Influence of humic acids on nutrient use efficiency of three phosphorus sources in a sandy soil

Rhine-Waal University of Applied Sciences
Faculty of Life Science
Bachelor thesis submitted for the degree of:
Bachelor of Science in Sustainable Agriculture
Declaration of Authorship

I certify that the submitted thesis is entirely my own work except where otherwise indicated. I am aware of the University's regulations concerning plagiarism. Any use of work of other authors, in any form, is properly acknowledged at their point of use.

Author’s signature:

Name:

Date of Submission:
Abstract

A pot trial was conducted under greenhouse conditions to assess the effect of humic acid (HA) application in combination with rock phosphate (RP) on the plant phosphorus (P) nutrient use efficiency (NUE), in comparison to the performance of struvite (ST) and triple superphosphate (TSP) amended plants. A sandy soil, exhibiting a low P content, was used in combination with a lignite derived, liquid humic acid product, with a HA content of 4.8 %. Nine groups of perennial ryegrass (Lolium perenne L.) were set up as experimental plants, in replications of six, each containing 4.5 kg air dry soil with a P supply of 27 mg P kg\(^{-1}\) soil, causing P deficiency during the experiment. HA was applied at rates of 1, 30 and 100 mg kg\(^{-1}\) soil, directly to the soil as coated RP or as foliar application. Aboveground biomass was harvested biweekly, five times in total. Amendment performance was evaluated by analysing plant biomass, soil pH, plant phosphorus-, carbon-, nitrogen-, iron-, zinc-, manganese- and boron contents. The label doses of 1 mg kg\(^{-1}\) soil as well as 100-fold increases in HA concentration didn’t result in a significant difference in plant shoot or root growth and P NUE compared to untreated plants. Positive trends were displayed at higher foliar application rates. Plant tissue micronutrient concentrations of HA treated samples were generally lower and partly significantly lower than the control. The detected effects were accredited to the hormone-like influence of HA on plants’ metabolism and the ability to alter soil conditions. The lack of calcium in ST amended samples resulted in better plant P availability, significantly higher P uptake and better shoot biomass development. Trends show that ST could be applied in combination with HA to further increase plants P NUE in the future.
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<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAL</td>
<td>Calcium acetate lactate</td>
</tr>
<tr>
<td>CA-P</td>
<td>Calcium phosphates</td>
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<tr>
<td>CEC</td>
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<td>DM</td>
<td>Dry matter</td>
</tr>
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</tr>
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<tr>
<td>HSD</td>
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<tr>
<td>ICP-OES</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Triple superphosphate</td>
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<td>WHC</td>
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1 Introduction

Next to nitrogen (N), phosphorus (P) is the most important macronutrient for plant growth. It is crucial for every organism on the planet, since it’s a major component of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in cells. Beside this, P is an essential element of adenosine triphosphate (ATP) - the foundation of animal’s and plant’s energy system (Weil and Brady, 2017, p. 662). In contrast to N, P is not an element, which is virtually abundant in the atmosphere or can be fixed to the soil by natural processes, i.e. biological N fixation or atmospheric deposition (Weil and Brady, 2017, p. 606). In nature P is usually found in its oxidised state, phosphate ($\text{PO}_4^{3-}$), in soil solution as $\text{HPO}_4^{2-}$ at acidic pH or $\text{H}_2\text{PO}_4^-$ at alkaline conditions. Throughout this paper the character “P” is used to describe the various forms P can produce in the environment. Today, the main P flux is mined from sedimentary rock deposits, which formed over millions of years, in the form of rock phosphate (RP), i.e. hydroxyl-, fluor- or chlorapatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH, F, Cl})$) (Butusov and Jernelöv, 2013, p. 38). Next, the mineral is typically processed and afterwards applied as fertilizer to agricultural systems. In the shape of biomass or animal products it is taken up by humans and ends up as human- or livestock waste. Foremost, P is lost at the end of this product chain as sewage sludge deposited in landfills or led into the sea. Nonetheless, losses from livestock, food production and land fertilization play a major role as well (Butusov and Jernelöv, 2013; Weil and Brady, 2017, pp. 670-675). The problematic arising from these facts is that at the current world P consumption, RP reserves are estimated to be depleted in 30 - 300 years, with declining availability after peak production in 2033 (Cordell and White, 2011). Consequently, P needs to be recycled to sustain crop yields for agricultural
systems. Although, effort is made to produce recovered P fertilizers from wastewater treatment facilities, e.g. struvite (ST) - \( \text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O} \), heavy metal, hormone and antibiotic contamination can be an issue (Butusov and Jernelöv, 2013, pp. 89-100).

Schulte and Kelling (1996) stated the concern that P could be relatively insoluble, hence plant unavailable, when added to soil in its pure, mined form RP, e.g. hydroxyapatite - \( \text{Ca}_5(\text{PO}_4)_3\text{OH} \), exhibiting a water solubility of \( \leq 1 \% \). On an atomic scale this is the result of stable bonds between multiple calcium (Ca) atoms and phosphate molecules (Weil and Brady, 2017, pp. 679-684). RP is processed using high amounts of chemicals, plus electric energy to manufacture highly water soluble conventional fertilizers, e.g. triple superphosphate (TSP) - \( \text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O} \), reaching a solubility in water of > 85 % (Schulte and Kelling, 1996; Butusov and Jernelöv, 2013, pp. 41-42). This usually yields phosphate molecules, bonded to few Ca atoms, thus creating a high P content and less stable compound. However, when fertilizers are added to soils, normally less than 20 % become plant available during the application year (Holford, 1997). The remaining P is fixed to soil colloids, chemically fixed to inorganic and organic molecules or immobilized by microorganisms. In addition, plant P availability is very pH dependent. At pH < 6, P is predominantly precipitated in combination with hydrous oxide metals, resulting in minerals such as vivianite (\( \text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O} \)) and variscite (\( \text{AlPO}_4 \cdot 2\text{H}_2\text{O} \)). Above pH 7, P solidifies when bonding with calcium, forming calcium phosphates (Ca-P), e.g. hydroxyapatite (\( \text{Ca}_5(\text{PO}_4)_3\text{OH} \)) or octacalcium phosphate (\( \text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O} \)) (Zhu et al., 2018). Therefore, a soil pH between 6 and 7 should be maintained to obtain maximum plant P availability. According to Weil and Brady (2017, pp. 690-692) plants acquire P by
different mechanisms. The simplest tool is the formation of lateral roots and additional root hair, thereby enhancing the roots' absorptive surface area. Secondly, they can exude organic acids that chelate with metal hydrous oxides, making P plant available. Moreover, $H^+$ ion release, decreasing the rhizosphere pH or enzyme secretion, especially phosphatase, leads to increased plant P uptake (Weil and Brady, 2017, pp. 690-692).

