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# The effects of struvite and sewage sludge on plant yield and the microbial community of a semiarid Mediterranean soil

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## Abstract

Phosphorus (P) limitation will play a key role in the productivity of agriculture in the coming decades. Struvite is

an ammonium magnesium phosphate mineral that can be recovered from wastewater-treatment plants and can

be considered as an alternative source of P. However, the impact of struvite on the plant yield and, particularly, on the soil microbial community is barely known. Here, we tested the impacts of struvite, sewage sludge, and their combination on the barley yield, soil macro and micronutrients, and biochemical and microbiological soil properties. Amendment with struvite alone and its combination with sludge increased the availability of P in soil, the plant uptake of P and Mg, and the barley yield. The analysis of phospholipid fatty acids (PLFAs) and the utilization of metaproteomics approaches revealed significant effects of struvite on the biomass of Gram-positive bacteria and, particularly, on actinobacterial and verrucomicrobial populations in soil.

*Keywords*: phosphorus; struvite; sludge; soil microbial community; PLFAs; metaproteomics

## Introduction

Phosphate rock is a non-renewable resource that will be exhausted 70-100 years from now (Cordell et al., 2009) and there is no substitute for phosphorus (P) in nature (US Geological Survey, 2005; Kim et al., 2018). In consequence, it is expected that P limitation will play a pivotal role in the agricultural productivity in the coming decades. Further, there are stringent regulations about contaminants from rock phosphate (i.e. cadmium) (Pizzol et al., 2014). If we take together the predicted limitations in P supply and the growth of the human population, the consequence is immediate: we need to explore sustainable sources of P in order to maintain agricultural productivity (Desmidt et al., 2015).

Sewage sludge has an abundant content of macro and micronutrients. However, it may also contain undesirable components such as heavy metals, pathogens, or some emergent pollutants. The application of sewage sludge has been proved to benefit soil quality in semiarid environments (Bastida et al., 2008; 2009; Torres et al., 2015), but ideally its application should follow some type of previous sanitization and stabilization treatment, such as composting. Due to the relatively high P content of sewage sludge, its addition to soil is one option for the potential recycling of P. Although the major part of sewage sludge is composted (60-90% of the sewage sludge produced in UK, Ireland, Spain, France, or Luxemburg; Kelessidis and Stasinakis, 2012), most of the remainder is incinerated. Consequently, substantial amounts of P from this organic amendment are lost with regard to agriculture. Additionally, P from fertilizers and detergents is directly discharged into water bodies and the excessive P content is causing pollution and eutrophication of some aquatic habitats (Chen et al., 2017).

Phosphorus can be recovered at municipal wastewater-treatment plants from the supernatant resulting from anaerobic sludge digestion, through the crystallization of struvite, an ammonium magnesium phosphate mineral with the formula $MgNH_4PO_4.6H_2O$ (Shu et al., 2006; Plaza et al., 2007). Struvite can be considered an alternative source of P, nitrogen (N), and magnesium (Mg) for agricultural purposes (Doyle & Parsons, 2002; Gilbert, 2009). Although it has been indicated that struvite has a low solubility - pure $MgNH_4PO_4.6H_2O$ has a solubility of around 0.2 g $L^{-1}$ (Cabeza et al., 2011) - several studies have found that struvite from sewage sludge is as effective as superphosphate alone for the promotion of dry matter production and P uptake in plants (Plaza et al., 2007; Massey et al., 2009; Uysal et al., 2010). This suggests that P solubilization from struvite occurs, at least partially, in soil.

In the sustainability of agroecosystems, the soil microbial community is critical because it plays a paramount role in soil fertility. For instance, microbes drive organic matter decomposition and fixation in soil (Paul, 2007), these reactions being catalyzed by a suite of enzymes related to the C, N, and P cycles (Bastida et al., 2008; Burns et al., 2013; Garcia et al., 1994). Furthermore, microbial parameters, such as those related to biomass, activity, or diversity, are often considered as highly sensitive (Bastida et al., 2008) and are influenced by fertilization practices (Moreno et al., 2008). Among the most novel approaches for the characterization of the diversity and functionality of the soil microbial community, metaproteomics provides more straightforward information about the active microbial populations and their functional roles in soil through the direct identification of microbial proteins (Keiblinger et al. 2012; Bastida and Jehmlich, 2015; Bastida et al., 2016; 2017; Hettich et al., 2013; Hultman et al., 2015).

