Plant phosphatase activity under cadmium stress and the impact of phosphatases on phosphorus recovery from sludge

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Abstract

Waste materials like sewage sludge and biosolids are becoming more common with global population growth, and they present both problems and opportunities. On the one hand, they contain significant quantities of dangerous toxins, like heavy metals, but they are also full of organic matter and important chemicals that can be used to fertilize crops.

Biosolids, a type of processed and dewatered sewage sludge, has great potential to be used as a soil amendment, but they often concentrate toxins like cadmium and lead, which makes farmers justifiably distrustful of its use. Phytoextraction, which consists of absorbing a matrix's contaminants into the above-ground organs of plants for subsequent removal, is widely used for heavy metal removal, often with great success. A little-explored factor that could influence plants' ability to tolerate and accumulate heavy metals is phosphatase activity.

Sewage sludge contains heavy metals which must be removed to avoid bioaccumulation in the food chain, and phosphorus that must be extracted to avoid eutrophication in waterways. Among the methods that have been applied to achieve the removal of phosphorus, electrocoagulation is becoming popular due to recent technological advances. In this technique, an electrical current pass between two iron or aluminium electrodes placed in a batch containing the sludge, releasing metal cations that react with phosphate to form an insoluble salt. It may be possible to accelerate electrocoagulation by degrading organic phosphorus at some point before or during the process, as these molecules take more time and more intense currents to degrade.

This project was divided in two parts:

(1) Two fast-growing, high-biomass crop plants, maize (*Zea mays*) and sunflower (*Helianthus annuus*), were grown under different cadmium concentrations to determine the impact the metal has on their early growth. Several parameters were measured, most importantly the activity of three classes of phosphatases in root exudates, root tissue, and shoots.

(2) Electrocoagulation was conducted with filtered suspended sludge either pre-treated with phosphatase or with the enzyme added at the start of the process, and outcomes were compared with chemical precipitation of phosphorus with iron salts.

Cadmium had a significant effect on all growth parameters, reducing shoot height, chlorophyll content, and root length and weight, while increasing root hair area in both species. Activity levels for all phosphatases in root exudates was increased, except for monoesterase at the highest cadmium concentration. Most of the increase in total root diesterase and phytase activities in the presence of cadmium was due to exudate activity, and not root tissue activity. Maize stems showed decreased activity of monoesterase and phytase, and no change in diesterase activity. Sunflower stems had no change in monoesterase activity and increases in diesterase and phytase, but at the highest cadmium concentration, all enzymes had decreased activity. Both root and shoot tissue accumulates cadmium, with a translocation rate from root to shoot higher than expected for young plants: shoot cadmium reached 39% of total plant cadmium in maize and 33% in sunflower. Phosphatase activity, unlike the other parameters, did not necessarily follow the same pattern under cadmium stress as in phosphorus deprivation.

Electrocoagulation combined with phytase was the only treatment that enabled the complete recovery of phosphorus dissolved in sludge. Pre-treatment with phytase did not have great influence on the result of the process compared to phytase added at the beginning of the process, but it did lead to more rapid precipitation for the first 20-30 minutes of electrocoagulation; phytase also lead to greater phosphorus recovery from chemical precipitation with two iron salts, but not complete recovery. Alkaline monoesterase did not lead to complete phosphorus recovery under any treatment, but it contributed to greater precipitation when combined with iron(II) sulphate.

This project provides the first comprehensive profile of phosphatase activity under heavy metal stress in two major crop plants, and the results are relevant for engineering lines that can tolerate and extract Cd from the soil. They also lend credence to the status of maize and sunflower as important phytoextractors. The electrocoagulation experiments provide the first evidence that enzymes can improve the rate of phosphate recovery from sewage

sludge; future research should focus on recovery efficiency with combinations of enzymes and with the conjugation of phosphatases and other promising phosphorus precipitation methods, such as ultrasonic cavitation.

Introduction

Cadmium toxicity

Cadmium (Cd) is one of the most toxic elements in the environment and is capable of causing severe growth inhibition and abnormalities even at low concentration. Soil Cd is absorbed by plants and the animals that consume them, where it bioaccumulates and endangers the food supply and human health (Ueno *et al.*, 2010). In humans, Cd has been shown to cause kidney and bone damage, and to contribute to diabetes, hypertension, cancer and overall mortality (Satarug *et al.*, 2010). In plants, long-term exposure causes roots to become mucilaginous, brown, and eventually to decompose; shoot and root elongation is reduced, with shorter roots being one of the earliest and most distinctive signs of Cd toxicity; leaves roll and become chlorotic and stunted, have decreased chlorophyll content and altered chloroplast structure; eventually they desiccate and necrosis sets in (He *et al.*, 2017; Tran and Popova, 2012). One way in which Cd harms plants is by reducing their uptake of polyvalent cations by competing for binding sites in proteins and transporters, especially in roots (Rodriguez-Serrano *et al.*, 2009). Atabayeva *et al.* (2016) found that soil Cd reduced the content of Mg, Mn, Fe, Zn and Cu in rice shoots; the benefit of being able to exclude Cd is made clear by the fact that the rice cultivars with the lowest Cd content were also the ones with the highest concentrations of the five cations mentioned above. Atabayeva *et al.* (2017) also showed that Cd reduces Fe content in all organs of rice plants, and Zornoza *et al.* (2002) found lower root concentrations of P, K, Fe, Mn, and Zn. The reduced cation uptake could affect the plants ability to respond to Cd stress, since one polyvalent cation in particular, Ca^{2+}, is important in stress signalling (Sandalio *et al.*, 2001). Muradoglu *et al.* (2015) analysed the effects of soil Cd in strawberry plants and found that increasing the heavy metal's concentration decreases chlorophyll content, root and stem growth, and causes chlorosis. It also causes increases in plant tissue Cd concentrations, especially in the roots. Gonçalves *et al.* (2009) found that medium Cd causes low chlorophyll content as well as decreased root and shoot elongation in cucumber seedlings. Studies conducted in peas have shown that Cd causes the accumulation of reactive oxygen species (ROSs) such as H_2O_2 and $O_2^{\cdot-}$ in leaf tissue (Romero-Puertas *et al.*, 2004); it also reduces activity of super-oxide dismutase (an antioxidant enzyme) and calcium content, and depresses nitric oxide (an important messenger in several metabolic pathways) metabolism,

although calcium treatment alleviates the symptoms (Rodriguez-Serrano *et al.*, 2009). Seth *et al.* (2008) demonstrated that Cd affects cell division in *Allium cepa* root meristem, where it increases the number of chromosome aberrations (such as chromosome breaks) and mitotic abnormalities (like multipolar anaphase). The significantly higher prevalence of mitotic abnormalities suggests that Cd harms cell division mainly by acting on the spindle apparatus.

Plant adaptation to cadmium toxicity

Plants have developed strategies to deal with most contaminants they come in contact with, and cadmium, as an increasingly common pollutant of agricultural soils and biosolids, impacts plants with different capacities to tolerate, absorb, and translocate it from roots to shoot. It has been shown that Cd-tolerant plants display some of the same symptoms as plants adapted to P-deficiency, such as (1) shorter roots, (2) shorter shoots, (3) greater root hair density, (4) heavier roots, (5) lower chlorophyll content, and (7) higher phosphatase activity (particularly monoesterase, the most commonly assayed subgroup so far). Understanding the effect of cadmium on a variety of plant phosphatases is the first step in deciphering the role these enzymes might play in certain plants' increased tolerance and accumulation of soil toxins.

However, these are not the only adaptations to Cd and other heavy metals in soil: these contaminants are often sequestered in vacuoles or excluded from the roots before they can cause harm to the cellular machinery or reach the shoots. It is even possible that elevated (but not highly toxic) concentrations of ROSs could function as a signal for the upregulation of Cd resistance genes, even when not caused by Cd (Rodriguez-Serrano *et al.*, 2009); this even though Cd reduces calcium content in leaves (Sandalio *et al.*, 2001). Seth *et al.* (2008) note that mitosis-related damage after cell exposure to Cd is reduced after 24 hours, and suggest this could be due to the presence of some or all of several antioxidant enzymes, such as glutathione, glutathione peroxidase, and catalase, and/or DNA repair enzymes, like DNA ligase, DNA polymerase, and endonuclease. Fagioni and Zolla (2009) investigated the

differences in the proteomic profile of basal and apical spinach leaves (*Spinacia oleracea*) under Cd stress, and found clues regarding a possible strategy to increase survival in the presence of the toxin. In basal leaves, they observed a decrease in chlorophyll *a* synthesis and in photosynthesis rates, followed by an increase in glutathione and phytochelatin synthesis, both of which chelate cadmium to prevent it from damaging other cellular structures. Finally, basal leaves experience an increase in ROS production, causing senescence and death, with the dead leaves falling and taking with them a large proportion of the absorbed and chelated Cd stored in their cell's vacuoles. On the upper end of the plant, apical leaves have increased chlorophyll content, photosynthetic complexes, and enzymes involved in CO_2 fixation and carbohydrate metabolism. These differences could reflect an active strategy by the plant to concentrate energy metabolism in areas further from the Cd source while focusing the energy of the lower part of the stem on getting rid of the contaminant. Zornoza *et al.* (2002) analysed the effect of medium Cd on white lupin (*Lupinus albus*) and found characteristics in that plant that had a protective effect against Cd toxicity: specifically, free movement of Cd was diminished by immobilization in cell walls and complexation with thiols, whose concentration increased in the presence of Cd. Ramos *et al.* (2002), using lettuce as their model, also recorded that a high percentage (64%) of absorbed Cd is located in the cell wall.

Ueno *et al.* (2010) have found that a single amino acid mutation in a transporter expressed mainly in the tonoplast of root cells causes lower Cd accumulation in rice plants. The transporter is functional in the mutated, low-Cd variety, but not in the high-Cd variety; it is responsible for sequestering Cd in root cell vacuoles and preventing its translocation to the above-ground tissues. Overexpression of this transporter under control of the maize ubiquitin promoter in a low-Cd accumulating rice cultivar caused even greater reduction in grain Cd content. However, it should be noted that even in the same species, Cd translocation from root to shoot can vary greatly between cultivars. For example, shoot Cd can vary between 13% and 37% of whole-plant Cd (Harris and Taylor, 2013). Ueno *et al.* (2009) found that shoot Cd concentration in rice can vary between 6 and 15 μg of Cd/g of dry weight after growing for 37 days in Cd-contaminated soil, depending on cultivar.

Cadmium contamination in soil

Cd is present in nature at varying concentrations, depending mostly on the type of soil. Eastern and Southern French, Southern Chinese and Swiss limestone (as well as Chinese sandshale) tend to have high concentrations of the metal, while granitic soils have much less Cd (Rambeau *et al.*, 2010; Zhang *et al.*, 2011). In most cases, background Cd concentrations are too low to cause concerns regarding human or environmental health, but anthropogenic activity has increased concentrations well above natural levels and is now the most important source of environmental Cd. In some parts of the world, soil Cd concentrations have become worrying enough that entire regions have been declared as having a high capacity to contaminate crop production for human and animal consumption (Retamal-Salgado *et al.*, 2017: two Chilean regions), and recalls have been issued for food due to high Cd levels (Stafford *et al.*, 2018: the recall was for spinach in California). This situation, combined with the fact that it is common for levels toxic to humans to be present in plants that show no signs of Cd toxicity disease (Ueno *et al.*, 2010) highlights the need for close monitoring of Cd content in the food supply. In most of the world, Cd contamination comes mostly from industry (mining activity, battery manufacture, non-ferrous metals industry) and agriculture (pesticides and phosphate fertilizers) (Zornoza *et al.*, 2002). A nationwide survey of arable land in China found that most hotspots for high Cd concentration were located near mining operations and smelting facilities, pointing to those industries as the major source of contamination (Zhang *et al.*, 2014). An investigation of soil around a highway system in the Agra district of India revealed that aerial deposition from transportation emissions is also a relevant source of heavy metal contamination (particularly Pb and Cd); some radish and spinach samples grown close to the road had Cd concentrations above what Indian authorities consider safe for human consumption (Sharma and Prasad, 2010). Outside of New Zealand, sewage sludge, due to its relatively high Cd content, is also becoming a major source of environmental Cd contamination (Ghosh and Singh, 2005). Due to the predicted increase in sewage sludge worldwide over the next few decades (mainly due to population growth), the proportion of soil Cd originating from sludge is likely to increase.

Cadmium contamination in New Zealand soil

McDowell *et al.* (2013) have shown that in New Zealand, soil Cd correlates with soil P more strongly than with any other parameter. That is probably due to the high anthropogenic contribution to soil Cd, most of which (in New Zealand) comes from the application of phosphate fertilizers to the agricultural land; the phosphorus in these products comes from mines with high levels of Cd (Lugon-Moulin *et al.*, 2006). Phosphatic shale, one of the sources of phosphate for fertilizers, can have Cd concentrations as high as 200 mg/kg, more than 70 times more Cd than the most contaminated New Zealand soil (McDowell *et al.*, 2013). Stafford *et al.* (2018) compared a Waikato farm and a Canterbury farm with different phosphate fertilizer application histories, and concluded that the difference in soil Cd concentration, which was three times higher in the Waikato farm, was explained by its historically higher fertilizer use. Regarding land use, dairy farms, pastures, and horticultural land had the highest amounts of Cd (up to 2.7 mg/kg); in most sampled sites, the anthropogenic contribution to Cd concentrations was estimated to be just over 50% (McDowell *et al.*, 2013). High Cd concentrations in these three types of soil is due to their being the ones most often treated with phosphate fertilizers. These chemicals may be a greater threat to the food supply chain than other Cd-containing contaminants, since there is evidence in the literature that plants may accumulate more Cd when its source are P fertilizers than from other sources (Azzi *et al.*, 2017).

Wastewater

Wastewater is the name given to the liquid- or water-carried waste from a variety of sources, including industrial and residential sites, as well as ground and storm water that might be present. Wastewater contains significant quantities of undesirable components, such as toxins and pathogenic micro-organisms, and its treatment can produce a number of substances, including sewage sludge and biosolids (Werther and Ogada, 1999). Sewage sludge is created by dewatering wastewater, resulting in a semi-solid material with high quantities of organic matter, pathogens, heavy metals, and organic micro-pollutants (Chen *et al.*, 2012). After certain treatments to remove water and several toxins and pollutants, sewage sludge can be turned into biosolids, a substance mainly used as a soil additive, from

which nutrients can also be removed for the manufacture of fertilizers (Egan, 2103). The amount of sewage sludge in the world has been rapidly increasing since the middle of the 20th century, especially in and around urban centres. Mostly due to the growing populations and the increase in the proportion of houses connected to treatment plants (Rosolen *et al.*, 2005; Werther and Ogada, 1999), and it is projected to grow substantially over the next 30 years (Van Drecht *et al.*, 2009).

Wastewater in the environment

The release of untreated wastewater into the environment poses several ecological and public health threats. First, the organic matter present in the water is decomposed in a process that consumes oxygen, which reduces the availability of this element for aquatic animals in the receiving waters (Anza *et al.*, 2014; Singh *et al.*, 2017). Second, the same decomposition process produces foul-smelling gases. Thirdly, the presence of heavy metals and other toxic compounds makes the wastewater dangerous to plants and animals (Liphadzi *et al.*, 2003). Among those toxic compounds are pharmaceuticals like antidepressants and oestrogens (Metcalfe et al., 2010; Ting and Praveena, 2017), many of which can be readily absorbed by humans, while retaining full biological activity (Hiemke and Härtter, 2000). Fourth, the large amounts of nitrogen and phosphorus increase the chances of eutrophication (Johansson *et al.*, 2008); 0.02 mg/L of P in water can be enough to trigger the process, which generates toxins and reduces oxygen content, causing significant damage to wildlife (Huang *et al.*, 2016). Finally, the presence of pathogens is a health hazard to human populations (Krzyzanowski *et al.*, 2014; Werther and Ogada, 1999), with even extensively treated wastewater containing high concentrations of helminth eggs, *E. coli*, and *C. perfringens* (Ajonina *et al.*, 2015). Due to the above, w~~W~~astewater must go through a treatment process ~~for several crucial reasons. It is for those reasons that~~and industrialized countries have set limits to the amount of dangerous substances that can be present in processed wastewater or treated effluent. Treatment plants typically employ three stages: mechanical, biological and a third one for the removal of nitrogen and phosphorus.

Wastewater treatment: first stage

The mechanical stage consists of the passage of wastewater through a series of screens that filter out large solids, followed by the removal of settleable and floating solids in sedimentation tanks. Between these two steps there is often a chemical step where substances are added to the wastewater to coagulate very fine suspended matter and a few dissolved substances, to facilitate sedimentation (Zabava *et al.*, 2016). This first stage can remove between 50% and 70% of suspended solids and between 25% and 40% of the biological oxygen demand (Werther and Ogada, 1999; Zabava *et al.*, 2016).

Wastewater treatment: second stage and third stages

In the biological stage, also called activated sludge (Chong *et al.*, 2012; Mailler *et al.*, 2014), micro-organisms (mostly bacteria) coagulate much of the remaining non-settleable solids and organic matter, incorporating them into their cell tissue. This new abundance of micro-organisms, in the form of flocs, can then be removed by sedimentation. Some of the organic matter is also turned into gas (Werther and Ogada, 1999). One type of activated sludge (aerobic granular sludge) has been shown to contain about 67% inorganic P and 33% organic P (Huang *et al.*, 2015), and the analysis of other samples shows there is wide variation in that proportion, with organic phosphorus representing between 3% and 52% of total phosphorus in sludge (O'Connor et al., 2004). Before the final stage, partial recovery of phosphate can take place in the anaerobic supernatant of the activated sludge by methods like precipitation with salts (such as iron and alum salts), biosorption by red algae (Rathod *et al.*, 2014), or electrocoagulation (Huang *et al.*, 2017). Precipitation with salts is a simple process, but relatively expensive (Machnicka *et al.*, 2008). Another option is the use of certain filamentous bacteria which accumulate more P than needed for growth, such as *Acinetobacter*, *Pseudomonas*, *E. coli* and *Mycobacterium*. These can then be disintegrated by a process called hydrodynamic cavitation, which involves centrifugation and application of high pressure to the foam that contains the bacteria (Machnicka *et al.*, 2008). In the final stage, nitrogen is removed by a two-step process of nitrification and denitrification, in which ammonia is converted into nitrate, and nitrate is converted into free nitrogen. Phosphorus is

removed by chemical precipitation or by incorporation into cell tissue, either of which is followed by sedimentation (Werther and Ogada, 1999; Zabava *et al.*, 2016).

Sludge treatment

After wastewater treatment is finished, what remains is the sludge, where most of the elements that make wastewater dangerous are concentrated. This makes the processing of sludge a crucial undertaking for environmental and human health, as it aims to remove pathogenic bacteria, reduce the content of organic matter, and reduce water content for greater ease of handling and disposal (Werther and Ogada, 1999). This can be achieved by digestion with micro-organisms, through the addition of lime to increase pH, vibration-assisted filtering, or by a two-step heating process, which sterilizes the sludge, coagulates the solids, and drastically reduces water content (Denisov *et al.*, 2016).

Sludge in agriculture

The use of sludge as agricultural fertilizer has the advantage of returning nutrients like nitrogen and phosphorus to their biological cycle. Due to their higher organic matter and lower heavy metal content, sludge from residential wastewater is more appropriate for this use than industrial wastewater (Werther and Ogada, 1999). By 1999, about a quarter of all the sludge in the United States, and over half of it in some European Union countries (Spain, Denmark and France) was used as fertilizer (Werther and Ogada, 1999). By 2012, the countries where this method was most common were Portugal (87%), UK, Ireland (both 70%) and Spain (64%) (Kelessidis and Stasinakis, 2012), while in the United States the proportion doubled (Verlicchi and Zambello, 2015). However, even residential (or communal) wastewater has higher heavy metal content than typical farming soil, meaning uncontrolled addition of sludge to agricultural soils could cause heavy metals to bioaccumulate along the food chain, adversely affecting crop quality and human health. For example, while an application of 12 kg m^{-2} of sewage sludge to a rice field increases yield by 137%, it also raises the Cd concentration in rice grains to over 4 times the safe limit for human consumption (Singh and Agrawal, 2010). In another study, French bean had improved growth parameters when grown in soil composed of up to 40% sewage sludge (compared to 0% sludge) (Kumas and Chopra, 2014). This potential for greater growth is the

reason sewage sludge is often used as an alternative to inorganic fertilizers (Frost and Ketchum, 2000; Kumas and Chopra, 2014). Even so, the concentration of heavy metals (specifically Zn, Cd, Cr and Pb) increases even as growth is promoted (Kumas and Chopra, 2014).

Landfilling

Landfilling is the most common method of sludge disposal in a number of countries, such as Italy (40%), Romania (75%) and Malta (100%) (Kelessidis and Stasinakis, 2012). However, this is considered the most problematic method of disposal, especially due to the high potential for groundwater pollution. For that reason, landfilling is being phased out as a disposal method for sewage sludge in many countries, such as in Italy, where in the 1990s it accounted for 85% of sludge disposal (Werther and Ogada, 1999). In New Zealand, biosolids are usually disposed of by landfilling, but some of it is also used as fertilizer (Opus International Consultants Limited, 2005). All of the sludge from the Christchurch Wastewater Treatment Plant (which processes wastewater from Christchurch City and four other small centres) is used as fertilizer in the rehabilitation project of the Stockton mine site, where it helps prevent erosion (Anon, 2017).

Incineration

Incineration is now the most common method of sludge disposal in several countries, such as the Netherlands (70%), Slovenia (62%), Belgium (55%) and Germany (50%) (Kelessidis and Stasinakis, 2012). It has several advantages when compared to other methods, especially in urban areas, where the amount of sewage generated is much greater. Incineration reduces by up to 90% the volume of sludge, which facilitates handling and disposal in densely populated areas. This process also inactivates several toxic compounds, sterilizes the biosolid, and reduces its odor (Werther and Ogada, 1999). Phosphate in sludge ash is present in forms that are sparingly soluble (Whitlockite, Hilgenstockite, Enstatite, Brushite), and is thus mostly unavailable to plants (Sturm *et al.*, 2010). A number of techniques have been developed to remove P from sludge ash, such as sequential leachings with acid (Levlin *et al.*, 2002), which is not mature enough to be marketable, and an adaptation of the Triple Superphosphate production process, which is already being commercialized (Weigand *et al.*,

2013). Sludge has also been proposed as a renewable source of energy, through a process that would convert it into gas (Matveev *et al.*, 2014). Compared to coal, sludge has high levels of moisture, volatile matter and nitrogen (Werther and Ogada, 1999).

Heavy metals in sewage sludge

Heavy metals in sewage sludge are generally bound to organic compounds and are thus less available to plants grown in sludge or sludge-amended soil (Frost and Ketchum, 2000). The correlation between uptake of heavy metals by plants through the roots and heavy metal concentration in the soil is weak, except for Zn. Uptake is much more strongly correlated to solubility of the metals, which increases with lower pH (to which the heavy metals themselves contribute) (Speir *et al.*, 2003). This makes some metals, like Cd, more bioavailable (Rieuwerts *et al.*, 1998). Phosphate is also more bioavailable under low pH (Hinsinger, 2001). EDTA, a chelating agent, has been found to increase heavy metal soil solubility and bioavailability (including Cd) for removal by sunflower (Liphadzi *et al.*, 2003). A second study found that soil EDTA did not affect non-essential heavy metal uptake by plant organs, while essential heavy metal uptake was greater with EDTA in the soil (Liphadzi and Kirkham, 2006). EDTA has also been shown to improve phytoextraction of Cd by eucalyptus (Luo *et al.*, 2015). Plants with the concentrated metals can be ashed and the ash placed in a confined disposal area. If the metals are valuable, they can be removed from the ash (Liphadzi *et al.*, 2003).

Phytoremediation and phytoextraction

Phytoextraction of heavy metals is a technique that uses plant growth to accumulate heavy metals in the plant's above-ground tissues, which can then be harvested and processed for the recovery of the metals. It can be applied to different types of substrate, including agricultural soil and sewage sludge, and it is an environmentally friendly and relatively cheap way of removing these pollutants from excellent nutrient sources for crop plants (Maryam *et al.*, 2015). Ghosh and Singh (2005) measured the Cd absorption capacity of five high-biomass weeds common in India (*Ipomoea carnea*, *Dhatura innoxia*, *Phragmytes karka*, *Brassica juncea*, and *B. campestris*). All five demonstrated significant phytoextraction potential, with *I. carnea* (pink morning glory) performing the best among them, with an

average Cd concentration in the above-ground organs of 310 µg/plant. Two tropical plants, *Acacia mangium* and *Jatropha curcas*, are capable of removing Cu from sewage sludge, suggesting growing heavy metal accumulators directly onto sewage sludge could be a new method for treating the biosolid (Maryam *et al.*, 2015). Heavy metal hyperaccumulators like tobacco or sunflower could be particularly appropriate for this process. Tobacco has a naturally higher Cd level in its leaves, compared to other plants (Evangelou *et al.*, 2007). Average Cd levels on tobacco leaves go from 3.5 to 164.5 mg kg^{-1}, compared to 0.6 to 1.5 in sunflower (whole plant) (Evangelou *et al.*, 2007). Corn (*Zea mays*) has higher absolute Cd output than tobacco, but since it also has greater biomass than tobacco, it probably leaves less nutrients on the soil. A transgenic variety of tobacco was created that had 90% higher accumulation of Cd on leaves and 50% lower accumulation on roots (Macek *et al.*, 2002), which is important because leaves are the harvestable part of the plant.

Phytoextraction in New Zealand

In New Zealand, phytoextraction with *Brassica juncea* (Indian mustard) has been successfully applied at the abandoned Tui mine in Waikato, which was contaminated with high levels of mercury (Hg), ranging from 1.3 to 4.5 mg/kg (Moreno *et al.*, 2005), as well as lead (Pb) and Cd; Hg was removed mainly by volatilisation, and a risk assessment study found that this did not represent a significant health risk for the local population; however, there was a small chance that some of the volatilised Hg could be deposited in nearby water streams and biomagnified. In addition, native plants such as *Hebe stricta*, *Leptospermum scoparium* and *Phormium tenax* were planted at the site to prevent leaching of heavy metals into nearby bodies of water without accumulating them at significant levels themselves (West, 2015).

Waikato had been the site of another successful phytoextraction project at the Kopu waste wood disposal site (West, 2015). The location had high levels heavy metals used in timber treatment, such as copper, chromium, and especially boron. These contaminants reached water streams during rains, when the leachate pond overflowed. In 2000, poplar, willow and *Eucalyptus* were planted at the site, and since then leachate has diminished and poplar leaves show high concentrations of copper and chromium, as well as extremely high

concentrations of boron (West, 2015). At some point, these trees could be harvested, and the boron retrieved from them could be in fertilizers.

New Zealand has been gaining an international reputation as a phytoremediation research power, with researchers from Massey University conducting phytoremediation projects in Australia, USA, China, Mexico, Brazil, Fiji, and South Africa, as well as New Zealand itself (Anderson, 2013). A New Zealand company has developed a method to remove metals, particularly gold and mercury, from artisanal gold mining sites. This method involves (1) planting fast-growing and high-biomass species in contaminated sites, (2) amend the soil to increase solubility of gold and mercury only once the plants reach maturity, and, after plants accumulate the metals for one or two weeks, (3) harvest them and process to recover the metals (Anderson, 2013). Recovery of gold is an important part of the method, since artisanal gold mining is common in relatively poor regions of the world, and the possibility of getting an important material back is likely to increase local participation in a process that would otherwise have much less immediately tangible benefits (Anderson, 2013).