Conventional farming systems typically use high P content, readily water soluble fertilizers. Excessive broadcasting or improper management can lead to P leaching into water bodies, causing eutrophication, hence severely damaging environmental systems (Butusov and Jernelöv, 2013, pp. 57-68). Due to this, as well as the vast inputs needed for production, the application of highly water soluble fertilizers is not permitted under organic cultivation (European Council, 2007; Bioland, 2017). At the same time, this restriction is linked to the problem of P deficient soils organic farms can face. A negative P nutrient budget, stemming from consistent soil P withdrawal by harvests and only limited P replenishment, is the main factor restricting plant growth. Supplying sufficient amounts of P by means of organically certified fertilizers can be difficult as an organic farmer, since mineral and organic fertilizers feature little P contents, in addition to low plant P availability of mineral ones (Weil and Brady, 2017, pp. 668, 803, 1041). A solution to this problem could be the application of biostimulants. Biostimulants are classified as amendments that possess no major amounts of plant essential nutrients themselves, but improve plants' metabolism, resistance to abiotic stress and their nutrient use efficiency at small application volumes (Calvo et al., 2014). Nutrient use efficiency (NUE) is described as the proportion of yield, e.g. biomass or assimilated nutrient, per quantity agricultural input, e.g.
fertilizer (Hawkesford et al., 2016). It comprises the plant’s ability to mobilize, take up, transport and store nutrients from its environment and finally convert these in biomass. In the past decades, biostimulants have been applied increasingly and demonstrated their ability to enhance plant characteristics (Ertani et al., 2012; Calvo et al., 2014). In this context humic substances (HS) have shown to increase plants’ P nutrient use efficiency (Wang et al., 1995; Sharif et al., 2002; Tufenkci et al., 2006; Nikbakht et al., 2008; Zhu et al., 2018). HS originate from degradation-, or accumulated metabolites from microbial decomposition, of dead organic substances i.e. plant or animal tissue (Asli and Neumann, 2010). Common sources from which they are extracted include leonardite, a soft brown coal, compost and sewage sludge (Ayuso et al., 1996; Jindo et al., 2012). HS are highly resistant to further microbial decomposition, made up of mostly aromatic structures and contain diverse, oxygen containing functional groups (Schnitzer, 1978). They are categorised according to their molecular mass and behaviour in solution as humin, humic- or fulvic acids, the former having the greatest and the latter the lowest molecular mass. Humins can’t be extracted from organic matter at any pH. HA are brown to black, feature a molecular mass between 50,000 and 300,000 amu, are soluble in alkaline solution (> 7 pH) and precipitate at a pH < 2. FA have a yellow to red colour, molecular masses up to 50,000 amu and are soluble in water, at neutral, or any other pH (Schnitzer, 1978; Weil and Brady, 2017, p. 567). Bocanegra et al. (2006) revealed that FA, in contrast to HA, would have a higher number of negatively charged functional groups, thus a higher cation exchange capacity (CEC). In particular, the role of FA as a natural chelating agent is discussed, because of their ability to complex micronutrients, as demonstrated by Lobartini et al. (1998) and their small molecular size, which
enables FA, to cross plant membranes, in opposition to HA (Bocanegra et al., 2006). However, generally the specific properties of HS products depend on the raw material, its HS content and extraction method (Ayuso et al., 1996; Nardi et al., 2005; Berbara and García, 2014). In this paper the term HA describes the action of HA and FA, since main properties are shared and the product used in the pot experiment, described later on, is a mixture of both. Piccolo (2001) demonstrated that HA are heterogeneous supramolecules, composed of comparably small molecules, varying in size. Besides this, it has been proven that supramolecules can interact with their environment by separation of smaller humic molecules. In this context Piccolo et al. (2002) suggested that plants can alter the size of HS by release of organic acids, thus changing their characteristics and impact on plant growth. Canellas et al. (2008a) found further evidence for this hypothesis. In regard to NUE, HA can enhance it in a direct way by modifying the metabolism of plants, specifically altering gene regulation and protein expression, consequently modifying nutrient ion uptake (Nardi et al., 2000; Quaggiotti et al., 2004; Aguirre et al., 2009). Especially hormonal, auxin-like behaviour of HA is linked to H⁺-ATPase stimulation in plasma membranes of plant roots, which in turn exude higher proton amounts and therefore increase nutrient transportation through plasma membrane proteins (Hager, 2003; Jindo et al., 2012). Moreover, elevated H⁺ secretion has shown to enhance plant root development and surface area as proposed by the acid growth theory (Rayle and Cleland, 1992; Zandonadi et al., 2007; Canellas et al., 2008a). Canellas et al. (2008a) also showed, that HA can elevate the release of organic acids into the rhizosphere. These have the ability to chelate metal ions and increase plant micronutrient and P availability (Grossl and Inskeep, 1991; Mackowiak et al.,
Further plant physiological improvement of HA is initiated by increasing plant water uptake and better seed germination (Chen and Aviad, 1990). Atiyeh et al. (2002) described the mitigation of salt stress by HA added to the soil, resulting from reduced root membrane leakage. On the other hand, NUE can be influenced indirectly by altering the conditions in the plants' rhizosphere. In the soil solution, especially at acidic pH, HA is capable of chelating Al and Fe, as a result releasing plant accessible P (Lobartini et al., 1998). Under neutral or alkaline conditions, HA have shown to increase plant available P by decreasing its amounts precipitated into less plant accessible CA-P (Delgado et al., 2002). In this case P would bind to HA, forming phosphohumates, from which it could be taken up readily by plants (Ayuso et al., 1996; Tan, 2010, p. 236). Additionally, complexion of micronutrients by HA functional groups impact their plant availability in the soil solution. Increases in accessibility, as well as decreases have been reported, particularly depending on the individual element and HA application rate (Stevenson, 1972; Chen and Aviad, 1990, pp. 171-173; Ayuso et al., 1996; Weil and Brady, 2017, pp. 741-744). Finally, Valdrighi et al. (1996) reported a positive effect of HA on soil microorganism diversity and numbers, which can alter plant nutrient availability by excretion of organic acids, nutrient immobilization as well as mineralization. Numerous studies demonstrate yield increases and additional positive effects on plant characteristics when HA are applied. For instance, Karakurt et al. (2009) demonstrated significantly higher pepper (Capsicum annum L.) yields and fruit quality in comparison to the control group, when HA was applied as soil drench or foliar application to organically grown peppers in greenhouse culture. Kirn et al. (2010) supplemented okra plants (Abelmoschus esculentus L.) with lignite
derived HA, amended to the soil, resulting in a significantly better nutritional value and higher number of fruits and shoot biomass in contrast to the control. Arjumend et al. (2015) reached, when compared to the control, significantly higher wheat (Triticum aestivum L.) shoot, root and grain yields, plus substantially higher N, P and potassium (K) leaf and grain contents by adding lignite derived HA to the growth substrate. An optimum HA application rate, to a point where plant performance starts to decline, is reported in several studies (Chen and Aviad, 1990; Ayuso et al., 1996; Atiyeh et al., 2002). However, these thresholds vary depending on HA properties, as previously pointed out. Having said that, non-significant or negative impacts on plant growth have been reported, too. Asli and Neumann (2010) stated that three different HA sources, at an application rate of 1 g L⁻¹, caused reduced shoot growth and a decrease in hydraulic conductivity, resulting in water stress for maize plants (Zea mays L.). Verlinden et al. (2009) reported mainly non-significant results in contrast to the control, when a leonardite derived HA product was applied, at a recommended rate of ~ 50 L ha⁻¹, as a foliar spray or in solid form to a grassland, maize, spinach or a potato crop. Jones et al. (2007) showed in a greenhouse experiment, that 1.7 kg HA ha⁻¹, the label dose of a HA product, didn’t yield any significantly different plant performance outcomes in comparison to the control.

Due to inconsistent results of scientific studies, leading to no consent concerning the effect of HA on plant performance, HA application in the field is not a common practice. However this might change in the future, since Chen and Aviad (1990) summarized overall positive effects of HA on plant characteristics, in addition to a recent meta-analysis by Rose et al. (2014), that concluded an average gain of 22 % shoot biomass of plants treated with HA, compared to untreated ones.
Furthermore, to enhance transparency of marketed HA products, a new standard method for their analyses has been introduced in accordance with the International Humic Substances Society (IHSS), producing consistent and precise readings for humic- and fulvic acid contents, which often wasn’t the case in earlier methods (Lamar et al., 2014). In the face of dwindling, minable P resources, solutions on how to increase the sustainable use of P in agricultural systems is urgently needed. Research studying the impact of HA on plant P availability is limited. Additionally, the obtained results don’t come to a consent. Therefore, the aim of this study is to further identify if HA could play a key role in increasing plant P availability, particularly in organic farming systems. In this context, the P supplying capacity of RP, the mineral, organic fertilizer, supplemented with a low, medium and high amount of HA, as either soil or foliar application, is compared to the recycled fertilizer ST and as a benchmark, to the conventional, readily water soluble fertilizer TSP in a low P content, sandy soil. As indicators for amendment performance plant biomass, soil pH, plant carbon (C), nitrogen (N), iron (Fe), zinc (Zn), manganese (Mn) and boron (B) contents are analysed.

It is hypothesised that:

- HA soil and foliar amendments increase plants’ NUE of P, Fe, Zn, Mn and B.
- HA feature an optimum application volume for the best plant performance between the medium and high application rate.
- ST yield higher plant P availability than RP as a result of absent Ca.
- TSP would supply slightly less plant available P in contrast to ST, because of Ca presence.
2 Materials and Methods

2.1 Experimental set-up

Design

A pot trial was conducted in the greenhouse climate chambers of Rhein-Waal University of Applied Sciences from 16th February to 02nd May 2018. As a ground structure for the experiment, bottoms of round, black containers, 21 cm x 16.4 cm (Diameter x height), volume: 4 L (TEKU®-MCI 21, Pöppelmann GmbH & Co. KG, Lohne, Germany) were lined with cut to size, black root mat to prevent later loss of soil during handling, especially by leaching. For the final filling process 2.25 kg of quartz sand (RKW, Falkenstein, Germany) was weighed (ECE50KGK20 scale, KERN & SOHN GmbH, Balingen-Frommern, Germany) into a sturdy plastic drum with screw-on lid. Next, the P-fertilizer, coated with HA where applicable, was distributed on the surface and silty loam soil B horizon from a paddock of the neighbouring village Neulouisendorf added to a total weight of 4.50 ± 0.02 kg. This mixture was then thoroughly shaken for 20 seconds and filled into the prepared pots. Afterwards the emptied crimp neck bottles, in which fertilizers were stored, were rinsed with distilled water and tipped on top of the corresponding pots. On three tables the containers were distributed in three rows composed of six containers each. The order was arranged according to a completely randomised design.

Seed

Perennial ryegrass (Lolium perenne L.) variety “Libronco” (Samenshop24, Aurich, Germany) served as experimental plants. A germination test (n = 100)
from 12th January to 15th January showed a germination rate of 84%. For this, seeds were distributed on an aluminium tray containing a soaked sheet of paper and stored in a plastic bag slightly opened for ventilation purposes. After four days seedlings were counted and the germination rate was obtained.

