The effect of fluoride pollution on soil microorganisms

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10 ECTS thesis in partial fulfilment of a
Baccalaureus Scientiarum degree in biochemistry

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Reykjavik, October 2011

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Abstract

The influence of fluoride pollution on soil microorganisms was investigated in an Icelandic Brown Andosol. A laboratory experiment was performed where soil cores were leached with fluoride solutions (NaF) of different concentrations and pH. Chemical analyses were performed on outlet solutions and a high fluoride retention capacity of the soil was detected. The effect of the fluoride pollution was evaluated by measuring microbial biomass carbon and phosphatase activity. Phosphatase activity significantly decreased at a fluoride concentration of 1000ppm, which confirmed the inhibitory effect of fluoride ions on phosphatase enzymes and showed that high fluoride concentrations are toxic for soil microbial communities. The microbial biomass did not show any response to fluoride pollution, which questions the reliability of this parameter in short-term experiments. Retained enzymatic activity due to fluoride pollution indicates that acute fluoride pollutions such as those known to follow some volcanic eruptions might have a negative influence on soil health and fertility.

Útdráttur

Áhrif flúormengunar á örverur í jarðvegi var rannsökuð í íslenskri eldfjallajörð, brúnjörð. Tilraun inn á rannsóknastofu var framkvæmd þar sem jarðvegskjarnar voru skolaðir með flúorlausnum (NaF) af mismunandi styrk og sýrustigi (pH). Flúor í útskolunarlausnum var efnagreint og þær niðurstöður nýttar til að meta flúorbindingu í jarðvegi. Áhrif mismunandi flúorstyrks á frjósemi jarðvegs var metinn með því að mæla jarðvegslífmassa og virkni fosfatasa í flúorskoluðum jarðvegskjörnum. Virkni fósfatasa var marktækt minni við hæsta flúorstyrkinn,1000 ppm, sem sýnir að hár flúorstyrkur getur hamlað virkni fosfatasa í jarðvegi og þar með minnkað frjósemi hans. Jarðvegslífmassi brást ekki við ábornum flúori á jarðvegskjarna, hvorki 100ppm né 1000ppm, sem bendir til þess að mæling á lífmassa, á stuttum tímaskala þessarar tilraunar, sé ekki ákjósanlegur mælikvarði á áhrif flúormengunar á jarðvegslíf. Minnkandi ensímvirkni við hækkandi flúorstyrk bendir hins vegar til þess að bráð flúormengun, sem fylgir sumum eldgosum, getur haft neikvæð áhrif á heilsu og frjósemi jarðvegs.

Table of Contents

List of Figures

List of Tables

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1 Introduction

The latest major eruptions of the volcanoes Grímsvötn (21st May, 2011) and Eyjafjallajökull, (14th April, 2010) on the south coast of Iceland, which caused a wide spreading of fluoride-containing ash over farming areas, has made research in the environmental influence of this very reactive and highly toxic compound increasingly relevant.

In spite of low abundance in nature, fluoride can enter the environment in several ways. Volcanic eruptions and weathering of fluoride containing minerals comprise the natural sources, and of anthropogenic sources, application of phosphate fertilizers, emission from aluminium smelters and phosphate fertilizer factories and burning of fossil fuels can be mentioned (Hedley *et al.* 2007, Arnesen 1997, Mirlean and Roisenberg, 2007).

When toxic chemicals are released in this way, it presents an immediate risk to the soil systems that life on earth depends on. The quality of soil determines the type of plant ecosystems and the capacity of land to support animal life and human society. In the future we will most likely be even more dependent on the soil quality since biomass grown in soil seems to become an increasingly important feedstock for fuels and manufacturing as the world supply of fossil fuels is being depleted. In addition, most of the fibres we use for lumber, paper, and clothing industries have their origin in soils of forests and farmlands (Brady and Weil, 2002).

One property of soil is that it works as Natures recycling system, where waste products and dead organic material are assimilated and the basic elements made available for reuse. The essential players in this recycling system are the soil microorganisms (Brady and Weil, 2002).

These microorganisms are a part of the biosphere that has received little attention in research so far, when it comes to fluoride pollution. Fluoride is very immobile in soil, which can be beneficial for groundwater resources but have a very opposite effect for the microbial community. Tscherko and Kandelar (1997) performed a study in the influence of atmospheric fluorine deposits on soil microorganisms and found that severe contamination would decrease microbial biomass up to 80%. Accumulation of organic matter close to the fluorine source further showed that the contamination inhibited microbial processes.

Iceland frequently experience volcanic eruptions, and often the ash have shown very high fluoride content (Flaathen and Gislason, 2007). Furthermore Icelandic soils have very high retention of phosphorous, so phosphate fertilizers, which contain a natural amount of fluoride, must be applied in large amounts (Arnalds, 2004). Finally there are three operating aluminium smelter plants in the country, which constitute a risk for fluoride pollution, so research is of especially great importance in Iceland.

2 Review of Literature

2.1 Soils in Iceland

Soils of the earth have a wide variety of compositions. The soil type, which is subject to study in this research, is classified as a Brown Andosol and is the most common soil type in Iceland along with Cambic Vitrisols. Brown Andosols normally have pH in the interval 5.5-7.5 and contain a considerable amount (>6%, typically 15-30%) of allophane, which is hydrous aluminium silicate clay. Ferrihydrite is a hydrous ferric oxyhydroxide mineral, which is also common in Andosols. Organic build-up is another characteristic of Icelandic soils. The main pathways for accumulation are formation of allophane-organic matter complexes and metal humus complexes and the cold Icelandic climate furthermore favours the build-up, as mineralization processes are slower at low temperatures. Andosols are generally fertile but a tendency to immobilization of phosphorous is a limiting factor (Arnalds, 2004)

2.2 Microorganisms in soil

Soil is a very complex and vital environment that offers a variety of microhabitats, and therefore the diversity of microorganisms is very large. In fact, in pristine organic soils, the amount of different genomes has been estimated to 11,000 per cm^2! (Brady and Weil, 2002) Microbes inhabit the pores between soil particles and are often associated with plants. The pore space is an ideal habitat because both water and oxygen is present (Ashman and Puri, 2002).

Soil microorganisms are important due to their fundamental role in biogeochemical cycles. In these cycles nutrients are transformed and circulated between reservoirs, and the soil microorganisms play their part with the process of mineralization, where nutrients are converted to inorganic forms that are easily taken up by plants. As a result of the large diversity, an extensive amount of different metabolic processes and enzymes exist, and this makes it possible for these communities to serve a variety of purposes in the modification of chemical species (Willey *et al.*, 2008, Burns and Dick, 2002). The important role of microorganisms in agriculture and in the maintenance of a good environment is therefore indisputable.

The microbial transformation of nutrients is metabolism-related, so a good estimate of soil fertility will be the activity of key enzymes. Enzymes are highly sensitive to environmental changes and have therefore been widely used in soil pollution research. (Tcherko and Kandeler, 1996, Acosta-Martinez and Tabatabai, 2000, Burns *et al*., 2002)

The measurement of soil phosphatases can be of relevance since phosphatases are present in all organisms as the enzymes responsible for dephosphorylations, which is one of the most important ways for regulating metabolic pathways. Additionally bacteria, fungi and some algae are able to secrete these enzymes outside the cell when they are in shortage of P substrate. As exozymes, phosphatases catalyze the mineralization of organic phosphates in the surrounding environment to inorganic forms (Wang *et al.*, 2011). In soil

microbiology, the phosphatases in question are phosphomonoesterases, which hydrolyzes phosphate monoesters, phosphodiesterases, which hydrolyze phosphate diesters and finally pyrophosphatases, which transfers pyruphosphate into orthophosphate (Wang *et al*., 2011). The conversions of organic phosphates to orthophosphates is necessary to make phosphorous available to plants and it is therefore an essential step in the phosphorous cycle, which wouldn't be a cycle without these hydrolyses taking place. The efficiency of the phosphorous cycle is very important since phosphorous often is the limiting nutrient in ecosystems (Manahan, 2000). Icelandic Andosols are good examples of P limited ecosystems, since they can have P retention reaching above 90% (Arnalds, 2004). The availability of soil P in Icelandic soils are therefore of importance in terms of soil fertility in Iceland with farmers often having to apply high amounts of P fertilizer on agricultural fields. Reduced soil phosphatase activity due to environmental contamination of e.g. F- can hence have great environmental and economical consequences (Guðmundson *et al.*, 2005).

Phosphatases are furthermore very relevant in relation to fluoride pollution since they are known to be inhibited by F^-. Activity measurements on these enzymes are therefore one of the methods that will be used in this study to evaluate soil health.

2.3 Basic chemical description of fluoride

Fluorine is the lightest halogen and the most chemically reactive non-metal. It is also the most electronegative atom, and therefore has the ability to make strong hydrogen bonds. The small size of element and ion makes high coordination numbers in molecular fluorides possible, and often there will be good overlap between orbitals, leading to short strong bonds. These can be reinforced by ionic contributions when differences in electronegativities are large (Housecroft and Sharpe, 2008).

Of all metal ions, Al^{3+} makes the strongest bonds to F^-, but also beryllium binds with high affinity (Li, 2003). The bonds in AlF_x–complexes are mostly ionic, and the coordination number and configuration can be different. The structure is influenced by pH; in acidic pH, the form will be AlF_4^- and in the pH range 7.5-8.5, AlF_3 will dominate. Furthermore the fluoride concentration may have an effect on configuration in such a way that as the fluoride concentration increase the coordination number will increase (Schlichting and Reinstein, 1999, Strunecka *et al.*, 2002). Fluoride has low abundance in nature, but is found in the minerals fluorospar (fluorite, CaF_2), cryolite ($Na_3[AlF_6]$) and fluorapatite ($Ca_5F(PO_4)_3$).

2.4 Fluoride in soil

2.4.1 The behaviour of anions in soil

The capacity of soils to store and release chemicals is largely due to electrostatic properties of colloidal particles. Within the mineral fraction of the soil, clay particles exhibit these properties, and within the organic fraction, humus is the charged species. The major sources of charge on soil colloids are 1) hydroxyls and other groups that can release or accept protons and thereby acquire negative or positive charges and 2) isomorphous substitutions resulting in charge imbalances. The charges associated with hydroxyl groups are pH dependent and are therefore called variable charges. The isomorphous substitution happens when cations of comparable size, but different charge is exchanged in crystals of clay minerals. Since there is no pH-dependence, this type of charge is called constant. These charged colloids have the ability to adsorb oppositely charged ions from the soil

solution. Ions in the soil solution are usually hydrated and since the electrostatic forces must act through this water coating they are often weak. Equally charged ions can therefore compete for the charged sites, which are consequently called exchange sites.

In most soils of temperate regions negative charges will be dominant and the soil will have high ability to retain cations, whereas anions will be more easily leached. Anions can be held in soil by the mentioned electrostatic forces and can furthermore react with surface oxides and hydroxides and form very tight bonds. When pH increases, fewer of the variable charge sites will have a positive charge so the anion exchange capacity of the soil decreases.

The space between the particles of solid material is just as important when it comes to movement of chemical species in soil. Both liquid and gaseous species occupy these pore spaces and make exchange processes possible. The liquid fraction is called the soil solution since it contains many different soluble compounds and acts as the intermediary in the ion exchange. Equilibrium will therefore always exist between the ions in the soil solution and the ions retained on the charged colloidal particles. The pH of the soil solution can have large influence on this equilibrium and for the form and structure of chemical species. Furthermore the soil solution usually has very good buffer capacity (Brady and Weil, 2002).

