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Soil microorganisms increase Olsen phosphorus from poorly soluble organic phosphate: A soil incubation study

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Abstract

The potential shortage of mineral phosphorus (P) sources and the shift towards a circular economy motivates the introduction of new forms of P fertilizers in agriculture. However, the solubility of P in new fertilizers as well as their availability to plants may be low. In this experiment, we incubated an agricultural soil poor in P (28 mg P_2O_5 kg^{-1}) for 63 days in the presence of a range of organic and inorganic poorly soluble P forms commonly found in new fertilizers: hydroxyapatite (P-Ca), iron phosphate (P-Fe), phytic acid (P-Org) and a combination of P-Ca and P-Org (P-Mix). Cellulose and potassium nitrate (KNO_3) were added to stimulate microbial activity at the beginning of the incubation. We included a positive control with triple superphosphate (TSP) and negative controls with no P application (with and without cellulose and KNO_3). We assessed the fate of the different poorly soluble P forms in $NaHCO_3$ extracts (Olsen P) over time as a proxy for plant-available P. Soil microbial biomass, fungal to bacterial ratio, soil respiration, enzymatic activities (β-glucosidase, arylamidase and acid and alkaline phosphatase), N mineralization and soil pH were also monitored. At the beginning of the incubation, TSP showed the highest Olsen P across all treatments and P-Fe showed higher levels of Olsen P than the other poorly soluble P forms ($p < .05$). During the incubation, the levels of Olsen P decreased over time for TSP (positive control). Contrastingly, Olsen P increased significantly over time for all the poorly soluble P forms and the negative controls, indicating an increase in plant-available P. Particularly, levels of Olsen P for the P-Org treatment roughly doubled (shifting from 16.5 mg kg^{-1} to 32.9 mg kg^{-1}) over the whole incubation period. The rate of increase in Olsen P was positively correlated with microbial biomass C:P ratio ($p < .01$) for all poorly soluble treatments. The higher levels of Olsen P for the P-Org treatment were also explained by a positive correlation with fungal biomass. Our results show that poorly soluble forms of P may be made available to plants under the influence of the microbial community, with a stronger effect on organic P forms.

KEYWORDS
incubation, iron phosphate, Olsen P, organic phosphorus, P forms, soil microorganisms

1 | INTRODUCTION

Phosphorus (P) fertilization is among the most common practices in agriculture worldwide. The utilization of mineral P fertilizers is accompanied by a series of issues which vary across the globe. From an economic perspective, the production of mineral P fertilizers is restricted to a handful of countries, posing a substantial dependency on imports (Schoumans et al., 2015). Moreover, P resources are limited and are currently listed as a critical raw material in the European Union (Bertrand et al., 2016; Van Vuuren et al., 2010). From an environmental perspective, P fertilization can incidentally cause P enrichment of water bodies leading to water eutrophication (Mekonnen & Hoekstra, 2018; Ortiz-Reyes & Anex, 2018), known to cause severe damage in water ecosystems (Dorgham, 2014). In addition, large areas of the world's soils contain an excess of previously applied P fertilizer that cannot be used by crops (MacDonald et al., 2011; Pavinato et al., 2020; Zhu et al., 2018). All these economic and environmental challenges motivate a more efficient utilization of P resources by using new forms of P fertilizers and more effective P solubilization and mineralization from P bound to soil particles (Faucon et al., 2015).

One solution to tackle P scarcity is the utilization of recycled P from different waste sources (Delgado Sancho et al., 2019; Hu et al., 2021). However, most of the recycled P fertilizers share a lower P solubility compared to mineral fertilizers (e.g. triple superphosphate) (Römer & Steingrobe, 2018). In recycled fertilizers, a large proportion of the P can be strongly chelated by iron (Fe), aluminium (Al) or calcium (Ca) cations or organic molecules, thus reducing P availability to plants. Large efforts are made during the production phase to reduce the formation of insoluble P molecules in chars (Han et al., 2022; Khalaf et al., 2022; Mercl et al., 2020), ashes (Ahmed et al., 2021; Kumpiene et al., 2016) or during the formation of P-salt precipitates such as struvite (Numviyimana et al., 2020) or vivianite (Prot et al., 2021). Unfortunately, it remains poorly understood how the solubilities of poorly soluble Fe-P, Al-P, Ca-P or organic-P compare to one another.