Prior to sowing, 0.75 g seeds were soaked in 5 ml liquid in covered crimp neck bottles overnight for each pot. Following product instructions, groups that would be treated with humic acid during the experiment, received a solution containing 9.09 % humic acid product dissolved in distilled water. All other groups were steeped in distilled water only. Twelve to fourteen hours later the seeds were tipped onto a 0.62 mm sieve, air dried for five minutes and separated on filter paper. Subsequently, they were distributed evenly on the corresponding pots’ soil surface utilizing an A4 paper sheet. Finally, all pots were sprayed with distilled water and covered with a transparent plastic sheet to keep a high humidity. To ensure homogeneous germination the seeds were misted with distilled water daily for four days.

**Abiotic factors during the vegetation period**

Conditions for an optimal plant growth were set as a goal. Target temperatures were defined to 16 – 21 °C during the night and 20 – 23 °C at daytime. As a consequence of extreme outside conditions these values deviated at times, to an extent of minimum 14.9 °C and maximum 36.3 °C. A light period of 16 hours, from 6 AM to 10 PM was selected. Sodium growth lights ensured adequate light provision when natural light intensity dropped below 20 kilolux. Irrigation was done twice a week gravimetrically (ENTRIS6202-1SUS scale, Sartorius Lab Instruments GmbH & Co KG, Goettingen, Germany). At project start total weight
of each pot including trivet at a water holding capacity (WHC) of 70% was noted on the corresponding pot label. During an irrigation event deionized water was carefully added until the desired weight was reached tolerating a variance of ± 5 g. Repeated leaching after watering led to an adjustment of the 70 % WHC from 929 g to 718 g pot⁻¹ 15 days after start of the pot trial. This was backed by laboratory measurements of the WHC conducted according to methods of Markus Raubuch (2001). Soil was sieved to < 2 mm and heated for 12 hours at 105 °C in a drying cabinet (Thermo Scientific, model UT 6420 Thermo Electron LED GmbH, Langenselbold, Germany). Glass columns including frits (n = 5) were weighed in a dry and wet state. After filling the columns half way with soil and noting down their weight, they were covered, placed in a water bath, water level adjusted to 1/5 of the soil level and incubated for three hours. Afterwards the water-level was risen to height of the soil surface for 15 minutes. The next step was to drain samples on filter paper for 30 minutes. Subsequently columns were weighed and the last procedure (placing the columns in the tub at maximum water-level) repeated for control purposes. Finally samples were dried for 12 hours at 105 °C, weighed and 70 % WHC calculated, for the soil mass of one container, according to following formulas (Eq. 1, 2, 3):

\[ \text{Oven dry soil weight (g)} = \text{DWC}_T - \text{DWC} \]
\[ \text{DWC}_T = \text{Total dry weight column, including soil (g)} \]
\[ \text{DWC} = \text{Dry weight column (g)} \]

\[ \text{Water holding capacity (g)} = \text{WWC}_T - \text{WWC} - \text{ODW}_{\text{soil}} \]
\[ \text{WWC}_T = \text{Total wet weight column, including soil (g)} \]
\[ \text{WWC} = \text{Wet weight column (g)} \]
\[ \text{ODW}_{\text{soil}} = \text{Oven dry weight soil (g)} \]
70 % Water holding capacity of 4.5 kg soil (g) = \( \frac{WHC \times 4500 \times \frac{ODW_{soil}}{ADW_{soil}}}{ODW_{soil}} \times 0.7 \)

WHC = Water holding capacity (g)
ODW_{soil} = Oven dry weight soil (g)
ADW_{soil} = Air dry weight soil (g)

Harvest

After an establishment phase of 24 days, plants were cut every two weeks in the morning hours. The fifth and final harvest took place nine days after the fourth one, since no further plant growth was detected. Pots were laid down horizontally on a tray and plant shoots cut off, 2 cm above the soil surface, using scissors. At final harvest all aboveground biomass was cut off at soil level. Ending the experiment, the soil surface was scraped off, removing all unwanted biomass as preparation for the root washing process. Pots that couldn't be handled the same day were stored in a cold room at 5 °C to slow down breakdown by enzymatic activity and processed the following day. To assess belowground biomass, containers were tipped onto a 4 mm sieve positioned on top of another 1 mm one. For separation, the sieves were placed in a tub and roots were washed out of the soil with the help of a garden hose nozzle. Subsequently, all plant matter was rinsed out of the meshes into a water bath. Here, the roots were separated from any remaining objects such as pebbles and particulate organic matter. For four minutes remaining roots were picked from the sieves and combed out of the water. Finally, they were dried on a laboratory paper towel. Above- and belowground biomass was stored in paper bags and dried for a minimum of 12 hours at 80 °C in a drying cabinet.
For the pot trial, in total nine treatments with six replicates each were established featuring an air dry soil mass of 4.5 kg and the following amendments (Table 1). Samples supplied with RP were also referred to as the control group.

Table 1. Composition of pot trial groups (n=6). †

<table>
<thead>
<tr>
<th>Treatment name</th>
<th>Humic acid (HA) application</th>
<th>Phosphorus fertilizer</th>
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<tbody>
<tr>
<td></td>
<td>form</td>
<td>rate (mg kg⁻¹ soil)</td>
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<td>RP*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HA1S*</td>
<td>Soil (S)</td>
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<td>HA30S*</td>
<td>Soil</td>
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<td>Foliar (F)</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TSP*</td>
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</tbody>
</table>

* RP = Rock phosphate, ST = Struvite, TSP = Triple superphosphate. HA1S, HA30S, HA100S = Humic acid 1, 30 or 100 mg kg⁻¹ soil as soil application, HA1F, HA30F, HA100F = Humic acid 1, 30 or 100 mg kg⁻¹ soil as foliar application.

† Soil weight on an air dry basis.
2.2 Growth substrate

Subsoil was collected in December 2017, air dried, aggregates broken down by a Jaw Crusher BB 200 (Retsch GmbH, Haan, Germany) and subsequently sieved (< 4 mm). The processed soil featured a powdery texture. Quartz sand was delivered in plastic bags and had a grain size of 0.1 to 0.5 mm. The final growth substrate was a mixture of 50 % soil and 50% sand. Its oven dry weight was identified by heating samples (n = 5) at 105 °C in a drying cabinet for 12 hours and adjacent cooling in a desiccator until weight constant was attained. Oven dry mass was subtracted from air dry soil weight. This resulted in a water content of 0.85 %. Correspondingly 1 kg of air dry soil had an oven dry weight of 0.9915 kg. When soil weight was mentioned in this paper it always referred to air dry mass. Previously, in a different experiment the subsoil extractable P was analysed according to Calcium Acetate Lactate (CAL) and Mehlich-3 procedures. The CAL-extractant comprised 0.3 M acetic acid and 0.05 M calcium acetate; the soil to extractant ratio was 1:20. Soil to Mehlich-3 extractant proportion was 1:10 and the solution contained 0.2 M acetic acid, 0.25 M NH₄NO₃, 0.015 M NH₄F, 0.13 M HNO₃ and 0.001 M EDTA. Subsoil CAL analysis yielded 4.41 mg P 100 g⁻¹ soil and Mehlich-3 extraction 15.39 mg kg⁻¹ soil (Watson, 2018, pers. comm., 16.08). Since the utilized medium was mixed 1:1 (soil: sand), CAL P- as well as Mehlich-3-readings were categorized as “low” (Havlin et al., 2005, p. 347; VDLUFA, 2008, p. 7).
2.3 Fertilizers

Phosphorus

As phosphorus sources rock phosphate (RP) - \( \text{Ca}_5(\text{PO}_4)_3 \text{OH} \), struvite (ST) - \( \text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O} \), and triple super phosphate (TSP) - \( \text{Ca}((\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O} \) were chosen. At different rates an anticipated P amount of 27.25 mg kg\(^{-1}\) soil was applied (Table 1). The P mass was selected in a way to be the limiting nutrient during the vegetation period. Rock phosphate (ICL Fertilizers, Ludwigshafen, Germany, 18.5 % P) and triple superphosphate 45 (ICL Fertilizers, Ludwigshafen, Germany, 24.57 % P) were crushed using mortar and pistil, then sieved to (< 0.2 mm) and (< 1 mm) respectively. Struvite (Soepenberg GmbH, Hünxe, Germany, 12.62 % P) was homogenised (< 1 mm) by sieving. For further processing the required fertilizer masses were weighed into crimp neck bottles using a microscale (ENTRIS2202I-1S, Sartorius Lab Instruments, Goettingen, Germany).