Phosphorus absorption in the soil

Phosphorus is an essential and limiting element for life, especially for the growth of plants; it is particularly important in legumes, as it is necessary for the conversion of N_2 into NH_4 in rhizobia (Lazali *et al.,* 2013). However, the application of P fertilizers on crop land is necessary because soluble Pi (inorganic phosphate) is present at much lower concentrations in soil than inside plant cells (1-10 µM versus 5-20 mM) (Tran *et al.*, 2010). In addition to being present at low concentration in that medium, soil Pi is also the least bioavailable of all macronutrients, and that is because it readily reacts with calcium salts (in alkaline soils) or with iron and aluminium oxides (in acidic salts) (Richardson, 2009; Richardson et al., 2009; Tran et al., 2010). The chemical form of phosphate in soil depends greatly on pH: in alkaline and neutral soils, inorganic P is mostly present in the form of calcium phosphate, while in acidic soils, it is predominantly bound to or occluded by iron or aluminium oxides (Spohn and Kuzyakov, 2013).

In addition to morphological adaptations to increase total root area and direct resources to root growth (as opposed to shoot growth), plants have evolved a number of physiological adaptations to deal with low P levels in soil, both to solubilize inorganic P and to mineralize organic P. To mineralize organic P, plants release acid phosphatases, and to solubilize inorganic P, they release protons and organic ligands (Spohn and Kuzyakov, 2013). P deprivation also causes de novo synthesis of purple acid phosphatases, responsible for scavenging extracellular phosphorus to be used inside cells (Tran *et al.*, 2010). P deficiency leads to reduced yields in crops, a situation that causes concern, given that mineral P reserves (the main source of P for fertilizers) are predicted to last only another 50 to 100 years (Huang *et al.*, 2015). It is for that reason that it is so important to find ways to recover P from sources other than mines, such as sewage. When turned into ash, the P content of sewage sludge can be as high as 11%, which is comparable to medium- to high-grade ore (Donatello *et al.*, 2010).

Microarray data suggests that in spite of P starvation being responsible for the induction of 600-1,800 genes, there is only a 25% overlap in induced genes between roots and shoots, suggesting adaptations to P scarcity are strongly tissue-specific. The same holds true for repressed genes. Wu *et al.* (2003) found that of all genes repressed by P deprivation in *Arabidopsis thaliana*, only 10% were the same between leaves and roots, while Mission *et al.* (2005), also analysing *Arabidopsis*, found that number to be 4.8% (Tran *et al.*, 2010).

To create efficient strategies for biotechnological enhancement of Pi acquisition by crops, it is necessary to understand Pi-inducible gene expression and the resulting biochemical effects on plants. Manipulating the expression of genes involved in the P starvation response can lead to the creation of cultivars that require less fertilizer due to increased capacity to absorb soil Pi and research suggests there is much room for improvement in crop plants. Leelapon *et al.* (2004) have shown that a single point mutation (serine to aspartic acid) in a storage protein that makes up 40% of total soluble protein in soybean leaves increases its phosphatase activity 20-fold (Tran *et al.*, 2010).

Phosphatases

Phosphatases catalyze the hydrolysis of esters and anhydrides of phosphoric acids, which releases inorganic P (also known as orthophosphate) (Khade *et al.*, 2010; Png *et al.*, 2017). These enzymes are classified into five groups: alkaline, low-molecular mass acid, high-molecular mass acid, purple acid and protein phosphatases (Guimarães *et al.*, 2006). When growing in soil with low availability of P, plants release carboxylates and phosphatases in their root exudates to solubilize the nutrient present in soil and absorb it (Gusewell, 2016). In acidic and P-deficient soils, acid root phosphatase action is greater than alkaline root phosphatase action (Khade *et al.*, 2010). Under these conditions, acid root phosphatase activity is also greatly increased by the presence of arbuscular mycorrhizae in the roots. Both secreted and intracellular phosphatases are upregulated under P deprivation (Duff *et al.*, 1994; Tran *et al.*, 2010), and they can be numerous in type: for example, the *Arabidopsis* genome codes for 50 putative phosphatases (Li *et al.*, 2002). These responses are part of the P starvation syndrome, which, in addition to the effects on root phosphatase activity, also causes changes in root architecture, specifically shorter primary roots, greater secondary root growth and greater root branching (Gaume *et al.*, 2001). Root traits that promote P acquisition are up-regulated under P deficiency, and are either reduced or completely suppressed under sufficient P. It is important that these traits be plastic, because the production of root structures entails a substantial energetic cost (Gusewell, 2016). Biological fixation of nitrogen entails a high P cost (Png *et al.*, 2017). Ethylene also appears to be related to the P starvation syndrome, as plants in P-deficient soil that are exposed to it have their acid root phosphatase activity and high-affinity phosphate transporters up-regulated (Li *et al.*, 2011). Despite their impact, plant root phosphatases are only responsible for a minority of phosphatase activity in the soil: microbial phosphatases play a greater role in that environment than the plant variety (Papatheodorou *et al.*, 2014). Plant roots produce mainly acid phosphatases, so the action of these enzymes is concentrated in the rhizosphere instead of in the soil as a whole (one important class of alkaline phosphatases, the alkaline monoesterases, is produced only by bacteria (Nakas *et al.*, 1987)); microbes, on the other hand, produce both acid and alkaline phosphatase at similar proportions, so action of alkaline phosphatases is spread out throughout the soil (Spohn and Kuzyakov, 2013). Even though plant roots do not produce much alkaline phosphatases, they still benefit from their

activity, since the phosphorus they mineralize is also available to them. The prevalence of microbial phosphatase in the soil may have caused plants to rely more heavily on those enzymes when soil P is at sufficient concentrations.

Even when phosphorus is present in the soil at high concentrations, its bioavailability may be affected by other factors. Microbial phosphatases (acid and alkaline) also have the power to increase phosphate availability to plants when applied to agricultural soils (Nalini *et al.*, 2015). This leads to the possibility of phosphatase-producing microbes being used as biofertilizers in soil, to facilitate P uptake by plants (Mahasneh and Tiwari, 1996; Marques *et al.*, 2013). On the other hand, it has also been shown that alkaline phosphatase activity in the soil decreases with increases in Cd concentration (Khan-Mohammadi and Nourbakhsh, 2011), although very small concentrations of both Pb and Cd can stimulate both acid and alkaline phosphatase activity (Stuczynski *et al.*, 2003). Microbial acid phosphatases also have some industrial applicability, with phytase, a member of that group, being produced by fermentation and then used in animal feed to convert organic P into inorganic P, decreasing the amount of phosphate in animal manure by up to 30% and aiding in preventing eutrophication (Guimarães *et al.*, 2006; Pandey *et al.*, 2001).

The presence of heavy metals in soil, like P starvation, generally leads to the increase of root phosphatase activity. Gubrelay *et al.* (2014) found that both acid and alkaline phosphatase activity increased in barley plants treated with a Cd solution twice-weekly; increases were greater in roots than in shoots. Yang *et al.* (2014) analysed acid phosphatase activity in the Cd hyperaccumulator *Sedum alfredii*, and also found an increase with the addition of Cd to the soil. Gonçalves *et al.* (2009) recorded opposing effects of medium Cd on acid phosphatase activity in roots and shoots of cucumber seed: activity was inhibited in the latter and promoted in the former. All the above studies explored the effects of only one class of plant phosphatases, namely monoesterases. In these and other cases there were also significant increases in heavy metal concentrations in plant tissues (Eid and Shaltout, 2016; Gubrelay *et al.*, 2014; Kumar *et al.*, 2016; Yang *et al.*, 2014).

Phytases

Phytases are enzymes that catalyse the sequential hydrolysis of phytic acid and release less phosphorylated myo-inositol derivatives and inorganic phosphate (Pi) (Oh *et al.*, 2014; Tran *et al.*, 2010). These enzymes fall in two groups, depending on their optimum pH: acid phytases have an optimum around 5.5 and alkaline phytases have an optimum around 7.5. However, histidine acid phytases can have optima as low as 2.5. (Zhang *et al.*, 2011). In *Phaseolus vulgaris* (common bean), phytases are particularly active in rhizobia, and activity increases under P-deprivation (Lazali *et al.,* 2013). In plants, phytases are most critically important in early development, where between 60% and 70% of seed phosphorus is in the form of phytic acid, their main substrate (Bhavsar and Khire, 2014).

Phytases have been approved for use in animal feed by the United States' Food and Drug Administration (FDA) since 1996, and in 2014, they had become the third most commonly used feed enzyme (Bhavsar and Khire, 2014). Phytases are used in this context as a way of making the undigestible phosphorus in phytic acid (and the essential minerals that bind to it) digestible (Persson *et al.,* 1998), which also has the benefit of reducing the amount of undigested phosphate in animal manure that would otherwise contribute to eutrophication and algal blooms (Chen *et al.*, 2007). Another promising strategy is to engineer plant lines to be used as feed that have high phytase activity in the seeds themselves: this has been accomplished with canola (Peng *et al.*, 2005), maize (Chen *et al.*, 2007) and soybean (Bilyeu *et al.*, 2007), for example, all expressing a bacterial or fungal phytase in their seeds.

Animal phytases tend to have high substrate specificity, while phytases produced by plants and by many bacteria show great variation in specificity (Oh *et al.*, 2006). More specifically, while plant acid phytases exhibit low substrate specificity, plant alkaline phytases exhibit high specificity (Belho *et al.*, 2015; Johnson *et al.*, 2010). Animal phytases are also less common and less essential to the organism that produces it than plant phytases, while microbial phytases are significantly more active and efficient than both (Bhavsar and Khire, 2014); fungal strains, even those with some industrial application like some *Aspergillus* strains, produce mostly low-activity phytase (Suleimenova *et al.*, 2016). However, there are exceptions: Grenier *et al.* (1993) reported two *E. coli* phytases that are closer in structure to

plant phytases than to other microbial phytases, with narrower substrate specificity than other members of their own group; Belho *et al.* (2015) reported a rice bean phytase with high activity and such broad substrate specificity that it is even able to act as an acid monoesterase (Tran *et al.*, 2010). Shah *et al.* (2017) reported that an *Aspergillus niger* phytase with broad substrate specificity was capable of degrading extremely toxic organophosphate pesticides like chlorpyrifos, methyl parathion and monocrotophos (all phosphotriesters). That phytase was even able to degrade chlorpyrifos in commercially available green chilli by 90%, suggesting a new way to degrade organophosphates before they enter the food chain (Shah *et al.*, 2017).

Advanced wastewater treatment

In addition to the usual stages of wastewater treatment, new techniques are being developed to reduce the dangers posed by the processed liquid and to recover substances that may still be present in it; many of these new techniques rely at least partly on enzymes. Sangave and Pandit (2006) applied ultrasound and cellulase treatment to distillery wastewater at various combinations (each alone, simultaneously, and one followed by the other). They found that applying ultrasound and then cellulase to wastewater achieved the highest reduction in chemical oxygen demand (COD) in the effluent. The effect of ultrasound is due to cavitation, which is the formation, growth, and collapse of cavities (microbubbles in a liquid) within mere microseconds (Sangave and Pandit, 2006). This phenomenon increases temperature and pressure at millions of locations simultaneously, resulting in partial or complete oxidation of many pollutants. For that reason, ultrasound has been used since the 1970s in the treatment of wastewater.

Locar *et al.* (2013) screened *Bacillus sp.* from several locations in Serbia and found that 3 strains exhibited high laccase activity, with optima at high temperatures (between 65^{o}C and 80^{o}C), while preserving stability. Laccase is a biotechnologically significant enzyme, due to its above-average temperature optima and broad substrate specificity. The genes coding for the enzymes were clones and expressed in *E. coli*. Laccase was extracted, and its activity was assayed against blue, green, and black dyes common in chemical industry wastewater, where its degrading power was found to be high.

Chiong *et al.* (2016) extracted peroxidases from soybean hulls and *Luffa acutangula* skin peels and used it to treat synthetic wastewater containing the largely non-biodegradable methyl orange dye, made to simulate effluents from the textile industry. These effluents are a human and environmental health concern, since unlike natural dyes, the synthetic dyes used by the industry are stable and can be degraded into mutagenic and carcinogenic compounds. For example, an azo dye (the same class as methyl orange) originating from a dye processing plant was identified as the mutagenic agent polluting a river in São Paulo, Brazil (Alves de Lima *et al.*, 2007). In addition to that, the colour they give to water is more than an aesthetic problem, since it decreases light penetration, which reduces photosynthetic activity and reduces available oxygen for aquatic wildlife. They found that maximum decolourisation efficiency was 75.3% for *L. acutangula* peroxidase and 81.4% for soybean peroxidase under their respective optimum conditions of pH, temperature, enzyme dosage and others.

Electrocoagulation

The need for alternative sources of phosphorus is increasing worldwide, due to the element's relative scarcity in most environments and the consequent need to use fertilizers and food additives to increase plant and animal food production (Bhavsar and Khire, 2014). Phosphorus fertilizers are becoming a less viable option (Bhavsar and Khire, 2014; Carpenter and Bennett, 2011), since depletion of phosphate mines worldwide is making their application too costly, and their continued use is likely to increase environmental levels of contaminants like cadmium (Mission *et al.*, 2005) (Tran *et al.*, 2010), uranium and thorium, and phosphate rock processing produces toxins like phosphogypsum (Bhavsar and Khire, 2014). Geopolitics is also a contributing factor to the need for new phosphorus sources, since only five countries (Morocco, China, South Africa, Jordan, and the United States) are home to about 90% of the world's phosphate rock mines (Bhavsar and Khire, 2014).

As previously mentioned, wastewater and the sludge it forms after treatment have high concentrations of phosphorus, and new techniques are being developed that compare favourably with, for example, chemical precipitation, which is still commonly used. Chemical

precipitation is the addition of salts like iron sulphate, iron chloride, and calcium hydroxide; the cations bind to phosphate ions and precipitate, removing P from solution.

Electrocoagulation is an electrolysis-based technique that has been in use in parts of the USA since 1911, but is only gaining popularity over the last decade, due to the development of new electrode materials and more compact reactors (Farhadi *et al.*, 2012; Huang *et al.*, 2015); this revival is also being driven partly by its flexibility, as it seems to be suited not only for large- but also for small-scale plants and decentralized treatment systems (Farhadi *et al.*, 2012). Electrocoagulation differs from chemical precipitation mainly in the way metal ions are introduced into the liquid. It consists of placing two electrodes made of iron or aluminium in a solution and letting a current pass between them and through the solution. Under the action of the current, the electrode serving as the anode releases metal ions that serve as active coagulant precursors, binding to the phosphate ions (and other dissolved pollutants) and precipitating them (Huang *et al.*, 2015). At the anode, hydrogen gas (H_2) is formed (Holt *et al.*, 2005), and depending on the reactor operating conditions, the it may form bubbles that will float some of the coagulated pollutants to the surface. (Farhadi *et al.*, 2012). Most studies have found the optimum initial pH for electrocoagulation to be between 3 and 6 (Irdemez *et al.*, 2006; Li *et al.,* 2013), with iron electrodes having more efficient precipitation rates at lower initial pH values (Irdemez *et al.*, 2006); however, it should be noted that as electrocoagulation progresses, pH rises due to the generation of hydroxyl ions on the cathode surface (Mollah *et al.*, 2004; Verma *et al.*, 2013). (The anode is also called “sacrificial anode” due to the degradation caused by metal ion loss.) Despite the variety of techniques available for P removal (such as struvite precipitation, and marine macro algae adsorption), electrocoagulation has received increased attention due to its high efficiency and simple operation, and it has significant advantages over chemical precipitation. The latter requires constant monitoring of pH, and it also demands that large quantities of stock solutions be prepared, transported and stored, making it harder to comply with clean production standards (Huang *et al.*, 2015).

The reactions happening in each electrode during electrocoagulation can be represented in the following way (“M” standing for “metal”) (Mollah *et al.*, 2004):

• **At the anode:**

$M(s) \rightarrow M(aq)_n+ + ne-$

$2H_2O(l) \rightarrow 4H+ (aq) + O_2(g) + 4e-$

• **At the cathode:**

$M(aq)_n+ + ne- \rightarrow M(s)$

$2H_2O(l) + 2e- \rightarrow H_2(g) + 2OH^-$

Electrocoagulation has been employed successfully to treat different types of wastewater, including from unusual sources like landscaping projects and liquid organic fertilizer factories (Huang *et al.*, 2015), and it has been used to remove substances like oil, chromium, arsenic, phosphate, dyes, and surfactants. Akyol *et al.* (2013) have used it for the treatment of liquid organic fertilizer manufacturing wastewater, which contains high concentrations of phosphorus. They employed electrocoagulation and electro-Fenton, a process in which H_2O_2 is added to the solution undergoing electrocoagulation to degrade organic phosphorus compounds, achieving complete P removal with electro-Fenton at 20 minutes of operation with a current density of 10 mA/cm^2. Drouiche *et al.* (2009) used electrocoagulation with aluminium electrodes successfully to remove fluoride from wastewater. NaCl was used as a supporting electrolyte, and it was verified that precipitation of the contaminant was dependent on electrolyte concentration.

Electrocoagulation was combined with ultrasound to remove dyes from water (Vianney and Muthukumar, 2015). It was theorized that the combination of ultrasound technology and electrocoagulation could improve P recovery rates. Ultrasound would create cavitation effects that would send small shock waves through the wastewater, which would be able to

remove compact layers of aluminium oxide and other products that accumulate in the electrode surface as electrocoagulation progresses, possibly solving the problem of electrode passivation (Vianney and Muthukumar, 2015). The application of ultrasound, with the ultrasonic probe placed on the water surface between the electrodes, was found to have a synergistic effect with electrocoagulation when applied to wastewater. While electrocoagulation alone achieved near complete P removal after 20 minutes, the use of ultrasound achieved complete removal in just 5 minutes. However, current densities above 40 mA/cm^2 were found to increase flocculation of wastewater particles, reducing P removal efficiency (Vianney and Muthukumar, 2015).

Verma *et al.* (2013) were able to precipitate 100% of chromium (Cr) in both synthetic and real effluent solutions using electrocoagulation with iron electrodes and electric current at 50 mA/cm^2. An initial pH of 4 led to 80% precipitation of Cr as chromium hydroxide ($Cr(OH)_3$) at 15 minutes of electrocoagulation, with higher initial pH reducing the rate of precipitation (at the same point, initial pH 8 led to only 18% precipitation). However, all initial pH values led to 100% recovery at around 60 minutes, except for pH 4, which reached completion at 45 minutes. Electrocoagulation was found to be much more efficient than chemical precipitation using ferric chloride, which removed less than half of dissolved Cr (Verma *et al.*, 2013).

Although we found no studies on the effect of electrocoagulation on enzyme activity, there is evidence that electric currents affect the activity of several enzymes in soil. Cang *et al.* (2009) have demonstrated that the application of electric currents to soil for the removal of heavy metals affects the activity of soil enzymes. Using electrokinetic remediation, which consists of applying a low-voltage current to soil for long periods of time to make heavy metals and some organic chemicals migrate to electrode chambers for removal, they achieved over 90% Cd removal. However, the current also increased the activities of invertase and catalase, while decreasing the activity of urease and acid phosphatase. By measuring the effect of the current on soil parameters, the authors concluded that variations in enzyme activity could be explained by changes caused by electricity, such as pH, electrical conductivity, and dissolved organic carbon. For acid phosphatase, most of the variation in its activity (72%) was explained by the increase in electrical conductivity.

Objectives and hypotheses

So far, there are few studies in the literature looking at the effect of soil parameters on phosphatases other than monoesterases; Güsewell (2017) produces a thorough analysis of two sedges, *Carex flava* and *Carex muricate*, finding that both species have decreased root exudate activity of monoesterases, diesterases and phytase under low soil P. This is the first project aiming at a more comprehensive understanding of the effect of the P-deprivation and Cd toxicity syndrome in the roots of major crop plants, which will be achieved by looking at the monoesterase, diesterase and phytase groups. Respectively, these groups of enzymes catalyse the release of phosphate from monoesters, diesters, and phytic acid (or phytate). We will determine how P deprivation affects the activity of these subclasses of enzymes in root exudates, root tissue, and shoots. We will also determine how Cd toxicity affects enzymes in those same places.

We will submit maize (*Zea mays*) and sunflower (*Helianthus annuus*) plants to growth in media with different concentrations of Cd and measure morphological and physiological parameters impacted by the treatments, comparing the results to the phosphate deficiency syndrome. Both *Z. mays* and *H. annuus* have been identified as non-hyperaccumulators with phytoremediation potential (He et *al.*, 2017). Additionally, we will determine the levels at which Cd accumulates in plant tissue during early growth of the two species, with the goal of determining whether they are good candidates for phytoextraction of Cd. As part of that analysis, we will also measure survival rates for seedlings transplanted at different stages after germination in 10% MS medium to media containing different concentrations of Cd.

One factor that reduces the rate of precipitation during electrocoagulation (and other P recovery methods) is the presence of organic forms of phosphorus. While inorganic phosphorus is readily oxidized at the anode, direct oxidation of organic forms is only possible at high electric potentials. Methods such as electro-Fenton and ultrasound are capable of oxidizing organic P and increase electrocoagulation rate, but it is possible that phosphatases can also achieve that result. We will determine the effect that phosphatases have on phosphorus recovery by electrocoagulation and chemical precipitation with iron electrodes and iron salts, respectively. In both cases, sludge will be treated with phosphatase either at

the beginning of the process or one hour before it starts. This will be accomplished by adding phytase from wheat and monoesterase from rabbit intestine (separately) to sludge samples from a Christchurch sewage treatment plant.

Based on our objectives and on current literature, we have formulated the following hypotheses:

1. Longer waiting periods between germination and transfer to a Cd-contaminated medium will increase survival rates for maize and sunflower.

2. Absence of medium P will cause maize and sunflower to have lower germination rates, shorter roots and shoots, greater root hair area, and lower chlorophyll content.

3. Absence of medium P will cause higher root exudate and total root activity of phosphatases to increase.

4. The presence of Cd in the medium will cause similar symptoms to P deficiency, including the lower germination rates, shorter roots and shoots, increased root hair area, and low chlorophyll. The highest Cd concentration, which is twice the maximum concentration of bioavailable Cd allowed by the European Union for crop plant growth, is likely to cause severe reductions in all parameters.

5. Medium Cd is also likely to cause higher phosphatase activity.

6. In both P-deficient and Cd-treated plants, increases in phosphatase activity are likely to be greater in root exudates than in shoots or in root tissue.

7. Electrocoagulation will cause a higher percentage of phosphorus removal than chemical precipitation.

8. The presence of phosphatases will accelerate the rate of precipitation of phosphate compounds in both chemical precipitation and electrocoagulation; wheat phytase will be more efficient than rabbit intestine monophosphatase at increasing phosphorus precipitation rates in both chemical precipitation and electrocoagulation.

9. Analysis of precipitated crystals formed during electrocoagulation will reveal high amounts of phosphorus and aluminium.

Materials and methods

10-day transfer survival rate

Seeds of corn (*Zea mays*, Golden Sweet variety) and sunflower (*Helianthus annuus*, Russian Giant variety) were gently shaken for 15 minutes in 5% sodium hypochlorite (NClO) for sterilization, and then washed in de-ionized water three times, each time for 3 minutes. Seeds were then transferred in an aseptic environment to 10 cm-tall containers with Murashige-Skoog (MS) medium diluted to 10%, so as not to inhibit germination.

Z. mays seeds germinated 3-4 days after being placed in the containers, while *H. annuus* took 6-7 days. Seeds were transferred to one of five media three, six, or nine days after germination. The media were:

1. 10% MS
2. 10% MS without phosphorus
3. 10% MS with 0.1 mM cadmium (Cd)
4. 10% MS with 0.5 mM cadmium (Cd)
5. 10% MS with 1.0 mM cadmium (Cd)

A new set of *Z. mays* and *H. annuus* seeds was planted directly into each of the five media.

In the no-phosphorus media, potassium dihydrogen phosphate (KH_2PO_4) was replaced with potassium chloride (KCl). In the cadmium-containing media, Cd was provided in the form of cadmium chloride ($CdCl_2$).

Survival was defined as the absence of necrosis on at least one leaf, and survival rates were measured ten days after transfer for the first set of seeds and ten days after germination for the second set.

Morphological response to medium Cd

As in the transfer survival rate experiment, *Z. mays* and *H. annuus* seeds were planted in 10% MS medium. That experiment showed that a three-day waiting period between germination and transfer was enough to ensure a high survival rate up to 0.5 mM of Cd, and at that stage the roots are short enough to be easy to handle without damage during the transfer. We used those results to determine the shortest waiting period that would for high survival rates in subsequent experiments.

After three days, seeds were transferred to the same five media as in the previous experiment:

1. 10% MS
2. 10% MS without phosphorus
3. 10% MS with 0.1 mM cadmium (Cd)
4. 10% MS with 0.5 mM cadmium (Cd)
5. 10% MS with 1.0 mM cadmium (Cd)

After ten days, plants were removed from the containers to measure the effect of the treatments on the following morphological parameters: root length, root fresh weight, lateral root area, shoot height, shoot weight and chlorophyll content. Roots were gently dried before weighting, and lateral root area was measured by pixels on Photoshop.

Root hair area

The parameter referred to as “root hair area” was determined by selecting root segments of the same length and from the same root part of plants under different treatments, photographing them and measuring the area occupied by non-black pixels on Photoshop. This parameter also takes root area into account because some root segments had no root hairs, making a percentage comparison impossible.

Chlorophyll content analysis

Leaf tissue was ground with an 80% acetone solution in a mortar, and then centrifuged at 2655g and 4^{o}C. The supernatant was diluted in acetone, and absorbance was measured in a spectrophotometer at 645 nm and 663 nm. Chlorophyll content was determined by the following equation (West, 2015):

$$[\text{Chl a + b}] = 17.76\ E645 + 7.34\ E663\ (\mu g\ g^{-1})$$

Root and shoot phosphatase activity

After removal from the containers and morphological measurements, roots were cut into subsections of about 100 mg each and transferred to test tubes. Into each test tube were placed 4 mL of one of three enzyme substrates:

1. 5 mM *p*-nitrophenylphosphate (for monoesterase activity)
2. 5 mM bis(p-nitrophenyl)phosphate (for diesterase activity)
3. 5 mM phytic acid (for phytase activity)

P-nitrophenylphosphate and phytic acid were buffered to pH 5.5 with acetate buffer 50 mM, and bis(p-nitrophenyl)phosphate was buffered to pH 8.0 with TRIS buffer 50 mM.

Test tubes were placed at room temperature (about 25^{o}C) and shaken gently every five minutes for one hour. The monoesterase and diesterase reactions were terminated by transferring 100 µL of the assays to new test tubes containing 2 mL of NaOH (0.5 M for monoesterase and 0.1 M for diesterase). Absorbance was measured at 410 nm on a spectrophotometer and calibrated with *p*-nitrophenol standards. Activity for both monoesterase and diesterase was expressed in µmol of *p*-nitrophenol released per g of fresh root weight per hour ($\mu mol.g^{-1}.h^{-1}$).