2.4.2 Fluoride in soil

In spite of being an anion, fluoride is very immobile in soil. Saeki (2008) investigated the adsorption sequences of toxic inorganic anions in a representative allophanic Andosol and found that fluoride was the species retained with highest affinity. The main factors that influenced mobility of fluoride is pH and formation of aluminium and calcium complexes (Pickering, 1985; The International Program on Chemical Safety, 2002). But also the chemical form, rate of deposition, soil chemistry and climate has an influence.

When studying the research that has been done on the adsorption of fluoride in soil, there seems to be a clear difference between the results for acidic soils and for calcareous neutral-basic soils.

Most research has been done on acidic soils and it has been found that in soils with pH< 6 fluoride is mainly bound in complexes with either aluminium or iron (e.g. AlF^{2+}, AlF_2^{+}, AlF_3, AlF_4^{-}, FeF^{2+}, FeF_2^{+}, FeF_3) (Elrashidi and Lindsay, 1986).

As mentioned aluminium is the metal with the highest affinity for fluoride and it is also the most abundant metal in the soil (Rayner-Canham and Overton, 2003). It is present in free hydrous oxides, aluminosilicates, and other minerals, and the possibility for fluoride binding therefore lies in the replacement of OH^- -ions in the free hydroxides, and in replacement of surface ligands in crystal lattices (Tinker and Nye, 2000).

The OH-displacement by fluoride in an acidic soil were investigated by Romar *et al.* (2009), and it was found that within the fraction of labile aluminium in the soil, the concentration of Al-OH complexes decreased when fluoride treatment was applied, and the Al-F complexes increased, especially AlF_3 and AlF_4^-. The study showed a correlation between the increase in pH and extractable aluminium, which indicated that the increase in pH was due to the substitution of F- ions for OH- ions (Romar *et al.*, 2009).

Arnesen (1997), investigated acidic Norwegian soils and came to similar conclusions when it was found, that a horizon, which contained more Al-oxides/hydroxides, sorbed

considerably more F than a horizon containing less of these compounds. In the same study it was found that the pH-value, at which maximum adsorption occurred, was pH= 4.8-5.5.

Hedley *et al.* (2007) further investigated the relation between pH, fluoride retention and aluminium content. In this study a comparison of different soil types was made, and it was found that as pH decreased a larger fraction of fluoride was complexed with aluminium. The reason for the decline was said to be that at higher pH, the electrostatic potential of the oxide coatings on soil particles increased and the fluoride ions were repelled.

Farrah *et al*. (1987) also made speculations about the decline in aluminium fluoride complexes when pH increased and hypothesized that when pH rose above 6.5, it seemed that the higher concentration of OH^- -ions won the competition for exchange sites and displaced F^- from solids so the amount of F sorbed or converted to complexes declined.

According to these studies a large amount of fluoride will be associated with aluminium and make aluminium fluoride complexes at acidic pH, while F will be much more abundant in the F- form at neutral-basic pH.

If enough calcium and free fluoride ions are present, formation of fluorite (CaF_2) is a possibility. The solid and free ions will exist in the following equilibrium:

$$Ca^{2+} + 2F^- \Leftrightarrow CaF_{2\,(s)}$$

When fluoride adsorption capacity is exceeded, and the fluoride and calcium ion activities exceed the ion activity product of calcium fluoride, the solid will be formed (Tracy *et al.*, 1984).

Turner et al (2005) studied fluoride removal by calcite, the most stable polymorph of calcium carbonate, $CaCO_3$. It was found that when a fluoride solution came into contact with calcite, adsorption immediately occurred over the entire calcite surface and fluorite precipitated. The amount of fluoride adsorbed was dependant on the pH and the surface area of the calcite particles, in such a way that the largest fluoride removal from solution happened at near neutral pH. It decreased as the pH rose and as the surface area declined.

If $CaCO_3$ is abundant in the soil, either naturally or as a result of liming, it is therefore very likely that fluoride ions will be removed from the solution and precipitate as calcium fluoride. Free calcium ions will have the same effect.

2.5 Biochemical description of fluoride

2.5.1 Toxicology of fluoride

The biochemical role of fluoride in larger organisms can be rather ambiguous. On one hand it is one of the most effective means of preventing caries in teeth, as it replaces hydroxyl ions in enamel, yielding an apatite crystal that is more resistant to acid. Fluoride ions also add a buffering capacity to the plaque fluid, so protons extruded by acidogenic bacteria becomes less damaging. Finally it can be incorporated into bones, where it has an activating effect on the proliferation of osteoblasts and thereby increases bone formation. (Gazzano *et al.*, 2010). Fluoride deficiencies have however never been documented and if the dose of fluoride is too strong (above 2mg/day) it can cause mottled teeth (dental fluorosis) and osteosclerosis. Doses of 20mg/day for a period of 10-20 years can lead to

skeletal fluorosis and renal toxicity. Furthermore intoxications has been put in connection with depletion of energy production through inhibition of the citric acid cycle, muscle atrophy, liver and kidney toxicity, allergy, hypersensitivity, gastrointestinal and skin irritation (Gazzano *et al.*, 2010). The probable toxic dose has been set to 5mg/kg and acute toxicities of fluoride have symptoms as nausea, vomiting, diarrhea and cardiac arrhythmia. (Hayes, 2001, Gazzano *et al*., 2010)

2.5.2 Biochemical effects of fluoride

The main ways, by which fluoride can affect microbial cells, are; 1) By direct inhibition of enzymes like enolase, urease, catalase or phophatase by either F^-or HF. 2) Through effects of aluminofluoride or berylliumfluoride complexes that can act as phosphate analogs and affect phosphate translocating enzymes such as phosphatases. 3) Finally by uncoupling of oxidative phosphoryation as a result of HF acting as a transmembrane proton transporter (Marquis *et al.*, 2002).

HF as an uncoupler of oxidative phosphorylation

HF is such a small and polar molecule that it should be able to cross biological membranes through water channels, including aquaporins (Marquis *et al*., 2002).

The presence of hydrogen fluoride depends on the position of the equilibrium:

$$H_3O^+ + F^- \Leftrightarrow HF + H_2O$$

The pKa for hydrogen fluoride is 3.45 in dilute solutions (Housecroft and Sharpe, 2008) and HF is therefore characterized as a weak acid according to the definition that strong acids have pKa-values below that of the hydronium ion (pKa=-1.7).

Thermodynamically, hydrogen fluoride is however highly non-ideal and the activity increase much faster than the concentration. Therefore HF is a very strong acid in concentrated solutions. Giguère and Turell (1979) studied the low acidity of hydrogen fluoride and it was shown that the ionization process of hydrogen fluoride is actually a double equilibrium:

$$H_2O + HF \Leftrightarrow [H_3O^+ \cdot F^-] \Leftrightarrow H_3O^+ + F^-$$

The first equilibrium does lie far to the right but the formation of the complex means that the activity of H_3O^+ is reduced and this result in the lowered acidity of the ion. If however the concentration of HF is high, another equilibrium will exist:

$$[H_3O^+ \cdot F^-] + HF \Leftrightarrow H_3O^+ + HF_2^-$$

This means that the F^- ion is stabilized and the result is a fast increase in activity of the hydronium ion (Giguère and Turell 1979, Housecroft and Sharpe, 2008). In dilute solutions HF will therefore behave as a weak acid, which means that at least a little HF will be present and the amount will ncrease with decreasing pH. (Giguère and Turell, 1979).

The permeability coefficient of synthetic membranes for HF has been found to be about 10^7 times higher than for F^-, so the predominant movement of fluoride into the cell is likely to be HF in acidic environments even when pH rises well above the pKa (Sutton *et al*.,

1987). In the cytoplasm, where pH is higher, HF will dissociate and give the enzyme inhibitor; F^- and furthermore acidify the cytoplasm with H^+ and cause a reduction in the electrochemical potential over the membrane. HF thereby works as a decoupler of the oxidative phosphorylation. Some studies have concluded that the effect on ΔpH is the most important factor in fluoride alterations of the physiology of microbial cells when pH is low (Sturr and Marquis 1990, Marquis 1995, Marquis *et al*., 2002).

The phosphate analogs

Fluoride forms strong complexes with aluminium or beryllium, and these complexes can mimic phosphate groups and inhibit phosphate-transferring enzymes such as phosphatases, GTPases, ATPases and phosphohydrolases.

The bond length of Be-F, Al-F and P-O is very similar (~1.55Å), and both F and O are electronegative atoms that make hydrogen bonds (Li, 2003). Because of the similarity to the phosphate molecule, the aluminium and beryllium fluorides can enter metabolic pathways and act as phosphate analogs and this can cause disturbances in a broad range of enzymes that act in phosphoryl transfer. Phosphoryl transferring enzymes carry out important reactions in many essential biochemical pathways involved in for example energy transduction, regulation of cell growth and signalling. The most studied type of phosphoryl transferring enzymes, when it comes to inhibition by aluminium fluoride, is guanosine nucleotide-binding proteins, or simply G-proteins. It was in these proteins the mechanism of inhibition by aluminium complexes was first discovered.

G-proteins are characterized by their intrinsic GTPase activity and they are especially important in bio-signalling pathways in larger eukaryotes. All G proteins have the same structural core, and can exist in an active conformation, where GTP is bound, and an inactive conformation, where GDP is bound. The unique property of the G proteins is that they are able to inactivate themselves via a build-in GTPase activity. The catalytic rate is rather slow and therefore all G proteins are equipped with a timer corresponding to this specific delay (Gilman 1994, Sprang 1997, Nelson and Cox 2008).

The GTPase activity can however be inhibited by the mentioned fluoride complexes and this realisation led to further investigation in the mechanism of inhibition. Sternweis and Gilman (1982) were the first to confirm the role of AlF_x-complexes in GTPase inhibition, and also found that Be^{2+} can play a similar role to Al^{3+}. The inhibiting effect of BeF_3 did however seem to be less than that of aluminium containing complexes.

Since it is the binding of either GDP or GTP that decides what conformation the protein assumes, it logically follows that the critical determinant is the γ phosphate of the GTP molecule. This phosphate group interacts with a region in the G protein called the P-loop and induces a conformational change by making hydrogen bonds to specific residues (Nelson and Cox, 2008).

The following figure (figure 2.1) shows the structure of the G protein G_i-alpha-1 mutant with bound GDP (inactive conformation). The P-loop is visible just below the phosphate groups of the GDP molecule.

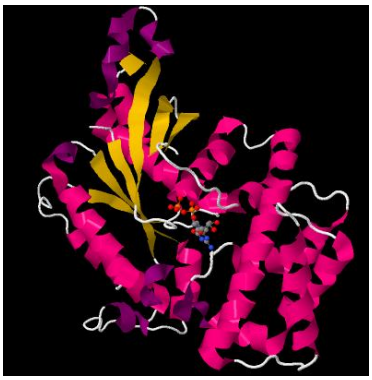

Figure 2.1. Structure of the G protein Gi-alpha-1 mutant in the inactive conformation with bound GDP. The P-loop is visible just below the phosphate groups of the GDP molecule (Kapoor et al., 2009).

As already mentioned, the aluminium and beryllium complexes can act as phosphate analogs and this gives them the ability to bind instead of the essential γ phosphate and render the enzyme into the active state. The binding inhibits the GTPase activity, which is supposed to turn the signal off again and the analogs can therefore seriously alter the pathway (Li, 2003).

The inhibition happens because GDP-AlF_4 not simply mimics GTP; it acts as a transition state analog and therefore binds with even higher affinity than the actual phosphate group. BeF_3, on the other hand, is an analog to the phosphate in its ground state (Li, 2003, Bigay *et al.*, 1987) and therefore Sternweis and Gilman (1982) found this inhibitor to be less effective. The contrast between the two analogs arises because of differences in the structures.