Nutrient solution

Three supplementing solutions were produced, one for each phosphorus fertilizer (RP, ST, TSP), to compensate for nutrients not provided by them. The aim was to provide all plants of the different treatments with the same amount of nutrients. Nutrient quantities supplied by the P fertilizers were taken into account and the proportions estimated, which would become plant available during the experiment. For this, 50 % of N and 66 % of Mg provided by ST were estimated to be plant available. In terms of TSP and RP it was 50 % of their calcium content. Next, these portions were deducted from the overall nutrient pool of the corresponding solution. Consequently, the ST fluid received about 75 % less Mg and 7 % less N. Ca content in RP and TSP liquid was reduced by 57 % and 30 % accordingly (Table 2). The nutrient solutions were produced from macro- and
Micronutrients supplied in form of stock solutions. Macronutrient salts used for this purpose were calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$ · 4H$_2$O) (Carl Roth GmbH + Co. KG, Germany, 99 - 103 %, ACS), magnesium sulphate heptahydrate (MgSO$_4$ · 7H$_2$O) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany, 99 - 100.5 %, Ph. Eur., USP, BP), potassium sulphate (K$_2$SO$_4$) (Merck KGaA, Darmstadt, Germany, ≥ 99.5 %, EMSURE® ACS, ISO, Reag. Ph Eur), ammonium sulphate ((NH$_4$)$_2$SO$_4$) (Merck KGaA, Darmstadt, Germany, ≥ 99 %, EMSURE® ACS, ISO, Reag. Ph Eur) and ammonium nitrate (NH$_4$NO$_3$) (Merck KGaA, Darmstadt, Germany, ≥ 95 %, EMSURE® ACS). Micronutrient solutions were made from iron sulphate heptahydrate (FeSO$_4$ · 7H$_2$O) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany, ≥ 99 %, ACS), manganese sulphate monohydrate (MnSO$_4$ · H$_2$O) (AppliChem GmbH, Darmstadt, Germany, 98 - 101 %, Ph. Eur.), zinc sulphate heptahydrate (ZnSO$_4$ · 7H$_2$O) (AppliChem GmbH, Darmstadt, Germany, 99 - 104 %, Ph. Eur., USP), copper sulphate pentahydrate (CuSO$_4$ · 5H$_2$O) (VWR International, Leuven, Belgium, 99 - 102 %, AnalaR NORMAPUR), boric acid (H$_3$BO$_3$) (Bernd Kraft GmbH, Duisburg, Germany, ≥ 99.5 %), ammonium heptamolybdate tetrahydrate ((NH$_4$)$_6$Mo$_7$O$_{24}$ · 4H$_2$O) (VWR International, Leuven, Belgium, 81 - 83 %, AnalaR NORMAPUR) and cobalt nitrate hexahydrate (Co(NO$_3$)$_2$ · 6H$_2$O) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany, ≥ 98 %, ACS). Amounts of N added with the three solutions were chosen in a way to obtain a NO$_3$-N- to NH$_4$-N-ratio greater than 1.2:1. Overall N-levels were roughly the same, respecting half the N-amount supplied by struvite. Each pot, containing 4.5 kg soil, was watered with 0.929 L of one of the three nutrient solutions, depending on the provided phosphorus fertilizer, prior to sowing.
Table 2. Elemental content of nutrient solutions (mg L\(^{-1}\)) and P-fertilizers (mg kg\(^{-1}\)). †

<table>
<thead>
<tr>
<th>Macronutrients</th>
<th>(\text{NO}_3^-\text{N})</th>
<th>(\text{NH}_4^+\text{N})</th>
<th>P</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
<td>218.52</td>
<td>180.26</td>
<td>-</td>
<td>484.66</td>
<td>142.35</td>
<td>141.06</td>
<td>467.24</td>
</tr>
<tr>
<td>Fertilizer</td>
<td>-</td>
<td>27.25</td>
<td>27.25</td>
<td>58.78</td>
<td>330.73</td>
<td>34.54</td>
<td>58.78</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Micronutrients</th>
<th>Fe</th>
<th>Mn</th>
<th>Zn</th>
<th>Cu</th>
<th>B</th>
<th>Mo</th>
<th>Co</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
<td>12.50</td>
<td>3.90</td>
<td>2.73</td>
<td>3.05</td>
<td>0.52</td>
<td>1.63</td>
<td>0.61</td>
</tr>
<tr>
<td>Fertilizer</td>
<td>12.50</td>
<td>3.90</td>
<td>2.73</td>
<td>3.05</td>
<td>0.52</td>
<td>1.63</td>
<td>0.61</td>
</tr>
</tbody>
</table>

* RP = Rock phosphate, ST = Struvite, TSP = Triple superphosphate.

† Displayed figures are calculated values.
2.4 Humic acid

The utilized product was a liquid solution derived from Canadian leonardite. Laboratory analysis was conducted by G.A.I.A. – Gestioni Analisi Indagini Ambientali laboratories, Udine, Italy. Results showed a dry matter content of 10 % and insignificant concentrations of essential or beneficial plant nutrients considering the application volume (Table 1). Humic and fulvic acid contents yielded 4.8 % and 0.046 % of total product mass (Eq.4) (Appx. Table A1).

Determination of HA and FA content in the product (Eq. 4):

\[ HA \text{ Content (\%)} = DM \times C_{org} \times HFA \]

DM = Dry matter content of product (%)

C_{org} = Organic carbon content (%)

HFA = Humic or fulvic acid content of organic carbon (%)

The digits of FA in the analysis report seemed to be transposed. Therefore, a FA content of 0.8 % of dry matter, which would conform with the sum of humic and fulvic acids, was assumed and inserted into the equation. In the pot trial, humic acid product (HA) solution was applied at three rates in two forms. Soil application (S), namely coating of a P-fertilizer featuring a high Ca content with the product was chosen since HA have shown to increase P availability most likely due to interruption of calcium phosphate (Ca-P) crystal formation (Grossl and Inskeep, 1991; Wang et al., 1995; Alvarez et al., 2004). The second application form was a foliar spray (F), in compliance with given instructions of the supplying company. Former research showed that HA foliar application enhanced plant yield and performance (Delfine et al., 2005; Yildirim, 2007). As for amendment volume, the lowest one originated from the instructions given by the supplying company, at two times 1 L ha\(^{-1}\) during the vegetative period of the crop. The second one, 58.5
L ha\(^{-1}\), was selected under consideration of previous scientific work (Atiyeh et al., 2002; Tahir et al., 2011) and instructions for liquid humic acid products from other suppliers. Recommended rates ranged from 25 to 45 L ha\(^{-1}\) for Liqhumus\(^{®}\) (Humintech, Grevenbroich, Germany) while volumes for Humifirst\(^{®}\) lay between 25 and 50 L ha\(^{-1}\) (Tradecorp, Brussels, Belgium). The highest selected rate, 195 L ha\(^{-1}\), was stated by various sources (Sharif et al., 2002; Arjumend et al., 2015) to achieve optimum yields, whereas others noticed adverse effects on growth parameters (Tahir et al., 2011). The dosage for one kilogram of soil was calculated by accounting for an average bulk density of 1.3 g cm\(^{-3}\) and a topsoil layer of 15 cm (Weil and Brady, 2017, pp. 182-183). Additionally, the product volume to weight ratio was adopted to be 1:1 as indicated by the product label (1 L ha\(^{-1}\) = 1 kg ha\(^{-1}\)). Accordingly, HA application rates numbered 1, 30 and 100 mg kg\(^{-1}\) soil (Eq. 5).

Calculation of HA application rate (Eq. 5):

\[
\text{HA application rate (mg kg}^{-1}) = \frac{HA_V}{A_{Ha} \times BD \times TS}
\]

\(HA_V\) = Humic acid product application volume (mg ha\(^{-1}\))

\(A = \text{Area of one hectare (m}^2)\)

\(BD = \text{Bulk density (g cm}^{-3})\)

\(TS = \text{Top soil layer (m)}\)

On the one hand, for three treatment groups, liquid humic acid was incorporated into the soil during setup of the experiment. Therefore, 663 mg rock phosphate was blended with 500 mg distilled water, plus 4.5, 135 or 450 mg HA solution. After heating for 12 hours at 40 °C in a drying cabinet all water was evaporated.
The compound was then homogenised using a spatula until the original powdery form of the rock phosphate was retrieved. Later this aggregation would be mixed into the soil.

The second measure was a two stage foliar application during the first half of the vegetation period. The first one was performed twelve days after sowing, at an average shoot height of 6 - 8 cm. The second spraying took place thirteen days after the first one, the day after the first harvest at a shoot length of 2 - 5 cm. As a carrier liquid distilled water was mixed in a 25 ml volume beaker with humic acid product. First, HA solution was weighed into a tared beaker on a microscale and distilled water was added. Then, 20 ml of H₂O were mixed with 2.25, 67.5 and 225 mg of humic acid product respectively. No additional mixing was conducted since the product easily dispersed in distilled water.

For the application the top part of a fine, 250 ml hand sprayer was used (Bürkle, Bad Bellingen, Germany). By pulling the lever water was sucked out of a container and forced through a nozzle, dispersing it into small droplets and vapour particles. At the start the sprayer tube was submerged in the liquid. The liquid was sprayed on in a circular manner, rotating the pot, aiming at moistening all leaf surfaces. After the rest of the solution was used up, for rinsing purposes 5 ml of distilled water were filled into the beaker and sprayed onto the soil. Care was taken not to wash off any HA solution from plant leaves. All groups not treated with HA as foliar application were sprayed with an equal amount of distilled water in the same way. The same quantities were used during the second procedure. Summing up to an identical HA rate compared to the soil application group: 4.5, 135 and 450 mg product per pot.
2.5 Laboratory analyses

After drying the biomass, it was cooled down in a desiccator for one hour until equilibrium weight was reached. Samples were weighed and average weights of dried, empty paper bags (n = 5) subtracted. On this basis mean values for each treatment group were determined. For further analyses, plant matter of one pot from different harvests was combined and homogenised in a Retsch ZM 200 centrifugal mill (Retsch GmbH, Haan, Germany).