Phytase activity was measured through the coloured product of the reaction between phosphate and the ammonium molybdate-ascorbic acid reagent, which yields a blue colour. 200 µL of the 5 mM phytic acid solutions were transferred to a test tube with 2 mL of the reagent, and were gently shaken every five minutes for a half-hour. Absorbance was measured at 880 nm and calibrated with KH_2PO_4 standards. The process was repeated with controls, or root solutions incubated in pH 5.5 acetate buffer instead of phytic acid, and the result was expressed as the difference between the phytate and control solutions, in µmol of phosphorus released per g of fresh root weight per hour (µmol g^{-1} h^{-1}).

Shoots were sampled from the top for sections of about 100 mg, placed in a mortar with 4 mL of acetate buffer at pH 5.5, and then crushed with a pestle. The liquid fractions of the resulting mixtures were transferred to test tubes and processed in the same way as the 100 mg root samples, with 500 µL of liquid fraction instead of 100 mg of root tissue.

Cadmium content in root and shoot tissue

Root and shoot samples were dried in an oven at 60^{o}C for two days and were then ground with mortar and pestle. For each sample, 100 mg were placed in a beaker, to which 5 mL of nitric acid (HNO_3) were added to start the digestion. The beaker was then heated to 120-130^{o}C for 14 hours, after which 2 mL of hydrogen peroxide (H_2O_2) were added. After the end of digestion, the sample was diluted to 50 mL, labelled, and sent to the Department Of Chemistry at the University Of Canterbury for analysis of heavy metal content by inductively coupled plasma mass spectrometry (ICP-MS).

Beakers used in this experiment were soaked overnight in a 10% nitric acid bath and rinsed several times with de-ionized water before use.

Phosphorus recovery through precipitation with metal salts

A sample of sewage sludge was obtained from the Christchurch City Council Bromley Wastewater Treatment Facility. The sludge was suspended in Milli-Q water and filtered through filter paper to prevent suspended solids from affecting the experiment.

Compared to other commonly used metal ions, Al^{3+} is the one that presents the highest phosphorus recovery rate at the optimum pH for phytase (5.5), at around 85% (Huang *et al.*, 2016). Fe^{2+} has the lowest recovery rate at that pH, at just 50% (it reaches 75% at pH 6.5). However, phosphate recovery through precipitation with Fe^{2+} salts is much cheaper than with Al^{3+} salts, at just 54% of the price. Given the financial advantage and the much greater room for optimization, Fe^{2+} salts were used in this experiment, provided as $FeSO_4.H_2O$. Fe^{3+} was also used, provided as $FeCl_3$. Optimum pH for Fe^{3+} is 4.5, at which P recovery reaches 83%, compared to 80% at pH 5.5 (Huang *et al.*, 2016).

100 mL of filtered sludge were transferred to 250 mL beakers, and a magnetic stirrer was placed at the bottom and set to stir at 150 rpm. In the iron(II) sulphate treatment, each beaker also received 0.1 g of sodium thiosulfate ($Na_2S_20_3$), to eliminate the oxygen in the wastewater and prevent the conversion of Fe^{2+} into Fe^{3+}. Iron salts were added at a molar ratio of Fe^{2+}:P equal to 1.25:1. Solution pH was adjusted to 4 (for phytase) or 10 (for alkaline monoesterase) with 0.1 M NaOH and/or HCl, and wheat phytase (Sigma Life Science; 1 unit/100 mL) or alkaline monoesterase (from rabbit intestine; Sigma Chemical Co.; 1 unit/100 mL) was added to the solution, either one hour before the start of electrocoagulation (pre-treatment) or at the same time.

The reaction was carried out for 60 minutes, with aliquots being taken at 5, 20, 35, 50 and 60 minutes of reaction to sample for total P content; 2 mL of NaOH were added to each sample to stop phytase action and the mixtures were allowed to settle for another 30 minutes. After that, the 5 mL samples were filtered through a 0.45 μm membrane for phosphorus content analysis.

This process was repeated with suspended sludge that had been left to react with phytase for one hour before the addition of the iron salts.

In the alkaline phosphatase experiments, pH was set to 8, which is close to the optimum PH of the enzyme, but not so high that the efficiency of P removal by Fe^{2+} is too low.

Electrocoagulation

The setup for the electrocoagulation experiments is shown in figure 1, while figure 2 gives a clearer schematic presentation; aluminium electrodes of dimensions 120mm X 10mm X 0.8mm were used, and a pH meter was added to the beaker as required. They were connected to a benchtop power source with capacity of 3 A of current through alligator clips and hung from a ring stand; they were placed inside a 1,000 mL beaker at a height of 10mm above the bottom. As in the chemical precipitation experiments, the beaker was placed atop a magnetic stirrer; as in Zhang *et al.* (2013), the gap between electrodes was set at 25 mm. Solution pH was adjusted to 4 (for phytase) or 10 (for alkaline monoesterase) with 0.1 M NaOH and/or HCl, and wheat phytase (Sigma Life Science; 1 unit/100 mL) or alkaline monoesterase (from rabbit intestine; Sigma Chemical Co.; 1 unit/100 mL) was added to the solution, either one hour before the start of electrocoagulation (pre-treatment) or at the same time. In some cases, after at least 30 minutes of treatment, conductivity in the solution became too low to create a strong enough current for our purposes. In these cases, NaCl was added to increase conductivity for the remainder of the experiment.

Optimum phytase and alkaline monoesterase pH

The optimum pH for phytase and alkaline monoesterase was determined by adding each enzyme to filtered suspended sludge at different pH values, allowing the reaction to occur for 30 minutes, and measuring the decrease in organic phosphorus concentration. The optimum pH was the one with the greatest decrease in organic phosphorus.

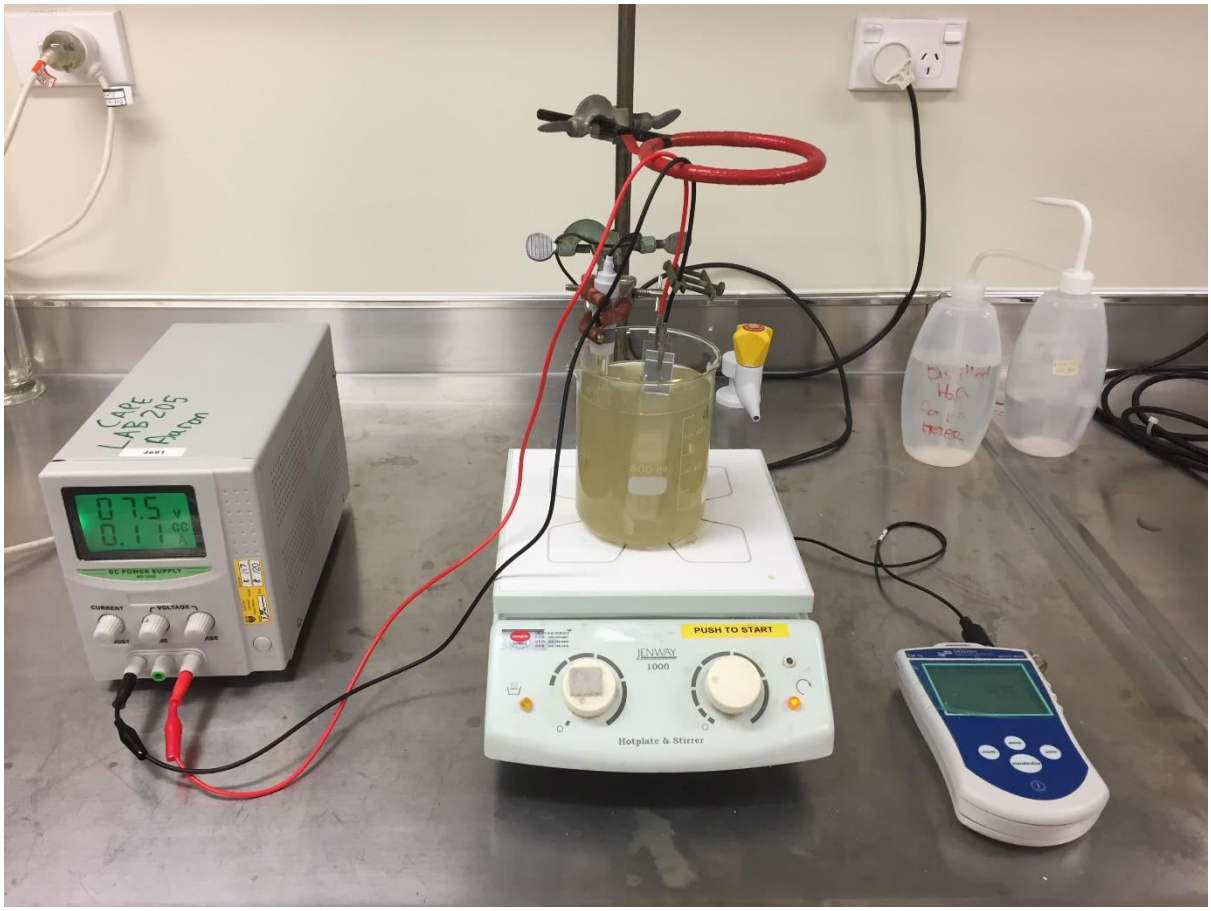


Figure 1: the setup for the electrocoagulation experiments.

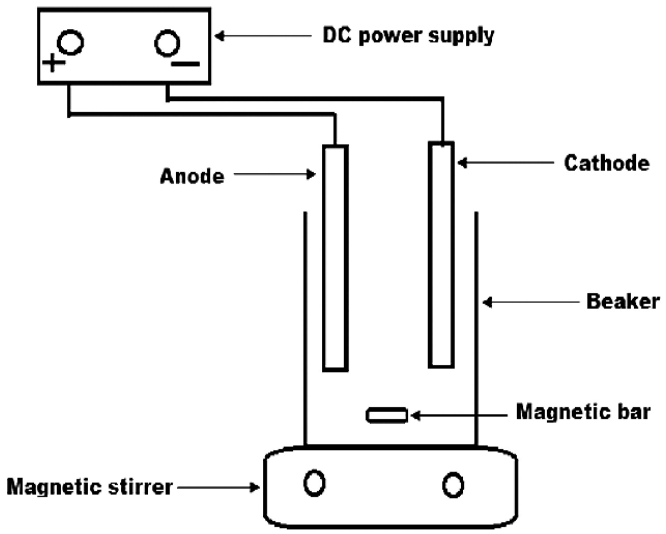


Figure 2: the setup for the electrocoagulation experiment. Image from Verma *et al.* (2013).

Total phosphorus analysis

Liquid samples were diluted to 50 mL and transferred to an Erlenmeyer flask, to which 1 mL of 11 N sulphuric acid (H_2SO_4) and 0.4 g of ammonium persulfate were added. This mixture was boiled for about 30 minutes or until about 10 mL were left, while being intermittently shaken gently. The resulting liquid was left to cool and diluted to 40 mL, then filtered. To remove excess iron, 5 mL of sodium bisulfite solution was added to the samples, which were then placed in a 95% water bath for 30 minutes. After cooling, they were diluted to 50 mL.

Hydrolizable phosphorus analysis

Liquid samples were diluted to 50 mL and transferred to an Erlenmeyer flask, to which 1 mL of 11 N sulphuric acid (H_2SO_4) were added. Samples were gently boiled for about 30 minutes, then left to cooled, diluted to 40 mL and filtered. To remove excess iron, 5 mL of sodium bisulfite solution was added to the samples, which were then placed in a 95% water bath for 30 minutes. After cooling, they were diluted to 50 mL.

Orthophosphate analysis

Liquid samples were diluted to 50 mL and transferred to an Erlenmeyer flask, to which 1 mL of 11 N sulphuric acid (H_2SO_4) and 4 mL of ammonium molybdate-antimony potassium tartrate were added. The mixture was mixed, then 2 mL of ascorbic acid solution was added, and it was mixed again.

All samples (orthophosphate (O), total (T) and hydrolizable (H) phosphorus) had phosphorus concentration determined by spectrophotometry at 650 nm after the reaction between sample P and the ammonium molybdate-ascorbic acid reagent (also containing potassium antimony tartrate), calibrated with KH_2PO_4 standards. Organic phosphorus was determined as T – (O + H).

Energy dispersive X-ray spectroscopy

After electrocoagulation was finished, samples from the foam that formed above the liquid were gathered. After the liquid was filtered, samples from the precipitate that remained in the filter membrane were also taken and were dried for 24 hours at 60^{o}C along with the foam samples. These samples were taken to the Mechanical Engineering Department at the College of Engineering, where they were carbon-coated and placed in a scanning electron microscope for chemical characterization through energy dispersive X-ray spectroscopy.

Statistical analysis

Basic statistical analysis (such as means and standard deviation) and graphics were performed using Microsoft Excel, while Student's t-test for comparison of means was carried out with R version 3.4.4.

Results

Note: Unless specified otherwise, the threshold for statistical significance was set at 95%.

Germination

Maize

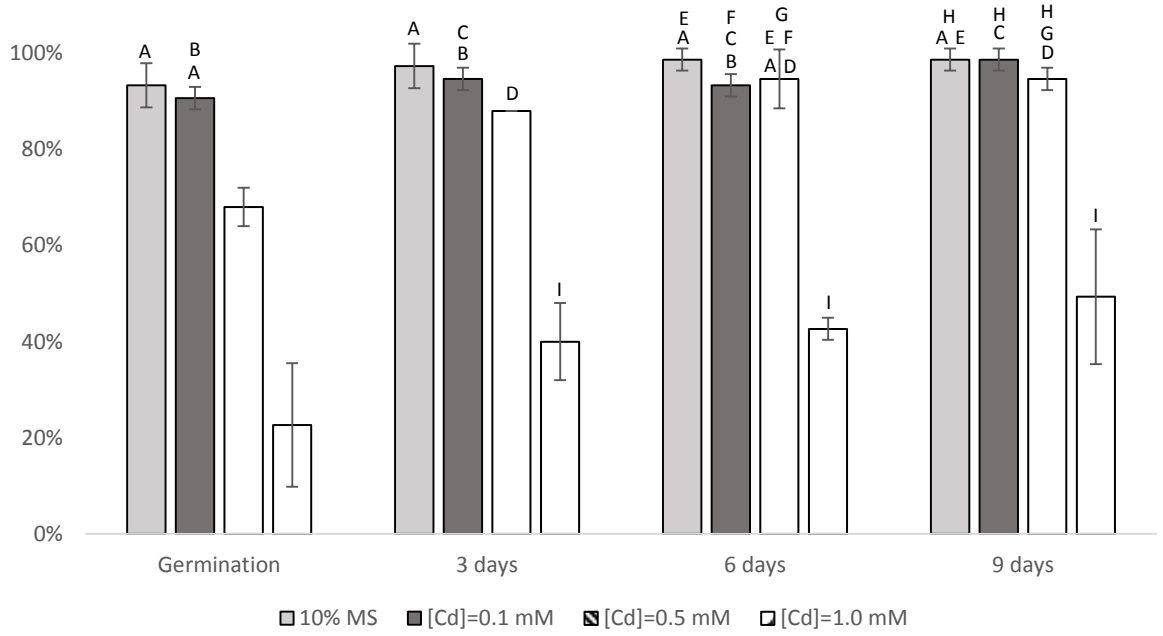


Figure 3: 10-day survival rate of **maize** seedlings transplanted into Cd-containing medium 3, 6, or 9 days after germination in 10% MS medium. Error bars indicate standard deviation. Statistical significance was calculated only between treatments that shared a Cd concentration or a transplant day.

Maize seedlings germinating in 10% MS medium or in [Cd]=0.1 mM showed no statistical difference in survival, both around 90% (figure 3). However, only 68% of seeds germinating in [Cd]=0.5 mM and 21% of those in [Cd]=1.0 mM survived for 10 days after germination. Seeds transplanted 3 days after germination had slightly higher averages of survival for control and [Cd]=0.1 mM, but without statistical difference from the germination treatments. [Cd]=0.5 mM and [Cd]=1.0 mM seedlings improved survival rates to 88% and 40%, respectively. 6-day and 9-day transplants showed no difference from 3-day transplant seedlings, although one group in the 9-day [Cd]=1.0 mM treatment reached 60% survival; in the 9-day transplants, only the [Cd]=1.0 mM treatment was below 10% MS controls, with just under half its average (49% versus 94%).

Sunflower

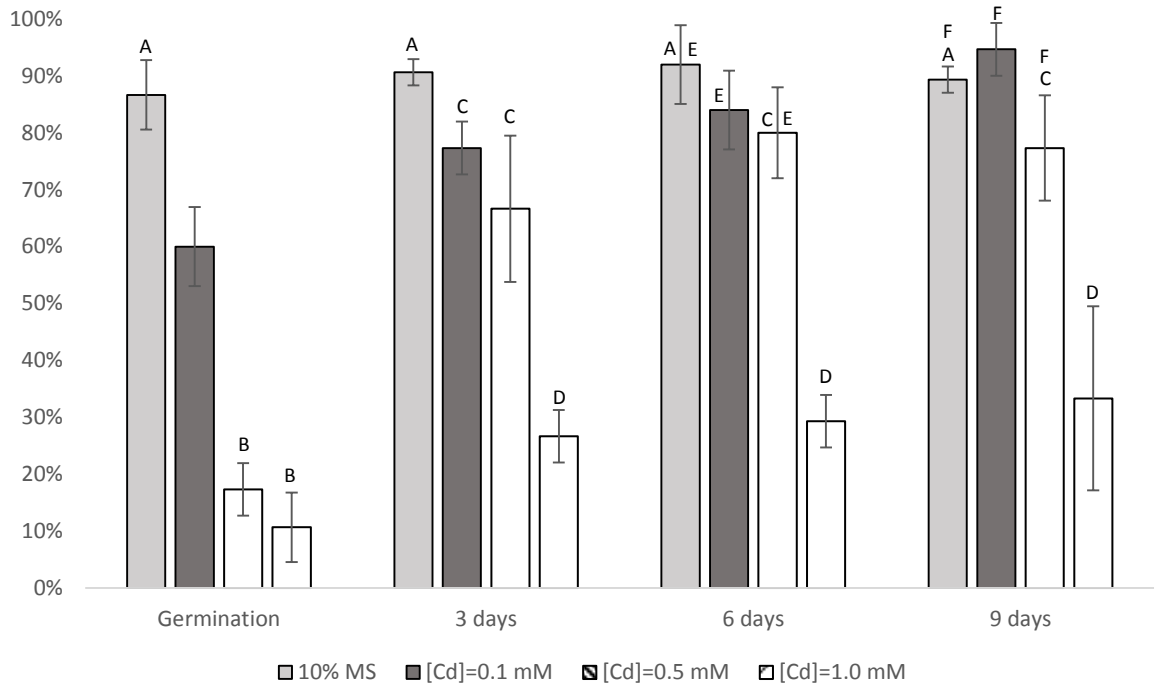


Figure 4: 10-day survival rate of **sunflower** seedlings transplanted into Cd-containing medium 3, 6, or 9 days after germination in 10% MS medium. Error bars indicate standard deviation. Statistical significance was calculated only between treatments that shared a Cd concentration or a transplant day.

Sunflower seedlings in the control treatments had survival rates around 90%, regardless of the transplant day (figure 4). Germination in a Cd-containing medium reduced average 10-day survival to 60% at [Cd]=0.1 mM, and to statistically indistinguishable 16% and 10% averages at [Cd]=0.5 mM and [Cd]=1.0 mM, respectively. Increasing time in 10% MS medium before transplant improved survival for the [Cd]=0.1 mM treatment to 76%, 84%, and 94%, all with statistical significance. In the [Cd]=0.5 mM treatment, transplant after germination greatly improved survival rates, with averages between 67% and 78%, but without statistically significant difference between transplant days. Transplant also improved survival rates for the highest Cd concentration, although, just as for maize, no statistical improvement was registered with increases in time before transplant, although the average increased from 27% to 33%; 9-day [Cd]=1.0 mM seedlings also presented much lower survival rates than all other treatments in the same day group (33% versus 95% for [Cd]=0.5 mM, the highest).

Root morphology

Length

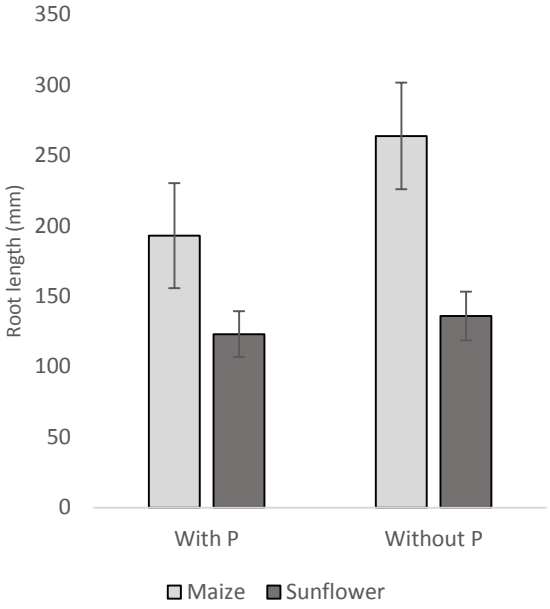


Figure 5: the effect of phosphorus deprivation on the root length of maize and sunflower plants. Error bars show standard deviation.

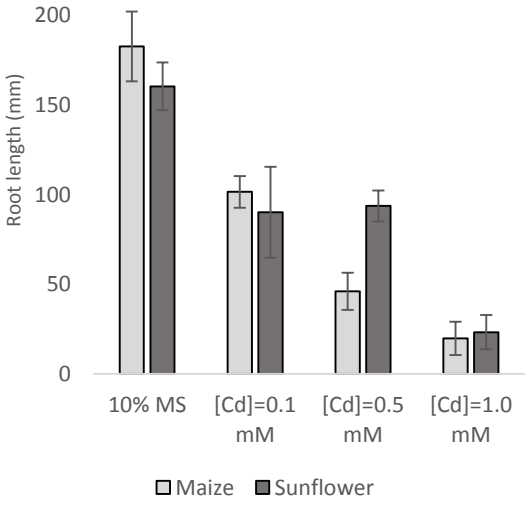


Figure 6: the effect of medium cadmium on the root length of maize and sunflower plants. Error bars show standard deviation.

Absence of P in the medium caused an increase in root lengths of both maize and sunflower (figure 5). Maize roots were longer than sunflower roots under both P deprivation and

control conditions, and the treatment caused a greater proportional increase in length of 34%, compared to only 12% elongation for sunflower.

Increasing Cd concentrations caused a sharp and steady decline in maize root length (figure 6). At [Cd]=1.0 mM, root length was only 11% of the control length. For sunflower, there was no statistical difference in root length between the [Cd]=0.1 mM and the [Cd]=0.5 mM treatments, where the decrease was to 74% of control. At [Cd]=1.0 mM, root length was statistically indistinguishable between the two species.

Weight

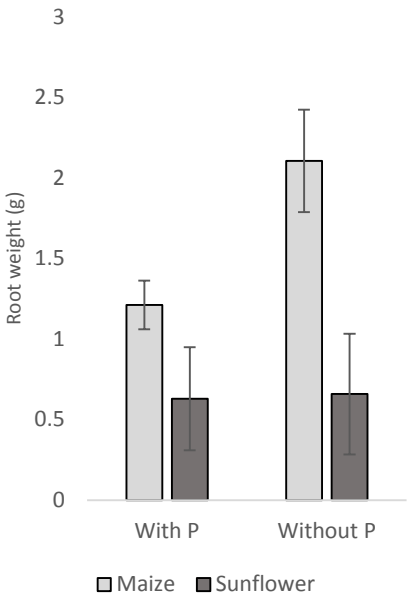


Figure 7: the effect of phosphorus deprivation on the root weight of maize and sunflower plants. Error bars show standard deviation.

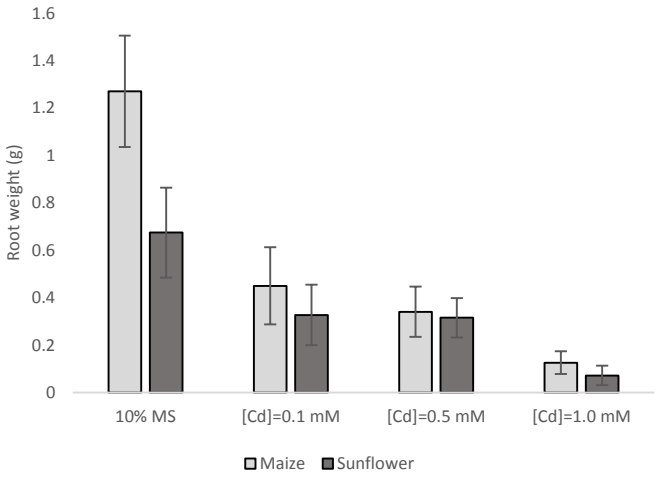


Figure 8: the effect of medium cadmium on the root weight of maize and sunflower plants. Error bars show standard deviation.

Maize roots were significantly heavier in the absence of phosphorus in the medium, showing a 68% increase (figure 7). Sunflower presented greater variance at both treatment and control, but no change in length was observed.

Cadmium stress had a very negative effect on shoot weight for both species (figure 8). At [Cd]=1.0 mM, both sunflower and maize roots were reduced to only 12% of the control weight. At the lower concentrations, sunflower suffered less significant decreases, with no statistical difference between [Cd]=0.1 mM and [Cd]=0.5 mM.

Root hair

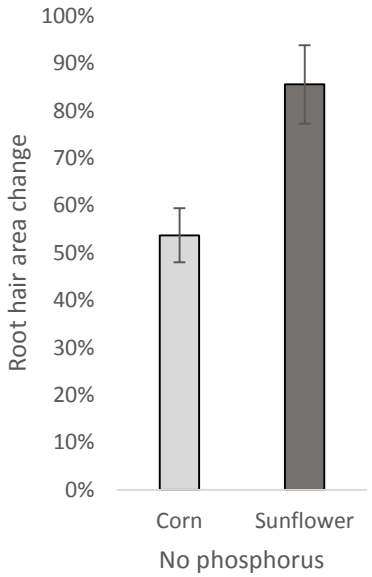
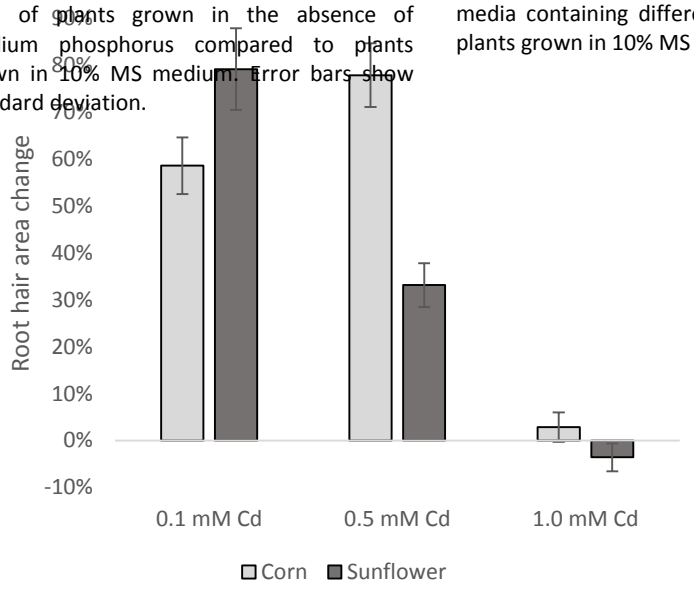


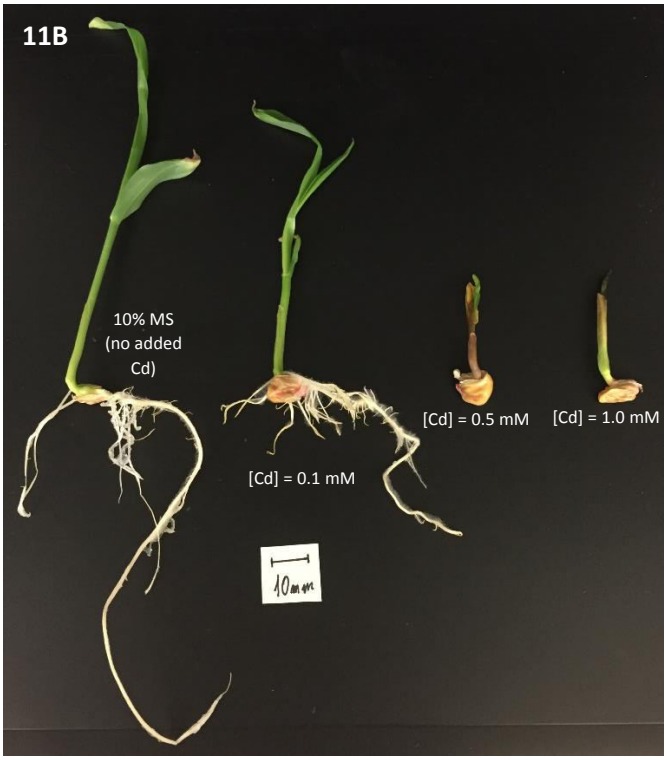
Figure 9: the percentage change in root hair area of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation.