Although a positive agronomic effect of struvite on the yield and P uptake has been observed for several crops (Plaza et al., 2007; González-Ponce et al., 2009; Cabeza et

al., 2011), its effects in the soil microbial community are not known. Here, we evaluate the impacts of struvite and its co-synergic effects with sewage sludge on the yield of barley (*Hordeum vulgare* L.) and on the soil microbial community. For this purpose, we applied a multimethod approach involving phospholipid fatty acids (PLFAs), enzyme activities, and metaproteomics. We standardized the soil amendment treatments (sewage sludge, struvite, and their combination) with respect to the P requirements of barley. Given the different contents of macro and micronutrients in sludge and struvite, we hypothesize that their combined use will benefit plant yield in a synergistic way, distinct from that of the single use of either of these materials, and will impact the overall soil microbial community. Further, the use of struvite alone, which is more "mineral" and more highly enriched in P, N, and Mg than sludge, should increase P and Mg accumulation in plants and affect specific microbial populations.

## Material and Methods

### *Experimental design*

A one-month pot experiment was carried out under controlled conditions in a plant growth chamber. Three amendment treatments (struvite, sewage sludge, and struvite plus sewage sludge) and a control (unamended soil) were tested, using barley as an indicator crop. Struvite can be considered as a potential mineral with a very high content of P, but also N and Mg; while sewage sludge is used in some tree crops as an organic amendment of soil due to its high contents of organic C and available N, besides a considerable amount of organic P. The soil utilized is classified as a Calcic Xerosol (FAO-UNESCO 2003) and it was sampled from an abandoned agricultural plot located in South-eastern Spain. This soil is characterized by a sandy loam texture and

the following main nutrient concentrations: organic C=2.73%, total N=0.17%, and available P=1.95 mg $kg^{-1}$. After sampling, the soil was sieved (2 mm) prior to being placed in plastic pots.

The struvite and sewage sludge were obtained from a wastewater-treatment plant. The dosage of these materials added to the soil was calculated on the basis of the P fertilization recommendation for barley crops (84 kg $P_2O_5$ $ha^{-1}$). Thus, the doses added were: 11.66 mg of struvite and 80.63 mg of sewage sludge per 100 g soil (dw) and one-half of these doses in the combined treatment with both materials. The chemical characterization of the struvite and sewage sludge is shown in Table 1. These materials were air dried and milled prior to mixing them with soil.

The plastic pots containing soil were pre-incubated at 50% of the water-holding capacity (WHC) for five days, at an air temperature of 25 ºC and an air relative humidity of 65%. This pre-incubation was performed in order to avoid confounding effects of humidity on soil microbial parameters. After that, ten barley seeds were sown in each microcosm and maintained under the same air and soil conditions of temperature and moisture. Periodically, distilled water was added to the soil to maintain the soil moisture at around 50% of the WHC. Two soil sampling times were established: one before sowing and another at the end of the plant growth experiment (after one month). At this time, the plant height and the number (%) of germinated seeds were measured. The barley plants of each pot were cut and the aerial biomass was weighed (fresh weight); after this, the biomass was dried in an oven at 60 ºC and then weighed again (dry weight). The dried plant tissues were placed in plastic bags, which were then sealed, and the soil samples were split into two portions: one was stored at 4 ºC for the chemical and biochemical analyses, and the other was stored at -20 ºC for the metaproteomic analysis.