Soil pH after cultivation was measured in 1:2.5, soil to 0.01 M CaCl₂ (Merck KGaA, Darmstadt, Germany, 99 - 102%, EMSURE® ACS, Reag. Ph Eur) solution, according to VDLUFA methods (Hoffman, 1991) in one sample from each treatment. One sample, which developed the greatest aboveground biomass in each treatment group, was chosen as representative, assuming it would also feature the greatest belowground biomass. Since root exudation of acids and plant cation uptake increase soil acidification (Weil and Brady, 2017, pp. 393-396), it was assumed to find the greatest pH differences in these plants.

Soil samples were taken during harvest by splitting the soil clump in middle after the container was removed. Next, three table spoons of soil were scraped off the uncovered inner soil surface in a consistent manner and filled into clear polyethylene bags. Samples were stored at 5°C in a cold room until further analysis. For pH determination samples were homogenised by sieving (< 2 mm) and particulate organic matter was manually removed. After drying for 12 hours at 105 °C and subsequent cooling, CaCl₂ solution was added and the mixture thoroughly stirred on a HS 501 digital reciprocating shaker (IKA, Staufen, Germany) for one hour. Finally the H⁺ concentration was assessed via a Seven Multi - glass electrode (Mettler Toledo, Gießen, Germany), while mixing on a VMS - C4 magnetic hot plate stirrer (VWR International GmbH, Darmstadt,
Regarding C- and N content examination, 5 mg of above- and belowground dry matter were weighed into separate tin capsules on a microscale and analysed using a vario PYRO cube (Elementar Analysensysteme GmbH, Langenselbold, Germany). Values were received as percentages of the dry matter. Based on this data C/N ratios were calculated (Eq. 6).

C to N ratios were calculated by the following formula (Eq. 6):

$$\frac{C}{N} \text{ Ratio} = \frac{C_{conc}}{N_{conc}}$$

$C_{conc}$ = Carbon concentration in plant matter (%)

$N_{conc}$ = Nitrogen concentration in plant matter (%)

For elemental analysis shoot dry matter was broken down in a three step wet digestion, "Acid digestion in a mixture of H$_2$SO$_4$ and 30 % H$_2$O$_2$" (Jones Jr, 2001, p. 206), using a behrotest K40 coupled with a behrosog 3 acid fume extractor (behr Labortechnik, Düsseldorf, Germany). Therefore, 0.5 g of previously ground plant material was filled into digestion tubes containing 4 boiling pebbles. Secondly 3.5 ml of 95 % H$_2$SO$_4$ (VWR International, Fontenay-sous-Bois, France, 96.5 %, AnalaR NORMAPUR) were added to tubes, 30 minutes later 3.5 ml 30 % stabilised H$_2$O$_2$ (VWR International, Leuven, Belgium, 30.2 %, Ph. Eur.). For the heating process all tubes were arranged in a completely randomized manner in the digestion block. The cooking process then ran for 80 minutes, 30 minutes at a temperature of 350 °C. After the liquid cooled below 60 °C, 2 ml of 30 % H$_2$O$_2$ were added and the cooking procedure repeated. This process was done once more to achieve a clear liquid. The digest was then diluted with distilled water and strained through acid resistant filter paper into 100 ml volumetric flasks. Finally,
the liquid was brought to volume with distilled H$_2$O and stored in 100 ml plastic bottles for later analysis. The elements P, Fe, Zn, Mn and B were analysed in an Optima 8000 inductively coupled plasma optical emission spectrometer (ICP-OES) (Perkin Elmer, Shelton, Connecticut, USA). In contrast to other elements P concentrations were measured in a 1:10, digest liquid to purified distilled water, solution. Obtained data were then converted to plant tissue concentrations (Eq. 7).

Conversion of ICP-OES readings to element contents in plant matter (Eq. 7):

\[
\text{Element concentration (mg kg}^{-1} \text{ DM}) = \frac{E_{\text{Sol}} \times DF \times V_{\text{Dig}}}{DM}
\]

\(E_{\text{Sol}}\) = Element content in solution (mg L$^{-1}$)

DF = Dilution factor

\(V_{\text{Dig}}\) = Volume of digest solution (L)

DM = Dry matter weight of plant sample (kg)

### 2.6 Statistical evaluation

For statistical evaluation the open source computer programs R-Studio (RStudio Team, 2016) and R (R Core Team, 2018) were utilized, supported by several software packages, comprised of: agricolae (Mendiburu, 2017), cowplot (Wilke, 2018), FSA (Ogle, 2018), lawstat (Gastwirth et al., 2017), MASS (Venables and Ripley, 2002), rcompanion (Mangiafico, 2018), plyr (Wickham, 2011), scales (Wickham, 2017a) and tidyverse (Wickham, 2017b). Graphical visualisations were plotted with ggplot2, which is part of the tidyverse package.
The values illustrated in the results are arithmetic means calculated from six replicates on an oven dry plant matter weight basis. To test for statistical significant differences between means of treatment groups, one-way analysis of variance (ANOVA) was performed for normally distributed data. Treatment was always the categorical variable and plotted against a continuous one. By means of a quantile – quantile and residuals vs. fitted plot it was determined if the data set followed a normal distribution and homoscedasticity. In other words, if values were arranged in the form of a bell-shaped probability distribution and there was a constant variance between data points, no modification was executed. Otherwise, a Box-Cox power transformation was applied to achieve normally distributed data. Next, if ANOVA resulted in significant differences between means, a Post-Hoc analysis using the Tukey HSD (“Honest Significant Difference”) test identified significance levels between treatment means with \( p < 0.05 \). For data that wasn’t distributed normally, even after a Box-Cox transformation, a Kruskal Wallis test, followed by a Dunn’s test including a Benjamini-Hochberg correction was carried out. In this way significance indicating letters were added to graphs created using the ggplot2 package. All experimental variables were plotted against each other and a correlation test conducted where a relationship between data points was visually observable. In that case the non-parametric Kendall rank correlation coefficient measure was used since data sets had a small sample size and didn’t follow a normal distribution, which was confirmed by a quantile – quantile plot. By the Kendall function delivered tau values, which describe the strength of relationship between two variables, were categorised according to Hopkins (2002): Figures 0.0-0.1 were classified as trivial, 0.1-0.3 low, 0.3-0.5 moderate, 0.5-0.7 high, 0.7-0.9 very
high and 0.9-1 sharing a nearly perfect correlation. Confidence intervals (CI) of 95 % were plotted in correlation graphs.
3 Results

3.1 Soil pH
Measurement of soil pH in CaCl₂ after cultivation revealed no major variation between the treatment groups. For each treatment soil pH was analysed in one representative sample. Values lay in the range of 4.9 to 5.1 indicating an acidic pH. A minor trend in soil pH from pH 5.1 over 5 to 4.9 was detected in HA1F, HA30F and HA100F respectively. Additionally there were no great decreases in pH as result of the growth period, since the value before the experiment was 5.1.

3.2 Biomass yield and C/N ratio
All above- and belowground biomass yields combined showed no significant differences of the HA groups compared to the RP control group (Table 3). However, a few trends were observed. Application of 100 mg HA kg⁻¹ soil as foliar spray produced the highest shoot dry weight with 7.6 g – a slightly greater yield than the control. In this respect all other HA treatments resulted in a lower or similar biomass compared to RP. On average, groups that were sprayed with HA produced more than 4 % shoot biomass in contrast to HA soil applied ones. The lowest aboveground biomass, 7 g – more than 6 % less compared to the control, was harvested from plants supplemented with a HA amendment of 30 mg kg⁻¹ soil applied to the soil. Greatest root mass was found in the control providing 2 g, in contrast to HA30S group, featuring roughly 15 % less root weight.
Table 3. Yield of above- and belowground dry matter of treatments. ANOVA showed no statistical significant differences between group means. †

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aboveground biomass (g pot⁻¹)</th>
<th>Belowground biomass (g pot⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP*</td>
<td>7.5 ± 0.1</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>HA1S*</td>
<td>7.3 ± 0.5</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>HA30S*</td>
<td>7.0 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>HA100S*</td>
<td>7.2 ± 0.6</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>HA1F*</td>
<td>7.3 ± 0.3</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>HA30F*</td>
<td>7.5 ± 0.4</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>HA100F*</td>
<td>7.6 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>

† Displayed values are arithmetic means ± SD (n = 6)

* RP = Rock phosphate, HA1S, HA30S, HA100S = Humic acid 1, 30 or 100 mg kg⁻¹ soil as soil application, HA1F, HA30F, HA100F = Humic acid 1, 30 or 100 mg kg⁻¹ soil as foliar application.