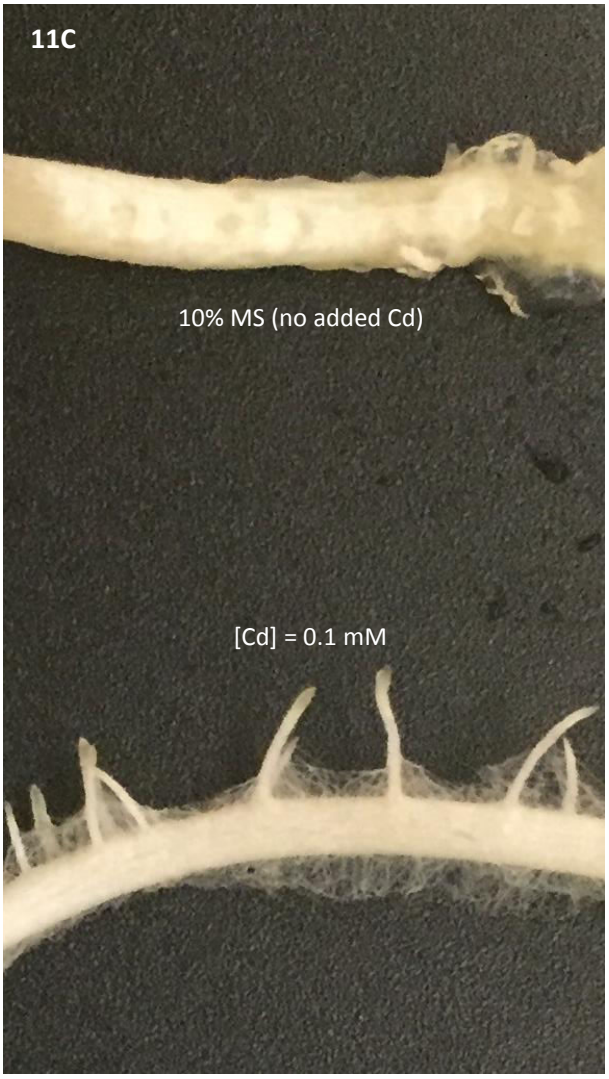
Figure 10: the percentage change in root hair area of plants grown in media containing different concentrations of cadmium compared to plants grown in 10% MS medium. Error bars show standard deviation.



Absence of phosphorus in the medium caused great increases in root hair area in both maize and sunflower seedlings (figure 9). The former's surface grew by 55% on average, while the latter grew by 85%.

Low and medium concentrations of Cd in the medium also caused root hair area to increase in both species (with one exception) (figure 10). However, while maize had more root hairs at [Cd]=0.5 mM than at [Cd]=0.1 mM (78% more surface area versus 59%), sunflower roots followed the opposite pattern (79% versus 33%), culminating in roots with less root hair area at [Cd]=1.0 mM than control plant roots. Maize roots at [Cd]=1.0 mM had only 5% greater root hair area than control plants. It should be noted that, as figure 11A shows, maize roots could not be removed from the medium together with the stems, as they easily detached from them when that was attempted.





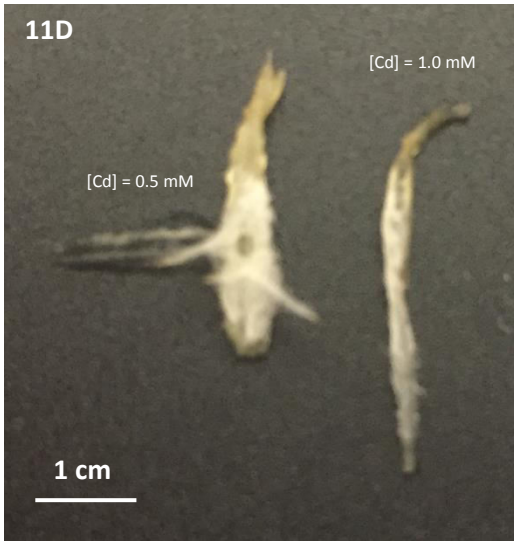


Figure 11: (A) Maize plants grown in the presence or absence of phosphorus; (B) Maize plants grown in presence of different concentrations of cadmium; (C) Closeup of maize root segments from plants grown in no-Cd medium or [Cd]=0.1 mM; (D) Maize roots grown at [Cd]=0.5 mM and [Cd]=1.0 mM.

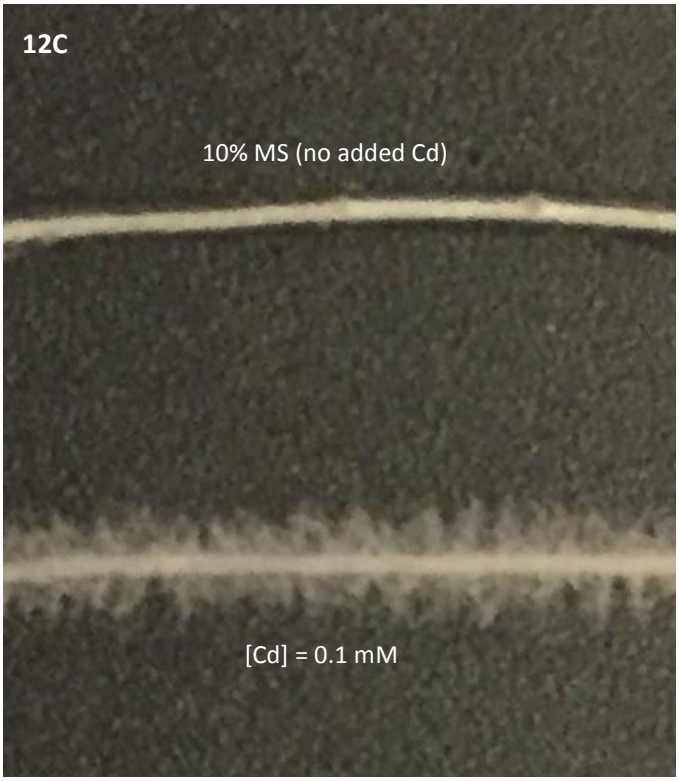


Figure 12: (A) Sunflower plants grown in the presence or absence of phosphorus; (B) Sunflower plants grown in presence of different concentrations of cadmium; (C) Closeup of sunflower root segments from plants grown in no-Cd medium or [Cd]=0.1 mM.

Root exudate phosphatase activity

Monoesterase activity

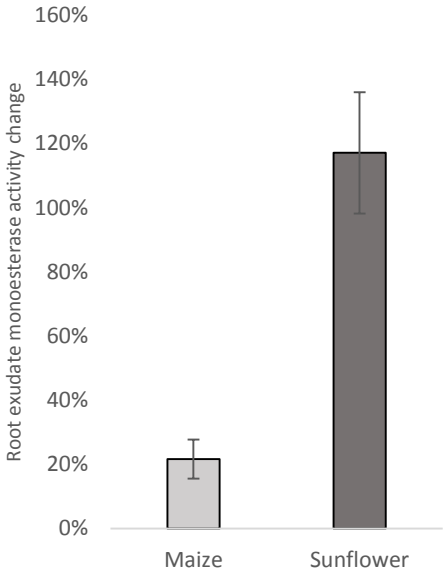


Figure 13: the percentage change in root exudate **monoesterase** activity of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation.

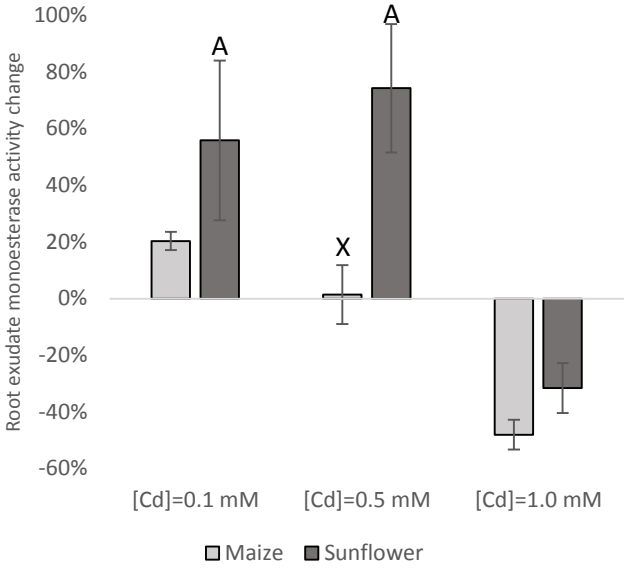


Figure 14: the percentage change in root exudate **monoesterase** activity of plants grown under different concentrations of Cd in the medium compared to plants grown in 10% MS medium. Error bars show standard deviation. X represents no statistically significant change. There is no statistically significant difference between bars that share a letter.

Sunflower root exudate monoesterase activity more than doubled in the absence of medium P (Fig. 13). Maize root exudates also showed increased activity of the enzyme, but that increase was almost six times smaller than that of sunflower roots.

A similar pattern was present under low medium Cd. [Cd]=0.1 mM caused a 20% increase in monoesterase activity in maize exudates, and an increase of 56% in sunflower (Fig. 14). Under [Cd]=0.5 mM, when maize plants were starting to show clear signs of Cd toxicity (Fig. 11A), monoesterase activity was statistically indistinguishable from control; sunflower exudates had a 72% increase, but like in the lower Cd concentration, variance was so high between samples that [Cd]=0.1 mM and [Cd]=0.5 mM were not statistically different. Under [Cd]=1.0 mM, both species showed lower exudate activity compared to control, with 28% and 48% decreases in sunflower and maize, respectively.

Diesterase activity

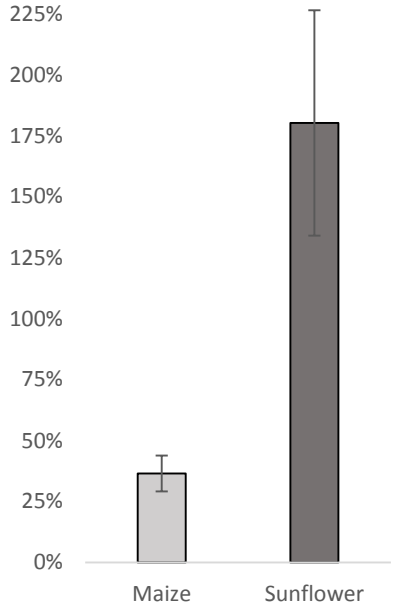


Figure 15: the percentage change in root exudate diesterase activity of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation.

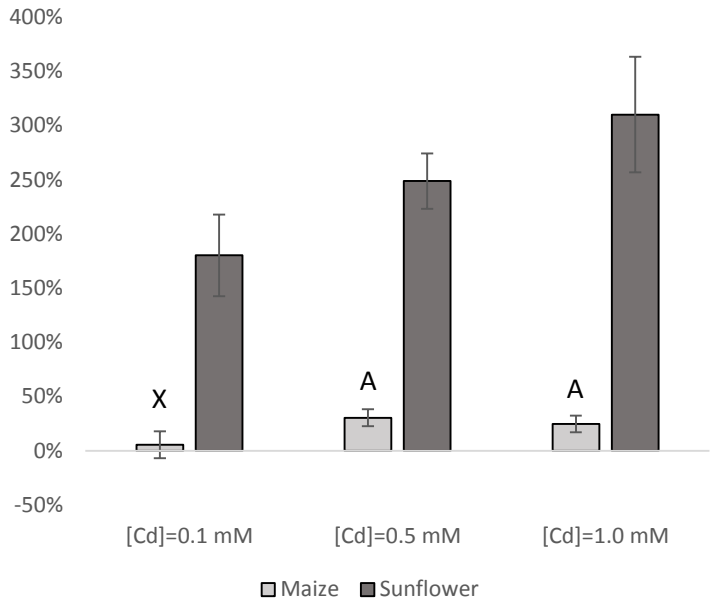


Figure 16: the percentage change in root exudate diesterase activity of plants grown under different concentrations of Cd in the medium compared to plants grown in 10% MS medium. Error bars show standard deviation. X represents no statistically significant change. There is no statistically significant difference between bars that share a letter.

The absence of phosphorus in the growth medium caused the same pattern of root exudate diesterase activity as it did for monoesterase activity: the enzyme more than doubled in activity in sunflower roots, and it had a significant but smaller increase in maize roots (Fig. 15).

Medium cadmium caused progressive increases in activity in sunflower, with diesterase activity almost doubling at [Cd]=0.1 mM, increasing by about 250% at [Cd]=0.5 mM, and finally more than tripling at [Cd]=1.0 mM (figure 16). The lowest Cd concentration had no statically significant effect on exudate diesterase activity in maize, but [Cd]=0.5 mM and [Cd]=1.0 mM increased by around 30%, with no statistical difference between the concentrations.

Phytase activity

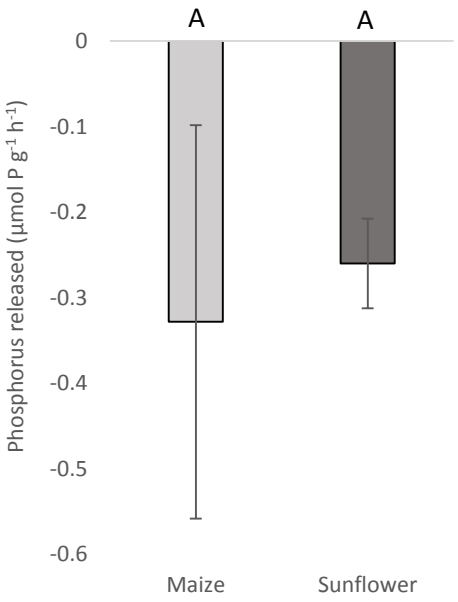


Figure 17: the change in **root exudate phytase activity** of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation. There is no statistically significant difference between bars that share a letter.

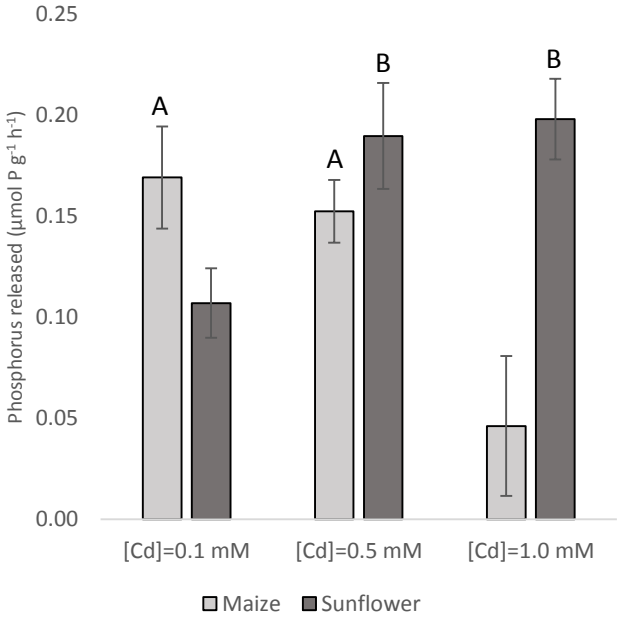


Figure 18: the change in **root exudate phytase activity** of plants grown under different concentrations of Cd in the medium compared to plants grown in 10% MS medium. Error bars show standard deviation. There is no statistically significant difference between bars that share a letter.

Lack of medium phosphorus decreased phytase activity by statistically identical amounts in both maize and sunflower plantlets; maize root exudates showed great variance, with decreases between 0.10 and 0.55 in phosphate released per gram of fresh per hour (figure 17). Sunflower exudates had much smaller variance, and had a smaller average decrease than maize exudates, despite the lack of significance.

Unlike absence of medium P, Cd caused increases in phytase activity in all treatments for both species (figure 18). Maize exudates had statistically indistinguishable averages of about 0.17 an 0.15 at [Cd]=0.1 mM and [Cd]=0.5 mM, respectively, but the increase compared to control was only a third of that value, with greater variance. Activities in sunflower exudates increased from 0.1 at [Cd]=0.1 mM to twice that amount at [Cd]=0.5 mM and [Cd]=1.0 mM.

Shoot parameters

Shoot height

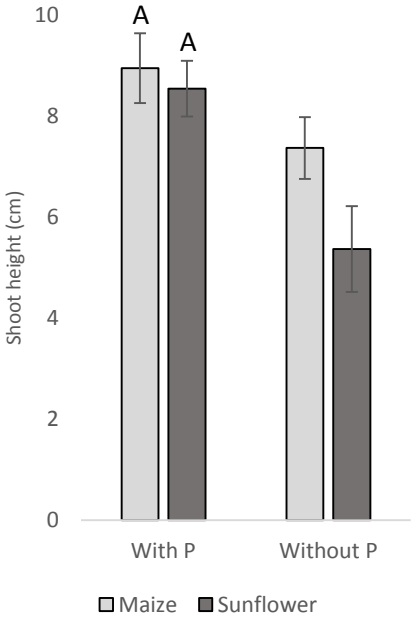


Figure 19: the effect of phosphorus deprivation on the **shoot height** of maize and sunflower plants. Error bars show standard deviation. Bars that share a letter are not statistically significantly different.

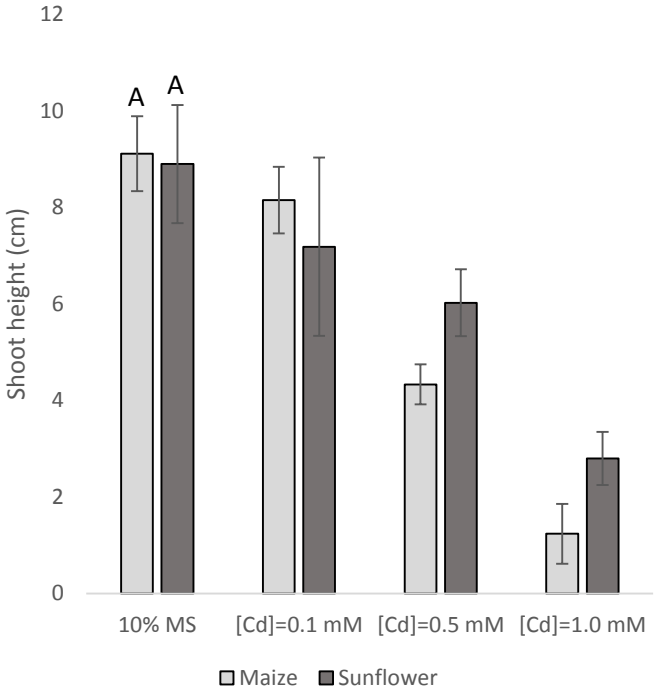


Figure 20: the effect of medium cadmium on the root weight of maize and sunflower plants. Error bars show standard deviation. Bars that share a letter are not statistically significantly different.

The absence of phosphorus in the medium had a negative effect on shoot height in both maize and sunflower (figure 19). The decline was greater in sunflower, where P-deprived shoots 35% shorter, against 17% in maize.

Medium cadmium also caused a significant decrease in shoot height (figure 20), but the decline was greater for maize than for sunflower. At [Cd]=0.1 mM, maize shoots were only 90% as tall as control, and at the highest Cd concentration, they were only 13% of that height. The decline for sunflower shoots was also sharp, but less so than maize: at [Cd]=0.1 mM they were 81% the height of control (with shoots taller than the tallest maize shoots at the same concentration), but had declined to just 31% of control at [Cd]=1.0 mM. As figure 11A shows, maize shoots had clear signs of necrosis at [Cd]=0.5 mM and [Cd]=1.0 mM.

Shoot phosphatase activity

Shoot monoesterase activity

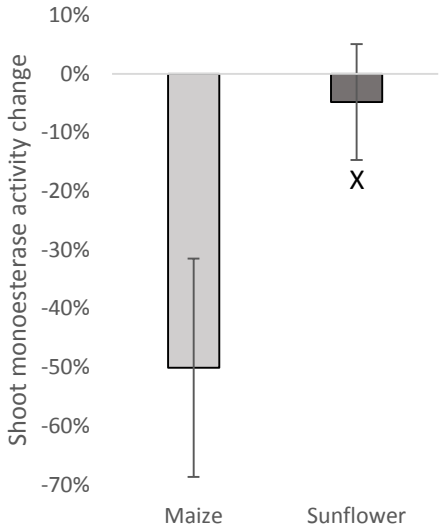


Figure 21: the percentage change in shoot exudate **monoesterase** activity of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation. X represents no statistically significant change.

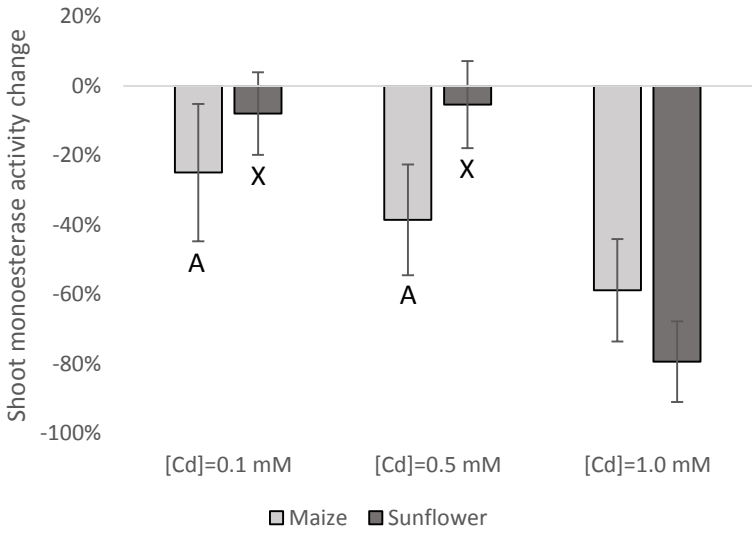


Figure 22: the percentage change in root exudate **monoesterase** activity of plants grown under different concentrations of Cd in the medium compared to plants grown in 10% MS medium. Error bars show standard deviation. X represents no statistically significant change. There is no statistically significant difference between bars that share a letter.

Absence of medium phosphorus had different effects on maize and sunflower shoot monoesterase activity (figure 21). Activity in maize shoots was cut in half. Some sunflower samples showed a decrease of up to 15% in activity, but some showed an increase, and there was no statistically significant change.

In maize shoots, the lowest concentrations of Cd also caused a decrease in enzyme activity, but not as pronounced as under P deprivation (figure 22). At [Cd]=1.0 mM, the decline was greater, of just under 60%. The lowest concentrations also did not affect sunflower shoot enzyme monoesterase activity to a significant level (although, like in the absence of medium P, there was an average decrease in activity), but at [Cd]=1.0 mM the enzyme lost 80% of its activity.

Shoot Diesterase activity

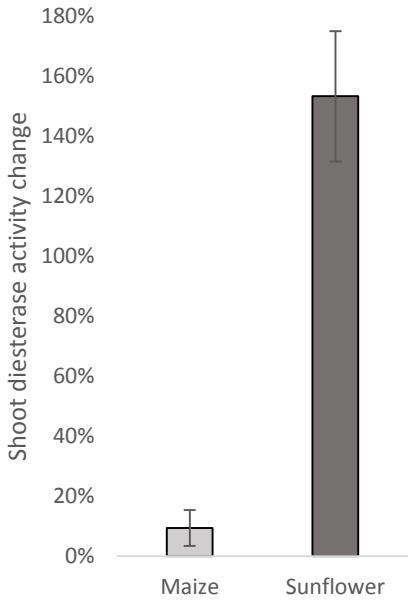


Figure 23: the percentage change in shoot exudate **diesterase** activity of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation.

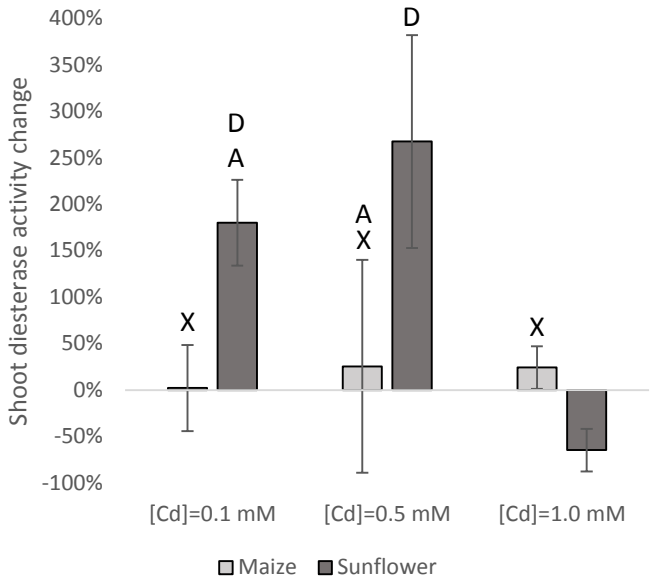


Figure 24: the percentage change in root exudate **diesterase** activity of plants grown under different concentrations of Cd in the medium compared to plants grown in 10% MS medium. Error bars show standard deviation. X represents no statistically significant change. There is no statistically significant difference between bars that share a letter.

Both maize and sunflower shoots had diesterase activities increased by the absence of medium P (figure 23). However, while the increase was of only 10% in maize shoots, activity in sunflower shoots more than doubled, reaching an average increase of more than 150%.

Medium cadmium caused no statistically significant changes in maize shoot diesterase activity. Individual sample values varied widely, with some samples at [Cd]=0.5 mM more than doubling activity and some showing a 90% decrease (figure 24). However, the average change was towards an increase under each cadmium concentration. The only treatment to produce a decrease in shoot diesterase activity was sunflower at [Cd]=1.0 mM, but at lower concentrations sunflower shoots showed nearly three ([Cd]=0.1 mM) and nearly four ([Cd]=1.0 mM) times the activity they showed under the control treatment.