Similarly, when P fertilizers are applied to soil, most of their soluble forms can be adsorbed into the soil mineral phase or immobilized into soil organic matter. Organic forms of P can represent up to 65% of the total P in soils (Blume et al., 2016). In particular, monoester forms such as inositol or phytic acid account for the majority of the organic P pool (up to 70%) and are considered relatively unavailable (Schneider et al., 2016; Turrión et al., 2001). Inorganic P forms are also numerous and their abundance depends strongly on soil physico-chemical properties. For example, P is precipitated by Ca cations at alkaline pH and adsorbed to Fe or Al (hydro-)oxides in more acidic conditions (Penn & Camberato, 2019).

Soil microorganisms play a crucial role in the release of orthophosphate from the organic and inorganic P pools (Amy et al., 2022; Richardson & Simpson, 2011). In doing so, soil microbes can improve plant growth by acting as an intermediary between unavailable P forms and plant uptake (Richardson & Simpson, 2011). Organic P is mineralized by the excretion of extracellular enzymes—phosphatases—that are able to hydrolyse organic molecules and release orthophosphate in the soil solution (Margalef et al., 2017; Richardson & Simpson, 2011). Solubilization of inorganic P can also be facilitated by soil microorganisms. By the excretion of low molecular weight organic acids (LMWOAs) such as citrate, oxalate, gluconate, malate or lactate (Mihoub et al., 2017; Richardson & Simpson, 2011) P can be released from Ca-P bonds (Brucker et al., 2020; Mihoub et al., 2017; Richardson & Simpson, 2011) and from Al-P and Fe-P molecules (Wang et al., 2021; Zhuo et al., 2009). The main pathways of LMWOAs to solubilize P are the direct dissolution of minerals, shifts in soil pH, alteration in the surface characteristics of soil minerals and the formation of complexes with Ca, Fe and Al (Wang et al., 2016).

The role of soil microbes in the release of poorly soluble P sources as plant-available P has been widely studied in the past. However, most of the experiments were conducted on culturable microorganisms or soil extracts. For instance, in culture media, P was shown to be solubilized from apatite (Amy et al., 2022; Kim et al., 1997; Monroy Miguel et al., 2020), from phytate (organic P) (Amy et al., 2022; Shulse et al., 2019) and from Fe phosphates (He et al., 2007; Jha et al., 2013). Similarly, tests were conducted on soil extracts in which the solubilization from apatite (Brucker et al., 2020; Efthymiou et al., 2018; Pastore, Kernchen, & Spohn, 2020) and Fe phosphates (Efthymiou et al., 2018; Pastore, Kaiser, et al., 2020) was assessed. Unfortunately, the percentage of soil microorganisms that strive in such laboratory conditions is far from the actual numbers that can be found in nature (Roesch et al., 2007). Studies that make use of direct soil incubations or pot experiments are few and use mostly radioactive ^{31}P isotopes (Chen et al., 2021; Pistocchi et al., 2018).

The availability to plants of P from poorly soluble sources is highly dependent on the availability of carbon (C) and to a lower extent nitrogen (N) (Brucker et al., 2020; Demoling et al., 2007; Pastore, Kaiser, et al., 2020; Pastore, Kernchen, et al., 2020; Pistocchi et al., 2018; Spohn & Kuzyakov, 2013). Indeed, in the soil, solubilization of P might be affected to meet stoichiometric homeostasis of the microbial biomass (Heuck et al., 2015; Spohn, 2016). Similarly, stoichiometry can also reveal the P limitation of soil microorganisms when looking at extracellular enzymes (Moorhead et al., 2016). It is expected that soil microorganisms will invest relatively more in producing enzymes that mediate the mineralization of the nutrients by which they are most limited (Allison et al., 2010).

Here, we aimed to (i) assess the release of P as sodium bicarbonate ($NaHCO_3$)-extractable P (Olsen P) over time from various forms of poorly soluble P often found in recycled P fertilizers and (ii) study the role of indigenous soil microorganisms in the change of Olsen P over time. To do this, we incubated a cropland soil with different organic and inorganic sources of poorly soluble P (hydroxyapatite, iron phosphate (III) and phytate) and measured at different dates Olsen P (a proxy for plant-available P) and microbial biomass and activity. To our knowledge, this is the first time these forms of P are compared to each other and studied directly in soil.