The fact, that aluminium fluoride is a transition state analog, was realised from the action of two amino acid residues that are essential for the catalysis, but do not assist in the binding of the γ-phosphate. These do, however, assist in the binding of the aluminium fluoride, which leads to the theory about the transition state analog.

Figure 2.2 shows schematic drawings of the phosphoryl transfer transition state, and the transition state with bound aluminium tetrafluoride and aluminium trifluoride. Both of the aluminium fluorides have a square planar geometry, similar to the phosphate, and are furthermore bound to oxygen ligands in the apical positions. The oxygen on the β-phosphate acts as the leaving group, and the other oxygen ligand acts as the attacking nucleophile. This second oxygen ligand is believed to come from a water molecule (Wittinghofer, 1997). The phosphoryl group therefore shows penta-coordinated bipyramidal geometry in the transition state. This geometry is not possible for the beryllium fluoride molecule, which is strictly tetrahedral. BeF_x complexes therefore only mimic the phosphate ground state (Chabre, 1990, Golicnik, 2010).

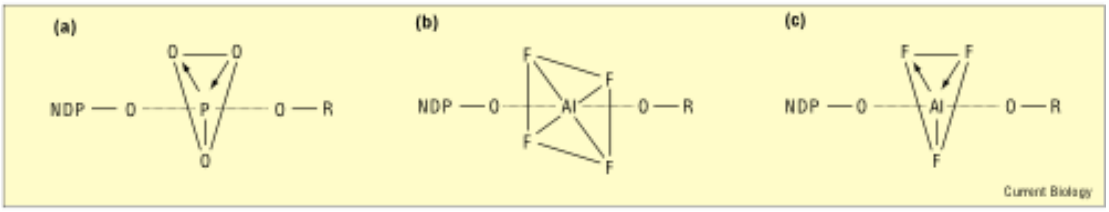

Figure 2.2. Schematic drawings of (a) a phosphoryl transfer transition state, (b) bound aluminum tetra fluoride and (c) bound aluminum trifluoride. Charges are not included; NDP stands for the nucleoside diphosphate and R for the attacking nucleophile (Wittinghofer, 1997).

Similar schematic drawings, with relevant amino acid residues included, are shown in figure 2.3(A) and 2.3(B). It is obvious that the same residues are active in the transition state binding of the phosphoryl group and in the binding of aluminium fluorides

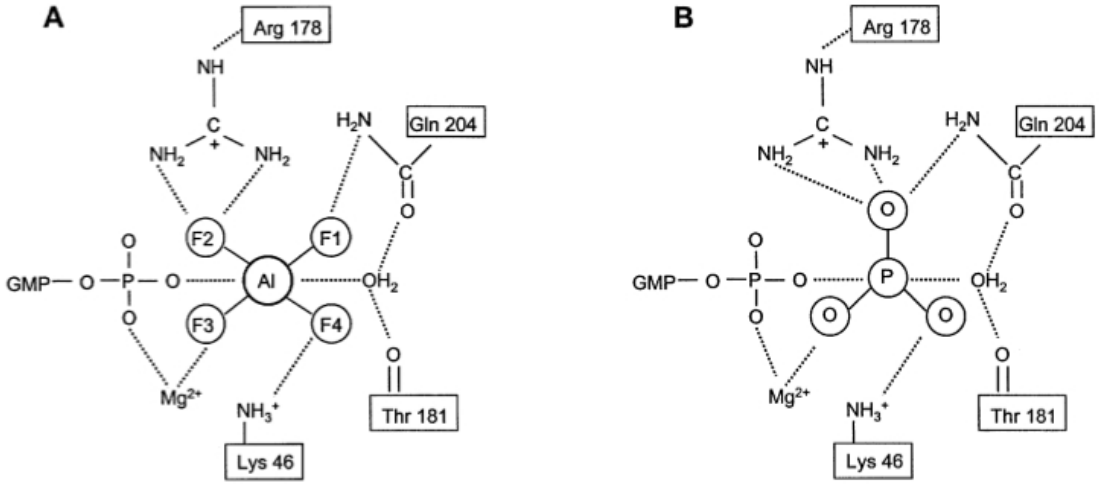

Figure 2.3. (A) Shematic illustration based on crystal structures of $G_i\alpha_1$·GDP·AlF_4^-. The associations of the AlF_4^- with active site residues, the β phosphate and magnesium ions are shown. [B] Schematic drawing of the active site of $G_i\alpha_1$ at the transition state (Li, 2003).

So far the heterotrimeric G-proteins have only been found in eukaryotes (Pandit and Srinivasan, 2003), and the inhibition is therefore less relevant for the microorganisms that are in focus in this study. The mechanism is however so well studied and results widely accepted that later studies on other phosphoryl-transferring enzymes are largely based on the knowledge of the inhibition mechanism in G proteins.

Shortly after the discovery of the inhibitory effect of aluminium fluorides on G proteins, focus was turned to one of the most essential enzymes in all aerobic organisms; the ATPases. Lundari *et al.* (1988) performed a study on both mitochondrial and bacterial F_1 type ATPases (eg. ATP phosphohydrolase, H^+-transporting) and found that micromolar concentrations of fluoride and aluminium ions along with ADP inhibited the ATPase activity. When aluminium ions were exchanged with beryllium ions an inhibitory effect was achieved as well. With the study by Sternweis and Gilman (1982) in mind, it was postulated that the AlF_4^- molecule because of structural similarities to PO_4^{3-} would mimic the γ-phosphate of ATP and that the inhibited fluoroaluminate-ADP-F_1 complex would mimic an intermediate formed during the course of the catalytic cycle of F_1 sector.

Using X-ray crystallography, Braig *et al*. (2000) confirmed the binding of aluminium fluoride in place of the γ-phosphate, when they determined the structure of bovine mitochondrial F_1 ATPase inhibited by the complex of Mg^{2+}ADP and aluminium fluoride. Figure 2.4 shows a schematic representation of the structure of the nucleotide-binding site that resulted from the study. The similarity to the schematic drawings of the nucleotide-binding site in G proteins is striking. The oxygen bindings to aluminium in the apical positions give the same penta-coordinated bipyramidal geometry and interactions with essential lysine and arginine residues are also found in both structures.

Enhanced binding affinity for Mg^{2+}ADP to the catalytic site in the presence of Al^{3+} and F^- ions, along with severity of mutation in residues that assist in the binding of the aluminium fluoride and the fact that the structure represents an intermediate between the known substrate bound form (ATP) and the product (ADP + P_i) complex, all pointed to the conclusion that the Mg^{2+}ADP-AlF_3 is a transition state analog (Braig *et al.*, 2000).

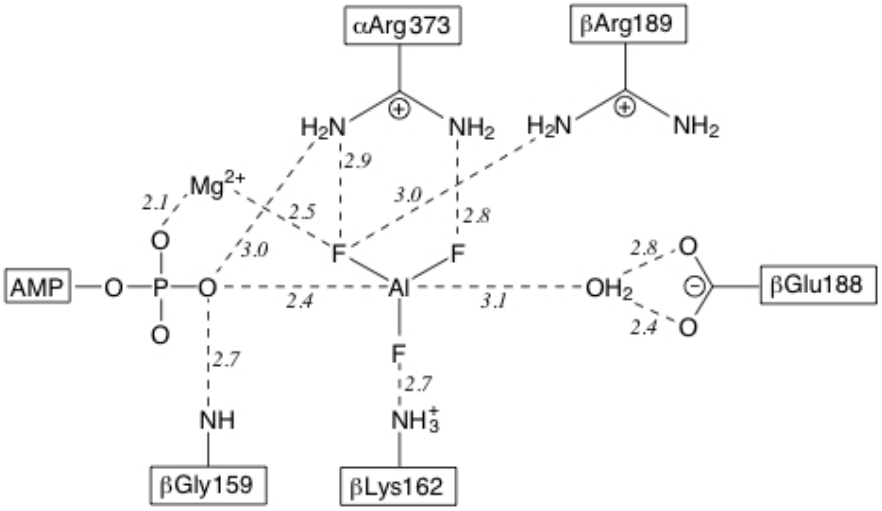

Figure 2.4. Schematic representation of the nucleotid binding site of the AlF_3-F_1 complex. The coordination of the aluminofluoride group is shown and possible hydrogen-bond interactions are shown as dotted lines. Furthermore bond lengths in Ångstrøms are shown (Braig et al., 2000).

With these two examples it has been established that AlF_x, and BeF_3 complexes can mimic the phosphate group and, by binding to nucleotide diphosphates (NDPs), act as transition state analogs. Inhibition by aluminium fluorides has also been shown for liver type-1 protein phosphatase (Bollen and Stalmans, 1988) and phospholipase D, an important signal transduction enzyme active in the conversion of phosphatidyl choline to phosphatidic acid (Li and Fleming, 1999).

The different studies establish the fact the that aluminium fluoride complexes can act as phosphate transition state analogs in a variety of enzymes and therefore are able to influence an array of biological pathways.

Enzymes inhibited by F^-/HF

Fluoride can also bind directly to and inhibit enzymes where the active site contains metal ions. One of the metalloenzymes that is affected by fluoride, and has been widely researched, is enolase. Enolase is a dimeric metalloenzyme, which uses two magnesium ions per subunit. The enzyme is part of the glycolysis pathway, where it is responsible for the conversion between phosphoenolpyruvate (PEP) and 2-phosphoglycerate (PGA). The enzyme can exist in three different conformations. The most closed conformation is assumed when PGA is bound, while the binding of PEP results in a slightly less closed conformation as a loop containing His157 changes position. When no substrate is bound the enzyme will exist in an open conformation.

Warburg and Christian (1941) were the first to realize that fluoride could act as an inhibitor of the glycolysis pathway and later studies confirmed that enolase was the point of action (Kashket *et al.*, 1977, Hata *et al.*, 1990, Guha-Chowdhury *et al*., 1997). In a study performed by Qin *et al*. (2006) it was confirmed that inhibition of the enzyme, and thereby the whole pathway, is the result of the assembling of a $P_i - F_2 - Mg_2$-complex in the active site.

In figure 2.5(A), a ligand assignment in the active site of the enzyme is shown. The inhibiting complex has been superimposed on the active site with the substrate PEP bound (light blue). The fluoride ions are shown in pale green, the inhibiting phosphate group in pink and the two magnesium ions in grey. Water molecules are red. The position of the phosphate group fits with the phosphate group of the substrate and the fluoride ions with the carboxylate oxygens and the complex will therefore have the same chemical characteristics as the substrate PEP. Furthermore, additional hydrogen bonds and a more closed structure are observed in the inhibited complex compared to the native structure. This could mean that the inhibitory complex resembles the transitions state and the extra hydrogen bonds are part of its stabilization. The difference in structure can be seen on figure 2.5(B), where a superposition of the enolase-$Mg_2F_2P_i$ inhibitory complex on the accepted "native" structure complex has been done. It is apparent that subunits A are very similar but the catalytic loop in subunit B of the inhibitory complex assumes a much more closed conformation. (Qin *et al.*, 2006)

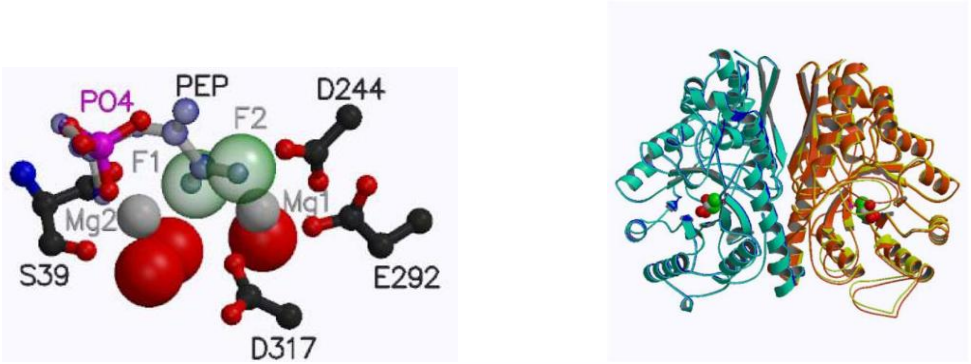

Figure 2.5. (A) Superposition of the enolase inhibiting phosphate/fluoride complex. The fluoride ions are shown in pale green, the inhibiting phosphate-group in pink and the two magnesium ions in grey. Water molecules are red. (B) Superposition of the enolase-$Mg_2F_2P_i$ inhibitory complex subunit A (cyan) and subunit B (orange) and the accepted "native" structure complex (hNSE•$2Mg^{2+}$•P_i/hNSE•Mg^{2+}•Cl^-) where subunit A is shown in blue and subunit B in yellow. The inhibiting complex is shown using the same colours as in (A). It is apparent that subunits A are very similar but large differences between catalytic loops are present in subunits B (Qin et al., 2006).