120 *Chemical analyses, enzyme activities, and phospholipid fatty acids (PLFAs) analysis*

121 The total N and total organic C (TOC) in the soil were analyzed using an Elemental
122 Analyzer (C/N Flash EA 112 Series-Leco Truspec). Bioavailable phosphorus ($P_{av}$) in
123 the soil was extracted with 0.5 M $NaHCO_3$ and measured by following the method
124 described by Olsen and Sommers (1982). The total contents of P, K, Ca, micronutrients,
125 and heavy metals in the soil, struvite, sewage sludge, and plant tissues were determined,
126 after nitric-perchloric acid digestion, using an ICP-OES spectrometer (ICAP 6500
127 DUO; Thermo-Scientific, Waltham, MA, USA).

128 Urease activity (URA) was determined as the $NH_4^+$ released in the hydrolytic reaction
129 using urea as substrate and borate buffer (pH 10), as reported by Kandeler and Gerber
130 (1988). The soil-substrate mixture was incubated in the buffer solution for 2 h at 37 ºC.
131 Alkaline phosphomonoesterase (APA) and β-glucosidase (BGA) activities were
132 analyzed by the methods of Tabatabai and Bremner (1969) and Eivazi and Tabatabai
133 (1988), respectively. Two milliliters of MUB (Modified Universal Buffer), pH 6.5 for
134 the β-glucosidase assay and pH 11 for the alkaline phosphomonoesterase assay, and 0.5
135 ml of *p*-nitrophenyl substrate (*p*-nitrophenyl-β-D-glucopyranoside for β-glucosidase and
136 *p*-nitrophenyl phosphate for alkaline phosphatase) were added to 0.5 g of soil. The
137 mixtures were incubated at 37 °C for 1 h. Then, the *p*-nitrophenol released was
138 measured by colorimetry in a UV-visible spectrophotometer (Helios Alpha, Thermo,
139 UK) at 400 nm.

140 Phospholipids were extracted from 6 g of soil using chloroform-methanol extraction, as
141 described by Bligh & Dyer (1959), and were fractionated and quantified using the
142 procedure of Frostegard et al. (1993). The phospholipids were transformed into fatty
143 acid methyl esters (FAMEs) by alkaline methanolysis and designated as described by

Frostegard et al. (1993). The complete dried FAME fraction was dissolved in isooctane containing 0.23 mg $mL^{-1}$ of 21:0 FAME as internal standard. The analysis was performed using a Trace Ultra Thermo Scientific gas chromatograph fitted with a 60-m capillary column (Thermo TR-FAME, 60 m x 0.25 mm ID x 0.25 μm film), using helium as carrier gas. The following fatty acids are characteristic bacterial fatty acids and were chosen as bacterial biomarkers: i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c, and 18:1ω9t. The fatty acid 18:2ω6 was used as an indicator of fungal biomass. The Gram-positive representative fatty acids used were i15:0, a15:0, i16:0, and i17:0. The Gram-negative fatty acids used were cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c, and 18:1ω9t. The 10Me-branched FAMES (10Me16:0 and 10Me18:0) were taken as specific actinobacterial biomarkers within the Gram-positive bacteria.

*Protein extraction from soil and mass spectrometric analysis*

Protein extraction was performed according to the method described by Chourey *et al.* (2010), which has been found to be suitable for semiarid soils (Bastida *et al.*, 2014). The cell lysis and disruption of soil aggregates were performed by boiling at 100 ºC for 10 min in sodium dodecyl sulfate (SDS) buffer. The proteins were separated by SDS-PAGE and, after electrophoresis, the gels were stained using colloidal Coomassie brilliant blue. The gel area containing the protein mixture of each sample was sliced into one piece. The samples were further processed by in-gel reduction and alkylation of cysteine residues, in-gel tryptic cleavage, and elution as well as desalting of tryptic peptides (Bastida *et al*., 2016). The peptide lysates were reconstituted in 0.1% formic acid prior to LC-MS measurement.