C/N Ratio

Computed C/N ratios displayed no significant differences among treatment groups of above- or belowground biomass (Figure 1). Proportions obtained from aboveground biomass ranged from 10:1 to 10.6:1, the lowest value representing the control and highest the HA100F group. Plants sprayed with HA solution showed an increasing trend of their C/N ratio, from 10.3:1 to 10.6:1, when HA concentrations rose. This was due to slightly declining N portions of the shoot biomass, from 4.3 % in HA1F to 4.2 % in HA100F. In belowground biomass, the RP treatment also showed the lowest ratio with 39.1:1, however here HA100S had the highest value of 42.8:1, a more than 9% increase compared to the
control. Increasing HA rates applied to the soil, led to root N concentration decrease. HA100S featured nearly 7 % less N in comparison to HA1S. Yet, this trend wasn’t reflected in shoot biomass. Nevertheless, by ANOVA these differences were not indicated as statistically significant.

Figure 1. Carbon to nitrogen proportion of above- (AB) and belowground biomass (BB) of treatment groups: RP = Rock phosphate, HA1S, HA30S, HA100S = Humic acid 1, 30 or 100 mg kg\(^{-1}\) soil as soil application, HA1F, HA30F, HA100F = Humic acid 1, 30 or 100 mg kg\(^{-1}\) soil as foliar application. Ratios shown as arithmetic means (n = 6). Error bars indicate the standard deviation (SD).
3.3 Phosphorus

P concentration in aboveground plant material varied across the treatments (Figure 2). Highest content was found in HA30S, numbering 2790 mg kg\(^{-1}\) DM. A proportion nearly 12 % greater than the control, which had the lowest concentration of 2500 mg kg\(^{-1}\) together with HA1F yielding 2490 mg kg\(^{-1}\). In HA foliar application groups a rising P content was observed when HA volume increased. HA30F and HA100F exhibited a roughly 4 % and more than 7 % greater P yield than HA1F respectively. Kendall correlation analysis coincided with these findings, verifying a moderate relationship (\(\tau = 0.34\)) (Figure 3). However, because of a scattered variance of the data, ANOVA validated no significant differences between group means. The same was true when aboveground biomass yield was factored in to determine overall mass of phosphorus withdrawn from soil by the experimental groups (Figure 4). Generally, roughly 157 mg P pot\(^{-1}\) in the form of added P fertilizers and existing soil nutrient levels (taking Mehlich-3 P values into account) were at most plant available. RP or HA treatments were able to extract between 11.6 % and 13.1 % of the theoretically plant available P. HA100F treatment aggregated the most phosphorus with 20.5 mg - about 10 % more than the control (18.6 mg). Here HA100S and HA1F numbered 18.5 mg and 18.2 mg hence the lowest values. Since the biomass yield of HA foliar application groups followed the same increasing trend as P concentration in biomass, this was also true in the cumulative phosphorus extracted by plants. In this case Kendall correlation test showed about the same moderate relationship (\(\tau = 0.35\)) (Figure 5).
Figure 2. P concentration in aboveground biomass dry matter measured in treatment groups: RP = Rock phosphate, HA1S, HA30S, HA100S = Humic acid 1, 30 or 100 mg kg\(^{-1}\) soil as soil application, HA1F, HA30F, HA100F = Humic acid 1, 30 or 100 mg kg\(^{-1}\) soil as foliar application. Arithmetic means (n = 6) are indicated by diamonds.
Figure 3. Relation of humic acid volume applied as foliar spray to P concentration in plant tissue. RP, HA1F, HA30F and HA100F displayed as: 0, 1, 30 and 100 mg HA kg$^{-1}$ soil. Analysis according to Kendall rank correlation. CI = 95 %, n = 6.
Figure 4. Phosphorus accumulated in shoot biomass per container and treatment: RP = Rock phosphate, HA1S, HA30S, HA100S = Humic acid 1, 30 or 100 mg kg⁻¹ soil as soil application, HA1F, HA30F, HA100F = Humic acid 1, 30 or 100 mg kg⁻¹ soil as foliar application. Arithmetic means (n = 6) are indicated by diamonds.
Figure 5. Relation of humic acid volume applied as foliar spray to overall P amount extracted by shoots. RP, HA1F, HA30F and HA100F displayed as: 0, 1, 30 and 100 mg HA kg⁻¹ soil. Analysis according to Kendall rank correlation. Confidence interval (CI) = 95 %, n = 6.

3.4 Micronutrients
ICP-OES analysis delivered micronutrient concentrations in shoot biomass for Fe, Zn and Mn. However, regarding B readings only 12 out of 54 values were above the limit of detection. Most of these concentrations lay in the range of 2 to 27.2 mg kg⁻¹ DM, in addition to one outlying value of 105.7 mg kg⁻¹ DM. This small amount of data made statistical analysis inaccurate and was therefore not conducted for B. Looking at the other nutrient concentrations the control group and HA100F stood out. In relation to Zn and Mn the RP group exhibited higher trace element proportions than any HA treatment. As for Fe contents, only HA100S had a 2 % greater yield. Plants of the HA100F treatment yielded significantly lower Fe, Zn and Mn concentrations in contrast to the control (Figure 6).
Within HA soil application groups, 1 mg kg\(^{-1}\) soil yielded with less than -28 % a significantly smaller Fe concentration than the 100 mg kg\(^{-1}\) soil amendment. However, HA soil amended groups held on average nearly a quarter more Fe per unit DM than the foliar application treatment. At rising HA soil application levels an increasing trend in Fe plant tissue concentrations was noticed. Fe concentrations with 241.5 and 251.9 mg kg\(^{-1}\) DM featured by the 1 and 100 mg HA kg\(^{-1}\) soil as foliar application group were not only the lowest, but also significantly smaller Fe accumulations than the control (370.6 mg kg\(^{-1}\) DM) and HA100S group (377.7 mg kg\(^{-1}\) DM). In this comparison biomass Fe concentrations were roughly one third smaller (Figure 6a). Fe concentrations in the foliar treatment group increased by about 10 % when HA concentrations rose from 1 to 30 mg kg\(^{-1}\) and dropped again by more than 5 % at HA volume elevation to 100 mg kg\(^{-1}\). For the Zn concentrations HA1S and HA100F accumulated more than 10 % less Zn per unit DM in contrast to the control which numbered 58.3 mg kg\(^{-1}\) DM – a significant difference. Data within the groups amended with HA ranged from 51.5 to 56.5 mg Zn kg\(^{-1}\) DM. The foliar application groups presented on average a slightly higher content of about 2 % (Figure 6b). Looking at Mn concentrations, the control featured the maximum value, at 359.3 mg kg\(^{-1}\) DM, closely followed by HA30F with 354.6 mg kg\(^{-1}\) DM. They held a significantly higher, at least 13 to 22 % greater Mn concentration in their biomass in contrast to other groups. The lowest content was found in HA1S with 288.8 mg kg\(^{-1}\) DM, having a similar value to HA30S at 292.4 mg kg\(^{-1}\) DM. Generally, HA foliar application groups had a nearly 10 % greater Mn accumulation than HA soil application groups (Figure 6c). Relations between applied HA volumes and microelement concentrations were discovered. Kendall rank correlation resulted
in moderate negative relationships between increasing foliar spray applied HA volumes and Fe ($\tau = -0.35$) and Zn ($\tau = -0.48$) concentrations in aboveground biomass (Figure 7a, b). With $\tau = -0.3$ correlation for Mn was weaker, but still classified as moderate (Figure 7c). When element concentrations were multiplied with aboveground biomass yields to estimate total micronutrient masses extracted, major trends and differences in the control, HA soil and HA foliar application groups didn’t change (Figure 8).
Figure 6. Fe, Zn and Mn concentrations in aboveground dry matter in respect of amendments. RP = Rock phosphate, HA1S, HA30S, HA100S = Humic acid 1, 30 or 100 mg kg\(^{-1}\) soil as soil application, HA1F, HA30F, HA100F = Humic acid 1, 30 or 100 mg kg\(^{-1}\) soil as foliar application. Arithmetic means (n = 6) are indicated by diamonds. Different letters represent significant differences (p < 0.05) between
treatment means. Fe and Zn data analysed with one-way ANOVA after Box-Cox-transformation, Mn with rank-based Kruskal-Wallis test.
Figure 7. Correlation of humic acid volume applied as foliar spray to Fe (a), Zn (b) and Mn concentrations (c) in plant tissue. RP, HA1F, HA30F and HA100F displayed as: 0, 1, 30 and 100 mg HA kg⁻¹ soil. Analysis according to Kendall rank correlation. Confidence interval (CI) = 95 %, n = 6.
Figure 8. Fe, Zn and Mn amounts accumulated in aboveground biomass per container in respect of amendments. RP = Rock phosphate, HA1S, HA30S, HA100S = Humic acid 1, 30 or 100 mg kg$^{-1}$ soil as soil application, HA1F, HA30F, HA100F = Humic acid 1, 30 or 100 mg kg$^{-1}$ soil as foliar application. Arithmetic means ($n = 6$) are indicated by diamonds. Different letters represent significant
differences \((p < 0.05)\) between treatment means. Fe and Zn data analysed with one-way ANOVA after Box-Cox-transformation, Mn with rank-based Kruskal-Wallis test.