Although exact structural analysis, as the one just presented for enolase, is hard to find for other metalloenzymes, data of inhibition analysis can be relied on as well.

The zinc-dependant aminopeptidases, which catalyze the hydrolysis of wide range of N-terminal aminoacid residues from proteins and peptides, is another metalloenzyme, which have turned out to be affected by fluoride. Pure uncompetitive inhibition over the pH range: pH = 6-9, was observed by Chen *et al.* (1997) and it was found that the fluoride ion binds instead of OH^-/H_2O in the active site containing two Zn^{2+} ions.

The same inhibitory displacement of water for fluoride in the metallocenter has been observed for urease of the bacteria *Klebsiella aerogenes* (Todd and Hausinger, 2000). This enzyme uses a dinuclear nickel active site to catalyze the hydrolysis of urea and the inhibition is pH independent and does therefore seem to involve F^- rather than HF.

Actually since the fluoride ion, as it does in soil, can bind as a replacement for OH^-, the compound is often used in mechanistic studies when trying to find out whether a hydroxide ion or water molecule is involved in the catalytic mechanism (Marquis *et al*., 2002).

Other studies have shown that F^- can act as a ligand of ferric heme (Winkler *et al*., 1996) and inhibition by fluoride have been confirmed for heme based peroxidases and catalases (Marquis 1995, Marquis *et al.*, 2002). Peroxidases use hydrogen peroxide as oxidizing agent for various substrates and are both important in prevention of oxidative damage and in various other processes as defence against pathogens and conversion of toxins. Catalase contains four porphyrin heme groups and it catalyzes the decomposition of hydrogen peroxide to oxygen and water. For aerobic organisms it is therefore a very essential enzyme in the defence against oxidative damage. The inhibition of catalase by fluoride has been shown to affect the capacities of bacteria to cope with oxidative damage in acidic environments (Phan *et al*., 2001).

The descriptions of the effect on these few selected enzymes show that fluoride can affect many different types of enzymes and not necessarily just the ones that have been subject to research.

F^- inhibition of Phosphatases

Especially relevant for this study on fluoride pollution, is the inhibition of phosphatase enzymes by fluoride ions, since phosphatase activity will be measured as a way to evaluate soil health.

From a mechanistic point of view the phosphatase enzymes can be divided into two groups. In phosphatases such as bacterial alkaline phosphatases, acid phosphatases and protein tyrosine phosphatases, the active site will contain a nucleophile (Ser, His and Cys respectively) which is used to displace an alcohol leaving group and form a phosphoenzyme intermediate, which is hydrolyzed by nucleophilic addition of water. In phosphatases such as protein phosphatases (specifically hydrolyses of serine/threonine phosphoesters) and purple acid phosphatases (PAPs) the attack of water happens directly without the intermediate being formed.

Alkaline phosphatase mechanisms furthermore differ from that of acid phosphatase and tyrosine phosphatase in using metallic cofactors. The three types of mechanisms are illustrated on figure 2.6. Figure 2.6a show the alkaline phosphatase mechanism, where a serine residue acts as the nucleophile and metal residues stabilize the phosphate group. Figure 2.6b shows the active site of protein tyrosine phosphatase where a cystein residue acts as the nucleophile and hydrogen bonding to other residues takes care of the stabilization. The acid phosphatase mechanism will be similar to this, except from the attacking residue being histidine. Figure 2.6c shows the active site of purple acid phosphatase. Here a binuclear metal centre with one divalent and one trivalent metal ion coordinated with 7 invariant amino acids activate a two metal ion bridging hydroxide for taking an active part in the substitution reaction.

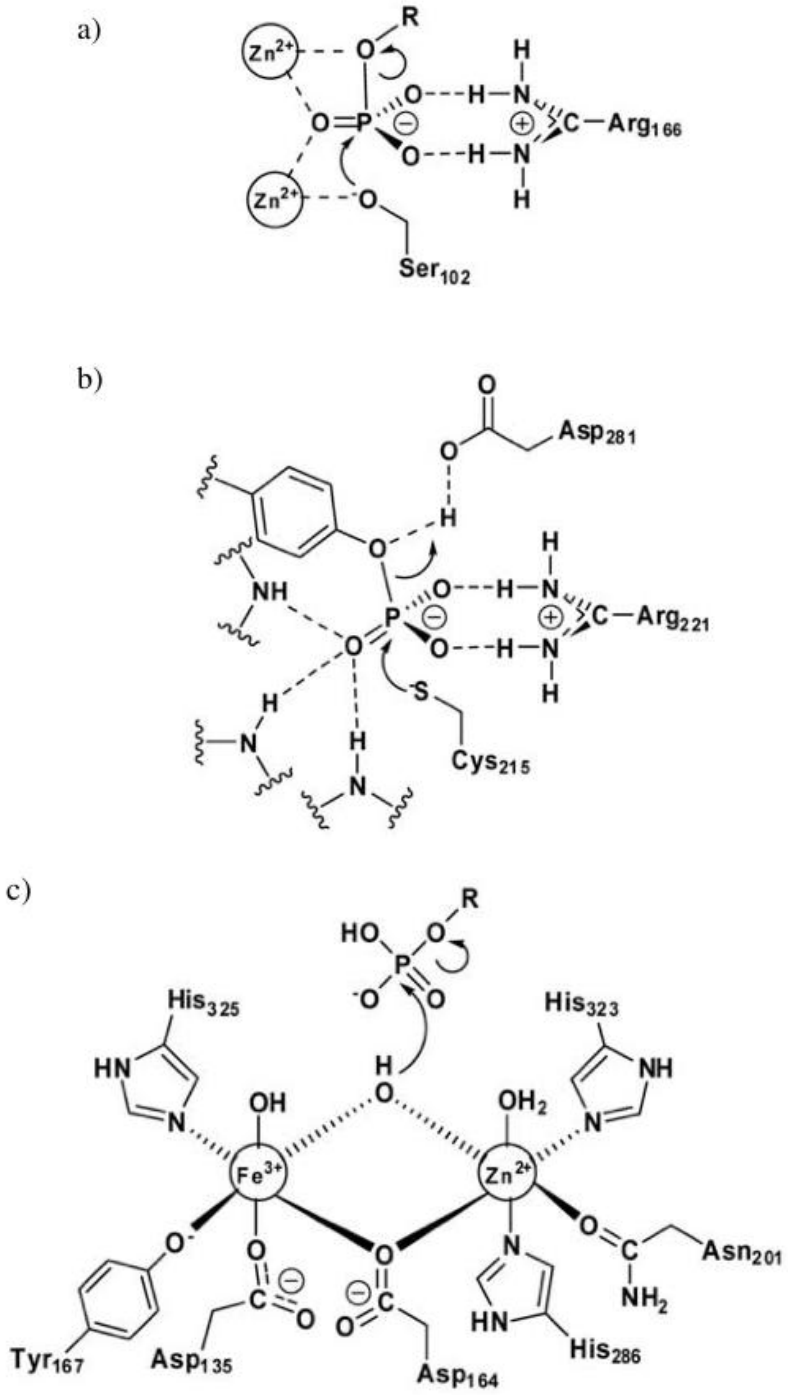

Figure 2.6. Schematic drawings of the active site of a) alkaline phosphatase b) protein tyrosin phosphatase and c) purple acid phosphatase (Golicnik, 2010).

The phosphatases that contain metallic centres (i.e. purple acid phosphatase and protein phosphatases) are inhibited uncompetitively by fluoride. Pinkse *et al.* (1999) investigated the inhibition of bovine spleen purple acid phosphatase by fluoride and obtained results that suggested inhibition as a result of fluoride binding to the trivalent metal ion instead of the hydroxy group (see figure 2.6c). Substitution of fluoride for the bridging hydroxide could however not be ruled out. Purple acid phosphatases have been found in fungi and DNA sequences for possible PAPs have been identified in prokaryotic organisms such as

cyanobacteria and mycobacteria. This type of phosphatase enzyme is therefore very relevant in connection to soil microbiology.

To the author's best knowledge the exact mechanism of the fluoride inhibition of the phoshatases without metal cofactors have not yet been deduced but the inhibitory effect has been found for acid phosphatase in human kidney, osteoblastic acid phosphatase, osteoclastic tartrate-resistant acid phosphatase, inorganic pyrophosphatase and alkaline phosphatase (Lau *et al.*, 1989, Partanen, 2002, Gazzano *et al.*, 2010).

2.5.3 The toxicity of fluoride

From the basic biochemical description of the different action of fluorides, it follows that the presence of F^- in our environment can have great influence on animal and microbial physiology.

However to evaluate the effect on organisms it is necessary to know how fluoride enters cells. Many of the enzymes that have shown to be affected by fluoride are well protected within membranes and cell walls, so the inhibition by fluoride is conditional on the entrance of the ion and, with the complexes of aluminium and beryllium, also on the presence of the metal ions. As it has already been suggested, the main form by which fluoride enter cells, is HF, so in that way intracellular effects will depend on the external pH and the availability of HF. The effect on exozymes is more straightforward and will depend of what fluoride species are found in the surrounding environment.

3 Aims of the project

The aim of the project is to study the influence of fluoride pollution on selected parameters that may be used as indicators on soil health. The evaluation will be based on measurements of microbial biomass carbon and phosphomonoesterase activity in soil cores subjected to different fluoride and pH treatments. Results will be analyzed in a biochemical perspective. It is hypothesized that the fluoride will have a toxic effect on the soil microbial community and therefore cause a decline in the mentioned parameters.

4 Materials and methods

4.1 Soil

The experimental soil has been classified as a Brown Andosol (Arnalds, 2004). Soils were sieved through a 2mm metal sieve for soil homogenization removing larger aggregates, roots and stones, which is important because the chemical processes of interest will occur under the 2mm scale.

