen in figure 4.6.

Table 7.2: Total inorganic P at EUROFACE site in 3 species of poplar under ambient and FACE, with and without fertilization, and the difference showing extra P in soils due to FACE effect.

Soil Fractions	Species	Ambient g P m^{-2}	FACE g P m^{-2}	Difference g P m^{-2}
			Non-fertilized	
P inorganic	*P. alba*	109.8 (±7.7)	173.3 (±37.4)	63.5
	P. nigra	116.7 (±5.9)	187.9 (±41.5)	71.2
	P. x euramericana	112.9 (±6.1)	183.5 (±25.5)	70.6
Total		113.1 (±6.6)	181.6 (±34.8)	68.4
			Fertilized	
P inorganic	*P. alba*	122.7 (±7.3)	145.1 (±17.1)	22.4
	P. nigra	122.3 (±5.8)	158.2 (±35.8)	35.9
	P. x euramericana	120.2 (±7.0)	163.8 (±34.7)	43.6
Total		121.7 (±6.7)	155.7 (±29.2)	33.9

Table 7.3: P turnover through biomass inputs showing contribution of leaf and fine roots in 3 species of poplar under ambient and FACE, with and without fertilizer treatment. The difference is FACE minus ambient concentration (g P m^2).

Plant material	Species	Ambient g P m^{-2}	FACE g P m^{-2}	Difference g P m^{-2}
			Non-fertilized	
Leaf	*P. alba*	0.92	1.12	0.20
	P. nigra	0.81	1.15	0.34
	P. x euramericana	0.95	1.30	0.35
Total		0.89	1.19	0.30
Fine root	*P. alba*	0.12	0.42	0.30
	P. nigra	0.36	0.34	-0.02
	P. x euramericana	0.14	0.44	0.30
Total		0.21	0.40	0.19
			Fertilized	
Leaf	*P. alba*	0.88	1.27	0.39
	P. nigra	0.76	0.98	0.22
	P. x euramericana	0.95	1.10	0.15
Total		0.86	1.12	0.26
Fine root	*P. alba*	0.09	0.46	0.37
	P. nigra	0.16	0.57	0.41
	P. x euramericana	0.22	0.48	0.26
Total		0.16	0.50	0.34

FACE significantly stimulated aboveground standing woody biomass up to 44.4% (P. nigra, in fertilized plots). Biomass production at the end of the third growing season did not significantly differ between the three species. Values ranged between 71.0 and 93.0 Mg ha^{-1}; 62.6 and 90.4 Mg ha^{-1}, and 76.2 and 92.8 Mg ha^{-1} for *P. alba, P. nigra and P. x euramericana*, respectively, depending on FACE and fertilization treatment. These values correspond with an average annual aboveground dry matter production ranging between 20.9 and 25.8 Mg ha^{-1} in control treatments and between 28.0 and 31.0 Mg ha^{-1} in FACE treatments, depending on species. There was no significant effect of fertilization on aboveground woody biomass production, although fertilization tended to increase biomass in the FACE treatments (not for *P. alba*). None of the interactions were significant.

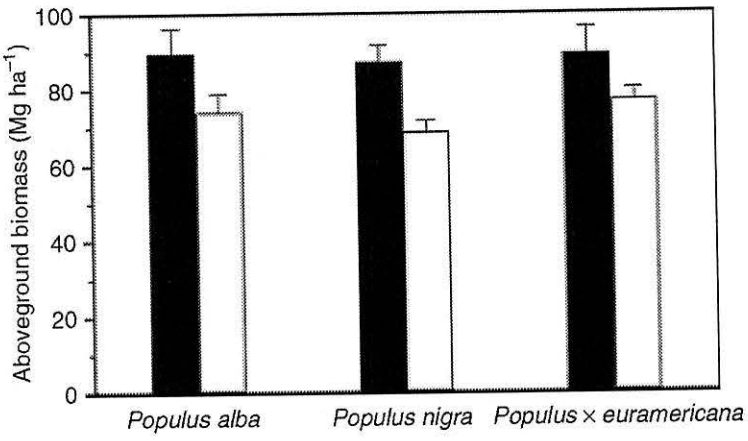


Figure 7.6: Final aboveground standing woody biomass (Mg ha–1) of three Populus species in elevated carbon dioxide (close bars) and control (open bars) treatments under fertilized and unfertilized conditions. As there was no fertilization effect, data are pooled across fertility levels. Trees have been growing for 3 years in the second rotation of a short rotation coppice. Values are means +SE (n = 3). (From Liberloo *et al.* 2006).