Shoot phytase activity

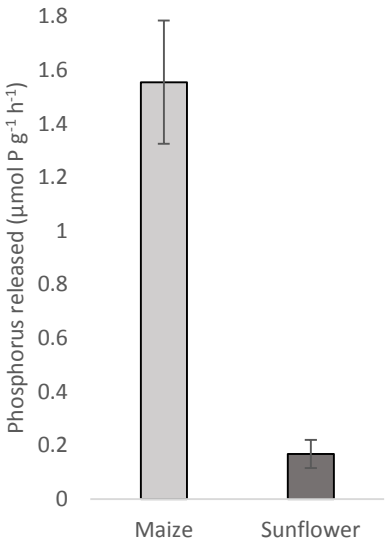


Figure 25: the change in shoot **phytase** activity of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation.

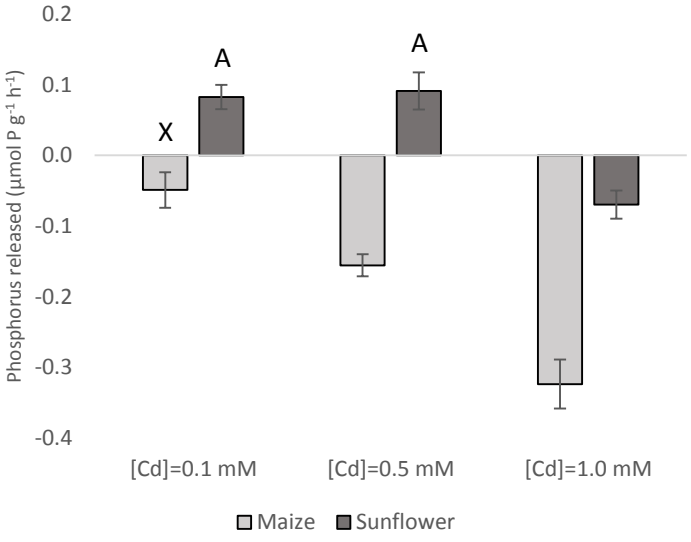


Figure 26: the percentage change in shoot **phytase** activity of plants grown under different concentrations of Cd in the medium compared to plants grown in 10% MS medium. Error bars show standard deviation. X represents no statistically significant change. There is no statistically significant difference between bars that share a letter.

Shoot phytase activity increased without phosphorus in the medium in both species (figure 25), but the increase was over nine times greater for maize than for sunflower shoots (just over 1.5 versus just over 0.15 µmol of P released per gram of fresh root per hour).

High cadmium caused significant decrease in maize shoot phytase activity (figure 26). While [Cd]=0.1 mM shoot showed no statistical change, all samples had lower activity than control, if only slightly so. [Cd]=1.0 mM caused a decrease of 0.3 µmol of P released per gram of fresh root per hour, while halving medium Cd concentration also halved the phytase activity decrease. Activity in sunflower shoots increased by the same amount at [Cd]=0.1 mM and [Cd]=0.5 mM, and decreased slightly at the highest Cd concentration.

Root/shoot activity ratios

Root/shoot ratio of changes in monoesterase activity

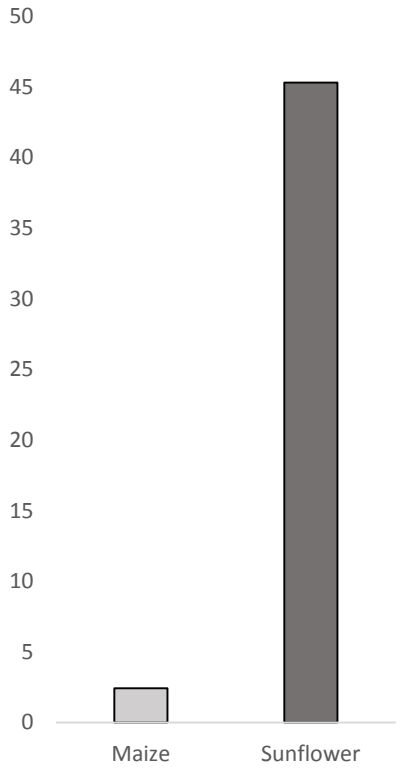


Figure 27: the relative difference in the change of monoesterase activity between roots and shoots in the **absence of medium phosphorus** compared to plants grown in 10% MS medium. Decrease in activity was considered as a fractional increase.

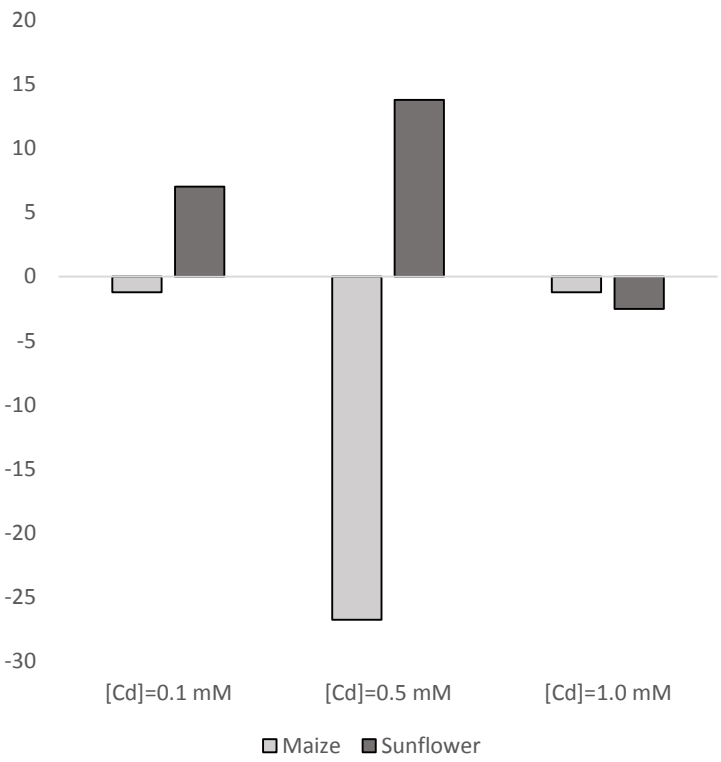


Figure 28: the relative difference in the change of monoesterase activity between roots and shoots in the presence of **different concentrations of cadmium** compared to plants grown in 10% MS medium. Decrease in activity was considered as a fractional increase. Negative values indicate a shoot/root ratio.

In maize plantlets, monoesterase activity in the roots increased 2.5 times more than in shoots when there was no P in the medium (figure 27). The increase was much greater in sunflower plantlets, where root exudate monoesterase activity increased more than 45 times compared to shoots.

In the presence of Cd, maize shoots had a greater increase in monoesterase activity than maize roots: levels rose 22% more at [Cd]=0.1 mM and [Cd]=1.0 mM, but they increased by a factor of almost 27 at [Cd]=0.5 mM (figure 28). In sunflower, root exudate activity of monoesterase increased by 7 and 13 times more than in shoots at the lowest

concentrations, but at [Cd]=1.0 mM shoots showed 2.5 times greater increased activity than roots.

Root/shoot ratio of changes in diesterase activity

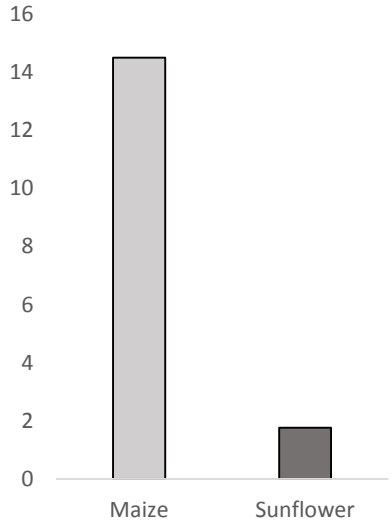


Figure 29: the relative difference in the change of diesterase activity between roots and shoots in the **absence of medium phosphorus** compared to plants grown in 10% MS medium. Decrease in activity was considered as a fractional increase.

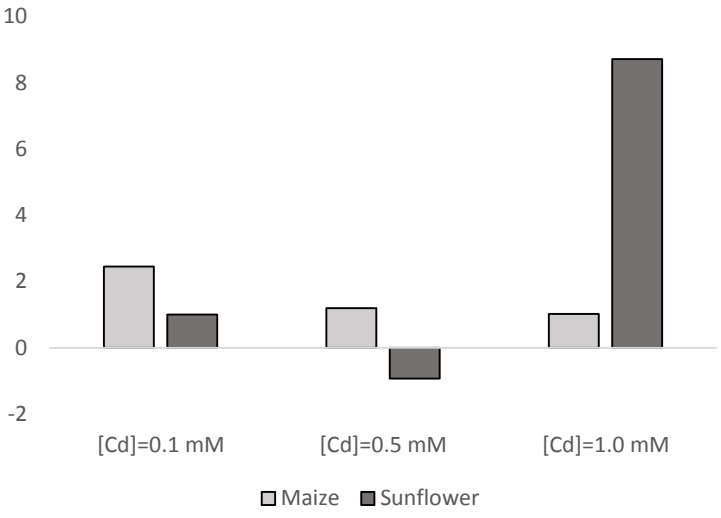


Figure 30: the relative difference in the change of diesterase activity between roots and shoots in the presence of **different concentrations of cadmium** compared to plants grown in 10% MS medium. Decrease in activity was considered as a fractional increase. Negative values indicate a shoot/root ratio.

In the absence of phosphorus, maize root exudates increased their diesterase activity over 14 times more than maize shoots, while sunflower root exudates increased it twice more than the plant's shoot (figure 29).

Cadmium led to greater increases in root exudate than in shoot monoesterase activity under all concentration for maize (figure 30). At [Cd] = 0.1 mM, exudates increased 2.5 times more than shoots in activity, and by 18% and 2% at [Cd] = 0.1 mM and [Cd] = 1.0 mM, respectively. There was no clear trend in the case of sunflower. At [Cd] = 0.1 mM, activity was almost identical in the two types of samples; at [Cd] = 0.5 mM, it increased 7% more in shoots than in roots; and at [Cd] = 1.0 mM, enzyme activity in exudates increased almost 9 times more than in shoots.

Root/shoot ratio of changes in phytase activity

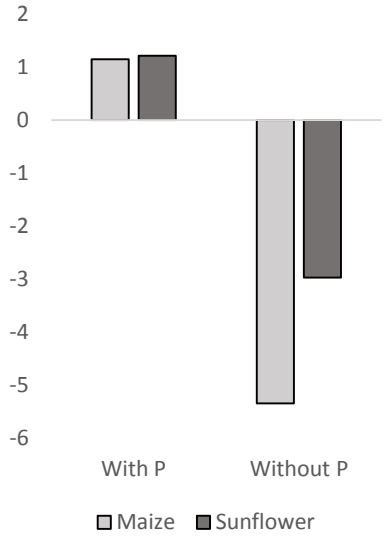


Figure 31: the relative difference in the change of phytase activity between roots and shoots in the **absence of medium phosphorus** compared to plants grown in 10% MS medium. Decrease in activity was considered as a fractional increase.

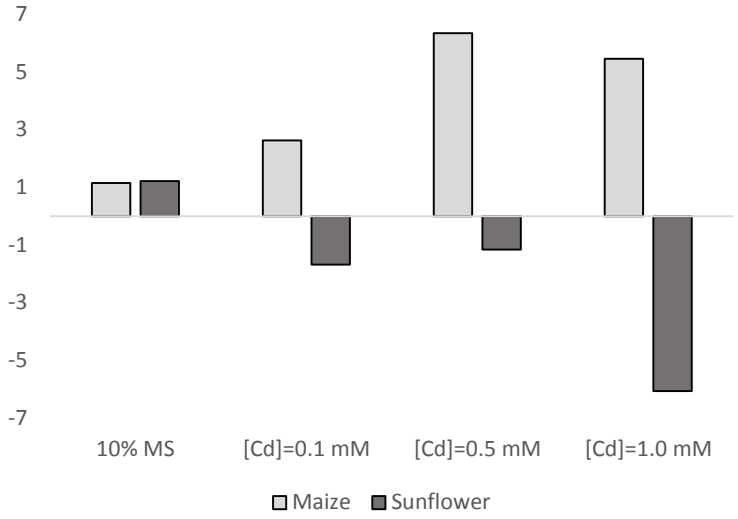


Figure 32: the relative difference in the change of phytase activity between roots and shoots in the presence of **different concentrations of cadmium** compared to plants grown in 10% MS medium. Decrease in activity was considered as a fractional increase. Negative values indicate a shoot/root ratio.

Under control conditions, root exudates from both species had only between 14% and 21% greater activity than shoots (figure 31). However, the absence of phosphorus caused maize exudates to decrease phytase activity over five times more than it increased in shoots, and in sunflower, almost three times more.

With cadmium in the nutrient medium, relative phytase activity was different between the species (figure 32). At [Cd] = 0.1 mM and [Cd] = 0.5 mM, maize root exudates had almost three and more than six times more phytase activity in the roots than in the shoots, respectively, while sunflower shoots had 67% and 15% higher increases in the shoots than in the roots. At the highest Cd concentration, maize continued to have greater increases in root exudates, this time over five times greater than in shoots; sunflower also continued in the same pattern, but this time shoot phytase activity increased six times more than in root exudates.

Total root phosphatase activity

Total root monoesterase activity

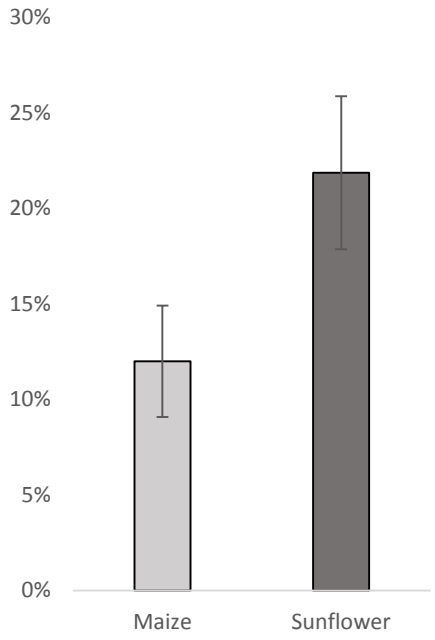


Figure 33: the percentage change in root exudate **monoesterase** activity of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation.

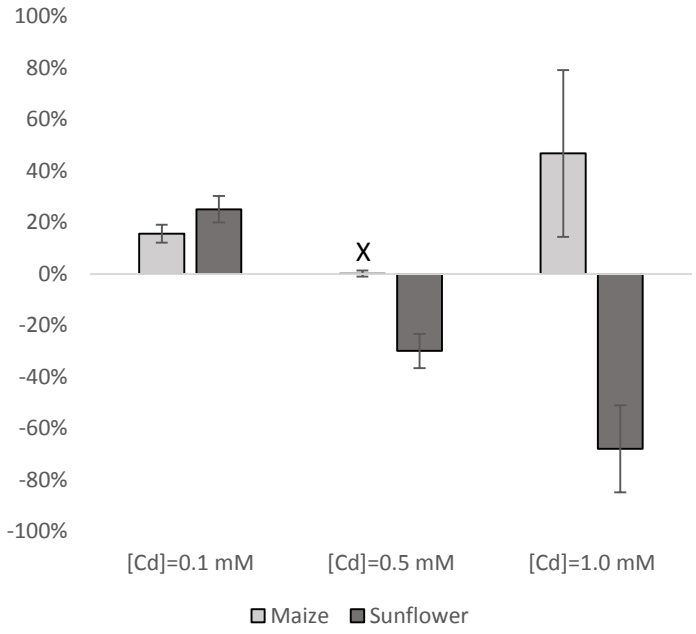


Figure 34: the percentage change in total root **monoesterase** activity of plants grown under different concentrations of Cd in the medium compared to plants grown in 10% MS medium. Error bars show standard deviation. X represents no statistically significant change.

In the absence of phosphorus, total root monoesterase activity increased in both species, by about 12% in maize and almost twice that amount in sunflower (22%) (Figure 33).

The lowest cadmium concentration had a similar but slightly stronger effect on the enzyme for both species, with increases of 16% for maize and 25% for sunflower (figure 34). In maize, enzyme activity at [Cd] = 0.5 mM was no different from control, but at [Cd] = 1.0 mM it increased to almost 50%, although individual samples were between 15% and 80% more active than control. In sunflower, monoesterase activity decreased by 30% with [Cd] = 0.5 mM and then by 68% at the highest concentration.

Total root diesterase activity

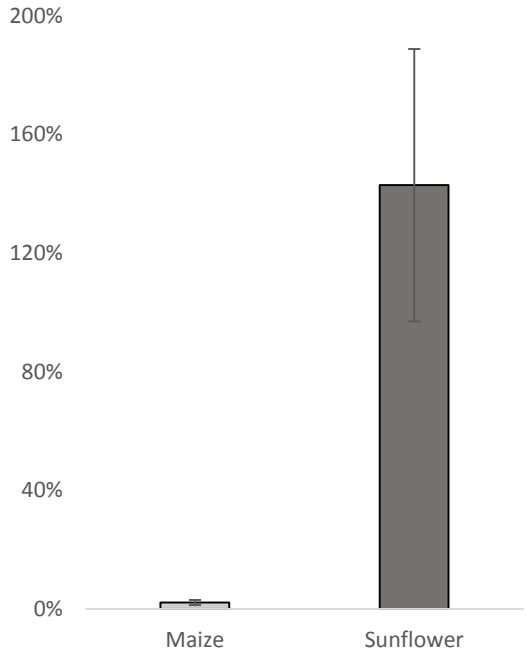


Figure 35: the percentage change in root exudate **diesterase** activity of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation.

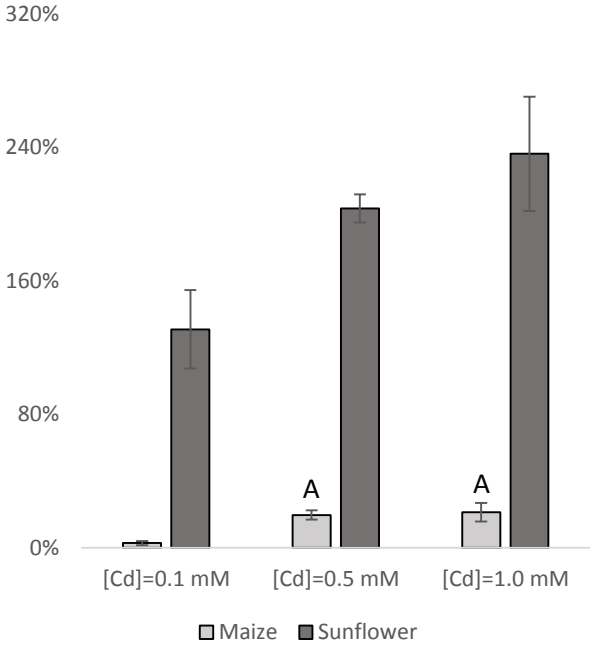


Figure 36: the percentage change in total root **diesterase** activity of plants grown under different concentrations of Cd in the medium compared to plants grown in 10% MS medium. Error bars show standard deviation. Difference between bars that share a letter is not statistically significant.

Phosphorus deprivation causes an increase of only 2% in total root diesterase activity (figure 35). Despite the small difference, the increase was consistent across samples. The increase is much larger in sunflower roots, at an average of 140% higher enzyme activity, with some samples having almost three times the average activity of control.

Much like in the case of monoesterase, both species had similar enzyme activities at [Cd] = 0.1 mM and in the absence of medium phosphorus: maize increased activity by 5% and sunflower by 180% (figure 36). At [Cd] = 0.5 mM and [Cd] = 1.0 mM total root diesterase activity for maize was between 25% and 30% more active than in the control treatment, while sunflower diesterase was 3.5 and 4 times more active, respectively.

Total root phytase activity

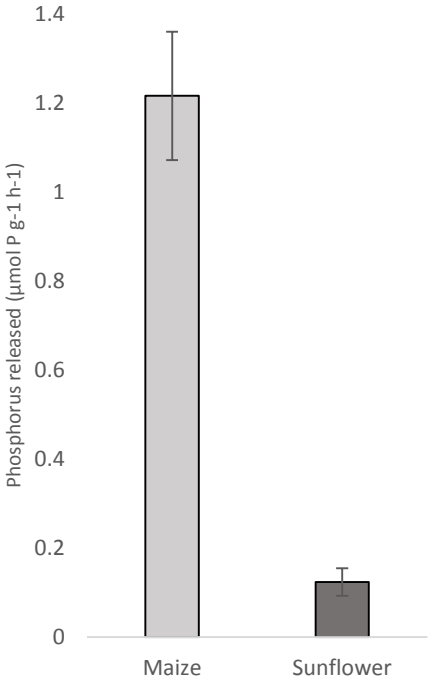


Figure 37: the percentage change in root exudate **phytase** activity of plants grown in the absence of medium phosphorus compared to plants grown in 10% MS medium. Error bars show standard deviation.

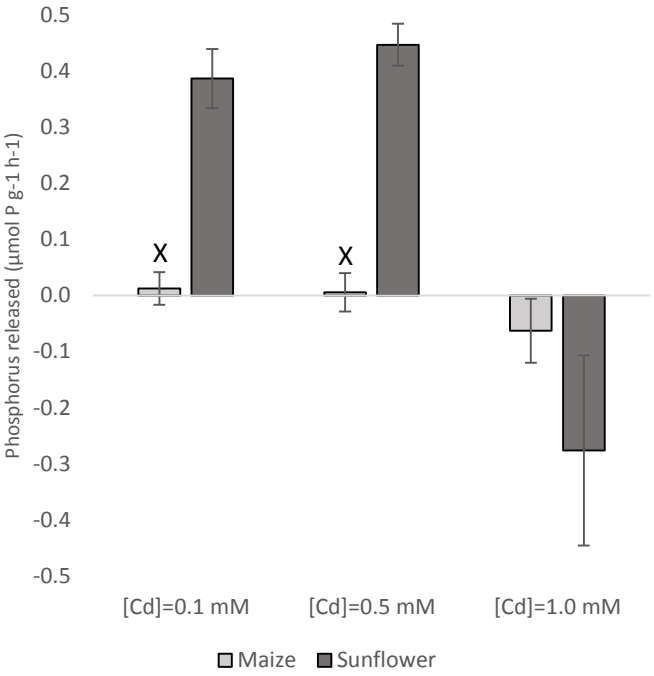


Figure 38: the percentage change in total root **phytase** activity of plants grown under different concentrations of Cd in the medium compared to plants grown in 10% MS medium. Error bars show standard deviation. X represents no statistically significant change.

Maize roots grown in the absence of medium phosphorus had total phytase activity higher than control roots by 1.2 µmol of phosphate released per gram of root per hour; the increase for sunflower roots was almost ten times smaller (figure 37).

Cadmium had a different effect on root phytase activity: apart from the highest concentration, there was no change in activity for corn roots compared to control, but there was a small decrease at [Cd] = 1.0 mM (figure 38). Enzyme activity in sunflower roots was increased at [Cd] = 0.1 mM and [Cd] = 0.5 mM by about 0.4 µmol, but it was decreased at [Cd] = 1.0 mM by 0.25 µmol, with great variance.

Chlorophyll content

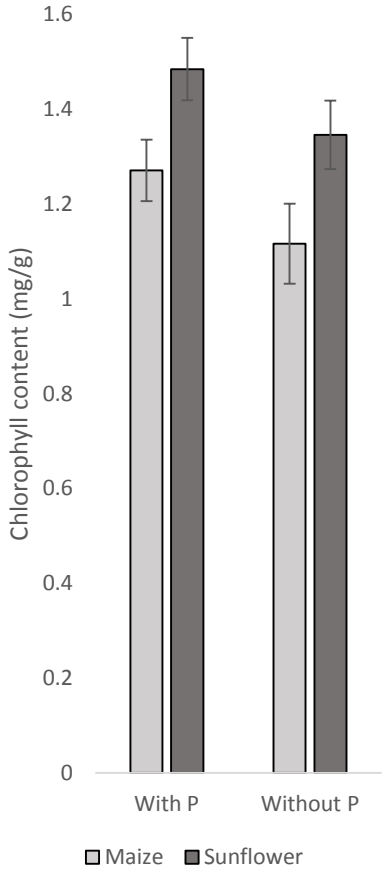


Figure 39: chlorophyll content in maize and sunflower in the presence and absence of medium phosphorus. Error bars show standard deviation.

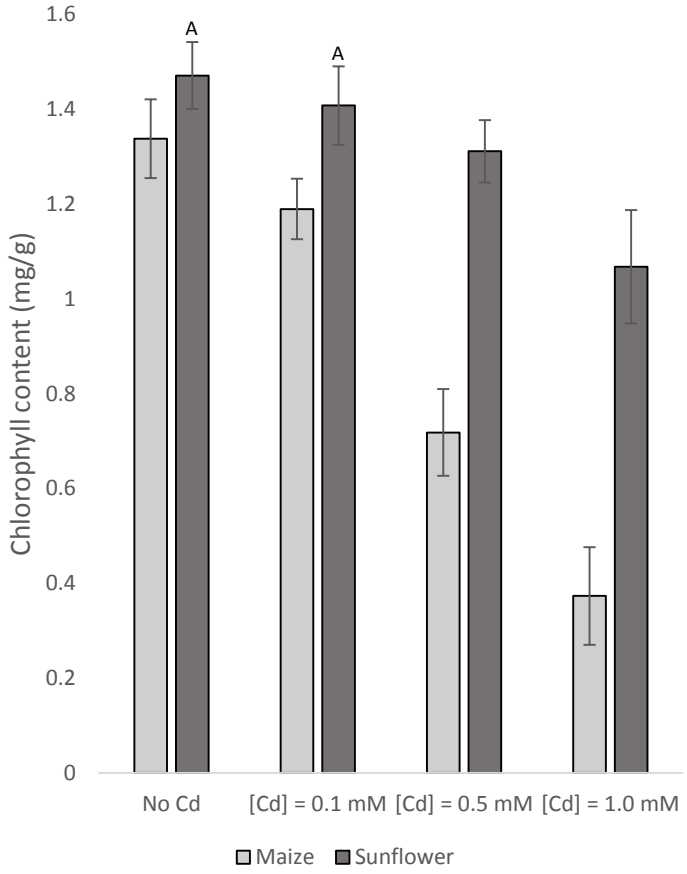


Figure 40: chlorophyll content in maize and sunflower in the presence of different concentrations of cadmium. Error bars show standard deviation. The difference between bars that share a letter is not statistically significant.

Chlorophyll content is slightly reduced in maize and sunflower plants in the absence of P, by 9% in the latter and 12% in the former (figure 39). It is also reduced in the presence of medium Cd, more so in maize than in sunflower: while control and [Cd] = 0.1 mM sunflower plants have comparable levels of chlorophyll, maize plants suffer a decrease of 11% at the same concentration, reaching 72% less chlorophyll at [Cd] = 1.0 mM (figure 40). By comparison, at [Cd] = 1.0 Mm, sunflower chlorophyll content declined only 27%.