4.2 Experimental setup

21 soil cores were used for the experiment. Each core was made from small PVC cylinders of 4cm in diameter, 10cm in length and with holes in the bottom covered with filter paper (S&S nr. 597). 50 g of soil was placed within the cores which where spiked with 3 different experimental treatments conducted at 3 different pH levels. Treatments included a 100 ppm NaF-solution (pH 3, 7, 10), which will be refered to as "treatment 1", 1000 ppm NaF solution (pH 3, 7, 10), which will be refered to as "treatment 2" and a control treatment where soil cores where leached with de-ionised water. The control treatment will be referred to as "treatment 3". All individual treatments where conducted in triplicates. The experimental setup can be viewed in figure 4.1. Addition of fluoride solution was performed 5 times over a period of 10 days and soil solution leachates collected for chemical analysis. The pH was adjusted with HCl and NaOH

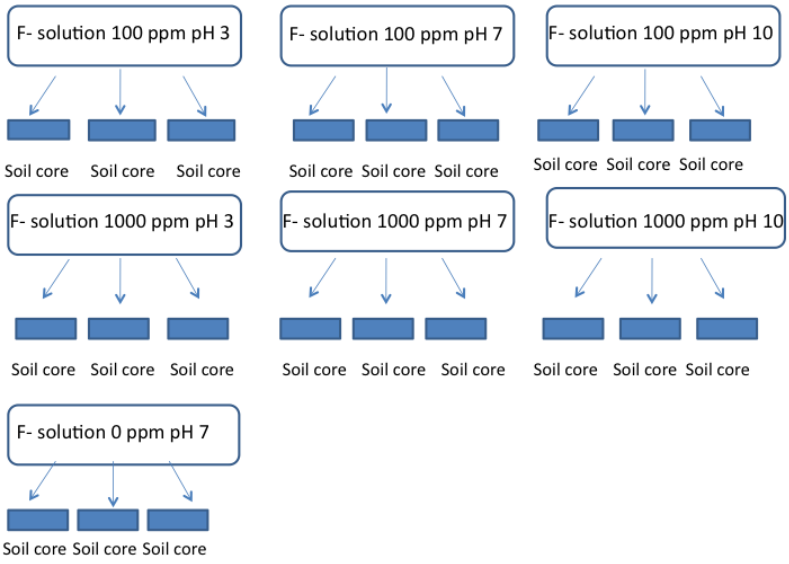

Figure 4.1. The experimental setup included for the 21 cores. Fluoride solutions of 100ppm (pH 3, 7, and 10) and 1000ppm (pH 3, 7, and 10) were used and addition of de-ionized water as a control was used for the reference samples. The experiment was conducted in triplicates.

4.3 Chemical analysis

4.3.1 Experimental soil pH

The pH of the soil was determined using 5g (sieved<2mm) of soil, which was shaken with 25 ml of de-ionized water for 2 hours. Afterwards the pH was measured with a glass calomel electrode (Oakton pH 1000), as well as soil solution pH after each leaching event (Blakemore *et al*., 1987).

4.3.2 Fluoride

The fluoride content of the leachates was found by a modification of the micro diffusion method described by Dabeka *et al*. (1979). To purify the samples and convert all fluoride to the F^- form, a newly designed micro-diffusion instrument was used. The fluoride was extracted from the sample and into a base (1.65M NaOH) by adding 5 ml of sample to an acid bath containing hexamethylenedisiloxane (HMDS) and incubated for 8-10 hours. During the incubation the HMDS complexed with the fluoride and evaporated in to the base.

Before the measurement, the extracted fluoride was diluted in HNO_3 and mixed 1:2 with the buffer "Total Ionic Strength Adjustment Buffer" (TISAB), to set the ionic strength and to add a minor amount of fluoride to weak samples to compensate for low sensitivity of the sensor at concentrations lower than 0.02ppm.

A WQ-FL fluoride sensor (NexSens Technology) was used for the measurement. This ion selective electrode measures the voltage in the solution, which is proportional to the concentration of fluoride ions. Standard solutions were used for the conversion of mV to ppm.

4.4 Physical analysis

4.4.1 Soil moisture content

The moisture content of the soil was determined by drying sieved soil samples at 105°C for >24 hours. The moisture content could thereafter be found as the difference in the mass before and after drying (Page, 1982).

4.5 Biological methods

4.5.1 Soil microbial biomass C

The soil microbial biomass carbon was determined by the chloroform fumigation method (Vance *et al*., 1987). Two 10g aliquots of moist soil were taken from each core and one of the two was fumigated with chloroform to lyse all cells. Fumigation took place in a desiccator with moist tissue paper and a 50 ml glass beaker containing 25 ml acid-washed chloroform (CHC_3) and boiling stones. The treatment was continued over 24 hours.

Both non-fumigated and fumigated samples were extracted with 30 ml of K_2SO_4 (0.5 M) for 30 min. and the amount of dissolved organic carbon (DOC) was analyzed in an aqueous carbon analyzer (LABTOC Pollution and Process Monitoring) with UV digestion and infrared detector.

The carbon analyzer gave results for the carbon concentration in ppm, which were divided by the dryweight of soil and multiplied with the volume of K_2SO_4 solution. This gave the unit mg C/kg of soil. Finally a correction factor, K_{EC} of 0.45 was used as for mineral soils (Sparling and West, 1988). The difference between the carbon content in the fumigated and non-fumigated samples expressed the organic biomass carbon.

4.5.2 Soil phosphatase activity

The measured soil phosphatases were phosphomonoesterases, which include acid phosphatase and alkaline phosphatases. The activity was found according to a modified method of Tabatatai and Bremner (1969) where phosphatase activity was measured at ambient soil pH in each soil core as the experiment was conducted at 3 different pHs (pH 3, 7, and 10). 1 g of field moist soil was placed in a glass test tube, to which 4 ml of de-ionized water, 1 ml of toluene and 1 ml of 0.031 M p-nitrophenyl phosphate (substrate) was added. A marble was placed on the top of the test tube and tubes were incubated at 37°C for 1h. Procedural blanks without soil additions were made. After incubation, 1 ml of 0.5 M $CaCl_2$ (to end the reaction) and 4 ml of 0.5 M NaOH extractant were added. The test tubes were then shaken for 30 s and filtered (S&S nr. 597). Absorbance was determined by UV-vis spectrophotometry at 400 nm (Amersham Biosciences: Ultrospec 2100 pro).

Standards of p-nitrophenol were used to determine sample concentrations. Multiplying the concentrations with total volume followed by division with dryweight and time of incubation then did the calculation of the activity. The activities of phosphatase enzymes were thereby found as mg of substrate converted to product per gram of soil per hour (mg/g/hr).

4.6 Statistical analysis

The statistical software package "R" was used to carry out statistical analysis on the experimental data. Analysis of variance (ANOVA) was used to study the differences between treatments and *t*-tests were used to compare individual treatments when the ANOVA showed significance. All levels of significance are expressed as $p<0.05$.

5 Results

5.1 Results of the chemical analysis

5.1.1 Experimental soil pH

The pH of the soil was measured to pH 7.11. The results for the pH measurements of the leachate collected after each watering are shown in table 1 (appendix 1) where the three replicas have been averaged and the standard deviation (*sd*) calculated. To illustrate the gradual change in solution pH during the treatments, scatter plots showing the pH as a function of added volume of F^- solution (NaF), can be found in figures 5.1-5.3. Figure 5.1 shows the treatments "pH 3, 100ppm" and "pH 3, 1000ppm", figure 5.2 shows the treatments "pH 7, 100ppm" and "pH 7, 1000ppm" and figure 5.3 shows the treatments "pH 10, 100ppm" and "pH 10, 1000ppm". Error bars representing the standard deviation of means have been added to all data points.

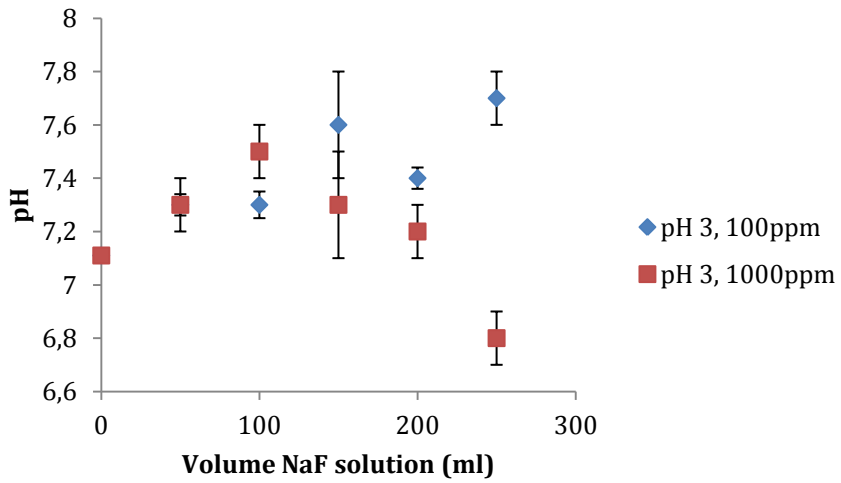

Figure 5.1. Plot of the pH as a function of the added volume of F^- solution (NaF) for the treatments "pH 3, 100ppm"(blue) and "pH 3, 1000ppm"(red). The pH has been averaged for the three replicas.

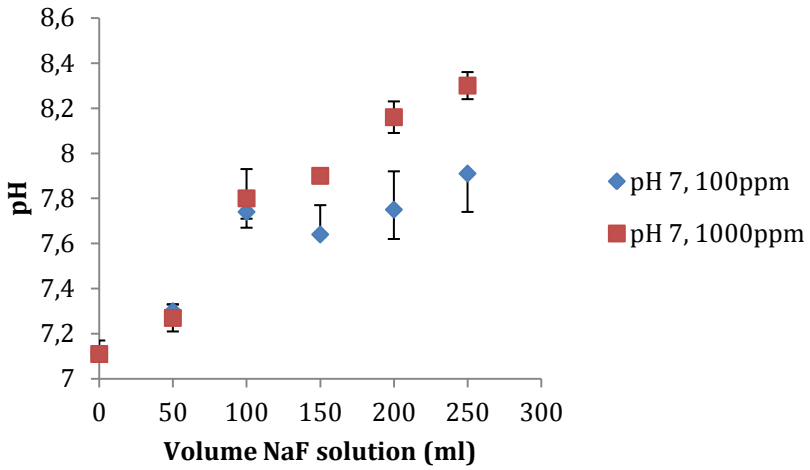

Figure 5.2. Plot of the pH as a function of the added volume of F^- solution (NaF) for the treatments "pH 7, 100ppm" (blue) and "pH 7, 1000ppm"(red). The pH has been averaged for the three replicas.

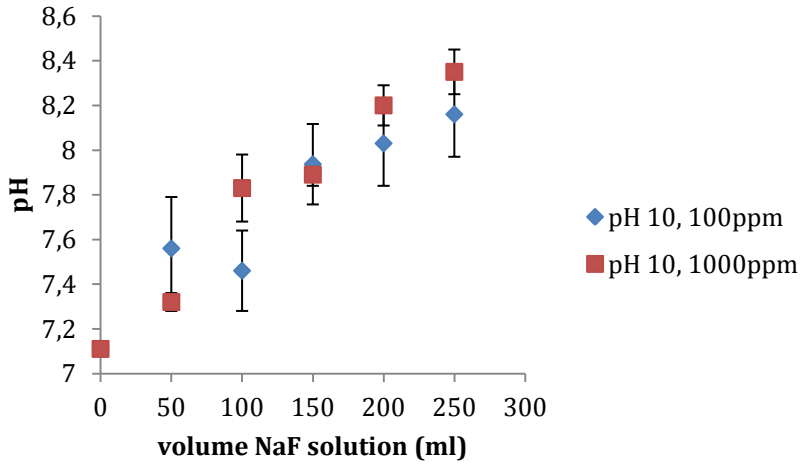

Figure 5.3. Plot of the pH as a function of the added volume of F^- solution (NaF) for the treatments "pH 10, 100ppm" (blue) and "pH 10, 1000ppm"(red). The pH has been averaged for the three replicas.

Treatment 1: Soil treated with 100 ppm F^- solution (NaF) at pH 3, pH 7 and pH 10.