2 | MATERIALS AND METHODS

2.1 | Soil sampling

We collected soil from the top 20 cm of a cropland in Haudricourt, Normandy, France (49°42′16.6″ N, 1°41′10.9″ E) on 26/04/2021. This soil is classified as a Cambisol (WRB) with a loamy texture (8.5% clay, 71% silt and 20.5% sand). The soil was characterized before the start of the experiment, showing a pH-H_2O of 7.1, plant-available P (Olsen P) of 28 mg P_2O_5 kg^{-1}, potassium oxide (K_2O) 177 mg kg^{-1}, magnesium oxide (MgO) 95 mg kg^{-1}, calcium oxide (CaO) 2198 mg kg^{-1}, K_2O:MgO 1.86, cation exchange capacity (CEC) 9.15 meq 100 g^{-1} and a soil organic matter content of 3%. We selected this soil because of its low P content and its neutral pH (COMIFER, 2019). Soil was homogenized, sieved at 5 mm and kept at 4°C before the start of the incubation experiments for no longer than 7 days.

2.2 | Incubation conditions and P treatments

Soil moisture was adjusted to 60% water holding capacity (15% gravimetric moisture content) and placed into Microbox©, Sac 0_2 boxes (540 mL), which contain a specific filter that allows air exchange while preventing dehydration. Water content was monitored on a weekly basis and water was added when necessary. Water holding capacity was determined by placing dry soil in a steel cylinder. The cylinder was then placed in a container where water was added to rewet the soil by capillarity and immersion. Then, the cylinder with wet soil was placed over a dry sand bed where soil was allowed to drain for 2 h. Afterwards, the gravimetric moisture content was measured, this value stands for 100% water-holding capacity.

Different forms of P were added to the soil at a rate of 100 mg of P kg^{-1} of dry soil: iron phosphate (III) (P-Fe) (CAS registry number: 13463-10-0), hydroxyapatite (P-Ca) (CAS: 7758-87-4), phytic acid (P-Org) (CAS: 14306-25-3) and a 50/50 mixture of P-Ca and P-Org (P-Mix). The total amounts added were: 75.83 mg for P-Fe, 62.9 mg for P-Ca and 44.67 mg for P-Org. The high rate of P was selected because of the potentially low solubility of the P forms. We also included a positive control with a regular P fertilizer, triple super-phosphate (TSP) and a negative control with no P application. The different forms of P were mixed thoroughly in powdered form (<0.5 mm) with 170 g of fresh soil. The boxes were placed in an air-ventilated incubator at 25°C for 63 days. Subsets of boxes were destructively sampled six times in total during the 63 days: immediately after the application of P and 7, 14, 21, 35 and 63 days after the application of the different P forms. We included four replicates per treatment and per sampling date in a completely randomized design, for a total of 168 incubation boxes.

In a previous incubation experiment, the same soil was sampled 4 months earlier (January) and amended with the same P forms. The selection of soil with a low P content (<30 mg P_2O_5 kg^{-1}) was aiming at inducing conditions of P limitation, in which P fertilization would be most useful in an agricultural setting (COMIFER, 2019). In those P-limiting conditions, we hypothesized that the addition of poorly soluble P sources would stimulate P acquisition strategies from soil microbes, thus allowing us to compare the effect of those strategies on the different P forms. Yet, we observed no differences between the P treatments in terms of changes in Olsen P over time or terms of microbial response (the results are briefly presented in Figures S1–S3). Therefore, in the present experiment, to alleviate C and N limitations and increase microbial demand for P, soil was amended with cellulose (α-cellulose, CAS: 9004-34-6) and potassium nitrate (KNO_3) at a rate of 2 g C kg^{-1} and 35 mg N kg^{-1}, respectively. Another control treatment (Amend) was made in which KNO_3 and cellulose were added without any P application. We used these rates to ensure no C or N limitation while limiting effects on the soil microbial community composition.