Plant tissue Cd content

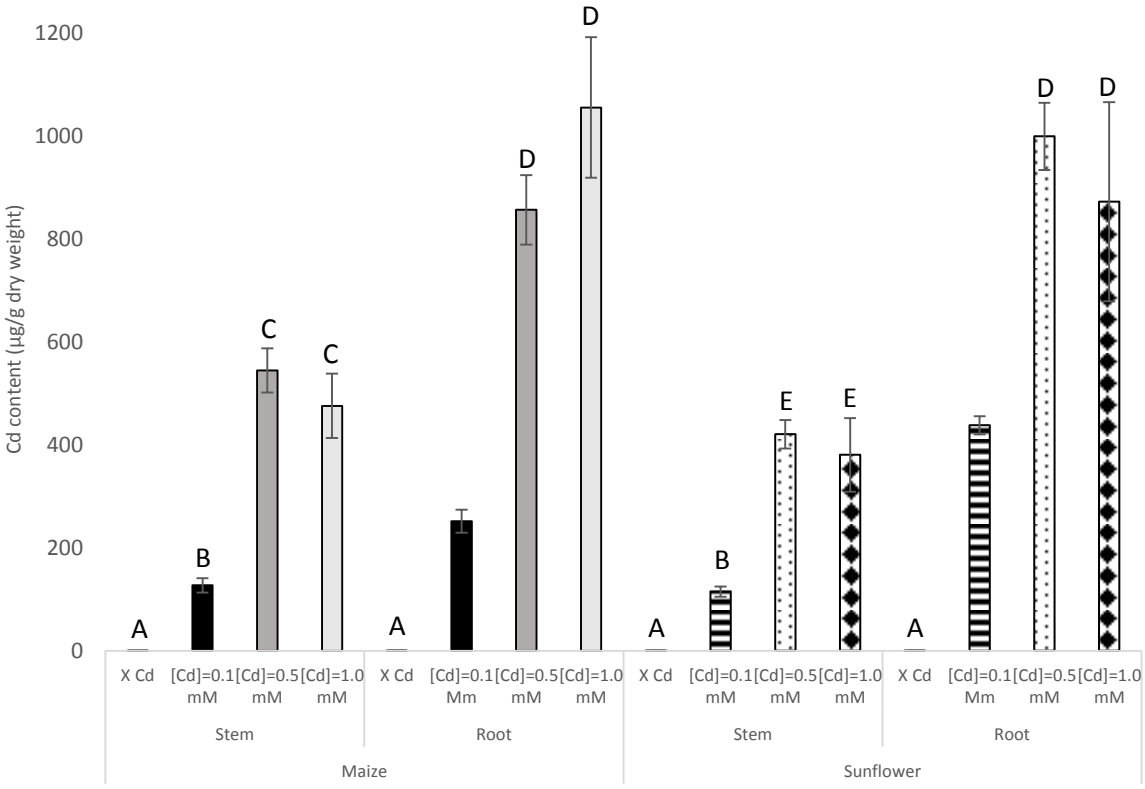


Figure 41: cadmium content in plant tissue after growth under different concentrations of cadmium. Error bars show standard deviation. Difference between bars that share a letter is not statistically significant.

In the absence of medium cadmium, only trace amounts were found in stems and roots of both maize and sunflower (figure 41). In maize roots, cadmium accumulated in high amounts as its concentration in the medium increased, rising to 250 µg per g of dry weight at [Cd] = 0.1 mM, and to 4 times that amount at [Cd] = 1.0 mM, although there was no statistical difference to tissue Cd at [Cd] = 0.5 mM. Stem Cd concentration was invariably lower than root Cd at the same concentration, but it was also greatly increased by medium Cd, reaching a maximum of 550 mg/g at [Cd] = 0.5 mM. It should be noted that in both organs, tissue Cd increased by 3 to 4.5 times from [Cd] = 0.1 mM to [Cd] = 0.5 mM, but there was no difference between [Cd] = 0.5 mM and [Cd] = 1.0 mM.

Sunflower reflects the same pattern seen in maize (figure 41). Root Cd reached 1 mg/g of fresh root weight at [Cd] = 0.5 mM, which was more than double the concentration it reached at [Cd] = 0.5 mM, but was not statistically different from the highest Cd concentration. In stems, the highest Cd concentration was also reached at [Cd] = 0.5 mM, but it was also statistically indistinguishable from [Cd] = 1.0 mM, and more than 3 times the amount at [Cd] = 0.1 mM.

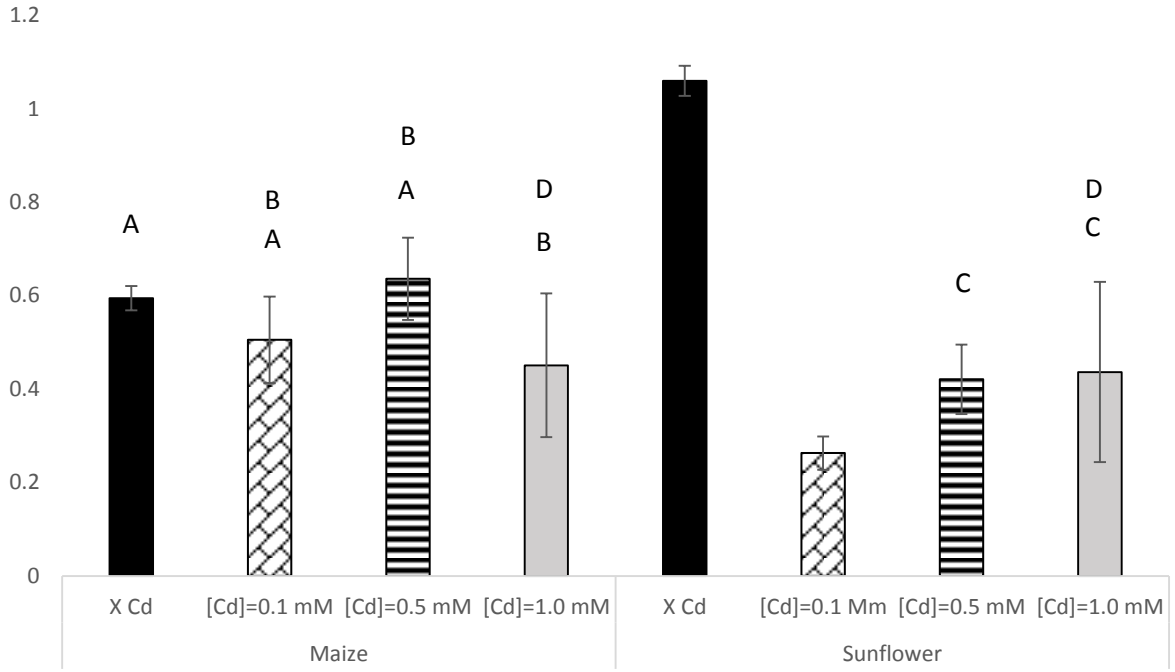


Figure 42: shoot/root ratio of plant tissue cadmium content after growth under different concentrations of cadmium. Error bars show standard deviation. Difference between bars that share a letter is not statistically significant.

Of all 8 treatments, only sunflower control plants had a higher concentration of Cd in shoots than in roots, indicated by the shoot/ratio above 1 (figure 42). In the presence of medium Cd, maize stems concentrated between 55% ([Cd] = 1.0 mM) and 37% ([Cd] = 0.5 mM) less Cd than roots (no statistical difference between three Cd treatments). For sunflower, the highest proportion of Cd accumulated in stems was at [Cd] = 1.0 mM, which was 56% less than roots; however, that was indistinguishable from [Cd] = 0.5 mM, which accumulated 58% less Cd.

Optimum phytase and alkaline monoesterase pH

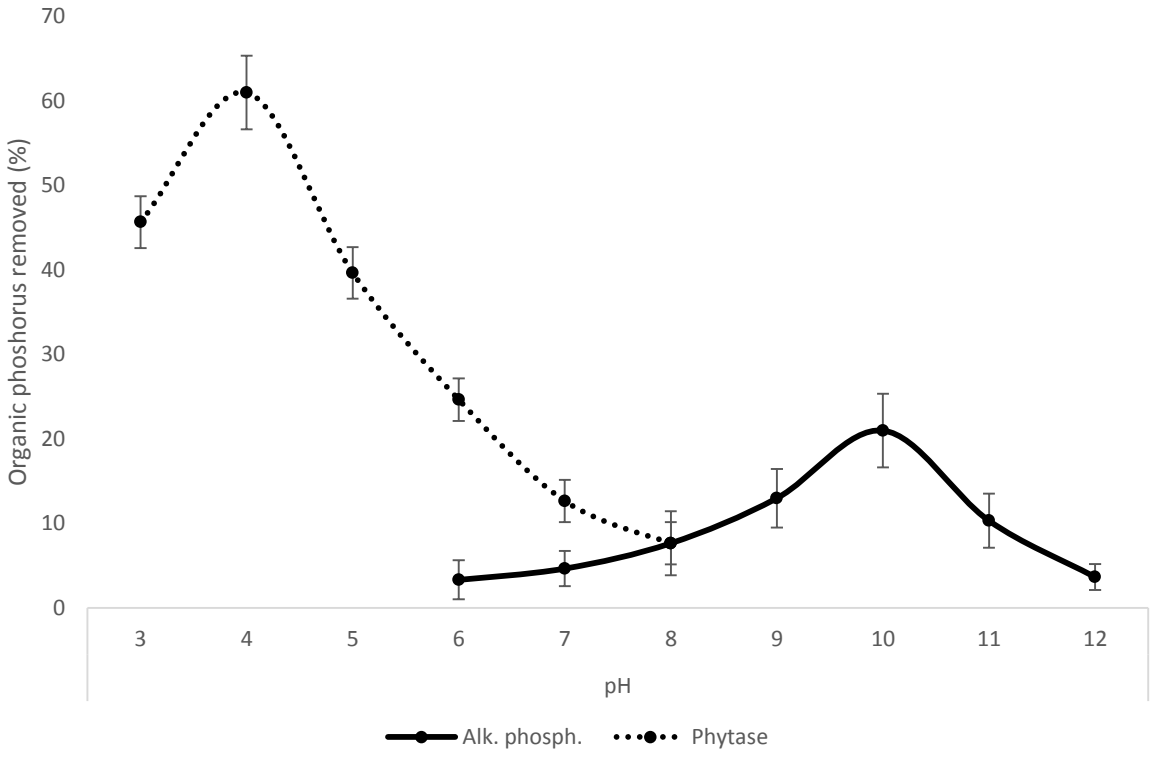


Figure 43: Optimum pH for wheat phytase and alkaline monoesterase, measured as organic phosphorus removed from suspended and filtered sludge. Error bars show standard deviation.

The optimum pH for phytase was found to be 4, while alkaline monoesterase performed best at pH 10 (figure 43). Phytase was able to mineralize three times more organic phosphorus from the liquefied sludge at its optimum pH than alkaline monoesterase at its own optimum (60% versus 20%).

Electrocoagulation

Phytase

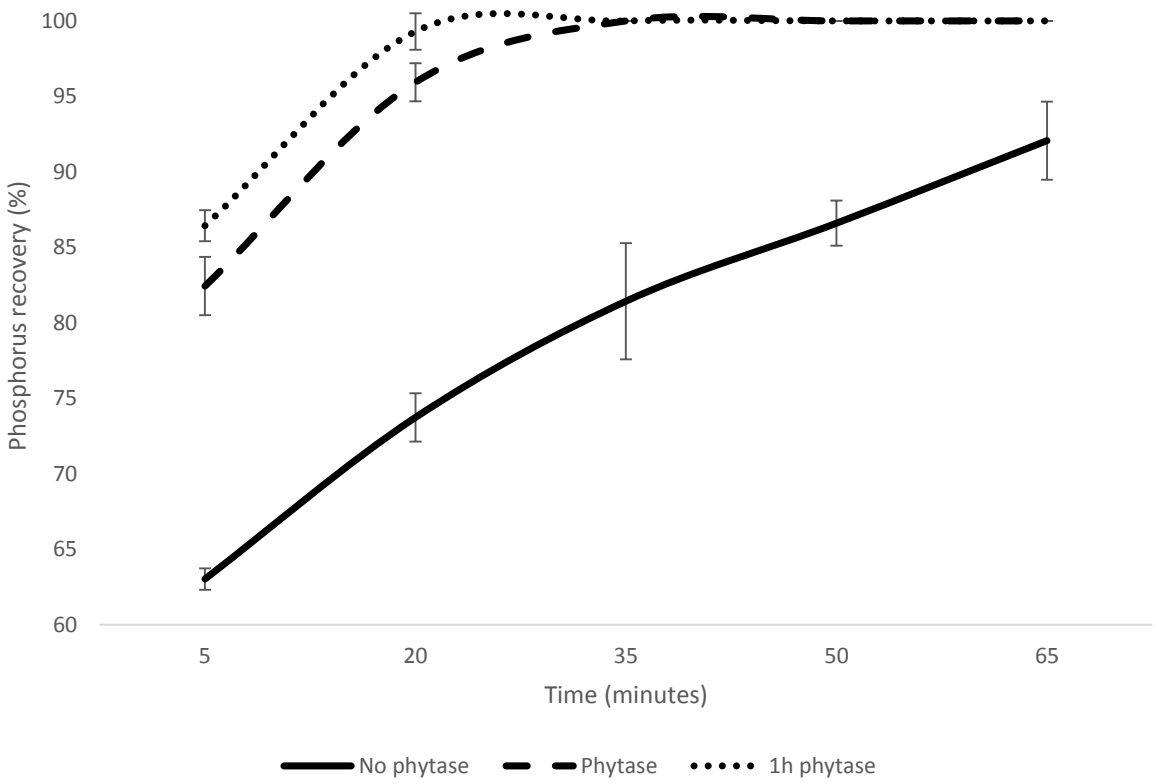


Figure 44: phosphorus recovery from liquefied sludge through electrocoagulation with aluminium electrodes in the absence or presence of phytase (pre-treated for one hour or not).

The addition of wheat phytase was responsible for a significant improvement in phosphorus recovery from liquefied sludge. 5 minutes after the power supply was turned on, both phytase treatments had reached a recovery efficiency of more than 80%, while in the absence of the enzyme that efficiency was only reached after 50 minutes of electrocoagulation (figure 44). Additionally, the pre-treated sludge had all phosphorus removed from it after only 20 minutes, with the added-phytase treatment reaching over 95% recovery at the same point. After 35 minutes, no changes were recorded in the phytase treatments. In the no-phytase treatment only 90% recovery had been reached by the end of the process, but there were no signs of a plateau being reached.

Alkaline monoesterase

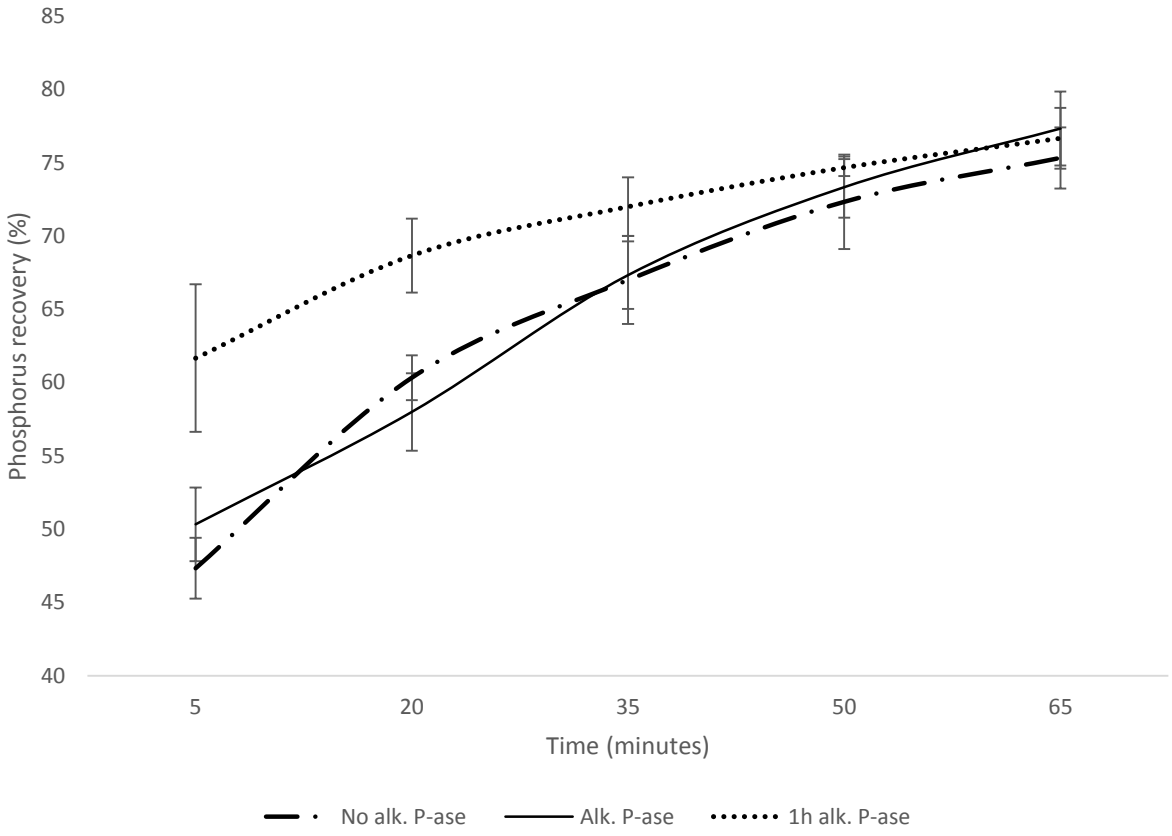


Figure 45: phosphorus recovery from liquefied sludge through electrocoagulation with aluminium electrodes in the absence or presence of alkaline monoesterase (pre-treated for one hour or not).

Pre-treatment with alkaline monoesterase had a significant impact on phosphorus recovery, but only for the first 50 minutes of electrocoagulation. After 5 minutes, the pre-treated sludge had 10% less dissolved phosphorus than the added-monoesterase and control sludge, which were not statistically different (figure 45). The percentage of phosphorus recovered after 50 minutes and at the end of the process was also not statistically different between any of the three treatments, reaching between 75% and 77%.

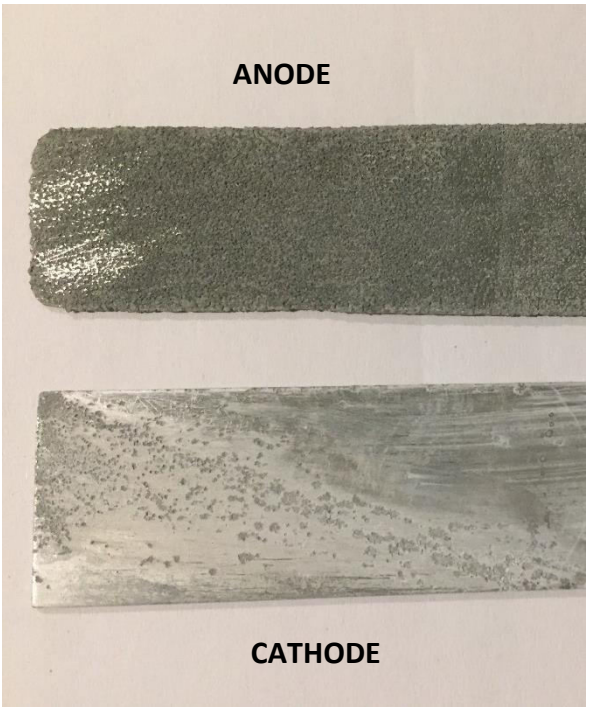


Figure 47: a pair of aluminium electrodes used in an electrocoagulation experiment with suspended and filtered sludge pre-treated for one hour with wheat phytase. Note the degradation of the sacrificial anode.

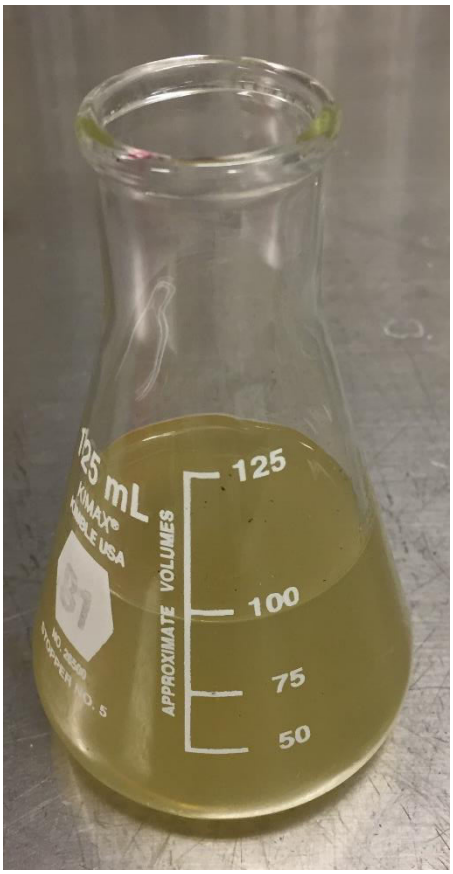


Figure 46: a sample of suspended and filtered sludge.

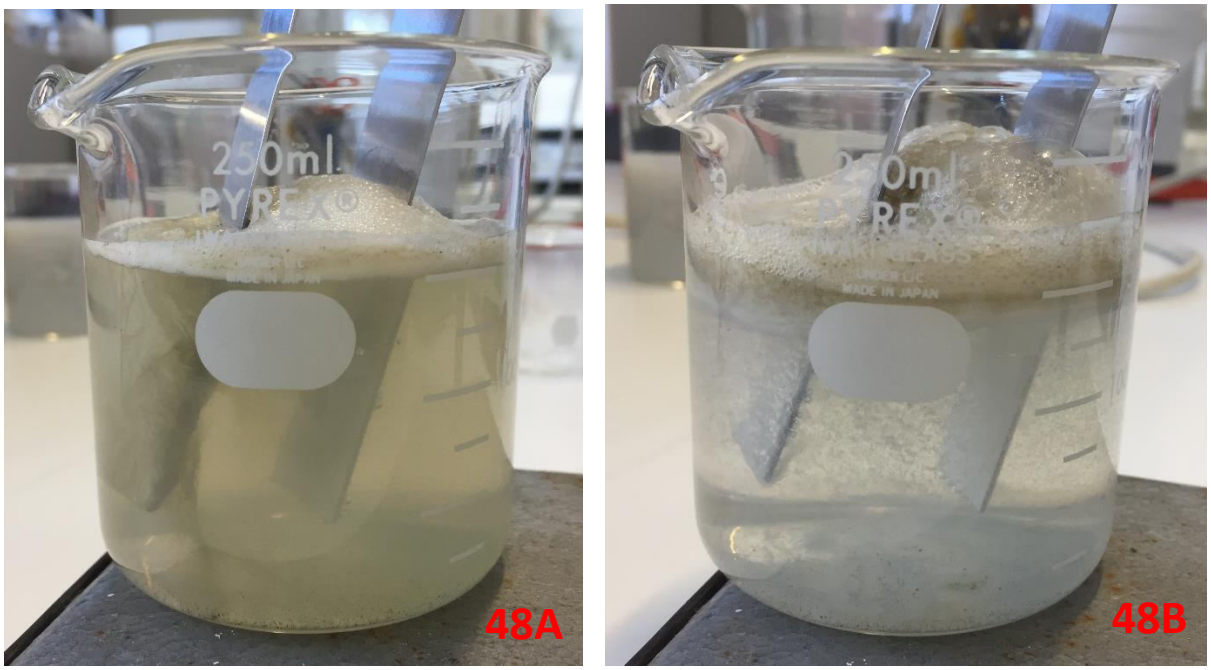


Figure 48: small-scale electrocoagulation experiment: (A) suspended and filtered sludge without phytase; (B) suspended and filtered sludge pre-treated for one hour with wheat phytase. Pictures taken 20 minutes after the start of electrocoagulation.

Chemical precipitation

Phytase

Iron(III) chloride

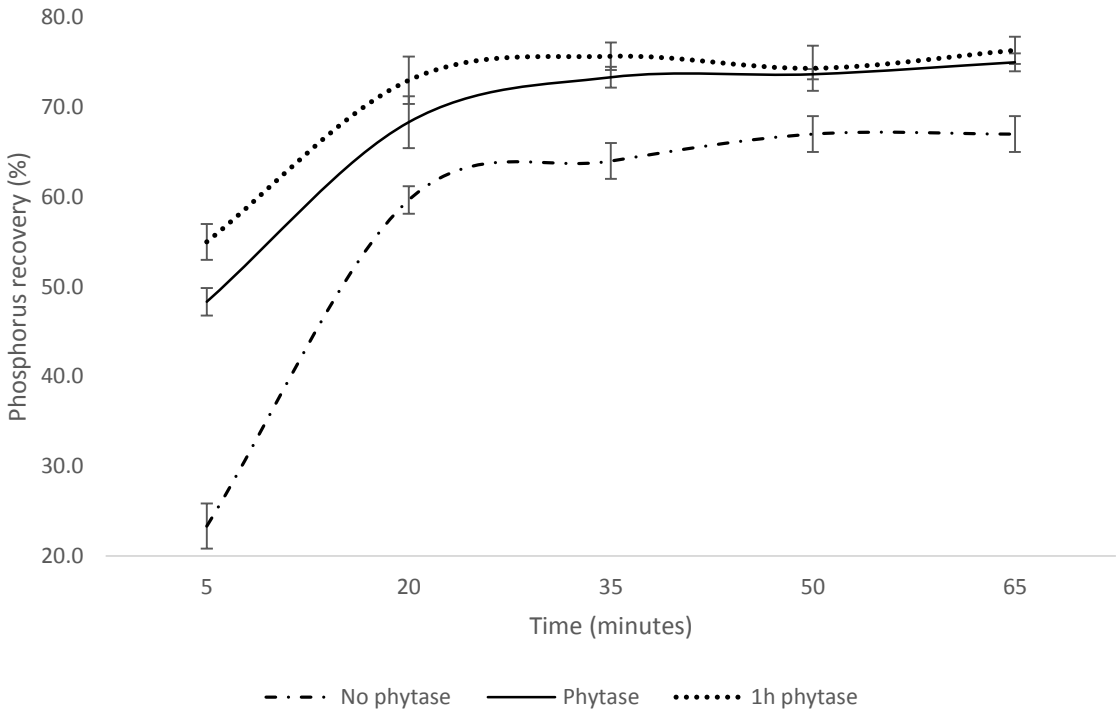


Figure 49: phosphorus recovery from liquefied sludge through chemical precipitation with Fe^{3+} ions (from $FeCl_3$) in the absence or presence of phytase (pre-treated for one hour or not).

The addition of phytase to filtered sludge improved phosphorus recovery by 8-9% compared to unaided chemical precipitation with $FeCl_3$: efficiency went from 67% to 75-76% (figure 49). Pre-treated sludge performed slightly better than sludge with phytase added at the same time as $FeCl_3$ for the first 20 minutes of the process, but there was no difference in outcome at the end of the hour.