As it can be seen both in table 1 (appendix A) and on the plots (fig. 5.1-5.3), the pH always increased from the initial soil pH of 7.11 to 7.7 at pH 3, to 7.9 at pH 7 and 8.2 in pH 10 solutions respectively.

When the final pH of the cores (250ml) was compared statistically for the three treatments ("pH 3, 100ppm", "pH 7, 100ppm" and "pH 10, 100ppm"), it was found that only "pH 3, 100ppm" and "pH 10, 100ppm" gave final pH-values that differed significantly from each other ($p<0.05$). There was no significant difference between the treatments "pH 3, 100ppm" and "pH 7, 100ppm" or between the treatments "pH 7, 100ppm" and "pH 10, 100ppm".

Treatment 2: Soil treated with 1000 ppm F^- solution (NaF) at pH 3, pH 7 and pH 10.

The treatment "pH=3, 1000ppm" was the only treatment that resulted in an overall fall in soil solution pH. The observed decrease was from the initial value of 7.11 to 6.68 (see table 1).

Table 1 further shows that the pH of the soil cores given the treatments "pH 7, 1000ppm" and "pH 10, 1000ppm" increased from the initial soil pH of 7.11 to 8.30 at pH 7 and 8.35 at pH 10 solutions respectively.

When an analysis of variance was done for the final pH-values (250ml) resulting from the three treatments ("pH 3, 1000ppm", "pH 7, 1000ppm" and "pH 10, 1000ppm"), a p-value below 0.05 indicated that at least one treatments induced a difference in solution pH that was significantly different from the population mean. Further analyses with students t-tests show that, as expected, it was the treatment "pH=3, 1000ppm" that gave a significantly lower solution pH ($p<0.05$) whereas there was no significant difference between the treatments "pH=7, 1000ppm" and "pH=10, 1000pmm" ($p>0.05$). When treatment 2 was compared to treatment 1 with student's t-test, no significant difference in final pH values was found ($p>0.05$).

Treatment 3: Control soil cores treated with de-ionized water (DI)

An increase in pH was also observed for the cores given the control treatment (DI-water). Here the pH changed from the initial value of 7.11 and up to 8.1 during the five additions (250ml). By using students t-test it was found that the final pH of the control soil cores differed significantly from the cores given the treatments "pH 3, 100ppm" and "pH 3, 1000ppm".

5.1.2 Fluoride analysis

The results for the fluoride analysis of the leachates collected after each watering are shown in table 2 (appendix 1), where the three replicas have been averaged and the standard deviation (sd) calculated. To illustrate the change in fluoride concentration in soil solution as the treatments proceeded, scatter plots, showing the fluoride concentration as a function of added volume of F^- solution (NaF), can be found in figures 5.4-5.6. Figure 5.4 shows the treatments "pH 3, 100ppm" and "pH 3, 1000ppm", figure 5.5 shows the treatments "pH 7, 100ppm" and "pH 7, 1000ppm" and figure 5.6 shows the treatments "pH 10, 100ppm" and "pH 10, 1000ppm". Error bars representing the standard deviation of means have been added to all data points

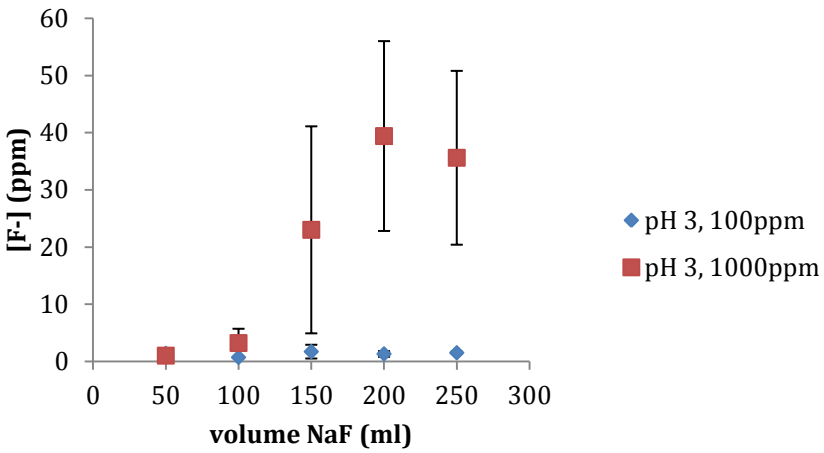

Figure 5.4. Plot of the fluoride concentration (ppm) as a function of the added volume of F^- solution (NaF) for the treatments "pH 3, 100ppm" (blue) and "pH 3, 1000ppm"(red). The fluoride concentration has been averaged for the three replicas.

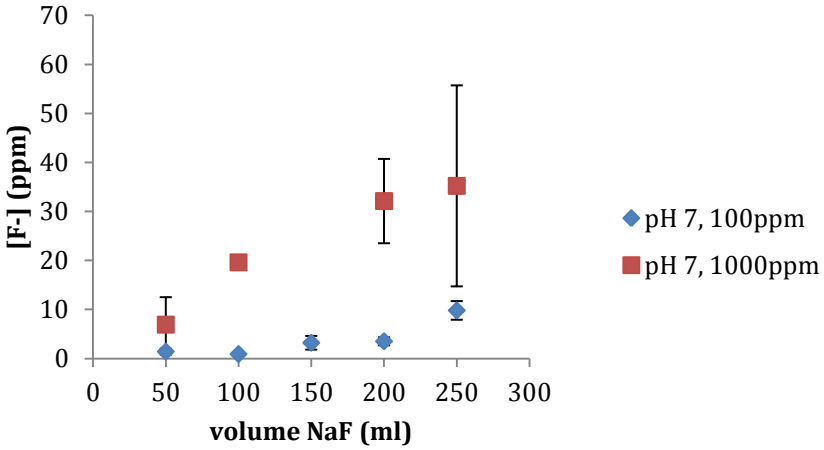

Figure 5.5. Plot of the fluoride concentration (ppm) as a function of the added volume of F^- solution (NaF) for the treatments "pH 7, 100ppm" (blue) and "pH 7, 1000ppm" (red). The fluoride concentration has been averaged for the three replicas.

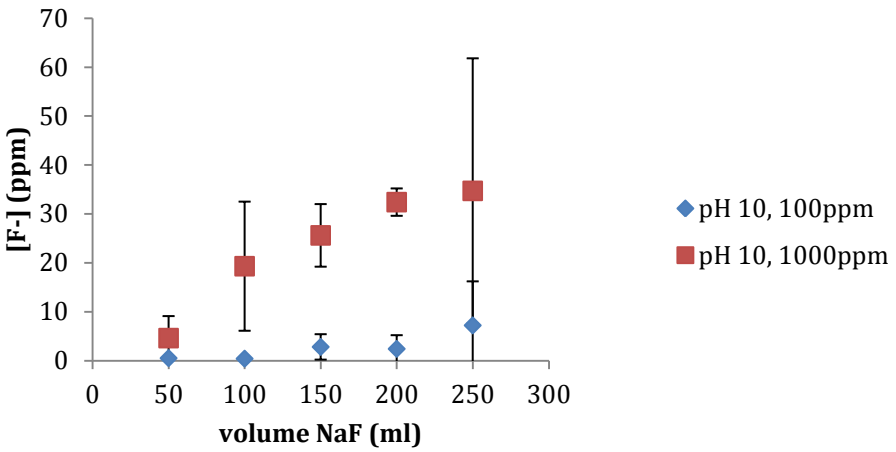

Figure 5.6. Plot of the fluoride concentration (ppm) as a function of the added volume of F^- solution (NaF) for the treatments "pH 3, 100ppm" (blue) and "pH 3, 1000ppm" (red). The fluoride concentration has been averaged for the three replicas.

Treatment 1: Soil treated with 100ppm F^- solution (NaF) at pH 3, pH 7 and pH 10

The data listed in table 2 (appendix A) show that the fluoride concentration in pH 3 leachates remained in the vicinity of 1ppm with no significant differences occurring throughout the experiment ($p>0.05$). All the fluoride that was added to the columns was therefore adsorbed to the soil particles.

At pH 7, the fluoride concentrations in the outlet solutions also remained low for the first four additions (50-200ml) with no significant differences from the pH 3 treatment. An increase to an average of 9.8ppm was however observed in the 250ml leachate (see table 2). The last increase is rather high and analysis of variance followed by student's t-tests showed that the concentrations detected after this final addition differed significantly from all other concentrations measured during the "pH 7, 100ppm"-treatment ($p<0.05$).

At pH 10 the fluoride concentrations in the outlet solutions was comparable to pH 3 and pH 7 treatments, with no significant differences being observed. As for the pH 3 treatment it can therefore be said that all added fluoride tended to be adsorbed to soil particles.

Treatment 2: Soil treated with 1000ppm F^- solution (NaF) at pH 3, pH 7 and pH 10

At pH 3, the fluoride concentrations increased from 1 ppm after the first 50 ml addition to 23 ppm after 150 ml of solution had been added (see table 2). The concentration thereafter remained above 30 during the rest of the experiment.

At pH 7, the fluoride concentration increased from 6.9 ppm at 50 ml to 32 ppm after 200 ml of solution and like the pH 3 treatments remained above 30 ppm during the remainder of the experiment.

The measurements, done after 150ml addition of the pH 7 treatment, showed a fluoride concentration in outlet solutions of 156ppm in average. There was good agreement between the replicas, which is shown by the standard deviation of only 9.9. The difference

from all other measurements is however so large that it has been chosen to leave these data out of account. The data point has therefore also been left out in the graph (fig. 5.5).

At pH 10 the fluoride concentration increased from 4.6 ppm at 50 ml to 25.6 after 150 ml of solution and remained elevated with values comparable to the pH 3 and pH 7 treatments.

Analysis of variance for the treatments "pH 3, 1000ppm", "pH 7, 1000ppm" and "pH 10, 1000ppm" for each of the additions (50ml -250ml), showed that no significant differences existed between any of the fluoride concentrations in the outlet solutions in treatment 2. There was therefore no significant difference in the fluoride retention capacities between the cores.

When treatment 1 (soil treated with 100ppm F^- solution (NaF) at pH 3, pH 7 and pH 10) and treatment 2 (soil treated with 1000ppm F^- solution (NaF) at pH 3, pH 7 and pH 10) was compared, a significant difference was found for the last three additions (150ml, 200ml and 250ml). After these, a significantly higher fluoride concentration was observed in the leachates following treatment 2 ($p<0.05$). This significant difference is also observable on figure 5.4-5.6, where comparisons of similar pH values but different concentrations have been done.

From the standard deviation listed in table 2, it is found that rather large variations often existed between the replicas in the different treatments. As an example the average concentration of fluoride was 39.4ppm after the fourth addition (200ml) of the treatment "pH 3, 1000ppm", but with a standard deviation of 16.6.

Treatment 3: Control soil cores treated with de-ionized water (DI)

The results for the fluoride measurements, performed on the leachates of soil cores treated with the control treatment, are shown in table 2. Low levels of fluoride (<1ppm) were actually detected in these samples although the treatment didn't include fluoride solutions. As an example, the leachate collected after the first addition (50ml) had an average fluoride content of 0.5ppm. The low level is constant throughout the experiment with no significant differences occurring.

When the control treatment was compared statistically to treatment 1 and 2, it was found that the control treatment gave a significantly smaller fluoride concentration in outlet solutions for all additions compared to measurements for the two fluoride containing treatments.

5.2 Biological analyses

Table 3 (appendix A) shows the results for the biological analyses where the activity of phosphatase enzymes and the microbial biomass were measured. The three replicas have been averaged and the standard deviation (*sd)* found. Apart from the treatments 1-3 spoken of earlier, the biological analyses were also done on soil that was completely untreated apart from sieving (2mm). This treatment will be referred to as "untreated".