The difference of P levels in soils between ambient and FACE is evident in fertilized and non-fertilized plots (Table 7.2). The higher difference in non-fertilized plots is further evidence of N fertilization effect on P availability. It is well documented that N fertilization creates P deficiency as the corresponding increase in nitrogen availability results in the decreased phosphorus availability (Mhorin *et al.*, 1986).

The past studies showed a significant increase in the aboveground biomass (Gielen *et al.*, 2005) could have been causing higher amounts of dust deposition on plant surfaces and ultimately adding more phosphorus into the soil. The seasonal nutrient input by dust to a forest ecosystem in Côte d'Ivoire (Africa) was estimated to be 0.11 kg ha^{-1} for P (Stoorvogel *et al.*, 1997). The atmospheric inputs of P at the EUROFACE site have not been determined, but if inputs similar magnitude are assumed the P content input through dust deposition are again to small to explain the total increase of P in soil.

As biomass and atmospheric inputs are sufficient to account for the increase in P in the 0-60 cm layer, a further possibility is upward movement of soil water. Mass flow of soil solution is thought to occur driven by canopy transpiration (Casper & Jackson, 1997). However, the two investigations of canopy transpiration under elevated atmospheric CO_2 carried out field conditions have both shown lower rates of canopy transpiration under elevated atmospheric CO_2 (Kellomåki and Wang 1998, Wullschleger and Norby, 2001). Again canopy transpiration has not been determined at the EUROFACE site, but the results of Kellomåki and Wang (1998) and Wullschleger

and Norby, (2001) are inconsistent with the idea that water flow form deeper soil layer is higher under FACE.

The increase in total Na seen under FACE is the same order of magnitude as the increase in the HCl extractable P fraction. This suggests that the P in the HCl fraction is composed of a Na and P containing mineral. Similarly a lower Ba/Sr ratio was also found in the HCl extractable P fraction under FACE. This is compatible with the idea that under FACE a higher movement of belowground water brings up P with Na minerals. However, the cause of this increased water movement cannot currently be determined.

Chapter 8

8 Concluding comments

8.1 Does elevated CO_2 have effects on P cycling?

The results obtained in this study after six years of elevated atmospheric CO_2 treatment on three poplar species, planted under ambient and elevated CO_2 levels, clearly showed that there is a FACE effect on P cycling. The effect is found opposite of our original hypothesis.

The investigation of soil analysis for all the species under study and different fertilizer treatments, clearly showed that there is more organic and inorganic P under elevated atmospheric CO_2 conditions. However, the amounts of phosphorus measured in different soil fractions showed that the experimental site is low in P. Elevated CO_2 had an impact on P quantity in the soil profile. The elevated CO_2 is clearly made changes in the soil phosphorus pools positively, especially in HCl extract. Higher levels of total soil inorganic P (+68 g P m^{-2}, P=0.007) and organic P (+26 g P m^{-2}, P=0.096) were found under elevated CO_2 compared to ambient, however there was no difference between tree species. Extracted fractions made different contributions to the available and occluded soil P pools and elevated CO_2 significantly (P=0.029) increased the contribution of both the NaOH extracted fraction, which contains both inorganic and organic P, and the HCl extractable fraction (P=0.001). Under FACE, the greatest increase was seen in the HCl extractable fraction, which contributed 67% of the P increase in soils. Water extractable fraction was not affected by elevated CO_2, while the HNO_3 fraction was marginally increased.

The results for P in different plant materials showed that there is no consistent trend in the samples. The leaves samples showed the only consistent trend that there is relatively higher P content under FACE but this difference is not found statistically significant. In roots non-fertilized samples showed the same trend in two species but the trend was different in *P. nigra* showing high phosphorus in ambient plots. While root samples showed a mix trend in fertilized plots. The wood samples showed a different trend as phosphorus was mostly found higher in ambient plots. The results were again not statistically significant. The P pools in living biomass, fine and coarse roots, stem and leaves was also increased by elevated CO_2, but not significantly (P=0.131). We can conclude from the results presented in this study that there is little or no effect of

elevated CO_2 on the different plant materials in EUROFACE experimental site.