Iron(II) sulphate

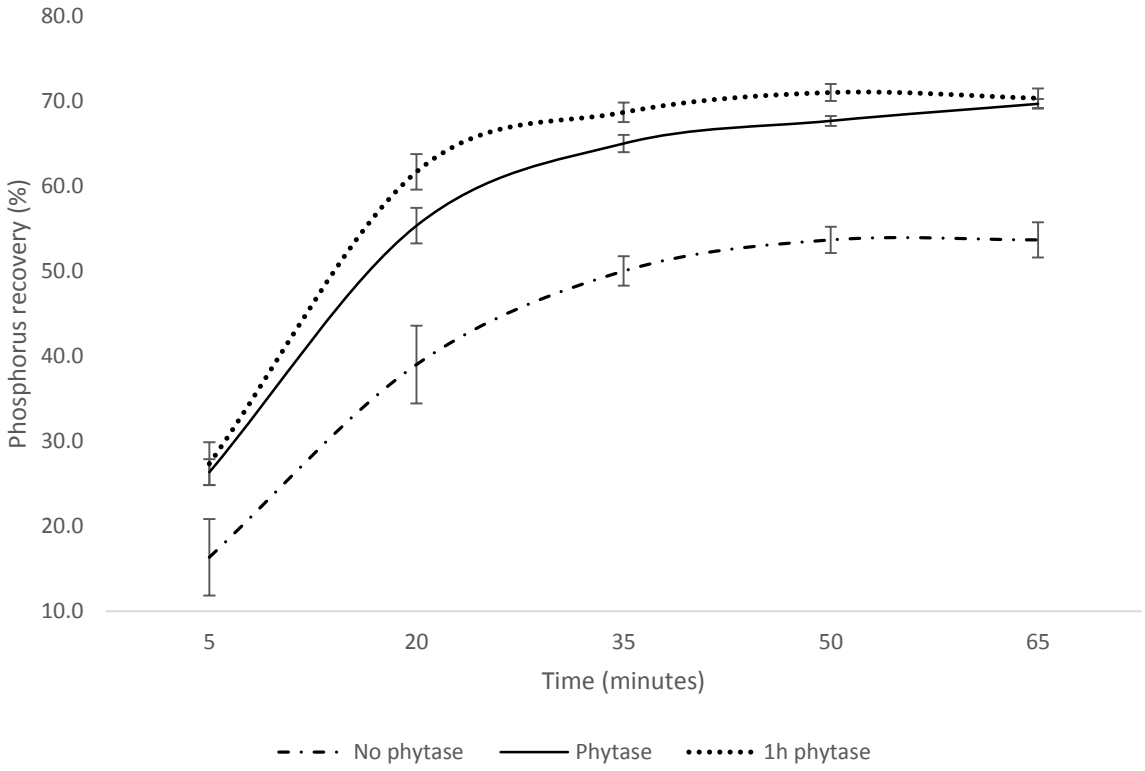


Figure 50: phosphorus recovery from liquefied sludge through chemical precipitation with Fe^{2+} ions (from $FeSO_4$) in the absence or presence of phytase (pre-treated for one hour or not).

As with iron(III) chloride, phytase enhanced phosphate recovery compared to control from 54% to about 70% (figure 50). There was no statistical difference between the pre-treated and jointly-added experiments at the beginning and end points of the treatment, but pre-treatment seems to have accelerated recovery slightly between 20 and 50 minutes after the process began.

Turbidity

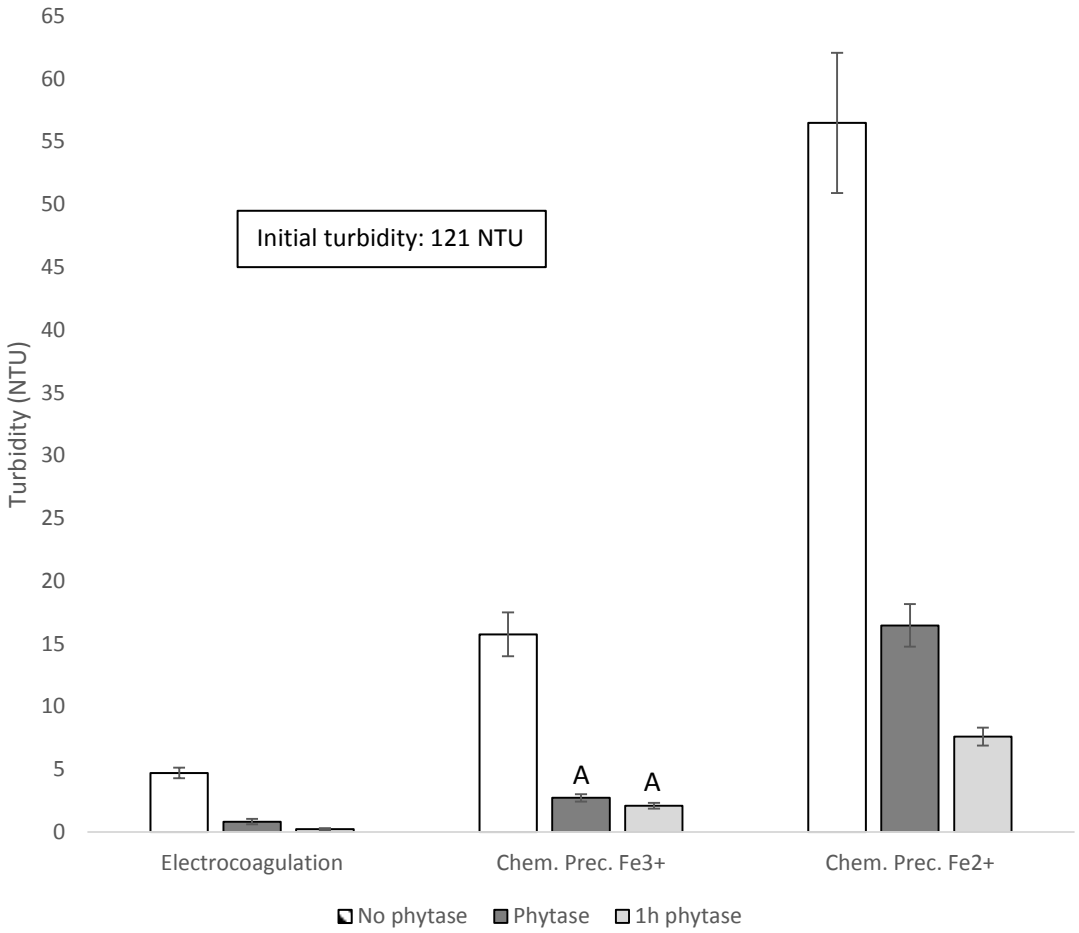


Figure 51: phosphorus recovery from liquefied sludge through chemical precipitation with Fe^{2+} ions (from $FeSO_4$) in the absence or presence of phytase (pre-treated for one hour or not).

Electrocoagulation and chemical precipitation with both iron salts decreased turbidity in filtered sludge, but they were not equally successful (figure 51). While precipitation with $FeSO_4$ reduced turbidity by 56%, $FeCl_3$ reduced it by 87%, and electrocoagulation by 96%. The addition of phytase decreased turbidity in all treatments even further, especially when the enzyme was used as pre-treatment. In those cases, turbidity decreased by 94% with $FeCl_3$, 98% with $FeSO_4$, and 100% with electrocoagulation.

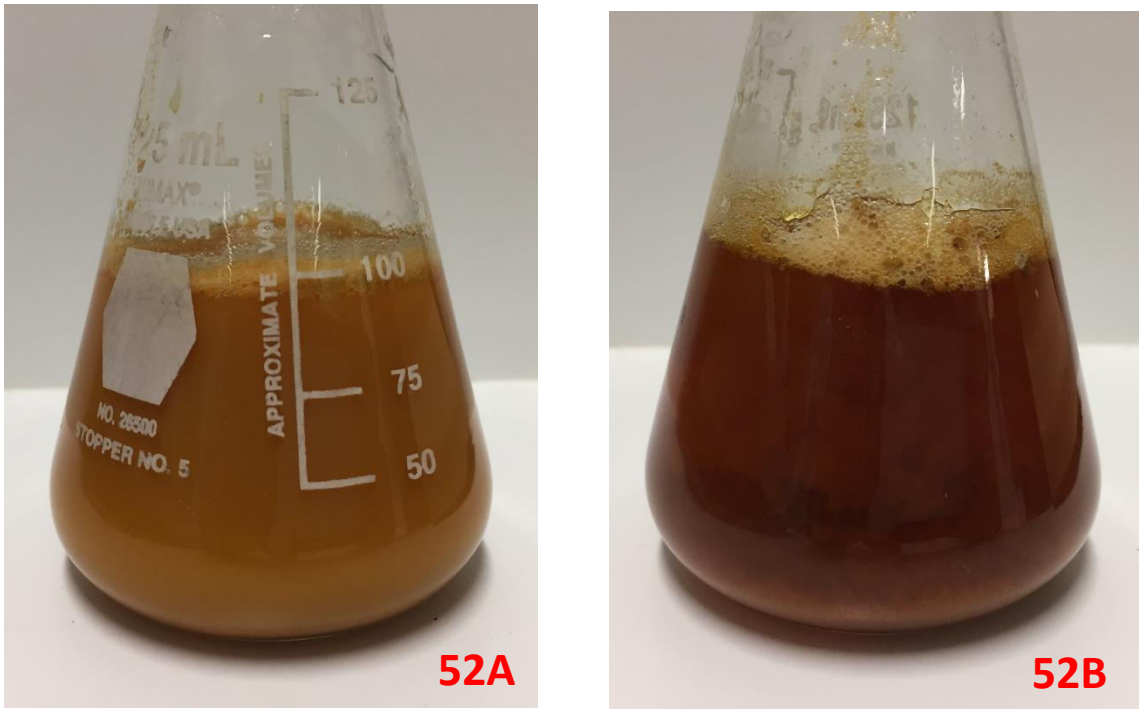


Figure 52: suspended and filtered sludge (A) after chemical precipitation with $FeSO_4$ and (B) after pre-treatment with phytase for one hour followed by chemical precipitation with $FeSO_4$. Both solutions were decanted for one hour after the end of chemical precipitation.

Figure 53: suspended and filtered sludge (A) after chemical precipitation with $FeCl_3$ and (B) after pre-treatment with phytase for one hour followed by chemical precipitation with $FeCl_3$. Both solutions were decanted for one hour after the end of chemical precipitation.

Alkaline monoesterase

Iron(III) chloride

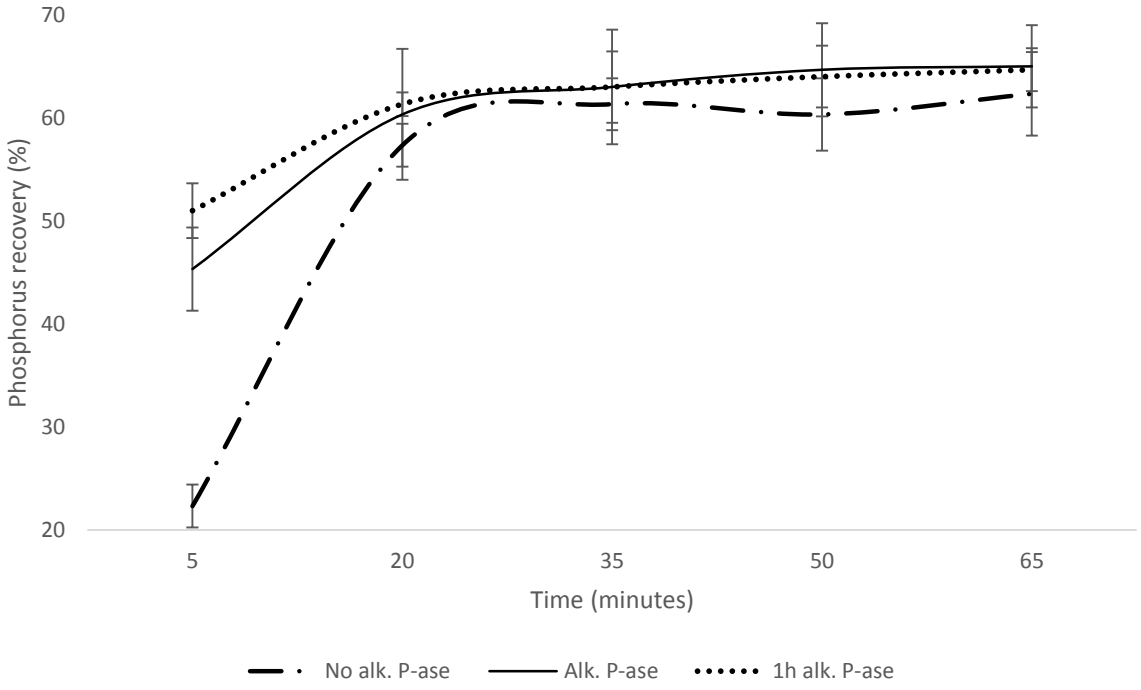


Figure 54: phosphorus recovery from liquefied sludge through chemical precipitation with Fe^{3+} ions (from $FeCl_3$) in the absence or presence of alkaline monoesterase (pre-treated for one hour or not).

5 minutes after the start of iron(III) chloride precipitation, control sludge had just over 20% less dissolved phosphorus; sludge containing alkaline monoesterase had between 45% and 51% less P (figure 54). 15 minutes later, all three treatments had removed around 60% of dissolved phosphorus, with no statistically significant difference between them. Average dissolved phosphorus declined slightly between 20 and 65 minutes in the presence of the enzyme, but again there was no statistical difference between time points or between enzyme and control treatments.

Iron(II) sulphate

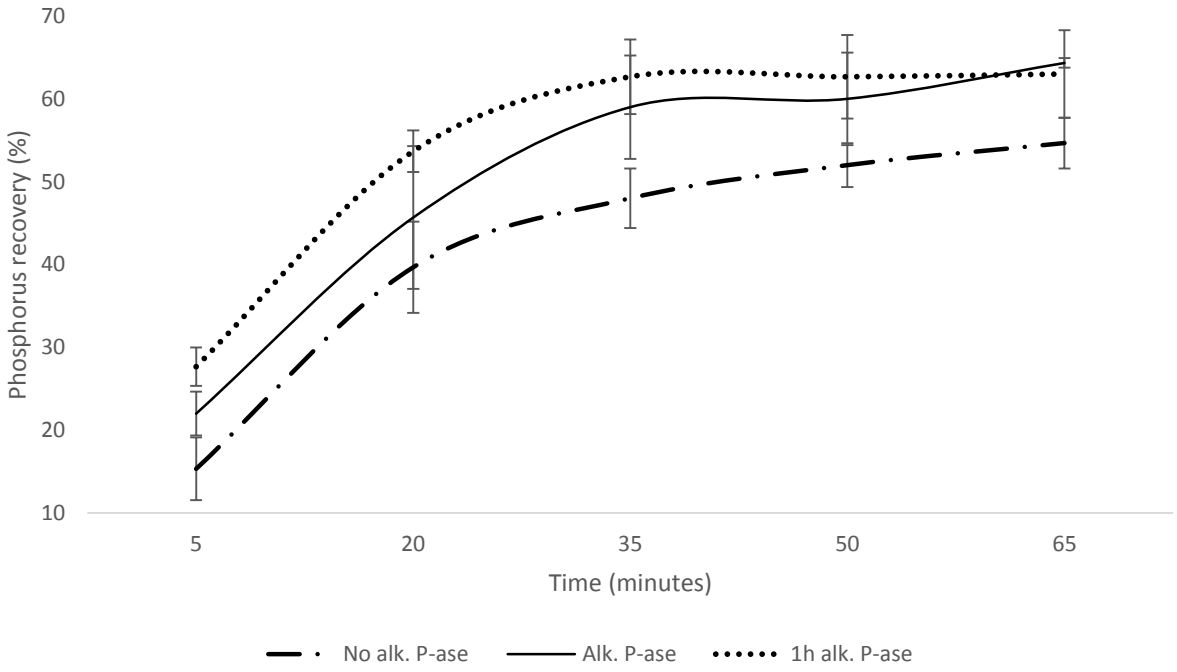


Figure 55: phosphorus recovery from liquefied sludge through chemical precipitation with Fe^{2+} ions (from $FeSO_4$) in the absence or presence of alkaline monoesterase (pre-treated for one hour or not).

5 minutes after the beginning of precipitation with $FeSO_4$, alkaline monoesterase-treated sludge had 22% less dissolved phosphorus, compared to 15% less in control and 28% less in pre-treated sludge (figure 55). That relative difference remains for another 45 minutes, at which point treated and pre-treated sludge have precipitated around 60% of solution P, compared to 52% for control. At the end of the hour, treated sludge had less dissolved phosphorus than pre-treated sludge, but without statistical difference.

Turbidity

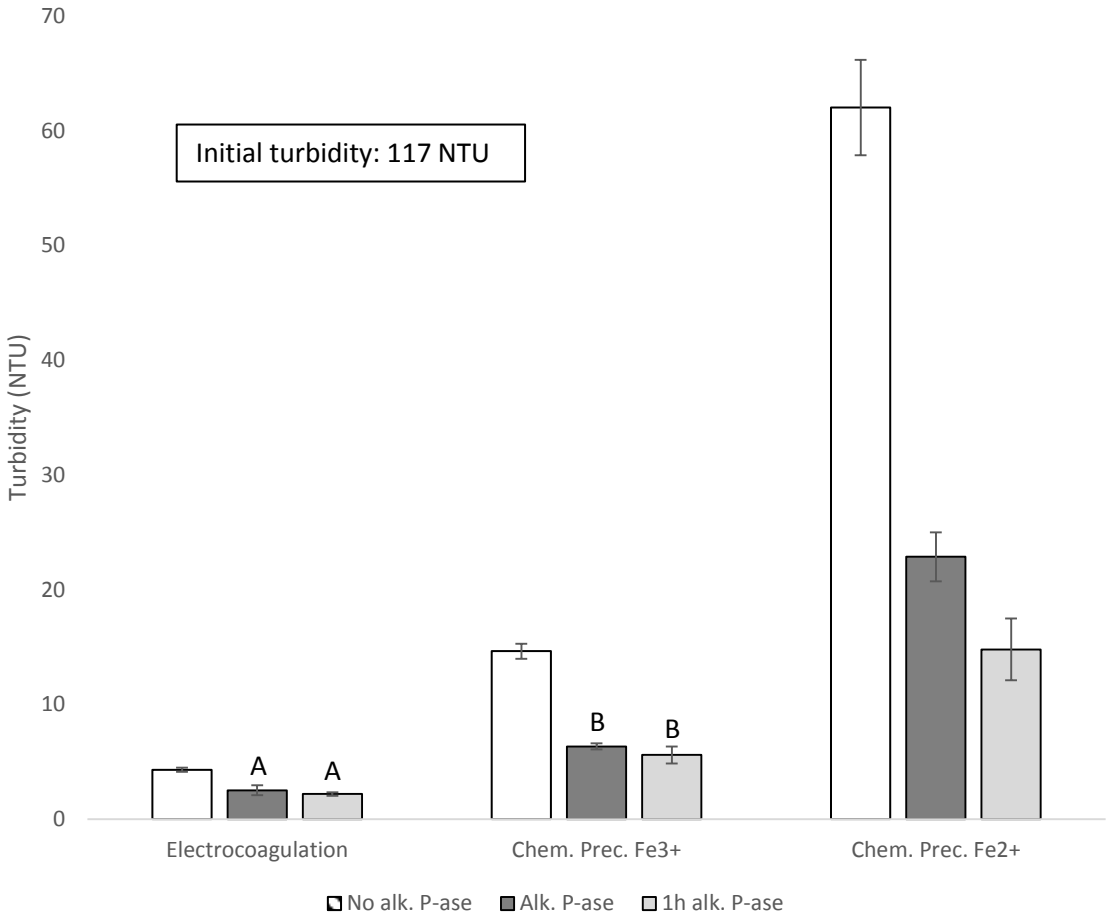
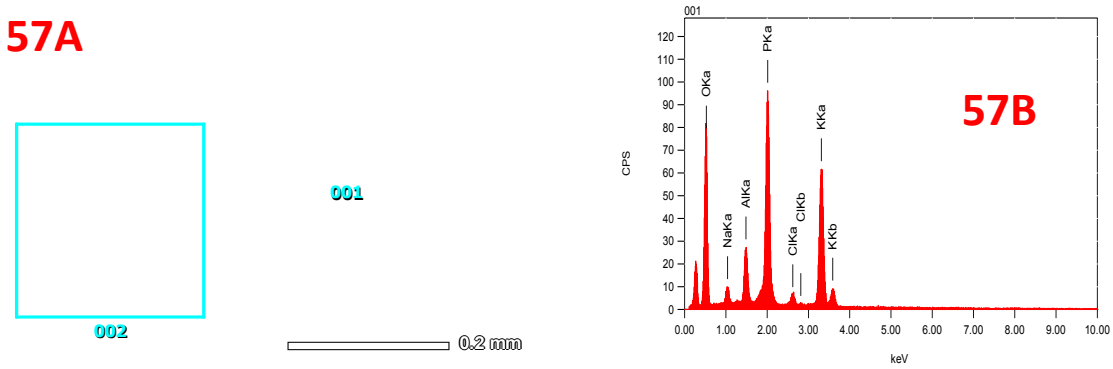


Figure 56: phosphorus recovery from liquefied sludge through chemical precipitation with Fe^{2+} ions (from $FeSO_4$) in the absence or presence of alkaline monoesterase (pre-treated for one hour or not).

As in the case of phytase, turbidity declined significantly in filtered sludge with each of the treatments (figure 56). Chemical precipitation with the Fe^{2+} ion was the least efficient in this regard, having had a 47% decrease; Fe^{3+} precipitation decreased turbidity by 87%, and electrocoagulation, by 97%. Pre-treatment with alkaline monoesterase caused an 87% decline in conjunction with Fe^{2+}, slightly more than when alkaline monoesterase was added with sulphate; for Fe^{3+} precipitation and electrocoagulation, the enzyme improved turbidity compared to untreated sludge by 95% and 98%, respectively, but the improvement was the same with or without pre-treatmement.

Energy Dispersive X-Ray Spectroscopy

Samples from the precipitate and from the foamy supernatant formed by H_2 gas were gathered from an electrocoagulation experiment with sludge pre-treated with phytase. These samples were dried at 60°C for 24 hours and analysed through energy dispersive X-ray spectroscopy to determine their elemental composition.

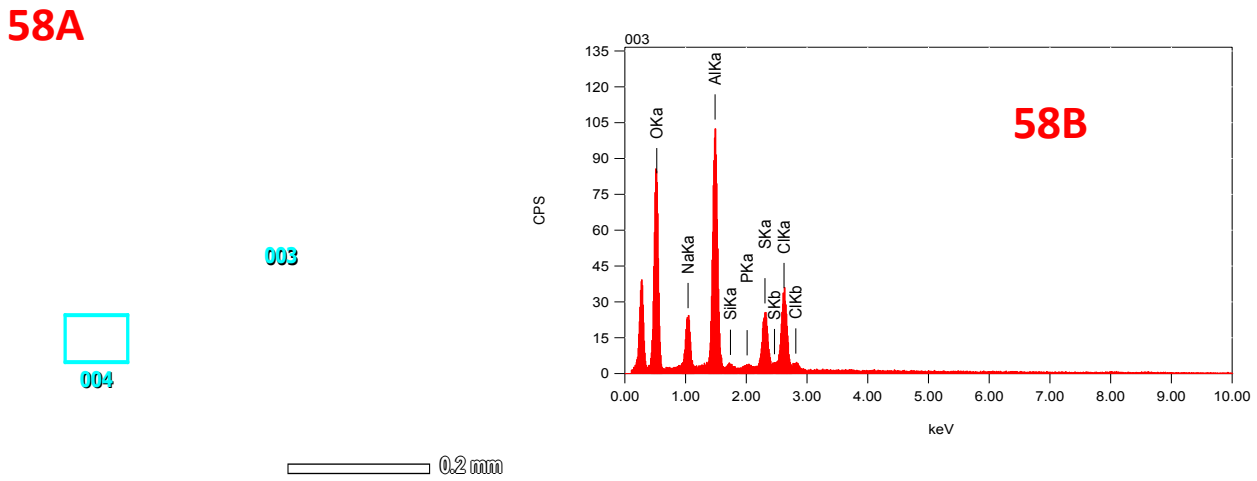


ZAF Method Standardless Quantitative Analysis

Fitting Coefficient : 0.1693

Element	(keV)	mass%	Error%	At%	Compound	mass%	Cation	K
O K	0.525	46.30	0.16	64.38				36.6119
Na K	1.041	1.80	0.11	1.74				1.5974
Al K	1.486	4.17	0.09	3.44				4.0659
P K	2.013	21.49	0.10	15.43				25.5963
Cl K*	2.621	1.48	0.12	0.93				1.7620
K K	3.312	24.76	0.17	14.08				30.3666
Total		100.00		100.00				

Figure 57: elemental analysis of precipitate crystals from liquefied and filtered sewage sludge after one-hour pre-treatment with wheat phytase and one-hour treatment with electrocoagulation.



ZAF Method Standardless Quantitative Analysis

Fitting Coefficient: 0.2125

Element	(keV)	mass%	Error%	At%	Compound	mass%	Cation	K
O K			0.525	46.08		0.26	60.66	48.1719
Na K			1.041	6.88		0.24	6.30	6.1794
Al K			1.486	23.77		0.22	18.56	21.2348
Si K*			1.739	0.65		0.28	0.49	0.5450
P K			2.013	0.39		0.28	0.27	0.3712
S K*			2.307	8.32		0.24	5.47	8.8570

Figure 58: elemental analysis of foam crystals from liquefied and filtered sewage sludge after one-hour pre-treatment with wheat phytase and one-hour treatment with electrocoagulation.

Phosphorus was a major component of precipitate crystals, making up 21% of total mass, while making up less than 1% of foam crystals (figure 57 and figure 58). Oxygen made up 46% of both crystal types, while potassium accounted for a quarter of precipitate weight; foam also contained high amounts of aluminium and chlorine, as well as some sodium and sulphur.

Discussion

Root morphology and chlorophyll content in the absence of P and in the presence of Cd

Survival rates according to transplant period show that moving maize seedling from 10% MS medium to [Cd] = 0.1 mM only 3 days after germination is enough to raise 10-day survival to parity with plants that germinate in 10% MS medium; the same transfer period raises survival to nearly the control rate for plants transferred to [Cd] = 0.5 mM medium. For the 6- and 9-day transfer periods, no statistical difference was found between control, [Cd] = 0.1 Mm and [Cd] = 0.5 mM plants, while [Cd] = 1.0 mM has a negative effect on survival at all transfer periods. It should be noted that despite only about 50% of maize plants surviving for 10 days under [Cd] = 1.0 mM in the 9-day transfer treatment, this represents a significant improvement over direct germination in [Cd] = 1.0 mM medium, where only 20% of plants survived, and over the shorter transfer periods, where approximately 40% survived. Sunflower appears to be more susceptible to Cd toxicity than maize, with germination in the toxin's presence reducing survival rates from almost 90% to 60% at [Cd] = 0.1 mM and 10% at [Cd] = 1.0 mM. A three-day waiting period increases survival at all concentrations, but without reaching parity with no-Cd seedlings; this happens after a six-day waiting period at [Cd] = 0.1 mM and [Cd] = 0.5 mM. As in the case of maize, [Cd] = 1.0 mM has a much more negative effect on survival than lower concentrations, but longer waiting periods also increases the rate significantly. These patterns suggest that days-old seedlings from both species could be used in phytoremediation projects; in addition, the fact that nine-day transfers at [Cd] = 1.0 mM presented an increase of 150% in survival for maize and 200% for sunflower suggests that waiting periods slightly longer than nine days could be enough to enable the use of both plants for phytoremediation even in heavily Cd-contaminated sites.