In order to get a more visual summary of the data, bar plots of both phosphatase activity and microbial biomass can be found in figure 5.7 and 5.6.

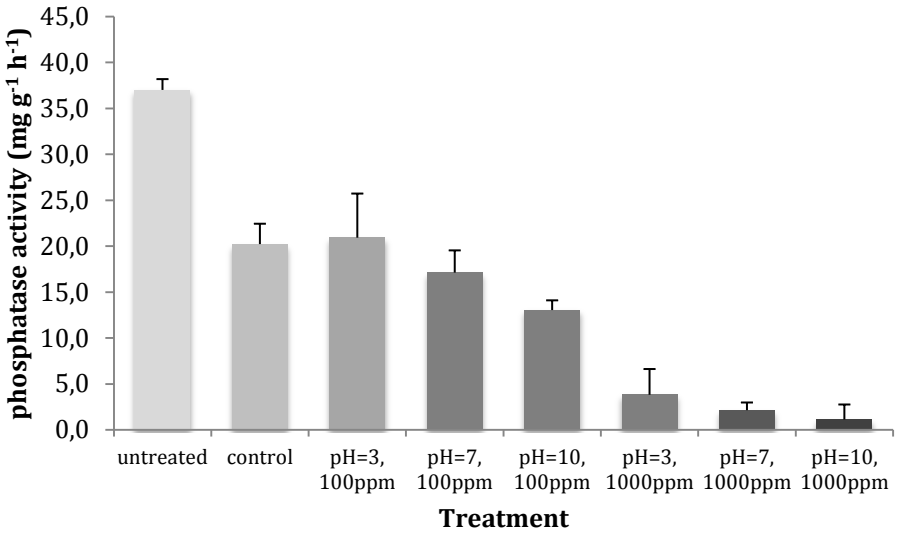

Figure 5.7. Bar plot of the phosphatase activity (mg/g/hr) for the different treatments

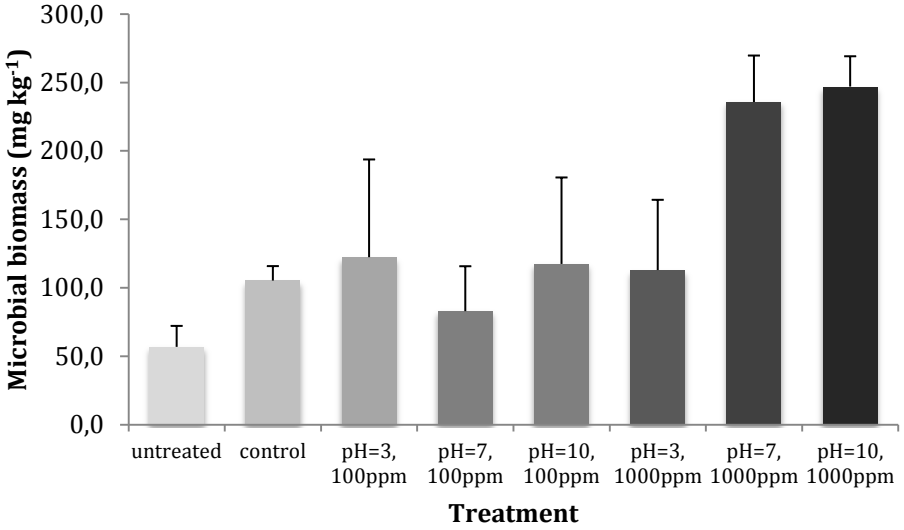

Figure 5.8. Bar plot of the microbial biomass (mg/kg) for the different treatments

5.2.1 Phosphatase activity

Treatment 1: Soil treated with 100ppm F^- solution (NaF) at pH 3, pH 7 and pH 10

As it can be seen in table 3, the cores that were given the treatment "pH 3, 100ppm" had an average phosphatase activity of 20.9mg/g/hr, with a standard deviation of 4.8. The treatment "pH 7, 100ppm" gave the average activity of 17.1mg/g/hr, while the treatment "pH 10, 100ppm" resulted in an average activity of 13.0 mg/g/hr (table 3). Although there is a tendency for the lowest activity to be found at the basic pH and the highest activity for the acidic pH, the difference between the three treatments is not significant ($p>0.05$).

Treatment 2: Soil treated with 1000ppm F^- solution (NaF) at pH 3, pH 7 and pH 10

Table 3 further shows, that the phosphatase activity in cores treated with "pH 3, 1000ppm" was found to be 3.8mg/g/hr in average, with a standard deviation of 2.8. The treatment "pH 7, 1000ppm" gave an average activity of 2.1mg/g/hr, while the treatment "pH 10, 1000ppm" resulted in an average activity of 1.1 mg/g/hr (table 3). As was the case with treatment 1, there is a tendency for the lowest activity to be found at the basic pH and the highest activity for the acidic pH, but again the difference between the three treatments is not significant ($p>0.05$).

When treatment 1 (soil treated with 1000ppm F^- solution (NaF) at pH 3, pH 7 and pH 10) and treatment 2 (soil treated with 1000ppm F^- solution (NaF) at pH 3, pH 7 and pH 10) was compared statistically, a significant difference in phosphatase activity between the two was found ($p<0.05$).

Treatment 3: Control soil cores treated with de-ionized water (DI)

As is visible in table 3, the cores that were given the control treatment had an average phosphatase activity of 20.2mg/g/hr with a standard deviation of 2.2. Statistical analyses show, that the phosphatase activity in the control treatment differ significantly from treatment 2, where soils were treated with 1000ppm F^- solution (NaF) at pH 3, pH 7 and pH 10 ($p<0.05$), but not from treatment 1, where soils were treated with 100ppm F^- solution (NaF) at pH 3, pH 7 and pH 10 ($p>0.05$).

Treatment 4: Untreated soil

The average phosphatase activity measured in untreated soil was 37.2 with a standard deviation of 1.2. This activity was significantly higher than what was found in any of the other treatments ($p<0.05$).

5.2.2 Microbial biomass

Treatment 1: Soil treated with 100ppm F^- solution (NaF) at pH 3, pH 7 and pH 10

As it can be seen in table 3, the cores that were given the treatment "pH 3, 100ppm" had an average microbial biomass of 122mg/kg, with a standard deviation of 71. The treatment "pH 7, 100ppm" resulted in an average microbial biomass of 83mg/kg with a standard deviation of 33, and the treatment "pH 10, 100ppm" affected the soil in such a way that the average microbial biomass was 117mg/kg with a standard deviation of 63 (see table 3). When an analysis of variance (ANOVA) was applied to the data, it did not show any statistical significant difference in microbial biomass between these three treatments ($p>0.05$).

Treatment 2: Soil treated with 1000ppm F^- solution (NaF) at pH 3, pH 7 and pH 10

Table 3 furthermore shows, that the cores, given the treatment "pH 3, 1000ppm", had an average microbial biomass of 113 mg/kg with a standard deviation of 51. The treatment "pH 7, 1000ppm" gave rise to an average microbial biomass of 236mg/kg and the treatment "pH 10, 1000ppm" resulted in the slightly higher number of 247mg/kg. There were no statistical significant difference between the microbial biomass found in cores

given the treatment “pH 7, 1000ppm” and “pH 10, 1000ppm”, but both of these treatments gave a microbial biomass that was significantly higher than what was measured in cores given the treatment “pH 3, 1000ppm”, “pH 3, 100ppm”, “pH 7, 100ppm” and “pH10, 100ppm” ($p<0.05$).

Treatment 3: Control soil cores treated with de-ionized water (DI)

As is shown is table 3, the control treatment resulted in a microbial biomass of 105 mg/kg with a standard deviation of 11. With the use of students T-tests it was found that the microbial biomass in the control soil cores only differed significantly from the treatments “pH 7, 1000ppm”, “pH 10, 1000ppm” and from the untreated samples ($p<0.05$).

Treatment 4: Untreated soil

The untreated soil samples showed an average microbial biomass of 57mg/kg with a standard deviation of 15. As was the case with the control treatment, students T-tests showed that the microbial biomass in the untreated soil only differed significantly from the treatments “pH 7, 1000ppm”, “pH 10, 1000ppm” and from the control treatment ($p<0.05$).

6 Discussion

6.1 Experimental soil pH

Increase in pH during the leaching experiment was observed for all treatments except "pH 3, 1000ppm". When a leaching experiment is performed, an increase in pH is expected as alkaline cations[1] are being leached (Guicharnaud and Paton, 2006).

There were no significant differences between the final pH value of the cores given treatment 1 and treatment 2. The fluoride concentration of the treatment therefore had no influence on the pH of the soil solution in this experiment. Increases in pH as a result of fluoride treatment have been reported in other studies (Arnesen, 1997, Romar *et al.*, 2009), and a larger increase in pH in the cores given treatment 2 would therefore have been expected. The change in pH has been put in connection with the replacement of hydroxide ions by fluoride and it could be, that the leaching didn't last long enough for this effect to be observable, since the buffer capacity of the soil wasn't exceeded in such short time. Andosols are known to have a very high buffering capacity (Guicharnaud and Paton, 2006), which can explain why the soil solution pH didn't respond to the treatment.

The treatments "pH 3, 100ppm" and "pH 3, 1000ppm" gave final pH values that were significantly lower than what was measured for the rest of the treatments. Due to the large buffer capacity of the soil, the difference from the other treatments was however little. When the final pH values of the cores (250ml) is compared, it becomes clear that the leaching didn't cause large changes in the pH of the soil solution, and all cores fall within the neutral to slightly alkaline categories (Soil Survey Division Staff, 1993.

6.2 Fluoride analysis

The results for the fluoride analyses of leachates confirmed that fluoride was largely retained in the investigated soil.

The fluoride concentration in treatment 1 was 100ppm, and measurements showed that essentially all fluoride was retained in the cores given this treatment. The fluoride concentration in treatment 2 was 1000ppm and the leachates from these cores had a significantly higher fluoride concentration than what was observed in treatment 1. The concentrations found in the outlet solutions were however still very low compared to the high concentrations that were added. As an example the absolute highest registered average value was 39.4ppm ("pH 3, 1000ppm", 200ml), which is still only 4% of the added concentration. High retention of fluoride in soil has been found in several other studies as well (Murray, 1984, Bellomo *et al*., 2007, Saeki, 2008).

Accumulations of fluoride means that the soil is a good buffer towards fluoride pollution of groundwater (Bellomo *et al*., 2007) but as Tscherko and Kandelar (1997) mentioned, the

[1] The alkaline cations usually present in soil are Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} and they are called alkaline because they form strongly dissociated bases such as potassium hydroxide ($K^{+} + OH^{-}$) (Brady and Weil, 2002)

high retention also means that soil decontamination rates will be very slow and long-term effects of fluoride pollution is likely.

In other studies the retention of fluoride in soil has furthermore shown a strong dependency on pH. Farrah *et al*. (1987) found that the maximum adsorption of fluoride in soil happened in the pH range 5.5-6.5 and concluded that the adsorption declined at lower pH due to preferential formation of AlFx complexes and declined at higher pH value due to displacement of F^- from solids by hydroxide ions. Other studies, like the ones done by Hedley *et al*. (2007) and Arnesen and Krogstad (1997) also mention pH as a determining factor for fluoride retention.

Due to the short experimental period and large buffering capacity of the soil, differences in final pH values between soil cores were not great enough to have any significant effect on the fluoride retention and therefore the experiment did not manage to show whether pH affected the fluoride retention. Short-term leaching experiments have been reported less stabile than long-term experiment by Hartley *et al.* (2004) who investigated arsenic and heavy-metal pollutions.