3.5 Struvite and triple superphosphate

HA product applied in selected quantities didn’t achieve any significantly greater yields in biomass or plant accumulated P. Even though some trends in biomass development, P and micronutrient accumulation were observed, much stronger differences could be observed in ST and TSP amended groups as compared to RP.

ST treated plants reached the highest aboveground biomass yield as well as extracted the biggest amount of P compared to all other groups. Values were significantly higher than in RP amended groups. Yields reached a more than 13 % greater shoot biomass and at least 57 % higher mass of extracted P (Figure 9). In comparison to TSP, ST DM yield was almost 5 % and plant extracted P more than 16 % higher. However, only the ST amount of plant accumulated P was significantly greater than TSP. Total theoretically plant available P was 156 mg pot\(^{-1}\), composed of 122 mg P from the respective fertilizer and about 34 mg P from the soil (assuming 7.5 mg kg\(^{-1}\) soil Mehlich-3 P). With 29.3 mg P pot\(^{-1}\), ST removed 18.7 % of the maximum plant available 157 mg P pot\(^{-1}\). RP (18.6 mg P pot\(^{-1}\)) and TSP (25.2 mg P pot\(^{-1}\)) took up 11.8 % and 16.1 % respectively. Plant tissue P-concentration which was taken into account for plant accumulated P numbered 2497 mg kg\(^{-1}\) DM for RP. ST and TSP displayed 3471 and 3121 mg kg\(^{-1}\) DM, a roughly 39 % and 25 % higher as well as significantly different P concentration to the RP treatment respectively.
Regarding this variable, TSP wasn't significantly different from ST.

Cumulative N amounts of above- and belowground biomass revealed that ST (364.1 ± 6.4 mg pot⁻¹) took up almost 3% more N from the growth substrate than the control, which extracted 352.9 ± 9.2 mg pot⁻¹. In this regard, TSP (348.6 ± 9.5 mg pot⁻¹) gathered a 1.5% lower amount of N, compared to RP. Nevertheless, in plant shoot tissue, RP (4.5 ± 0.1%) N concentrations were at least 9% higher in respect to ST (4.1 ± 0.2%) and TSP (4.1 ± 0.2%). Also in root tissue, N content of the control was higher in contrast to ST and TSP, at 1.2 ± 0.1% compared to 1.1 ± 0.04 and 1.02 ± 0.1%.

Figure 9. Plant shoot (a) and respective accumulated P yields per container (b) of RP in comparison to ST and TSP amendments. RP = Rock phosphate, ST = Struvite, TSP = Triple superphosphate. Arithmetic means (n = 6) are indicated by diamonds. Different letters represent significant differences (p < 0.05) between treatment means. Both data sets analysed with one-way ANOVA. Data (b) previously adjusted with Box-Cox-transformation.
4 Discussion

4.1 Soil pH

Recorded pH values after cultivation were classified as strongly acidic. Furthermore, observed minor changes in soil pH values after plant cultivation were most likely a result of plant root induced alteration by proton exudation (Weil and Brady, 2017, pp. 393-396). Although former studies suggested an impact of HA on soil pH by increasing the root mass of treated plants, leading to elevated amounts of root exudates and consequential pH decrease (Canellas et al., 2008b), this wasn’t reflected clearly in the presented results. Even though pH declined slightly with increasing HA foliar application rates, overall HA application volumes didn’t result in a significantly rising soil acidity. For a meaningful picture it would have been beneficial to analyse all soil samples.

4.2 Plant biomass

The most important plant characteristic looking from a practical, economic point of view, shoot biomass yield, didn’t show any substantial differences between plants amended with any selected HA rate compared to the control group (Table. 3). Nevertheless, a trend of increasing shoot biomass was observed when HA foliar application amounts were risen. Since this trend was only observed in HA foliar spray treated plants, whose surfaces came in greater product contact than HA soil applied groups, hormone like activities of HA might have been the driving factor. HA have shown to exhibit auxin like functions: causing cell elongation, enhanced formation of lateral roots and root hairs (Nardi et al., 2002; Zandonadi et al., 2007; Canellas et al., 2009; Canellas et al., 2010).
HA would also stimulate H⁺-ATPase synthesis, following H⁺ excretion (Aguirre et al., 2009; Canellas et al., 2009; Zandonadi et al., 2010), leading to an acidification of cell walls and consequential root cell multiplication, demonstrated by the acid growth theory (Rayle and Cleland, 1992). An intensification of the root structure, resulting in an enlarged root surface area with the same root mass, could have applied and caused the improved shoot biomass development of HA foliar application groups by enhanced nutrient uptake. However, given that root morphology wasn’t examined, this can only be theorised. At the same time, greater exudation of H⁺ ions by H⁺-ATPase into the rhizosphere would increase the electrochemical difference of the soil solution in contrast to the root cell cytosol, increasing nutrient diffusion into the cell (Canellas et al., 2002). Simultaneously, this would enhance the secondary ion transport system across the cell’s plasma membrane, both processes leading to improved nutrient uptake (Jindo et al., 2012). Higher HA foliar spray doses promoting H⁺ release and subsequently improved nutrient uptake would explain the trend of a rising shoot biomass. Conversely, only P was absorbed at higher rates. N portions decreased slightly, but Fe, Zn and Mn concentrations declined significantly at the highest amount of foliar amended HA. Nonetheless, since P was the plant growth limiting nutrient in this pot trial, the displayed increments in P concentrations (Figure 2) were most likely the decisive factor for the increasing trend in shoot biomass. At insufficient P amounts, plant growth would be limited as an effect of P lacking in structural and metabolic processes (Weil and Brady, 2017, p. 662). Contradictory to these assumptions is the case that HA30S yielded the highest P concentration in plant tissue (about 4 % higher than HA100F), but the lowest shoot biomass of all treatments. N and Zn concentrations were similar, the Fe level about 16 %
higher and Mn concentration significantly lower compared to HA30F and HA100F. Even though in this case Fe and Mn concentrations varied, they were most likely not the cause for the differing shoot DM mass, since their levels were neither in the deficient nor toxic range (Marschner, 2012, pp. 200, 204, 205). It could also be possible that HA soil application increased P solubility in solution, but nutrient uptake through root membranes was favoured by HA foliar amended treatments. This way P concentration in HA30S treatment was high, but couldn’t be turned into biomass as a consequence of missing nutrients that couldn’t be taken up through the membrane.

**C/N ratios**

Generally, C to N proportions were much higher in plant roots (~ 40:1) compared to shoots (~ 10:1), being in line with the theory of a decreasing C/N ratio plant upwards (Hicks, 1928). In contrast to his findings, the above-average root C/N contents might be due to N relocation into shoot biomass as a result of repeated harvesting. Mobile elements would always be translocated to plant parts of new growth (Hicks, 1928). HA amendments didn’t affect C or N concentrations significantly. Increasing HA soil application amounts led to higher C/N shoot ratios. Declining plant tissue N concentrations might be a result of reduced N absorption, because of its fixation onto HA molecules, thereby competing with plant roots. This can be linked to the fact that partially, NH₄-N can be fixed as NH₃ to functional groups of organic matter and become plant unavailable (Johansson, 1998; Weil and Brady, 2017, p. 609). Another factor that influenced decreased plant N uptake could have been N immobilisation by soil microorganisms from the soil solution. HA usually featured a high C content. In spite of the fact that
they are rather resistant to microbial decomposition, partial breakdown by microorganisms causing concurrent N withdrawal from the soil solution, would explain lower plant root N levels (Weil and Brady, 2017, pp. 554-556).

4.3 Phosphorus

Measured P accumulation in shoot biomass in respect to theoretically plant available P conformed to the guideline of 10 - 15 % P of applied fertilizers that would be taken up by plants (Weil and Brady, 2017, p. 662). During the experiment, conditions for optimal plant P availability were not met, since soil pH$_{CaCl_2}$ was about 5. Ideal P availability would be in the region of pH 6 - 7. In spite of this, the acid pH favoured solubility of the Ca containing fertilizers RP and TSP (Weil and Brady, 2017, pp. 685, 689). By supplying 27 mg P kg$^{-1}$ soil, the goal of causing P deficiency in treatments was achieved. Plant shoot biomass P concentrations were below 3000 mg kg$^{-1}$ DM, a threshold for optimal P supply (Marschner, 2012, p. 164).