The work on different element ratios clearly showed that different Ca/Sr ratios are obtained depending on the analytical method used. Determination by either AAS or ICP resulted in an almost 10 fold difference in Ca/Sr ratio in the soil fractions. The values were again found higher in FACE plots with significant differences. It is evident that elevated atmospheric CO_2 changes the element ratios found in different soil extracts. In most cases the biggest differences between ambient and FACE was found in the HCl extract of the soil. However, HCl extraction is representative of a poorly available fraction to plant roots, but it can be concluded that the elevated atmospheric CO_2 is clearly affecting the mineral composition of the P extracts.

Thus, in contrast to the widely accepted suggestion that P deficiency will limit the response of plants to elevated CO_2, in this study the P in plant available fractions in soils and tree biomass was shown to increase under FACE. Johnson *et al.* (2004) found in sweetgum under elevated CO_2 a decrease in easily available soil P, but no change in the HCl extractable P fraction. Blum *et al.* (2002) have shown that, in Ca depleted conditions, ectomycorrhizal trees are able to access less available $CaPO_4$ pools. We therefore hypothesised that increased demand for easily available P under elevated CO_2 would in turn decrease the stock of less available P. Contrary to our hypothesis, the HCl extractable pool increased under FACE, with smaller increases or no changes observed in the water, NaOH and HNO_3 extractable pools. The increase in the HCl extractable pool suggests either a transfer of P from occluded soil P pools or from other ecosystem inputs such as P transfer from deeper soils layers or input from atmospheric deposition.

Turnover of plant biomass results in formation of biogenic opaline silica, which more soluble than primary silicates (Kelly *et al.* 1998). Lukac *et al.* (2003) showed an increased fine root turnover and rooting depth of all the poplar species under FACE. Higher rates of turnover of fine roots and associated mycorrhizal hyphae could result in formation of biogenic P fractions, but also movement of P taken up in lower soil layers to higher soil layers. However, the amount of extra P returned to the soil via root turnover in FACE (ca. 1.1 g P m^{-2} for the 5 year enrichment period) is not sufficient to account for the increase in the soil P pools. Similarly, additional P return via leaf litter in FACE (ca. 1.5 g P m^{-2}) is insufficient to account for the increase in soil inorganic P pools. Adding these two biomass turnover inputs together can explain about 50% of the increase of organic P, but not the 45.5 g P m^{-2} increase in the HCl fraction.

Total soil C content decreased under FACE during the first rotation of the

EUROFACE experiment, but more new C was incorporated than under ambient CO_2, indicating a loss of 220 and 819 g m^{-2} of old soil organic matter under ambient and FACE respectively (Hoosbeek *et al.* 2004). Based on the soil organic matter contents, soil phosphorus content and assuming that soil organic matter contains approximately 50% C, the release of P from turnover of organic matter increased by FACE is between 0.4 and 0.7 g P m^{-2}. Again, this is clearly less than the P found in the HCl extractable fraction. Gielen *et al.* (2005) have shown a significant increase in the aboveground biomass under elevated CO_2, which due to larger plant surfaces could lead to a higher amount of dust deposition and ultimately add more phosphorus into the soil. The seasonal nutrient input by dust into a forest ecosystem was estimated to be 11 mg P m^{-2} $year^{-1}$ in Côte d'Ivoire (Africa) (Stoorvogel *et al.* 1997) and 25 mg P m^{-2} $year^{-1}$ in Japan (Tsukuda *et al.* 2006). The atmospheric inputs of P at the EUROFACE site have not been determined, but, if inputs of similar magnitude are assumed, the P input through dust deposition is again too small to explain the increase of total P in the soil.