2.3 | Soil biological analyses

2.3.1 | Soil microbial respiration

The activity of soil microorganisms was derived from measurements of soil carbon dioxide (CO_2) emissions (R_s) (AFNOR XP U44-163). In parallel to the microboxes, separate closed containers were used for these measurements. Fresh soil samples (equivalent to 25 g of dry soil) were mixed with the P forms and amendment to reproduce the different treatments described above and placed together with a flask with 10 mL of 0.5 M sodium hydroxide (NaOH) solution, and another flask filled with water to maintain the moisture content. The soil, water and NaOH solution flasks were placed in a 1 L closed container. The bottles followed the same experimental conditions as the main incubation and were sampled on the same dates. We included four repetitions per treatment, and the NaOH flasks were sampled destructively at each measurement date and replaced with freshly made NaOH solution. The containers were kept in the same incubator as the soil samples, in total randomization. A container with no soil was also included as a blank to account for the background CO_2 content. Containers were opened for analysis twice a week in the first 2 weeks and then weekly for the rest of the experiment, ensuring air ventilation. Carbon dioxide (CO_2) efflux was calculated by measuring the electrical conductivity (EC) in the sodium hydroxide (NaOH) solution. EC was measured at days 3, 7, 10, 14, 21, 28, 35, 42, 49, 56 and 63 after the start of the incubation. Results are expressed as μg CO_2–C g^{-1} dry soil.

2.3.2 | Microbial biomass

Microbial biomass was quantified in two ways: by the chloroform fumigation-extraction method and by qPCR (total microbial DNA and genes of 16S and 18S rRNA subunits separately for bacterial and fungal biomass, respectively).

Carbon, N and P in the microbial biomass were calculated following the chloroform-fumigation protocol of Jenkinson and Powlson (1976) and the standardized protocol AFNOR ISO 14240. Briefly, 30 g of fresh soil were placed in a beaker inside a desiccator under vacuum conditions. Inside the desiccator, a beaker with chloroform is also placed. Samples are left in the dark for 24 h and then C and N are extracted with 0.05 M potassium sulphate (K_2SO_4). The measurement was then performed in a TOC analyser (Shimadzu). Microbial C and N biomass (Microbial C and Microbial N) is then calculated. A blank of each sample, unfumigated, was also included. The values of Microbial C and Microbial N are then calculated by subtracting the blank values from the fumigated ones. Results are expressed in mg kg^{-1} of dry soil. No correction values were used. For the determination of microbial P biomass (Microbial P), we also fumigated soil samples with chloroform (Brookes et al., 1982). This protocol is very similar to the one previously described. Yet, in this case, only 5 g of fresh soil was fumigated. Phosphorus was subsequently extracted with 0.5 M $NaHCO_3$ (Olsen P). Results are also expressed in mg kg^{-1} of dry soil. No correction factor was applied. Different ratios of nutrients in microbial biomass were calculated, including C:P, C:N, N:P and C:N:P.

Total nucleic acids were extracted from 0.5 g of fresh soil using a FastDNA SPIN Kit (MP-Biomedicals). The soil used was stored at −80°C before analysis. Total DNA was quantified by fluorimetry using the Fluorescent DNA quantitation Kit Hoechst 33258 (Biorad). The results were expressed in mg kg^{-1} dry soil.

Fungal and bacterial biomass were quantified by performing 18S and 16S rDNA amplification respectively. Real-time qPCR was performed in a total volume of 25 μL. Briefly, 18S primers (FU18S1 5′-GGAAACTCACCAGG TCCAGA-3′ and Nu-SSU-1536 5′-ATTGCAATGCYCTA TCCCCA-3′) or 16S primers (63f 5′-CAGGCCTAACACAT GCAAGTC-3′ and BU16S4 5′-CTGCTGCCTCCCGTAGG-3′) were mixed with 5 ng of soil microbial DNA, 0.5 μM of 25 μL of LightCycler1 480 DNA SYBR Green I Master mix (Roche) and 0.25 mg mL^{-1} BSA (GeneON Bioscience). Standard curves were obtained using serial dilutions of linearized plasmids containing the cloned 18S rRNA gene of *Fusarium graminearum* or 16S rRNA genes from *Pseudomonas aeruginosa*. The amplification consisted of 40 cycles of PCR, 20 s at 95°C, 30 s at 62°C and 30 s at 72°C. It was performed using LightCycler 480 real-time PCR system (Roche). The results are expressed as 18S or 16S rDNA gene copy number per gram of dry soil.

2.3.3 | Potential enzymatic activities

Four different enzymes from the C, N and P cycles were analysed in this experiment. β-Glucosidase (E.C. 3.2.1.21) (Bglu), arylamidase (E.C. 3.4.11.2) (ARYLN) and acid and alkaline phosphatase (E.C. 3.1.4.1) (PAC) and (PAK), respectively.