One theory for increased P uptake of foliar and soil applied HA groups, was the development of a more advanced root system by the influence of HA stimulation. Although a higher root mass couldn’t be found in HA groups compared to the control, increasingly complex lateral root and root hair formation could have expanded the root surface area and resulting P absorption, while featuring a constant root mass. Besides this, elevated P uptake in HA foliar application treatments was suggested to stem from elevated H$^+$-ATPase creation and H$^+$ excretion, as mentioned in the previous section. Also, elevated amounts of organic acid exudates, caused by hormone like activity of HA, as stated by Canellas et al. (2008a), could have been a factor for increased plant P availability.
The cause for a P increase in HAS treatments might be due to interaction of HA with Ca-P precipitation, resulting in declined P fixation, thus better plant availability. HA was found to decrease precipitation of amorphous calcium phosphate to more insoluble forms (Alvarez et al., 2004). In a different study Wang et al. (1995) found an increase in water soluble P when HA were added to an alkaline soil, in addition to a strong reduction in plant unavailable P forms. This mechanism could have been partly responsible for an increased plant material P concentration in HA soil application groups, since P was added in the form of a Ca containing fertilizer. Given the acidic soil pH, the dominant form of P fixation in the soil solution occurred by Fe, Mn and Al hydrous oxides (Weil and Brady, 2017, p. 682). By bonding with functional groups of HA applied to the soil, Al and Fe could have been chelated and previously fixed P released back into soil solution (Antelo et al., 2007). The chelation process would explain the highest P tissue concentration of the HA30S group. An optimum for an increase in plant biomass P levels was reached at a HA amount between 30 and 100 mg kg\(^{-1}\) soil. Exceeding this threshold, plant tissue P concentration decreased. Ayuso et al. (1996) proposed that HA and P in soil solution formed phosphohumates, additionally validated by Tan (2010, p. 236), which were taken up by the plant and led to increasing P concentration in shoot biomass. Consequently, in this pot trial, adverse effects on plant yield could have been induced by negative hormonal activities as a result of HA molecules within plant cells, taken up through the roots. The P decline in HA100S plant biomass could be linked to a possible competition between HA molecules and plant roots.
4.4 Micronutrients

Plant shoot B values, which were below the detection limit of the ICP, were probably a result of the acid wet digestion method. The fault must have been of an analytical nature, since B was supplied in the nutrient solution and plants didn’t show any signs of B deficiency. According to Jones Jr (2001, pp. 206-207) B could be lost during the digestion process.

Although micronutrient plant tissue concentrations varied significantly between treatments, their levels lay in the normal range (Weil and Brady, 2017, p. 729). Practically all plants treated with HA contained lower micronutrient levels in their shoot biomass than the control. This might be a result of the metal complexing potential of HA applied to the soil. Ayuso et al. (1996) found a similar negative relationship between micronutrient levels and rising HA amounts. Levels of 50 and 100 mg C L\(^{-1}\) nutrient solution, roughly comparable to 100 mg HA kg\(^{-1}\) used in this trial, which caused the reduction of Fe, Mn and Zn contents in plant tissue. They hypothesised an excess of chelators would complex micronutrient metals in the soil solution and lead to reduced absorption. This would also conform with findings reported by Chen and Aviad (1990, p. 172), who linked HA products, which would be relatively insoluble in the soil solution, to their property of inhibiting micronutrient plant uptake. Weil and Brady (2017, pp. 741-744) illustrated that plant micronutrient availability could be reduced by functional groups of organic matter, in addition to organic compounds that could create insoluble chelates with metal ions. The results showed, that the lowest HA soil or foliar application rate resulted in lower shoot micronutrient concentrations than the control. Since soil in HA foliar applied treatments came in contact with HA as well, complexion of metals in the soil solution is possible likewise. However, since
metal chelation should have increased at higher doses of HA, plant tissue micronutrient concentrations should have decreased accordingly. This theory is not valid for HAS, since metal levels increased in shoot biomass with rising applied HA amounts.

For plants treated with HA foliar spray this explanation is highly unlikely, although metal concentrations decreased with higher HA application doses, since only a fraction of applied HA solution infiltrated into the soil. Here, hormonal activities might have caused the decline of micronutrients in plant tissue at increasing application volume. Aguirre et al. (2009) demonstrated that HA would have a hormone like effect on the up- and down-regulation of enzymes as well as transport proteins involved in mobilization and uptake of Fe. Quaggiotti et al. (2004) and Aguirre et al. (2009) suggested that HA would influence gene expression in roots and thereby regulate the synthesis of proteins that influence plant nutrient uptake. A similar process could have caused the down-regulation of proteins associated with micronutrient assimilation in root cells of HAF groups, since hormone like activities were of greater relevance in this treatment than in HAS as mentioned before in the plant biomass section.

4.5 Struvite and triple superphosphate

The significantly higher biomass production in the ST compared to RP group and 5 % greater yield in contrast to the TSP treatment, was most likely due to increased plant shoot P concentrations. Of the applied fertilizers ST treatment showed the greatest phosphorus plant availability. P in RP and TSP was more insoluble than ST, as a consequence of dominant calcium phosphates (Weil and Brady, 2017, pp. 682-685). It should be clarified that plant P availability of RP
treated plants benefited from the acid pH. At a pH between 6 and 7, which is usually aimed for in arable soils, RP would be less effective (Schulte and Kelling, 1996). Even though the soil pH was acidic, P could still be retained from the soil solution in certain forms of CA-P e.g. Octacalcium phosphate (Ben-Nissan et al., 1995, as cited in Alvarez et al., 2004). In contrast to RP, the TSP group yielded elevated biomass as well as plant tissue P concentrations, since P was fixed as Ca(H₂PO₄)₂ · H₂O, a less stable and highly soluble CA-P in comparison to RP (Ca₅(PO₄)₃ OH). In theory, the higher ST treatment shoot biomass yields might have been due to an increased total nitrogen availability. An underestimation of the plant availability of N supplied with the ST fertilizer, could have caused a higher total N supply of the ST- in comparison to the RP- and TSP group. On the contrary, the ST group’s overall plant extracted N was only 3 % higher than N accumulated by the RP treatment. The fact that ST accumulated, as a sum, more N than RP was most likely a consequence of an elevated shoot biomass, produced as a result of higher P availability. This was supported by the shoot biomass N concentration, which revealed that RP fixed at least 9 % more N per unit DM than ST and TSP. This elevated N proportion could have been an outcome of deficient plant P uptake, preventing the conversion of N into biomass (Weil and Brady, 2017, p. 662). As a result excessive N might have been stored in the plant’s vacuoles (Marschner, 2012, p. 142).

As a consequence of an often negative P nutrient balance, low P contents in organic fertilizers (e.g. manure) and low plant availability of mineral ones, P is frequently a plant growth limiting nutrient in organic farming systems (Weil and Brady, 2017, p. 1041). Furthermore, as demonstrated in the above section, sufficient N fertilization wouldn’t increase yields, conforming to the law of the
minimum (Weil and Brady, 2017, pp. 805-806). Research regarding the potential of HA as a biostimulants to increase plant P availability in organic agricultural systems, didn’t yield consistent results. Further studies have to be conducted to come to a conclusion concerning the specific mechanisms how HA increase plant P availability.

On the other hand ST presented promising results regarding P availability and biomass production. Since minable world P reserves are shrinking, the introduction of a recycled P fertilizer in organic agriculture, but also in conventional agriculture, is urgently needed (Cordell and White, 2011). Therefore, struvite could be a solution for a more sustainable P utilization in the future (Kataki et al., 2016; Talboys et al., 2016).
5 Conclusion

Perennial ryegrass (Lolium perenne L.) grown in a low P environment in sandy soil was treated with HA. The instructed application rate of 1 mg kg\(^{-1}\) soil did not have any significantly positive effect in plant P NUE, shoot or root growth compared to the control. Interestingly, even a 100-fold increase in HA concentration did not show any statistically meaningful results. On the contrary, measured plant tissue micronutrient concentrations of HA treated samples were in general lower and partly significantly lower than the control, especially for Mn. With rising HA doses applied as a foliar spray, increasing trends in shoot dry matter and aboveground biomass P concentrations were observed. The described effects are certainly due to the hormone-like impact of HA on plants’ metabolism or related to HA alterations of the growth substrate. Additional studies are needed to confirm the validity of these findings for different plant species and if demonstrated trends continue at higher HA amendment rates. Theoretically, higher HA foliar application doses could be advised to increase plants’ P nutrient use efficiency in agricultural systems utilizing RP. As a result of higher plant P utilization, the overall amount of P inputs could be reduced and the build-up of plant unavailable soil P stores diminished. Consequently, positive environmental effects such as a decrease in waterbody contamination through P erosion and leaching, as well as a reduction of eutrophication can be achieved. In addition, farmers could benefit financially, since less fertilizer would need to be purchased, transported and distributed. However in reality, applying more than 100-times the recommended application rate of HA wouldn’t be economically sustainable. Nevertheless, since the effect of HA is dependent on the raw material, HA content, manufacturing method, plant species and application method, further
investigations could find an commercially viable solution in the future.

Yet, to solve the problem of agricultural P input scarcity due to the inevitable end of fossil RP, the recycled fertilizer ST demonstrated feasible results. In contrast to RP and TSP, ST amended samples yielded significantly higher P contents and more shoot biomass. This was attributed to a better P availability of ST as a consequence of absent Ca. ST could be applied to reduce the dependency on minable P and thereby closing the gap in the anthropogenic P cycle. Despite its positive traits, phosphorus pollution in watersheds is still possible if managed improperly. Additionally, future studies focussing on the effect of HA in combination with ST are worthwhile to conduct in order to evaluate a further increase in P nutrient use efficiency.
6 References


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Table A1. Laboratory analysis of humic acid product.

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