A common response of ecosystems to elevated atmospheric CO_2 is an increase in soil CO_2 efflux, on average between 16 and 39 %, while the EUROFACE site soil CO_2 efflux increased by 31% to 50% (Norby *et al.* 2002, King *et al.* 2004). The increase in both autotrophic and heterotrophic soil respiration results in an increase of partial pressure of CO_2 in soil air (Oh & Richter, 2004), and subsequently in an increase in soil water carbonates (Andrews and Schlesinger, 2001). Andrews and Schlesinger (2001) showed that the increase in carbonic acid concentration in soil water leads to a 25% increase of concentration of Si. A potential source of P forming the HCl soluble material could be P weathering of occluded P pools. The source of elements dissolved in soil water and then taken up by trees can be traced by measuring element ratios (Blum *et al.* 2002., Bullen & Bailey, 2005). We have found changes in the amount of Na and in the Ba:Sr and Ca:(Ca+Na) ratios in the HCl extractable fraction, where a significant decrease was shown in the Sr:Ba and Ca:(Ca+Na) ratio as well as a ca. 5 fold increase in the levels of Na in the soil. The increase in total Na seen under FACE is of the same order of magnitude as the increase in the HCl extractable P fraction. Sodium was the only base element that showed a significant change in the HCl extractable fraction (Khan, unpublished). This suggests that the P in the HCl fraction is may be associated with release from a Na and P containing mineral, or that high carbonate concentration in soil water traps Na. While the element ratios do not allow us to determine the mineral source of P in the HCl fraction, they give a clear indication that it

originates from different soil minerals under ambient and FACE conditions. This process appears to be the only one of sufficient magnitude to explain the increase of extractable P pools under elevated CO_2.

This investigation shows that increased tree growth under elevated CO_2 has not resulted in depletion of P pools in soils, but rather in replenishment and increased storage of P in the rooting zone. Phosphorus limitation will therefore not reduce tree growth in a high CO_2 world. Furthermore, this work suggests that biogenically driven weathering of primary minerals in the rooting zone is sufficient to maintain the replenishment of plant available inorganic P. Since future levels of elevated CO_2 will stimulate biomass production in a diverse range of forests (Norby *et al.* 2005), this increase of phosphorus availability is of global consequence. We have observed a significant increase of available P caused by elevated atmospheric CO_2. At the present we are able to only speculate as to the origins of this extra phosphorus, but our finding suggest that future levels of CO_2 will not necessarily lead to phosphorus deficiency in plants.

8.2 Areas of future research

The experimental site was originally a forest 60 years ago and developed into an agricultural land before the plantation of three poplar species. In future research, to have a better understanding of the effects of elevated atmospheric CO_2 on P cycling, we could select a native forest site, which will give us more accurate information on this topic. The diversity of plant species should have been increased to determine the effect of elevated CO_2 on different forest plants. We would also like to increase the number of plots to study more replicates and variability, but not possible due to financial constraints. We would also try to measure the organic P in all the fractions to get a total organic P in the soil. In future research it is also important to bear in mind that any effect of elevated CO_2 on soils must be done by also finding the soil mineralogy of the site through X-ray diffraction or similar techniques.

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Appendix 1

Hyphal biomass turnover

Introduction

Most terrestrial plants form symbiotic associations between their roots and mycorrhizal fungi, of which AM fungi are the most common (Smith & Read 1997). Ectomycorrhizal (EM) fungi are important for the uptake of nutrients, because the external mycelium extends into the soil and increase the soil volume exploited by the tree roots (Harley, 1989; Smith & Read, 1997). The importance of external mycelium in plant nutrient uptake and carbon flow into the soil is well known, but the number of studies on the production of external mycelium in soil is small (Read, 1992). Most such studies have been performed in the laboratory, while few studies have been performed in the field (Coutts & Nichol, 1990; Lussenhop & Fogel, 1999). In laboratory experiments, it is possible to follow the growth of the external mycelia from mycorrhizal roots by visual examinations (Bending & Read, 1995). Another approach is to measure fungal-specific biochemical markers (Olsson, 1999). For EM fungi, the signature fatty acid 18:2w6, 9 (Olsson *et al.*, 1995), ergosterol (Nyland & Wallander, 1992) and chitin (Ekblad & Nasholm, 1996) have been used.