Separation of peptide lysates was performed using an 85-min, non-linear gradient from 3.2% to 80% acetonitrile, in 0.1% formic acid, on a C18 analytical column (Acclaim PepMap100, 75 μm inner diameter, 25 cm, C18, Thermo Scientific) in a UHPLC system (Ultimate 3000, Dionex/Thermo Fisher Scientific, Idstein, Germany). Mass spectrometry was performed on a Q Exactive HF MS (Thermo Fisher Scientific, Waltham, MA, USA) with a TriVersa NanoMate (Advion, Ltd., Harlow, UK) source in LC chip coupling mode. The mass spectrometer full scans were measured in the Orbitrap mass analyzer within the mass range of 350–1,550 *m/z*, at 60,000 resolution, using an automatic gain control target of $1\times10^6$ and a maximum fill time of 100 ms. An MS/MS isolation window for ions in the quadrupole was set to 1.4 *m/z*. The MS/MS scans were acquired using the higher energy dissociation mode at a normalized collision-induced energy of 28%, within a scan range of 200–2,000 *m/z* and using a resolution of 15,000. The exclusion time to reject masses from repetitive MS/MS fragmentation was set to 30 s

Proteome Discoverer (v1.4, Thermo Scientific) was used for protein identification and the MS/MS spectra acquired were searched with Sequest HT against the specific semiarid soil metagenome database (containing 48,094,830 protein-coding sequences) (Bastida et al., 2017). Enzyme specificity was selected to trypsin with up to two missed cleavages allowed, using 5 ppm peptide ion tolerance and 0.02 Da MS/MS tolerances. Oxidation (methionine) was selected as a variable modification and carbamidomethylation (cysteine) as a static modification. Only peptides with a false discovery rate (FDR) <0.01, calculated by Percolator (Käll *et al.*, 2007), and a peptide rank of 1 were considered as identified.

191 The mass spectrometry data were deposited with the ProteomeXchange Consortium via
192 the PRIDE partner repository (Vizcaino *et al.*, 2013), with the dataset identifier
193 XXXXXXX.

194 The "PROteomics results Pruning & Homology group ANotation Engine"
195 (PROPHANE) (http://www.prophane.de) was applied to assign proteins to their
196 phylogenetic and functional origin. The composition of the bacterial community was
197 calculated based on the normalized spectral abundance factor (NSAF) at the phylum and
198 order levels. In total, 1,995 protein groups with 3,889 peptides were identified in the
199 soil samples. In metaproteomics studies, the number of unique peptides and also the
200 peptide count per protein are generally low. All peptides analyzed here were measured
201 in high-resolution mode, which means that the quality and confirmation of identity are
202 of high fidelity.

203 *Data analysis*

204 One-way ANOVA was used to determine significant effects of the soil amendment with
205 struvite and sewage sludge on soil parameters. The Tukey post-hoc test was used for
206 multiple comparison of the average values of each variable across the soil treatments
207 and the sampling times, and to determine significant differences at $P<0.05$.

208

209 **Results**

210 *Macro and micronutrients in sludge and struvite*

211 The contents of N, P, Mg, and Mn were greater in struvite than in sewage sludge (Table
212 1). The P, Mn, and Mg contents of struvite were nearly 7, 3, and 11-times greater,
213 respectively, than in sludge. In contrast, the rest of the elements (including organic C)

were more abundant in sludge. The contents of heavy metals and other trace elements were all lower in struvite than in sludge.

*Soil macronutrients, enzyme activities, and PLFA content*

At the initial sampling time, the available P ($P_{av}$) content was significantly higher in the soil amended with struvite (St) than in the soil amended with both sludge and struvite (Sl+St) ($P<0.05$) (Table 2). The soils of these two treatments had a higher $P_{av}$ content than the control soil and the sludge-amended soil (Sl). The BGA of the soil receiving the combined amendment was lower than that of the control and sludge-amended soils at the initial time.

At the second sampling time (one month), the $P_{av}$ content was highest in the St soil, followed by the combined treatment (Sl+St). The control and sludge-amended soils showed the lowest $P_{av}$ contents (Table 2). At this time, the control and St samples had the highest N contents. The soil APA was lowest in the struvite treatment and highest in the control soil without amendment. There were no significant differences between treatments in the case of soil URA and BGA at the second sampling time.