Potential soil enzymatic activities were measured colorimetrically following a microplate technique (ISO 20130:2018) (Cheviron et al., 2021). Briefly, for Bglu and ARYLN activities, 4 g of fresh soil was homogenized with 25 mL of ultrapure water for 10 min at 250 rpm. For PAC and PAK a solution of Trizma buffer (50 mM) adjusted to pH 5.5 and 11, respectively, was used. In total, 125 μL were pipetted into 96-well microplates and mixed with their respective substrate solutions. 4-nitrophenyl β-D-glucopyranoside (CAS N°: 2492-87-7) for Bglu, L-leucine β-naphthylamide hydrochloride (CAS N°: 893-36-7) for ARYLN and 4-nitro-phenylphosphate disodium salt hexahydrate (CAS N°: 333338-18-4) for PAC and PAK. The final concentrations of the substrate solutions were 0.05, 0.008 and 0.05 mol L^{-1}, respectively. Microplates were incubated at 37°C for 1 h for Bglu, 2 h for ARYLN and 30 min for PAC and PAK. The reaction was then stopped by the addition of 25 μL 0.5 M calcium chloride ($CaCl_2$) and 100 mM 100 μL Trizma at pH 12 for Bglu, PAC and PAK. Then plates are centrifuged for 5 min at 1500 g. For ARYLN, the reaction was stopped by the addition of ethanol 96% and the colouration was revealed after the addition of 100 μL of acidified ethanol and 100 μL of DMCA. ARYLN's plates were incubated in the dark for 20 min at room temperature before measurement. The measurements were performed in a Varioskan Flash-Thermo microplate reader. Absorbance was measured at 405 nm for Bglu, PAC and PAK and 540 nm for ARYLN. Results of potential enzymatic activities were expressed in nmol PNP (paranitrophenol) $min^{-1} g^{-1}$ of dry soil for Bglu, PAC and PAK activities and in β-naphthylamine $min^{-1} g^{-1}$ of dry soil for ARYLN activities.

Stoichiometric ratios of enzymes were also calculated based on the vectorial approach proposed by Moorhead et al. (2016). Vector length and angle were calculated as in Equations (1) and (2):

$$\text{Vector length} = \sqrt{(\text{Bglu}/\text{PAC})^2 + (\text{Bglu}/\text{ARYLN})^2} \quad (1)$$

$$\text{Vector angle} = \text{DEGREES}\left(\text{ATAN2}(\text{Bglu}/\text{PAC}), (\text{Bglu}/\text{ARYLN})\right) \quad (2)$$

In general terms, the steeper angles are associated with higher P limitation of soil microorganisms and the longer vectors are associated with a more important C limitation. Yet, the interpretations from these approaches should be carefully considered as recent papers have raised debate on their utilization (Mori et al., 2023; Rosinger et al., 2019).

2.4 | Soil chemical analyses

We used the Olsen method (ISO 11263) (extraction in 0.5 M sodium bicarbonate ($NaHCO_3$)) as a proxy for plant-available P. In brief, 5 g of fresh soil was mixed with 100 mL of 0.5 M $NaHCO_3$ at pH 8.5 for 30 min. One gram of P-free and pH 7 active charcoal was added to the mix. This was done to reduce the interference of soil organic matter in the colour development. After the extraction, the solution was filtered with a P-free 5 μm paper filter. Two millilitres of extract was mixed with 8 mL of sulfomolibdic reagent and was incubated for 60 min. After, colouration was revealed after heating in a water bath for 10 min at 90°C. P was measured in the 825 nm wavelength using a spectrophotometer (Varian Cary 50 Scan UV–Visible Spectrophotometer).