The plants gain a number of benefits due to wide range of functions performed by mycorrhizas. The advancement in scientific techniques now allow investigation of different functions performed by AM and EM. These functions include nutrient capture (Chalot *et al.*, 2002), water uptake (Auge, 2001) and protection of host plant against pathogens (Duchesne *et al.*, 1988). The nutrient status of the plant also effects mycorrhizal growth as shown by reduced growth of the external mycelium after N addition (Nilsson & Wallander, 2003) and increased mycorrhizal production during plant P starvation (Wallander & Nyland, 1992). However the high abundance of AM in nutrient rich soils (Nilsson *et al.*, 2005) and increased EM growth after apatite additions (Nilsson & Wallander, 2003) suggests the relationship between plant, mycorrhizal and nutrient status is more complex. The ability of EM to capture and transport nutrients is believed to be strongly related to the explorative ability and function of the extramatrical mycelium (Godbold, 2005). The biomass of mycelia can be large (500-700 kg ha^{-1} as suggested by Wallander *et al.* (2001). The researchers have suggested high proportion of EM biomass in forest ecosystem is mycelia (30-87% by that a

Treseder & Allen, (2000); 80% by Wallander *et al.* (2001). In addition to nutrient uptake, another function performed by mycelia is the formation of common mycorrhizal networks between different forest plant species and mycorrhizal species through which nutrients may be transported (He *et al.*, 2003). This group of fungi, colonizes plant roots, forms association with 80% of plant species and is found in nearly every habitat in the world (Smith & Read 1997).

There were no reliable measurements of fungal hyphae death rates until Friese and Allen (1991) estimated a rapid hyphal turnover rate of 5-7 days and more recently Staddon *et al.* (2003), observed rapid turnover of external hyphae (5-6 days) using 14C labelling technique. The mycorrhizal mortality occurs throughout the growing season (Fitter *et al.*, 1997) and these organic matter inputs enter mineral soil directly. Therefore, mycorrhizal along with fine root mortality make a substantial proportion of the organic substrates entering soil, often equivalent to or greater than inputs from above ground plant tissues (Vogt *et al.*, 1986). The biomass of soil bacteria, actinomycetes and fungi is small i-e 1-3% of C and N on an ecosystem basis (Wardle, 1992). However, the flow of substrates through microbial biomass is a key factor in C storage. Carbon derived from mycorrhizal tissue can account for a significantly sized pool within ecosystems (Rillig & Allen, 1999). A sizeable amount of C can be allotted to mycorrhizal tissues but significantly, some of this is long lived in the soil (Graham, 2000). Not all the C derived from mycorrhizas stays in the soil for long- dead tissue is distributed between active and slow soil pools as a function of tissue quality (Parton *et al.*, 1988). While slow mycorrhizal matter consisting of components such as chitin and glomulin might last decades or centuries in the soil, active substrates, which make up 10% of the total soil organic matter, are often lost rapidly to microbial respiration (Mellilo *et al.*, 2002).

To estimate the hyphal biomass turnover we need to determine both total standing fungal biomass and fungal production. Total standing AM biomass can be measured using a marker molecule, the fatty acid 16:1w5 (Olsson *et al.*, 1995), which is not present in other organisms in significant amounts (Baath *et al.*, 2004). EM fungal biomass is more difficult to measure as current marker molecules, ergosterol and phospholipids fatty acid 18:2w6,9 are also present in saprophytic fungi (Baath *et al.*, 2004). One method to control for saprophytic fungi is to disconnect EM fungi from tree roots and measure the reduction of live fungal biomarkers (normally ergosterol or fatty acid 18:2w6,9). The reduction of fungal biomarker is assumed to represent the biomass

of mycorrhizal fungi as only saprophytic fungi will continue to thrive (Wallander *et al.*, 2004). This method can only be a rough estimate of total biomass as a conversion factors used to form biomass values can be unreliable due to variation in marker content in hyphae (Baath *et al.*, 2004). Also, other matter containing the marker may decompose during incubation. This may explain the large estimation of mycorrhizal biomass in Wallander *et al.* (2004). Despite these drawbacks, there are no other methods currently available for EM biomass estimation (Baath *et al.*, 2004).