The fluoride leaching was largest from the cores treated with strongest concentration (1000ppm), which showed that the retention capacity could be exceeded and high concentration increased leaching. This is in agreement with Tracy *et al*. (1984) and Arnesen and Krogstad, (1997), who also suggested that fluoride retention mechanisms could be saturated. Furthermore Arnesen and Krogstad (1997) reported that as their investigated soil became more polluted, a higher proportion of F was easily soluble. This is in accordance with the experimental result that fluoride concentration in leachates generally increased as more and more of treatment 2 was applied.

Large standard deviations seemed to be a characterizing trait of the fluoride measurements. This could be due to insensitivity in the micro-diffusion method, which was still under development when used, but more likely it is due to preferential flow of water in the columns. Preferential flow happens when water is able to move faster in certain parts of the soil profile and thereby bypasses a large part of the pore-space. This results in a much-decreased interaction with the soil and insufficient time to equilibrate with more slowly moving resident water (Simunek *et al.*, 2003). In other leaching experiment preferential flow has been shown to have a great influence on solute movement and decrease retention of ions (Camobreco *et al*., 1996).

6.3 Fluoride species in the soil

Based on the chemical measurements it should be possible to come with suggestions to what fluoride species are found in the soil.

The soil type generally has a high content of allophane and because of the high affinity of aluminium for fluoride much of the fluoride will probably be found adsorbed to these complexes. Dissolved aluminium fluoride complexes will be of the structure AlF_3, since the pH is neutral to basic in the investigated soil (Strunecka *et al.*, 2002). The concentration of these species in the soil solution is however likely to be minimal since fluoride has been found to exist mainly in the F^- form in neutral-basic soil environments (Elrashidi and Lindsay, 1986) and only complexed with aluminium at lower pH (Hedley *et al*. 2007). The high pH of the soil solution (compare to Arnalds, 2004) indicates that $CaCO_3$-species

are present and since adsorption of fluoride to calcite species is well known (Tracy *et al.*, 1984, Turner *et al.*, 2005), much fluoride will be bound in this form as well. Calcium fluoride, CaF_2 will precipitate from the soil solution and be adsorbed by soil mineral surfaces. The most abundant fluoride species in the soil will therefore be fluoride ions, F^-.

The observation that calcium is a common ion in the soil, makes it possible to argument for leaving out the unusually high fluoride concentration measurement at 150ml of the treatment "pH 7, 1000ppm". Although the equilibrium between CaF_2 and it's constituent ions in soil will be a multiple equilibrium problem to solve, an approximated simple equilibrium calculation with $K_{sp}(CaF_2) = 3.08 \cdot 10^{-11}$ (Garand and Mucci, 2004) and $[F^-]$=150ppm for the equilibrium: $Ca^{2+} + 2\ F^- \Leftrightarrow CaF_2$, shows that the calcium concentration should be as low as 0.01ppm for the solution to remain unsaturated without precipitate formation and decrease in fluoride concentration. A calcium concentration this low is unlikely in a soil with pH 7.11. A disturbance in the soil cores must have made soil particles move down through the column. If these particles escape the filter and enter the sample vial, they will be registered in the fluoride analysis.

6.4 Phosphatase activity

The activities of the measured phosphatase enzymes were affected by the applied treatments.

Phosphatase activities were significantly lower in soil treated with the higher fluoride concentration (treatment 2). The inhibiting fluoride species for phosphatases are fluoride ions, F^- (Gazzano *et al.*, 2010) and as it was rationalized above, the abundant fluoride species in the soil solution were fluoride ions. The inhibition of the enzymes can therefore be explained biochemically. Furthermore it was found that treatment 2 gave a larger amount of soluble fluoride in the soil solution, so the possibility for influences on microbial species is logically largest here. The inhibitory effect of fluoride on soil phosphatase activity is in agreement with results obtained by Polomski (1985) and Wilke (1987).

The fact that the phosphatase activity was unaffected by the lower fluoride concentration in treatment 1 indicates, however, that high doses are necessary before the inhibition is considerable. This is in agreement with conclusions made by Tscherko and Kandelar (1997) who investigated fluoride polluted soil near an aluminium smelter. They found that inhibitory effects of fluoride on soil enzymes and biomass only was significant in highly contaminated soils where F exceeded 100mg kg^{-1} soil.

The highest activity of the measured phosphatase enzymes was found in the untreated soil (treatment 4), where the activity differed significantly from all other measurements including the activity in the control soil cores. This shows that the leaching in it-self also had a negative effect on the phosphatase activity. In a study by Walker (2010) a decrease in phosphatase activity following a trend of increased leaching, was also observed.

Enzyme measurements are generally thought of as good indicators of biological activity in soil as they have a direct relationship to soil biology, are easy to measure and show rapid responses to changes in the environment (Singh and Kumar, 2007). The good quality of data and easily interpretable results of the phosphatase measurements in this study support this idea.

6.5 Microbial biomass

The microbial biomass showed no sign of being affected by the fluoride treatment.

The lowest microbial biomass was observed for the untreated soil. This is not unexpected as a leaching experiment easily results in an increased biomass because water is applied and the increased moisture content is beneficial for microbial growth (Iovieno and Bååth, 2008). Furthermore, the higher temperature in the lab will have a positive influence (Bárcenas-Moreno *et al.,* 2009).

Two treatments gave significantly higher microbial biomass than all other samples. These two treatments were "pH 7, 1000ppm" and "pH 10, 1000ppm". None of the experimental variables (pH and ppm) can explain why this difference exists and the experiment therefore did not confirm any relationship between fluoride pollution and microbial biomass. Effects of pH were not observed either. In a study by Pietri and Brookes (2008) it was found that the microbial biomass was unchanged in this pH interval so no larger variations in biological parameters are expected as a result of different soil solution pH.

A possible reason for the higher microbial biomass in the two mentioned treatments could be that these two samples contained more DOC (C in unfumigated samples) than the rest of the cores (data not shown), so the higher biomass might have something to do with DOC/F chemistry.

As mentioned in the literature review, the direct toxic effect of fluoride on microbial cells will be dependent on entry of fluoride into the intracellular space. As it was proposed in the review, the main way by which fluoride can enter cells is as hydrogen fluoride (Marquis *et al*., 2002), but with the high pH of the soil solution it is unlikely that that this form of fluoride is present. The lacking penetration of fluoride into the cells is a probable reason for the absent response in microbial biomass to the fluoride treatment.

The lowered direct toxicity of fluoride at higher pH values fits with conclusions made by Gazzano *et al.,* (2010) where it is stated that the fluoride toxicity increases at lower pH. It is furthermore speculated that this probably happens because fluoride enters cells faster by diffusion as HF when the pH is low. The phosphatase measurements however showed that extracellular enzymes are very easily affected and this is likely to cause a response in the microbial biomass in the long run, as available nutrients are depleted.

Apart from the missing direct response, another drawback of the microbial biomass measurements is that the chloroform fumigation method counts all microbes, vital or not, as part of the microbial biomass as long as the cell is intact. The method therefore says nothing about the "health" of the microbes.

These factors question whether microbial biomass is a proper variable to include and measure when trying to evaluate soil health and fertility. Problems with the biomass measurements have also been reported in other studies. Dilly and Munch (1995) measured enzymatic processes and microbial biomass in a comparison of different ecosystems and found the two parameters to be uncorrelated. Lehtinen (2010) measured microbial biomass as one of the parameters for evaluating soil health after polychlorinated biphenyl (PCB) pollution and also got a poor response towards the pollution. Enzyme measurements therefore seem more valuable when performing short-time experiments.

A good alternative to the measurements of biomass could be growth rate measurements of soil bacteria and fungi with TdR/leu incorporation which have proved to be very sensitive to changes in environmental conditions including toxins (Rousk and Bååth, 2011)

7 Conclusions

The investigation supports the hypothesis that fluoride has a toxic effect on soil microorganisms. There was a negative correlation between high concentrations of fluoride and the activity of phosphatase enzymes, which is indicative of a reduced microbial activity and thereby reduced soil health. The fluoride concentration did however have to be high before the reduction was significant. The inhibition of phosphatase enzymes could be biochemically rationalized as the abundant fluoride specie in the soil solution was found to be F^-. The microbial biomass showed no response to the fluoride treatment, which questions the validity of this parameter in evaluating soil health in short-term experiments. Finally the applied fluoride was largely retained in the soil, which shows that accumulation of fluoride is possible and long-term pollution effects likely. Deposition of fluoride have followed volcanic eruptions and based on the results in this experiment, it seems likely that retained enzymatic activity due to fluoride pollution from such eruptions can have a negative effect on soil fertility and health.

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Appendix A

Table 1: Results for the pH measurements for each treatment and each 50ml addition of F^- solution. The three replicas have been averaged and the standard deviation (sd) listed.

pH

	50ml	Sd	100ml	sd	150ml	sd	200ml	sd	250ml	Sd
pH 3, 100ppm	7.32	0.04	7.34	0.05	7.64	0.21	7.44	0.04	7.74	0.12
pH 7, 100ppm	7.30	0.06	7.52	0.13	7.64	0.02	7.75	0.07	7.91	0.06
pH 10, 100ppm	7.56	0.23	7.74	0.18	7.94	0.18	8.03	0.19	8.16	0.19
pH 3, 1000ppm	7.31	0.08	7.46	0.12	7.35	0.22	7.17	0.06	6.82	0.08
pH 7, 1000ppm	7.27	0.06	7.77	0.03	7.90	0.01	8.16	0.13	8.30	0.17
pH 10, 1000ppm	7.32	0.04	7.83	0.15	7.89	0.05	8.20	0.09	8.35	0.10
Control	7.62	0.15	7.78	0.09	7.91	0.06	7.91	0.01	8.14	0.05

Table 2: Results for the fluoride measurements in ppm. The three replicas have been averaged and the standard deviation (sd) listed.

$[F^-]$ (ppm)

	50ml	Sd	100ml	Sd	150ml	sd	200ml	sd	250ml	sd
pH 3, 100ppm	1.4	0.6	0.7	0.1	1.7	1.2	1.3	0.5	1.5	0.2
pH 7, 100ppm	1.4	0.6	0.9	0.2	3.2	1.4	3.5	0.8	9.8	1.9
pH 10, 100ppm	0.5	0.02	0.4	0.1	2.8	2.6	2.4	2.8	7.2	9.0
pH 3, 1000ppm	1.0	0.6	3.2	2.5	23.0	18.1	39.4	16.6	35.6	15.2
pH 7, 1000ppm	6.9	5.6	19.6	0.5	156	9.9	32.1	8.6	35.2	20.5
pH 10, 1000ppm	4.6	4.5	19.3	13.2	25.6	6.4	32.4	2.8	34.7	27.1
Control	0.5	0.04	0.3	0.1	0.4	0.0	0.4	0.1	0.6	0.0

Table 3: Results for measurements of the activity of phosphatase enzymes (mg/g/hr) and microbial biomass (mg/kg). The three replicas have been averaged and the standard deviation (sd) found.

	phosphatase activity (mg/g/hr)	***sd***	**Biomass (mg/kg)**	***sd***
pH=3, 100ppm	20.9	4.8	122	71
pH=7, 100ppm	17.1	2.4	83	33
pH=10, 100ppm	13.0	1.1	117	63
pH=3, 1000ppm	3.8	2.8	113	51
pH=7, 1000ppm	2.1	0.8	236	34
pH=10, 1000ppm	1.1	1.6	247	22
Control	20.2	2.2	105	11
Untreated	37.0	1.2	57	15