There were no differences in the fungal PLFA content between treatments. However, the bacterial PLFA contents of the amended soils were higher than that of the control ($P<0.05$) (Table 3). The bacterial PLFA content was highest in the St soil, followed by the Sl soil. The content of Gram-positive PLFAs was higher in the St soil than in the Sl+St and Sl soils ($P<0.05$). The control soil showed the lowest PLFA content. In the case of Gram-negative PLFAs, the content was higher in the St and Sl soils than in the control and Sl+St soils.

*Plant productivity and plant elements*

The highest fresh and dry weights of barley were observed in the combined treatment ($P$<0.05). The control plants had a lower fresh weight than the rest of the treatments ($P$<0.05) (Table 4).

The plants in the Sl-amended and St-amended soils, and in the combined treatment, had higher P contents than the plants in the control soil (Table 5). Moreover, plants growing in St-amended soil had a significantly higher Mg content than those of the other treatments. The analysis of the elemental contents in the plant tissue revealed no significant differences between treatments in the case of N, C, and K.

*Composition of the microbial community, analyzed through metaproteomics*

Regarding the protein content, the bacterial community at the phylum level was dominated by *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Planctomycetes* (Fig. 1A). The amendments only affected significantly the abundance of proteins from *Verrucomicrobia* (the protein content was greatest in the St treatment) and *Cyanobacteria* (the protein content was greatest in the Sl treatment).

At the order level, soil amendment influenced the protein abundance of *Nitrosomonadales* (the lowest content was in the combined treatment), and particularly several Actinobacterial orders (P<0.05) (Fig. 1B). The abundance of *Micronosporales* proteins was higher in all the amended treatments in comparison to the control; the abundance of proteins from *Pseudonocardiales* and *Corynebacteruales* was greatest in the combined treatment; and, conversely, the abundance of *Rubrobacterales* proteins was lower in the combined treatment and in the Sl-amended soil than in the control.

At the genus level, proteins from *Streptomyces* were more abundant in St-amended and Sl-amended soil than in the control (Fig. 1C). The abundance of proteins from

*Methylobacterium* was higher in Sl+St than in the control or Sl treatments. *Pseudomonas* and *Gemmatirosa* proteins were more abundant in the Sl treatment.

## Discussion

The availability of P was highest in struvite-amended (St) soil, followed by the combined treatment (Sl+St). The availability of P was lower at one month, but followed the same pattern, with St and Sl+St samples having the highest values. The reduction of P availability in both treatments at one month could be the result of P precipitation as Al, Fe, and Ca phosphates (Paul, 2007; Kruse et al., 2015), immobilization in clay particles, and plant uptake. In agreement, plant P uptake from St-treated soil was double that in the control. Indeed, it has been indicated that struvite-derived P can be easily taken up by plants (González-Ponce et al., 2009; Cabeza et al., 2011).

Several studies have observed higher plant fresh weight and P, Mg, N, K, and Ca uptake in struvite-amended soils, in comparison to other sources of P, such as superphosphate (Plaza et al., 2007). Nevertheless, some authors found that the higher yield and plant P uptake obtained with struvite addition to soil, in comparison to superphosphate, were attributable to the greater Mg content and uptake in plants and its synergic effects with P uptake in plant tissues (González-Ponce et al., 2009). In our study, the plant Mg uptake was highest in the St treatment, followed by the Sl+St combination. Magnesium is part of the chlorophyll molecule, which is essential for photosynthesis and hence for plant growth (Mengel and Kirby, 2004). A synergic effect of struvite and sludge has been observed here for barley yield (both dry and fresh weight). These results can be explained by the synergy between the higher Mg content of the struvite and the elevated content of other macro and micronutrients - such as K, Ca, Na, Fe, and Cu - provided

284 with the sludge. In other words, struvite can palliate a potential deficiency of Mg in soil
285 and benefit plant yield (Choudhury and Khani, 2001; González-Ponce et al., 2009).