Ergosterol and chitin analyses for estimations of fungal biomass have become more common (Salmanowicz & Nylund, 1988). Ergosterol is specific to the fungal kingdom (Weete & Gandhi, 1996) occurring mainly as a membrane constituent therefore its content is correlated with the amount of metabolically active fungal biomass (Nyland & Wallander, 1992). This is as ergosterol is broken down or leaked after cell lyses and should be considered a marker for living fungus rather than total fungal biomass (Ekblad *et al.*, 1998). Erroneous estimates are possible in studies of fungal turnover as ergosterol is not found in many AM (Olsson *et al.*, 2003). As recaltricent component of the cell wall (Lezica & Quesada-Allue, 1990; Goody, 2004), chitin content remains after cell death. Therefore, it can be correlated with total fungal biomass, alive or dead. The ratio of chitin to ergosterol can give an indication of the living fraction of fungal biomass (Ekblad *et al.*, 1998). In previous study, Ekblad *et al.* (1995) observed good correlation between chitin-ergosterol ratios and visual estimates of mycorrhizas. The ergosterol contents of both extraradical mycelium and colonized roots are measured by using a HPLC separation and specific detection of ergosterol using a UV detector (Nyland & Wallander, 1992) or a more recently used method utilising tandem mass spectrometry (Larsson & Saraf, 1997). Chitin contents are also measure using HPLC separation technique. The reliability of these methods relies to an extent on the use of correct conversion values to convert chemical marker values to biomass value. This is difficult because with all chemical markers for mycorrhizas, ergosterol content differs between fungal species (Olsson *et al.*, 2003).

There are certain factors affecting mycorrhizal biomass and fungal biomass markers concentrations. Plant communities have strong effects on soil microbial diversity (Godbold, 2005). The groups of mycorrhizal fungi also differ morphologically and functionally. Therefore variations in the turnover and volume of biomass are expected to occur between fungal species and communities. A dichotomy most likely exists between purely mycorrhizal fungi that must associate with roots for long periods

and saprophytic fungi that exist as free-living structures for long periods (Peachey, 2005). For example, *Paxillus involutus* has a high degree of saprophytic and mycorrhizal ability and broad host range (Donnelly *et al.*, 2004). Many common mycorrhizal species do not have extensive mycelium implying other as yet unknown ecological functions (Godbold, 2005). Seasonal variations in chitin and ergosterol were observed in a range of morphotypes and families of mycorrhiza (Wallander *et al.*, 1997). However, many mycorrhizal species growing in natural ecosystem are not well studied. Ectomycorrhizas hyphae can be a large part of the belowground biomass in soils (Wallander *et al.*, 2001), and however of hyphae contributes significantly to soil C (Godbold *et al.*, 2006). As hyphae contains large amount of P, their turnover may be important for P dynamics in soil. In this study we have tried to measure the hyphal biomass turnover through visual analysis and biomass markers, ergosterol and chitin.

Materials and Methods

The soil was collected from Abergwyngregin farm in November, 2003, to investigate the hyphal biomass turnover. The soil was sterilized in an autoclave (Prestige Medical 2100 Classic), then filled in two sets of rhizotrones. The first set of four rhizotrones was placed without plants. The second set was planted with *P. alba* species seedlings placed in a growth room and irrigated twice weekly. The plants were grown for six months and spread roots and mycorrhizae. The plant side of the rhizotrones was separated from another rhizotrone by a 41 μM mesh which allowed only fungal hyphae to pass to the second rhizotrone. Starting from July, 2004 soil samples were taken from both sets of rhizotrones. Weekly 3 soil samples were taken from each rhizotrones randomly using cork borer. The samples were thoroughly mixed and freeze dried. The dried samples were stored in Petri dishes at 4°C for further analysis. The sampling was carried out for 12 weeks.