Changes in Olsen P were investigated in absolute and relative values. The relative values of Olsen P were calculated by dividing each value of Olsen P by the mean of the initial value (beginning of incubation) of the corresponding treatment (Equation 3), where P_a is the content of P at a given time and treatment, P_{a1} is the level of P at the beginning of the experiment for that particular treatment. This was done to assess the changes in Olsen P over time with respect to their initial values.

$$\text{Relative P} = P_a / P_{a1} \quad (3)$$

Soil pH was measured in 1:5 v:w in water extracts using a glass electrode (Mettler Toledo). Soluble C and N were determined in a 0.05 M potassium sulphate (K_2SO_4) extract performed on fresh soil (Makarov et al., 2013). Carbon and N were extracted from 30 g of soil in 100 mL 0.05 M K_2SO_4 solution for 2 h. The extract was centrifugated at 5000 rpm for 5 min to remove solid particles. Soil extracts were frozen at −20°C before measurement. The measurement was performed in a TOC device (TOC-V CSH and TNM-1; Shimadzu). Nitrate (NO_3) and ammonium (NH_4) were also extracted from 25 g of soil in 75 mL 1 M potassium chloride (KCl) solution for 1 h. The extract was centrifugated at 5000 rpm for 5 min to remove solid particles. Soil extracts were also preserved at −20°C before performing the measurement. NO_3 and NH_4 were determined colorimetrically in a Gallery device (Thermo Fisher Scientific).

2.5 | Statistical analyses

All the statistical analyses were done using RStudio software (v4.0.2).

For each measured variable, differences between the P-form treatments were estimated by performing an analysis of variance (ANOVA). The assumptions of homogeneity, normality and heteroscedasticity were inspected by analysing the residuals of ANOVA models. Homogeneity was breached due to higher variance in the TSP treatment. Therefore, when ANOVA results showed significant differences, pairwise comparisons between treatments were performed using Games Howell's post hoc test, which accounts for heterogeneous variances between treatments (Hilton & Armstrong, 2006).

Differences over time in absolute and relative changes in Olsen P between the different P-form treatments were analysed by performing an analysis of covariance (ANCOVA). ANCOVA was performed using the function 'lm' in RStudio (R Core Team, 2022). The assumptions of homogeneity, normality and heteroscedasticity were inspected by analysing the residuals. Similarly, as for the ANOVA analyses, higher variability in the TSP treatment led to heterogeneity of the residuals. It was therefore decided to study the changes in absolute and relative Olsen P over time for TSP separately from the other treatments. Model assumptions were checked again on those separate models.

To assess changes in microbial activity over time cumulative soil respiration (R_s) was analysed using an asymptotic exponential function of time (Equation 4).

$$y = a \times (1 - \exp(-b \times \mathrm{Time})) \quad (4)$$

where parameter a describes the asymptote of the curve (the maximum value of R_s) and parameter b describes the shape of the curve (how quickly R_s reaches its asymptotic value).

To assess the differences in microbial activity over time between treatments, models fitting different parameters to the different treatments were compared (using AIC) with models in which parameters were constrained to be the same for all treatments using the R 'gnls' function (Generalized Least Squares) (Pinheiro et al., 2022).

To test for the effects of soil chemical and biological properties on the changes in Olsen P, a multiple regression analysis was conducted following the procedure used by Moinet et al. (2016). A candidate set of models was established to identify which of the soil properties best explained the changes in Olsen P (using relative values of Olsen P). The set of models was created using different combinations of potential explanatory variables with and without accounting for the interaction with the different P-form treatments. In total, over 160 models were evaluated. The models were ranked using the AICc to determine the Kullback–Leibler (KL) best model (Burnham & Anderson, 2002). The AICc identifies the model(s) most strongly supported by the data and is based on bias-corrected, maximized log-likelihood (LogLik) of the fitted model with a penalty for the number of parameters used. The model with the smallest AICc ($AICc_{min}$) is the most strongly supported. The ΔAICc value is calculated for each model i as $\Delta AICc = AICc_i - AICc_{min}$. Following convention, models with $\Delta AICc < 2$ are substantially supported by the data; whereas models with $\Delta AICc > 2$ indicate considerably less or no support (Anderson, 2007). A measure of the strength of support for either model is described by the model probability (Akaike weights, A_{wi}). This is the probability that model i is the KL best model, given the data and candidate set of models (Anderson, 2007). The sum of A_{wi} of the models in a candidate set equates to 1.

All data files are present in the Zenodo online repository (Velasco-Sánchez et al., 2022).

3 | RESULTS

3.1 | Soil biological activities

The addition of cellulose and KNO_3 increased largely soil respiration, yet the different P forms had a limited effect on soil respiration (Figure 1). TSP and P-Fe treatments showed slightly different soil respiration curves. Following Equation (3), P-Fe showed different ($p < .05$) a and b parameters, and TSP showed a significantly different b parameter compared to the rest of the treatments showing slightly increased activities at the beginning of the incubation. However, these differences were very small and no difference was observed in total C respired between the P treatments ($p > .05$) (Figure 1).