In the absence of medium P, the Golden Sweet maize cultivar used in this project showed a 20% increase in root length and an 80% increase in weight, as well as a 15% decrease in shoot height. Previous research by Magalhães *et al.* (2011) analysed the impact of phosphorus deficiency on eight maize genotypes. Their report suggests that there is wide variation in responses to environmental cues within a species, since differences between

cultivars were seen in all parameters: for example, under low P, root length in one genotype decreased by 27%, while increasing by 60% in another and remaining unaltered in a third genotype; patterns were similar for shoot height, shoot weight, root weight, and root surface area. In addition to increased root length, Wen *et al.* (2017) found that low shoot P (caused by low soil P) also causes an increase in root hair density. It should be noted that this intra-specific variation may be particularly pronounced in cultivated species, which have been subject to artificial selection for thousands of years. Sunflower showed no variation in root length and weight, but it had an 84% increase in root hair area in the absence of P and a more pronounced shoot height decrease than maize (35%); previous research suggests that sunflower roots become shorter and heavier under low medium P (Fernandez and Rubio, 2015), which means the difference from our results could also be due to the effects of long-term artificial selection.

The presence of Cd in the medium had the same effect on maize and sunflower root weight and length. Both parameters suffered reductions: for maize, the lowest concentration of Cd resulted in 70% less weight and 45% shorter length, and for sunflower, roots were lighter and shorter by about 50% compared to control; root hair area increased for both species at [Cd] = 0.1 mM and [Cd] = 0.5 mM by at least 30%. Shoot height at [Cd] = 0.1 mM was similar to shoot height in the absence of P, with slight decreases in both species. These results are in accord with established literature. Bahmani *et al.* (2016) found that medium Cd alters expression levels of several genes involved in root hair morphogenesis in Arabidopsis, causing increases in root hair length and density; Kucerova *et al.* (2014) state that the majority of reports on the phytotoxicity of Cd includes lower root lengths, while finding that the pattern is valid for Arabidopsis as well. Wang *et al.* (2017) report that wheat seedlings suffer significant reductions in shoot and root length as Cd concentrations are increased in the medium. Xue *et al.* (2013) found that soybean seedlings had lower root weight and length, and shorter shoots. As expected, [Cd] = 1.0 mM seems to be too high a concentration for early plant growth: declines in all the parameters above were of more than 80%, except for sunflower shoot height. Also, with the exception of root hair area, maize was more sensitive to Cd than sunflower, with greater relative declines in all other parameters.

Absence of P in the MS medium reduces chlorophyll content in maize and sunflower, as does the presence of Cd. Both species seem equally sensitive to the lack of phosphorus, having about 10% less chlorophyll under that treatment when compared to control. However, Cd has a more intense effect on maize than on sunflower: at the lowest Cd concentration, the decline in chlorophyll content is statistically insignificant for sunflower, and of only 13% for maize, but at [Cd] = 1.0 mM, sunflower loses 25% percent, compared to 70% for maize. Lower chlorophyll is a common effect of heavy metal toxicity: Kucerova *et al.* (2014) observed it in Arabidopsis grown with Cd in the medium, while Arshad *et al.* (2016) and Wang *et al.* (2017) reported the same pattern in wheat. Xue *et al.* (2013) found that soybean seedling leaves had 40% less chlorophyll at medium concentrations of [Cd] = 0.1 mM. Jiang *et al.* (2007) investigated the effect of Cd and Zn stress on the ultrastructure of maize chloroplasts through scanning and transmission electron microscopies. They found that both metals altered the shape of chloroplasts and reduced the number of thylakoid and grana inside them; Cd and Zn also reduced chlorophyll levels in maize leaves. Xue *et al.* (2013) also reported lower growth rates and decreased chlorophyll content in soybean seedlings grown in Cd-containing medium.

As expected, we found that shoot P concentration was much lower in P-deprived plants, and previous research suggests that low shoot P is the direct trigger for increased expression of P-deprivation traits, as Shane *et al.* (2003) demonstrated on *Lupinus albus* experiments. However, medium P does have an effect on shoot P (Güsewell, 2017), so it is more accurate to say that shoot P serves as a mediator for the effect of medium P on P deprivation traits. On the other hand, there were no changes in shoot P concentration, meaning no association could be established between that parameter and enzyme activity.

Part of the changes in root morphology reported here (decreasing length and increasing root weight and root hair density caused by the presence of Cd in the medium) are in sharp contrast with Florijn *et al.* (1993), who found that six maize lines grown in Cd-contaminated medium did not differ from control in root morphology, despite differences in tissue Cd distribution. Root morphology seems to be the most variable of this toxin's effects, with wide variation being observed between and within species (He *et al.*, 2017). Wei *et al.* (2012) found that small concentrations of Cd have no effect on root length, surface area,

diameter and volume of *Rorippa globosa*, but decrease root length in *R. palustris*. Li *et al.* (2009) found that Cd caused increases in root length, volume, and surface area of one variety of the hyperaccumulator *Sedum alfredii*, while causing decreases in all the same parameters for another non-hyperaccumulator variety of the same species. One possible pathway for the increase in root length and surface area in some plants is the conversion of glucobrassicin into auxin (Jakovljevic *et al.*, 2013). The absence of a decrease in the aforementioned root parameters seem to be correlated with a species' or cultivar's ability to accumulate Cd: *R. globosa*, for example, is one of the most promising candidates for large scale heavy metal phytoextraction projects (Wei *et al.*, 2009). It has been classified as a rare Cd-hyperaccumulator, being capable of concentrating the metal to over 100 mg/kg in the above-ground organs (Wei *et al.*, 2009). This places sunflower cultivar "Russian Giant" and maize cultivar "Golden Sweet," which were used in the present project, in the non-hyperaccumulator group.

Cadmium accumulation in maize and sunflower seedlings

Due to all our measurements being conducted in 10-day old maize plants, we expected Cd concentrations in the straw (the above-ground tissues) to be quite low, as most translocation from root to straw would not have happened yet. Retamal-Salgado *et al.* (2017) found that in 6-month old maize, Cd tended to accumulate in the straw at rates between 73% and 99% of total plant Cd. Our results show straw Cd making up between 23% ([Cd] = 1.0 mM) and 43% ([Cd] = 0.5 mM) of total plant Cd, suggesting high rates of Cd translocation start soon after germination.

It also seems that translocation rates are significantly higher in maize and sunflower than in other crop plants. Xue *et al.* (2013) found that ten-day old soybean seedlings grown under [Cd] = 0.1 mM transport a maximum of 28% of the Cd they absorb from roots to above-ground tissues; factoring in the weights of the different parts of the plant, that means roots had 50 times more Cd per gram than leaves and 20 times more than stems. This contrasts

with our results, where the highest root/shoot Cd ratio was slightly above 3 (sunflower at [Cd] = 0.1 mM) and supports sunflower's and maize's potential as efficient phytoextractors.

Phosphatase activity in the absence of P and in the presence of Cd

Both the absence of medium P and the presence of Cd at low and medium concentrations caused an increase in root exudate monoesterase activity. Increases were greater for sunflower than for maize, 5 times so in the no-P treatment and 3.5 times at [Cd] = 0.1 mM. Maize had no change at [Cd] = 0.5 mM, and both species had lower monoesterase activity at the highest concentration, once again pointing to a greater tolerance of sunflower and a toxicity threshold for medium Cd between [Cd] = 0.5 mM and [Cd] = 1.0 mM. The pattern for diesterase activity was similar, with greater increases for sunflower than for maize in most cases; the differences between the two enzymes were, for diesterase, the absence of a decline in activity for sunflower at [Cd] = 1.0 mM and a progressive increase for maize as Cd concentration increased. Phytase showed lower activity in the absence of P and higher activity in the presence of Cd: in maize exudates, activity decreased as Cd concentration increased, while the opposite happened for sunflower.

Compared to the *Carex* (sedges) analysed by Güsewell (2017), sunflower and maize had significantly higher phosphatase activity increases in root exudates for monoesterases and diesterases, as well as decreased phytase activity (*Carex* had decreased activity of all enzymes). Both species utilized in the present study are known as potent accumulators of heavy metals and are able to tolerate high concentrations of these pollutants in the soil, neither of which is true for *Carex*. It is possible that this pattern of activity involving these three enzyme groups could be used as an indicator for phytoremediation potential during screening studies. More intense responses to P deprivation in maize and sunflower when compared to sedges (Güsewell, 2017) may be due to the fact that the former two are crop plants that have been subjected to artificial selection for centuries and have perhaps become capable of responding to different environments in a way that would maximize yield.

A comparison between phosphatase activity in root exudates and in total root tissue shows that phosphatases may have different roles in the management of P deficiency and in Cd resistance. For both species, monoesterase in most cases and diesterase in all cases had a greater relative increase in activity in root exudates than in total root tissue. This suggests that the most important function of the newly-synthesized enzymes was to mineralize organic phosphorus in the root zone. The same can be said for phytase under Cd stress. On the other hand, the absence of P caused a clear relative decrease in phytase activity in maize and sunflower, which could mean that under that particular stress, phytase is more important inside the root (possibly scavenging extra-cellular P) than mineralizing organic P. Shoot activity patterns also support the hypothesis that different phosphatases may have different roles: while monoesterase activity in sunflower shoots was not affected by either the absence of P or the presence of Cd (except at the highest concentration), it fell sharply in maize shoots under both treatments. Conversely, while all treatments more than doubled diesterase activity in sunflower shoots (except at the highest concentration), P deficiency affected maize only slightly and Cd had no effect on it. Phytase had increased activity in both species' shoots in the no-P treatment, but Cd only increased its activity on sunflower.

Due to its relative scarcity in many soils and the resulting importance of phosphorus acquisition for plants, it may be thought that changes in activity of P metabolism enzymes in the presence of highly toxic contaminants (like Cd) would be part of a mechanism to maximize the absorption of P to enhance the performance of energetically-demanding detoxification pathways. However, it is possible that P plays a more direct role in Cd resistance by chelating the metal and storing it in vacuoles. Jiang *et al.* (2007) analysed the effect of phosphorus on Cd- and Zn-stressed maize; they found that adding P to the medium repaired damage done to chloroplasts by the metals and increased chlorophyll content, supporting the indirect role of P in heavy metal stress resistance and the need to enhance its acquisition from the growth medium. However, they also found large amounts of phosphorus complexed with Cd and Zn in vacuoles and in root cell walls: in fact, increased medium P had the effect of increasing Zn uptake into the roots while lowering translocation to the stem. This suggests that some of the absorbed P is not used as part of metabolic pathways, but instead works as a chelator of heavy metals that prevents much of it from

reaching above-ground organs and diminish the impact of the smaller amounts that reach leaves and stems (Jiang *et al.*, 2007).

In experiments on the effects of Cd wheat (*Triticum aestivum*), Arshad *et al.* (2016) obtained similar results to ours: Cd caused lower plant biomass, higher chlorophyll content and increased tissue Cd. In addition, its presence caused disparate changes in enzyme activity, even between enzymes with similar physiological functions. Superoxide dismutases (SODs), peroxidases (PODs) and catalase (CAT) are all involved with protection against ROS, but while SODs and PODs were more active in the presence of Cd, CAT was less active (Arshad *et al.*, 2016). As in Jiang *et al.* (2007), the addition of P to the soil improved biomass and chlorophyll content while decreasing tissue Cd. This has potential applications if the possibility arises to grow a crop in a region that would generally be considered suitable apart from heavy metal contamination, which is true for many soils with a history of phosphate fertilizer use: *in situ* heavy metal immobilization through the use of phosphate fertilizers from sources other than Cd-contaminated phosphorus mines could allow for healthy plant growth with minimal heavy metal content in the edible parts.

Oberleas (1983) states that phytate, the main substrate of phytase, is not present in stems and leaves. However, of all groups of phosphatases, phytases seem to be the ones with the broadest substrate specificity, so they could still be necessary for reactions involving other organic phosphate molecules, like monoesterases (Belho *et al.*, 2015). Their increased activity under P deprivation may be linked to the need to scavenge extracellular phosphorus to perform essential tasks, but their role under Cd stress is likely different. P has an indirect role in heavy metal stress resistance as an essential element in energy and DNA metabolism, but it has been shown that it can function in a more direct role as a chelator of Cd and Zn, transporting them to vacuoles and cell walls (Jiang *et al.*, 2007). Shoot phytase decreased with increased Cd concentration in maize and total root activity decreased in activity, while root exudate phytase increased activity; this could be an indication that this enzyme is not needed in any of phosphatases' putative functions as P scavenger or part of metabolic stress pathways under Cd, but is still required to mineralize soil P for its chelating properties. Phytase is also known to play a role in phosphorus storage and retrieval in all plant tissues

(Lazali *et al.,* 2013), which might help explain our findings, especially with relation to phytase activity in the stem.

Involvement of phytase in Cd resistance is not the first instance of that enzyme group's participation in the response to stimuli other than shoot P. George *et al.* (2014), noting the better performance of traditional landraces of oats, barley and rye grown in the Western Isles of Scotland (a region with low availability of nutrients in the soil), hypothesized that their hardiness could be partly due to greater root exudation of phytases, which might be able to release Mn bound to their primary substrates, inositol phosphates. The three species were grown under Mn starvation and sufficiency, and their roots were assayed for phytase activity. The enzyme's activity varied between plants grown under Mn-deprivation, but in all of them it was upregulated, suggesting root phytases may play a role in Mn acquisition in alkaline soils with low Mn availability. On the other hand, while exuded phytase was positively correlated with plant tissue Mn in the landraces, the correlation was negative in commercial varieties, suggesting increased phytase exudation through the roots might be secondary to another Mn acquisition trait that has been lost in other cultivars through breeding.

Rejmankova *et al.* (2011) found that wild plants from wetlands (an environment with a high proportion of P as phosphodiester) have root diesterase activity usually higher than root monoesterase activity (up to twice as high), in contrast to plants grown in monoester-rich soils, such as ryegrass, radiata pine (Chen *et al.*, 2002) or wheat (George *et al.*, 2008). We found that diesterases had a greater increase in activity in root exudates in both species compared to monoesterases, even though most crop soils have a predominance of monoesterases as a source of organic phosphorus (Rejmankova *et al.*, 2011). This suggests the association between phosphorus source and phosphatase activity may not be as strong as an analysis of only wild species would lead us to believe; it could also be a character that has not yet been selected for since the domestication of maize and sunflower, which could come from regions where diesterases predominate. Whatever the reason, it provides an opportunity to select for cultivars that are better able to mineralize the organic phosphorus sources in most crop soils.

Phosphorus recovery by chemical precipitation and electrocoagulation

We found that both acid and alkaline phosphatases are capable of appreciably increasing the efficiency of phosphate recovery by and by chemical precipitation. However, alkaline monoesterase only increased efficiency with the use of Fe^{2+} as the cation, while wheat phytase increased efficiency with the use of Fe^{2+} and Fe^{3+}. When iron(III) chloride was used as the metal salt, monoesterase did not improve final amount of precipitation, but it increased its rate in the first twenty minutes. Whether iron(III) chloride was used instead of iron(II) sulphate made no statistically significant difference to the final amount of phosphorus recovered when monoesterase was added to the solution (around 62% of the initial total), and it had only a small effect when phytase was added: with iron(II) sulphate, 69% of phosphorus was recovered, and with iron(III) chloride, 72%. Pre-treatment with enzyme for one hour made no difference to recovery amounts, but it had a small positive impact on rates of recovery in the early stages of treatment. Analysis shows that all treatments were able to reduce turbidity more than control conditions, with phytase causing a greater reduction than monoesterase in both electrocoagulation and chemical precipitation. While phosphorus recovery was similar for both iron cations, turbidity suffered greater reductions when Fe^{3+} was used. This could be due to Fe^{3+} being part of more competing reactions than Fe^{2+}.

The most efficient treatment for the recovery of dissolved phosphorus was electrocoagulation with phytase (either pre-treating the sludge or being added at the start of the process). The addition of monoesterase at the beginning of electrocoagulation produced the same result as control at all points in the curve; however, while monoesterase pre-treatment had no effect on the end amount of precipitated phosphorus, it accelerated the rate substantially for the first 50 minutes of reaction. The addition of phytase effected the complete removal of P from water in 20 minutes, while in the absence of phytase it took 2.5 times longer. Adding phytase at the beginning of the process leads to complete removal of phosphorus slightly later than adding it one hour before it begins. However, it should be noted that this does not necessarily make pre-treatment more economically viable than the

alternative: it is possible that in industrial applications waiting one hour before beginning electrocoagulation would result in high enough costs for the addition of phytase at t=0 to be more feasible.

The best comparison that can be made based on our results is between unaided electrocoagulation and electrocoagulation with phytase pre-treatment, with the latter decreasing the time required for complete phosphorus recovery by 30 minutes, from 50 to 20. With an average current of 0.09 A and average voltage of 7.7 V, our electrocoagulation experiments used energy at a rate of 0.69 W. Applied for 20 and 50 minutes, that is equivalent to 0.83 and 2.08 kWh, respectively. At a price of 26c/kWh, the total cost of each round of electrocoagulation comes to NZ$ 0.22 and NZ$ 0.54, or NZ$ 0.28/L and NZ$ 0.68/L (Energy Efficiency and Conservation Authority, 2018). In summary, the addition of phytase to phosphate recovery by electrocoagulation saves NZ$ 0.40/L.

Savings on electricity may not be enough to make enzymatic electrocoagulation a viable alternative to regular electrocoagulation, considering the cost of phytase production. While it is possible that mass production of phosphatases in bioreactors, as happens with phytase to be used in animal feed, could increase economic viability for the use of these enzymes in small-scale electrocoagulation plants, it is also the case that, as with most commercial enzyme production, the large-scale production of phytase suffers from high production costs and low yields (Bhavsar and Khire, 2014). Up to 70% of the cost of phytase production comes from the recovery and purification stages, where the systems involved are complex and biological activity cannot be compromised. However, research to increase efficiency is moving at a fast pace. Shah *et al.* (2017) have reported greater production of phytase by *Aspergillus niger* through a switch in the inoculum method, from the common spore inoculum to a vegetative inoculum (in which a freeze-dried strain is regenerated and allowed to multiply in a medium prior to inoculation).

Comparison of our results with similar experiments suggests potential for enzyme-aided electrocoagulation. Huang *et al.* (2015) applied chemical precipitation and electrocoagulation to filtered sludge: in the chemical precipitation experiment, molar ratios of metal:P varied between 1:1 and 1.6:1, while ours was 1.25. They also employed a

magnetic stirrer. As in this project, in the experiment using iron(II) sulphate (Fe^{2+}), $Na_2S_2O_3$ (sodium thiosulphate) was applied at 1 g/L to consume the oxygen in the water and prevent Fe^{2+} from turning into Fe^{3+}. The pH was kept at 7.5. In the sludge sample used in our study, the concentration of P was 216 mg/L (31% of total P), while in Huang *et al.* (2015) the concentration of P was 148 mg/L, and of K was 164 mg/L. Even though P concentrations in our filtered sludge were significantly higher, we achieved complete P recovery in 20 minutes at a constant current density of 9 mA/cm^2, while in the absence of phytase removal of P was achieved at 70 minutes at a maximum current density of 6.25 mA/cm^2. Huang *et al.* (2015) also note that higher initial pH increases the initial rate of P precipitation, but it is quickly surpassed by the rate at more acidic initial pH treatments. This is in accordance with our results for monoesterase, which, when added to electrocoagulation at pH close to its optimum, causes a rapid increase in precipitation, but eventually levels with control rates. In the case of phytase, complete recovery of phosphorus happens before parity is reached. An economic analysis by the authors revealed that electrocoagulation was a much cheaper process than chemical precipitation. In our case, we would need to factor in the addition of small quantities of a possibly mass-produced enzyme (phytase) and the reduced costs in energy use, due to complete recovery being achieved in a much shorter time.

Akyol *et al.* (2013) employed electrocoagulation and electro-Fenton to treat wastewater from a fertilizer manufacturing plant, which had P concentrations more than twice as high as Christchurch sludge samples. Complete removal was achieved in the same period as our most successful treatment (20 minutes), using a current density only slightly higher than ours, at 10 mA/cm^2 (Akyol *et al.*, 2013), compared to 9 mA/cm^2. The cost of hydrogen peroxide, the key reagent in electro-Fenton, is significantly lower than the cost of commercial enzymes, but our results suggest there is room for optimization of enzymatic electrocoagulation, especially regarding phosphatase production and screening and batch dynamics; for example, Huang *et al.* (2015) report that aeration can improve P recovery rates by up to 20% in 60 minutes.

A common problem in some electrochemical reactions that can impact operational costs in industrial settings is electrode passivation or blocking. This occurs when the electrode surface is isolated from the reactions occurring in the fluid around it by the species being

produced, and it is more common in aluminium electrodes (Mechelhoff *et al.,* 2013). The species produced, usually an oxide, prevents metal dissolution and electron transfer. Over time, the layer thickens and turns the electrode useless (Mechelhoff *et al.,* 2013). Periodic polarity reversal of the electrodes has been suggested as a technique that can reduce losses to passivation (Mao *et al.,* 2008), but, while promising, it has not been developed enough to have a significant impact; blocking is still problematic and seen as a potential limiting factor for the application of electrocoagulation in situations that require a low-cost and low-maintenance method. However, it has the potential to be another way to reduce the cost of electrocoagulation and increase its commercial viability (Mao *et al.,* 2008).

In any phosphorus recovery technique, it is necessary to know where in the solution the phosphorus is located after the process is finalized, so it can be effectively removed. In the case of chemical precipitation, P is located at the bottom, as part of the precipitate. However, in electrocoagulation, there is a possibility that at least some of it is in the supernatant formed by the action of hydrogen gas (from the anode reaction). Energy dispersive X-ray spectroscopy showed that there was no P in crystals from the foam, but it was a major component of precipitate crystals. Apart from P, the precipitate had large amounts of oxygen (from the phosphate ion); only foam crystals had a high concentration of aluminium.

Conclusion

This project yielded important information to answer all the hypotheses posed at the outset:

1. Waiting three days before transferring maize and sunflower seedlings from the 10% MS, Cd-free medium where they germinated to a Cd-containing medium was enough to ensure their survival for the earliest period of their lives when the Cd concentration was below 1.0 mM; that was enough to bring survival rates to parity with the control group. Waiting up to nine days increased survival with a Cd concentration of 1.0 mM, but longer periods are probably required to achieve parity

2. Absence of phosphorus in the medium caused in maize and sunflower the same effects that are observed in most plants, which constitute the P deficiency syndrome, including decreases in shoot height and root length, increased root area and root hair growth, lower chlorophyll content, and low germination rates.

3. Absence of medium P caused an overall increase in phosphatase activity both in root exudates and in total root tissue, but there was an exception: phytase activity decreased in root exudates, even though it increased in total root tissue, suggesting that in maize and sunflower this enzyme is not as important in the mineralization of organic phosphorus in soil as it is in one or more other functions inside the root, possibly scavenging of extra-cellular phosphorus.

4. Morphological symptoms of medium Cd were similar to the P deficiency syndrome, including the shortening of roots and shoots, lower germination rates lower chlorophyll content, and increased root hair growth. [Cd] = 1.0 mM caused toxicity symptoms in both plants, but they were more extreme in maize.

5. The effect of Cd on phosphatase activity varied widely between enzyme sub-group, plant organs, and plant species. For example, diesterase activity increased in root exudates and total root tissue in sunflower and maize at all Cd concentrations, but it did not change in maize shoots; low Cd increased monoesterase activity in both species' root exudates, but it caused no activity changes in sunflower shoots and

decreased it in maize shoots; at low and medium Cd, phytase activity increased in sunflower root tissue, but did not change in maize root tissue. Taken together, these results suggest that while root exudation od phosphatases is an important element of Cd resistance, phosphatase subgroups have different roles in that process, some of which are inside the plant.

6. In most cases, phosphatase activities increased more in root exudates than in total root tissue, except for phytase in the absence of medium P, which was less active in exudates but more active in root tissue. This suggests that mineralization of organic P in soil is the most critical function of root phosphatases in the presence of medium Cd and in the absence of medium P, but it is likely that phytase has a more important role inside root tissue in the latter case.

7. Both electrocoagulation and chemical precipitation removed significant amounts of phosphorus from liquefied and filtered sewage sludge, but electrocoagulation removed more of it. In the absence of enzyme treatments, phosphorus recovery from chemical precipitation was between 50% and 65%, and from electrocoagulation it was between 72% and 90%, depending on initial ph.

8. The addition of phytase to chemical precipitation and electrocoagulation increased phosphorus recovery compared to control. When added to sludge mixed with iron salts, an extra 5%-20% of the initial phosphorus concentration was removed, and when added to an electrocoagulation solution, extra P removed was 10% of the initial amount; electrocoagulation with phytase was the only treatment that completely removed solution P. Alkaline monoesterase improved the rate of recovery, but only increased the final amount of P recovered when added to sludge with iron(II) sulphate, from 50% to 60%. In every treatment, the amount of P recovered with electrocoagulation was higher than with chemical precipitation.

9. Precipitated crystals contained high amounts of phosphorus, aluminium, and oxygen; foam crystals formed in the supernatant by the action of hydrogen gas produced during electrocoagulation contained aluminium but very little P.

Future research

Future projects should determine whether phosphatases in general and phytases in particular are compatible with other promising techniques for recovering phosphorus from sewage sludge. Adding EDTA to electrocoagulation has been found to increase P precipitation (In *et al.*, 2004), and the same result was obtained with aeration (Huang *et al.*, 2015).

Electro-Fenton was found to be more efficient at removing phosphorus from wastewater than electrocoagulation alone (Akyol *et al.*, 2013), but it was also more expensive. It is possible that adding enzymes to perform the function of H_2O_2, while just as efficient at consuming P, would run into the same difficulty. If phytases are to be used in P removal from sewage by electrocoagulation, it will be necessary which commercial phytase is the most economically viable under the process' conditions, as their performance can vary greatly. Menezes-Blackburn *et al.* (2015) demonstrated this variance in an analysis of seven commercially available phytases, which also found that optimum pH varied between 3 and 5.5.

We have found that phosphatases, as well as morphological traits, respond to Cd toxicity, but it is also important to know if other P-efficiency traits are impacted by Cd, such as P transporters (Sawers *et al.,* 2017) and plant signals for the formation of mycorrhizae (such as flavonoids and strigolactones) (Akiyama *et al.,* 2005; Requena *et al.,* 2007). Screening processes could also benefit from the establishment of one or more indicators of phytoremediation potential, such as the pattern of enzyme activity reported in the present study.

With the new understanding of phosphatase activity patterns in two efficient accumulators, it becomes possible to compare different lines of maize and sunflower and determine whether monoesterases, diesterases, or phytases could be used as an indicator of Cd tolerance, as well as whether increased activity of any of these is a trait worth manipulating in breeding programs to produce plant lines efficient at extracting large amounts of soil Cd, or capable of growing in contaminated soil while excluding it from their edible parts.

One significant limitation in this project is the difficulty in sampling, manipulating, and measuring the area of plant roots. Future projects could employ WinRhizo, a software deisgned specifically for root analysis. It is able to determine several aspects of root morphology, topology, architecture, and colour, and in recent years has been applied with positive results in several studies (Deguchi *et al.*, 2017; Magalhães *et al.*, 2011; Pang *et al.*, 2011).

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