We determined ergosterol, chitin and visual estimates hyphal biomass were measured for all the samples. The ergosterol was determined by using extraction method adapted from Nyland & Wallander (1992) followed by determination on HPLC system (Varian ProStar). 1g freeze-dried soil was dissolved in 1 mL of cyclohexane and 4 mL of 10% (w/v) KOH in methanol (HPLC grade). Vortexed to desolve and sonicated in pre-heated sonic bath at 70° C for 15 minutes. After sonication heated in water bath for 90 minutes at 70° C. An additional 2 mL of deionized water and 2 mL of cyclohexane was added and vortexed again for 30 sec. Centrifuged at 1500 g for 5

minutes. The top cyclohexane fraction was collected before 2 mL cyclohexane again was added, vortexed and centrifuged as above. The collected cyclohexane was evaporated completely using tap evaporation and extract dissolved in methanol by heating in the oven at 40° C for 15 minutes. Ergosterol is light and air sensitive. The samples were immediately put into a HPLC system to determine ergosterol concentration. The HPLC system comprised Varian ProStar 230 pump, 310 UV-VIS detector with a reverse phase 5 µm HPLC column (15 cm by 4.6 mm).

The HPLC analysis of glucosamine, the main hydrolysis product of chitin, was used to determine chitin content. Soil and chitin preparation and HPLC determination of glucosamine was carried out by using method suggested by Ekblad & Näsholm (1996) with some adaptations for the use of soil samples rather than pure fungus. We hydrolysed 10 mg of freeze-dried soil or chitin (from crab shells, pure Sigma-Aldrich) was acid hydrolysed in 6 N HCl at 100° C for 7 h to yield glucosamine. Glucosamine from hydrolysed soil and chitin was converted to 9-fluorenylmethylchloroformate (FMOC-Cl) derivatives. Separation and detection of FMOC derivatives were carried out according to Ekblad & Näsholm (1996). Excess FMOC reagent and FMOC derivatives are highly fluorescent and appear in the same retention period as glucosamine. They were removed to some extent by vortexing and pipette-washing the sample with 1 mL n-heptane (Gustavsson & Betner, 1990). All solutions were HPLC standard (Sigma-Aldrich).

Visual estimates of hyphal lengths were made according to Koske & Gemma (1989). 0.5 g of soil was homogenised with 30 cm^3 deionised water. The sample was sonicated for 15 minutes, shaken for 5 minutes and then vortexed. The suspension was decanted onto a membrane filter (cellulose nitrate, 45µm), and vacuum filtered. Membranes were flooded with acid glycerol trypan blue solution for 1 h, rinsed with deionised water and vacuum filtered. The membrane was allowed to dry, and a 1 cm^2 , 10x10 square girded square eyepiece formed 11 horizontal and 11 vertical lines intercrossed perpendicularly, incorporated into the lens (x10) of a compound microscope (Axioplan 2 imaging and Axiophot 2, Ziess, Germany). The hyphae on the membrane filters were viewed at x20°, and the intersections between the hyphae and the gridlines were counted. The hyphal lengths were estimated in 30 fields of view on the each membrane. Then these hyphal intersections were used to quantify the hyphal lengths by the method proposed by Newman, (1966) to quantify root lengths.

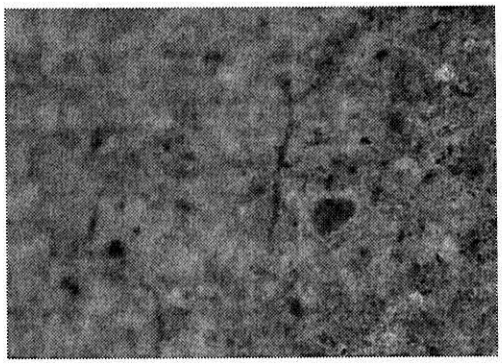

Figure 8.1: Hyphae on membrane filter stained with trypan blue

Results

Extraction of chitin and ergosterol from soils containing organic matter has a number of problems (Ref.). Despite numerous trials and changes in the methodology and HPLC conditions, extraction of chitin and ergosterol could not be carried out successfully. Only the results of the estimates of hyphal production are given which were calculated from visual analysis of hyphal lengths.

The results hyphal lengths calculated by visual method as mentioned above are presented in figure 7.2 (A & B) for the different rhizotrones. The results showed that there is clear difference between the two treatments, the blanks showed very low estimates of hyphal count with mean values in 4 replicates ranging from 1.14 to 5.21 cm g^{-1} of soil during the 12 weeks. While, the 4 mycorrhizal rhizotrones showed much higher hyphal count with average values from 4.07 to 12.56 cm g^{-1} of soil over the same experiment time. The count values remained low in the first 4 weeks and gradually increased from week 5 and maximum count was calculated in week 9. The analysis of variance also confirmed that there are significant differences between two treatments (P= 0.005).