286 In spite of their importance in soil fertility and functionality, and crop yields, there is
287 little knowledge (if any) about the impacts of struvite in the soil microbial community.
288 Interestingly, in this work the amendments had no effect on the urease and β-
289 glucosidase activities; this indicates that struvite has no potential impact on the cycles of
290 N and C, respectively, which are, in part, related to these enzymes (Shi, 2011).
291 However, the phosphatase activity was inhibited by struvite. The synthesis and activity
292 of extracellular hydrolases in soil are regulated by feedback mechanisms (Allison &
293 Vitousek, 2005; Burns et al., 2013; Sinsabaugh & Moorhead, 1994), so the higher P
294 availability in soil amended with struvite would explain the reduced phosphatase
295 activity. Indeed, we found a negative correlation between phosphatase activity and the
296 $P_{av}$ concentration (r=-0.79; P=0.002).

297 The PLFAs were used as indicators of microbial biomass. Fungal biomass was not
298 influenced by any treatment. However, bacterial biomass was positively influenced by
299 all amendment treatments. Organic amendment can stimulate the growth and biomass of
300 the soil microbial community as a consequence of the supply of C and N (Bastida et al.,
301 2008; Torres et al., 2015). In particular, the Gram-positive to Gram-negative biomass
302 ratio was higher for the St and Sl+St treatments. This trend in the G+/G- PLFA ratio
303 matched the higher P availability in these treatments, as reinforced by the positive and
304 significant correlation between Pav and the Gram+ PLFA content (r=0.66; P=0.018).

305 Despite their value as indicators of the microbial biomass, fatty acids do not provide
306 accurate information about the composition of the soil microbial community. In the last
307 few years, metaproteomics has been suggested as an alternative approach to track the

composition and diversity of the microbial communities, with special focus on the active populations (Keiblinger et al., 2012; Bastida et al., 2014; 2017; Hultman et al., 2015). Here, we found that, at the phylum level, the addition of agronomic doses of sludge or struvite did not influence the composition of the dominant soil bacterial phyla. However, when looking in detail at the order level, it was obvious that our treatments, and particularly struvite, had a notable impact on the abundance of Actinobacterial populations. For instance, the application of struvite increased the abundance of *Streptomycetales*, in comparison to the other treatments, while the joint application of struvite and sludge increased the abundance of *Pseudonocardiales* and *Corynebacteriales*. So far, it has been generally assumed that *Actinobacteria* are important as P solubilizers, both in wastewater plants (Bond et al., 1999) and in soil environments (Mander et al., 2012; Wakelin et al., 2012). A recent article by Zheng et al. (2017) concluded that more than 90% of the inorganic-P-solubilizing bacterial community in soil corresponds to *Actinobacteria* and *Firmicutes*, including *Streptomyces*.

Interestingly, we found evidence that some Actinobacterial populations were related to greater plant yield. For instance, we found positive correlations between the amount of Actinobacterial proteins and the plant fresh (r=0.58; $P$=0.019) and dry (r=0.50; $P$=0.048) weight. Among the Actinobacterial proteins, those of Micromonosporales (r=0.73; $P$=0.001) and Pseudonocardiales (r=0.72; $P$=0.002) were strongly correlated to the plant fresh weight.

## Conclusions

Soil amendment with struvite - alone or in combination with sewage sludge - increased the available P concentration in soil, the uptake of both P and Mg by barley, and the barley yield. These findings corroborate the potential usefulness of struvite as a mineral P-fertilizer, together with an organic amendment (i.e. sewage sludge), in agriculture.

Moreover, the study reveals the microbial mechanism behind the effects of struvite application to soil. A positive effect of struvite, when added to soil alone or jointly with sludge, on the Gram-positive to Gram-negative biomass ratio was demonstrated. Among the Gram-positive bacteria, metaproteomics revealed that struvite had a strong impact in some actinobacterial populations that may play a role in P solubilization and plant growth.

Further studies with these alternative P-resources - as mineral and organic fertilizers, respectively, at the field scale and with different doses and crops - will be necessary to determine the practical applications of struvite in agriculture.

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