Microbial biomass C and N increased over time over the incubation but no differences across treatments were found (Table 1). Microbial P showed no difference across treatments and sampling dates. The different ratios of microbial biomass nutrient concentration showed slight differences between treatments, only P-Ca showed statistically different intercepts for microbial C:N and microbial C:N:P ratios. Over time, only C:P showed a positive slope, and the other ratios studied showed no change over time.

Total DNA in the soil showed no difference across treatments or over time (Table 1). Similarly, 16S gene copies

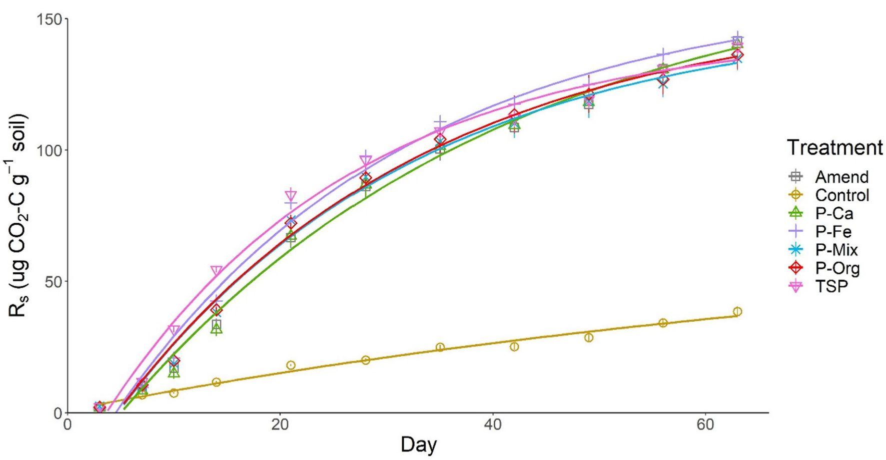

FIGURE 1 Cumulative soil respiration (R_s). P-Fe showed significantly higher growth and asymptote and TSP showed higher growth rate than the rest of the amended treatments. No effect was observed on cumulative respiration across the amended treatments.

TABLE 1 Analysis of covariance (ANCOVA) on the different soil biological variables studied.