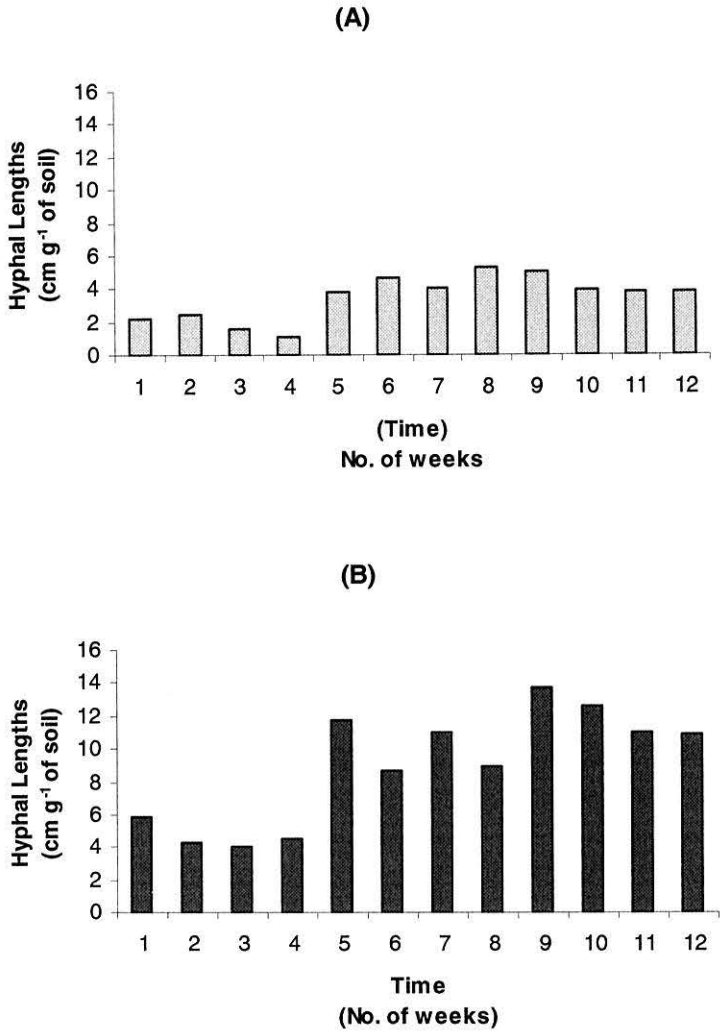

Figure 8.2: The lengths of hyphal biomass in the 12 weeks of trial in blank (A) and mycorrhizal (B) rhizotrones. Each bar represents the mean values calculated in 4 replicates of both treatments.

Discussion

Visual estimates of hyphal lengths suggest that the rhizotrones attached with *Populus* grown rhizotrones started colonizing fungi from the very first week. The population started increasing from the 1st sampling after one week and kept going until the 9th week. The maximum hyphal lengths were achieved in 9th week of sampling and after that it started coming down. This trend of increase in mycelium growth to a peak and followed by a decrease is common with saprophytic mycelia (Boddy, 1999). The visual estimation of hyphae was a relatively cheap technique to employ. However, the cross-grid method is time consuming and is susceptible to human error and bias.

Despite the identification of the glucosamine peak the resulting estimates did not correlate with visual estimates of hyphae. This is not in confirmation with the many

studies in past. Ekblad and Näshlom (1996) and Ekblad *et al.* (1998) found a good correlation between chitin and pure fungal biomass. Wallander *et al.* (1997) found a good correlation between chitin and ergosterol in soil samples. These results suggest that either there is some problem with the method or it was not suitable for the soil used. The results also suggest that either chitin method is measuring chitin from other sources or other substances in the soil are producing fluorescent peaks at the same time such as micro-arthropods and amino acids. However, this was another attempt to find out hyphal biomass turnover and the problems faced are needed to be overcome in future research.