Variable		Control	Amend	TSP	P-Fe	P-Ca	P-Org	P-Mix
Microbial biomass C ($mg\,C\,kg^{-1}$)	I	54.25	38.75	52.97	64.21	56.21	53.11	46.88
	S	1	2.04	1.68	1.54	1.86	2.16	2.64
Microbial biomass N ($mg\,N\,kg^{-1}$)	I	23.29	21.11	24.24	26.72	28	28.62	26.07
	S	0.46	0.67	0.83	0.55	0.61	0.62	0.68
Microbial biomass P ($mg\,P\,kg^{-1}$)	I	7.75	8.67	9.02	8.15	9.11	9.16	10.21
	S	ns	ns	ns	ns	ns	ns	ns
Microbial C:N	I	3.67 **(b)**	3.24 **(b)**	2.43 **(b)**	3.01 **(b)**	7.99 **(a)**	2.89 **(b)**	3.15 **(b)**
	S	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**	−0.13 **(b)**	ns **(a)**	ns **(a)**
Microbial C:P	I	8.05	5.1	4.48	9.49	8.68	9.74	6.14
	S	0.09	0.22	0.13	0.07	0.1	0.14	0.22
Microbial N:P	I	2.86	3.04	3.82	3.74	3	3.9	2.79
	S	ns	ns	ns	ns	ns	ns	ns
Microbial C:N:P	I	0.57	0.58	0.14	0.47	1.34	0.55	0.48
	S	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**	−0.03 **(b)**	ns **(a)**	ns **(a)**
Total DNA ($mg\,DNA\,kg^{-1}$)	I	120.02	122.94	126.95	122.94	126.93	126.73	128.03
	S	ns	ns	ns	ns	ns	ns	ns
Fungal biomass (18S gene copies)	I	1.81×10^{7}	8.95×10^{7}	8.66×10^{7}	7.7×10^{7}	13.84×10^{7}	9.5×10^{7}	9.6×10^{7}
	S	0.13×10^{6}	2.76×10^{6}	2.23×10^{6}	3×10^{6}	2.4×10^{6}	4.48×10^{6}	4.55×10^{6}
Bacterial biomass (16S gene copies)	I	3.2×10^{9}	3.35×10^{9}	3.12×10^{9}	3.18×10^{9}	3.41×10^{9}	3.45×10^{9}	3.81×10^{9}
	S	ns	ns	ns	ns	ns	ns	ns
Bacterial:Fungal biomass (16S:18S)	I	175.3 **(a)**	50.68 **(b)**	44.44 **(b)**	69.02 **(b)**	47.84 **(b)**	62.68 **(b)**	58.85 **(b)**
	S	−0.58	−0.78	−0.64	−1.24	−0.72	−1.05	−1.03
β-Glucosidase (PNP $min^{-1}g^{-1}$)	I	23.37 **(b)**	26.33 **(a)**	26.49 **(a)**	26.32 **(a)**	26.38 **(a)**	26.4 **(a)**	24.11 **(a)**
	S	ns	ns	ns	ns	ns	ns	ns
Acid phosphatase (PNP $min^{-1}g^{-1}$)	I	48.99 **(a)**	50.77 **(a)**	46.71 **(b)**	51.2 **(a)**	49.84 **(a)**	50.66 **(a)**	50.14 **(a)**
	S	−0.11	−0.1	−0.08	−0.1	−0.09	−0.12	−0.05
Alkaline phosphatase (PNP $min^{-1}g^{-1}$)	I	45.51	44.71	42.58	45.25	44.27	45.76	44.8
	S	ns	ns	ns	ns	ns	ns	ns
Arylamidase (β-naph $min^{-1}g^{-1}$)	I	2.39	2.59	2.66	2.57	2.52	2.64	2.47
	S	ns	ns	ns	ns	ns	ns	ns
Enzymatic vector length	I	9.38	10.26	10.12	9.85	10.6	9.73	9.87
	S	ns	ns	ns	ns	ns	ns	ns
Enzymatic vector angle	I	86.61	87.04	86.7	87.14	87.09	87	87.17
	S	0.02 **(a)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**

Note: Values represent the estimates for intercepts (I) and slopes (S) of all treatments. Letters in bold indicate significant differences between treatments ($p<.05$). When I or S estimates are not significantly different from 0, ns (non-significant) is indicated.

(bacterial biomass) did not reveal differences between treatments and time. Fungal biomass (18S gene copies), showed no difference across treatments but it significantly increased over time. The intercept of bacterial to fungal biomass ratio (16S:18S) was significantly higher for the unamended control (175.27 ± 10.2, $p<.01$) compared to the amended treatments.

Enzymatic activities were partially affected by the addition of cellulose and nitrogen but not by the application of different poorly soluble P forms (Table 1). The addition of cellulose and KNO_3 slightly increased the intercept of Bglu activities compared to the unamended control (23.37 ± 0.89 PNP $min^{-1}g^{-1}$). The intercept of PAC activity was decreased by the addition of TSP (46.71 ± 1.54 PNP $min^{-1}g^{-1}$) compared to the rest of treatments. No treatment effect was found in PAK and ARYLN activities. The activities of Bglu, PAK and ARYLN did not increase nor decrease over time. PAC activities slightly decreased over time for all treatments. Regarding enzymatic vectors (length and angle) no treatment effect was observed. Both length and angle showed no significant slope for the amended treatments.

3.2 | Soil chemical changes

Application of the different forms of poorly soluble P had a small impact on the soil chemical variables studied (Table 2). Carbon in K_2SO_4 extracts increased over time for all treatments but showed no statistical difference between treatments. NO_3 and N in K_2SO_4 extracts increased over time for the unamended control (2.58 and 0.24, respectively) but showed no significant slope for the rest of treatments. Intercepts for NO_3 and N in K_2SO_4 extracts were also significantly lower for the unamended control (40.17 and 11.46 mg N kg $^{-1}$, respectively) (Table 2). C:N ratio in K_2SO_4 extracts presented a different intercept and slope for the unamended control. No NH_4 was detected over the course of the incubation. Regarding soil pH, we found no significant change over time. TSP intercept had a significantly different intercept compared to the poorly soluble P forms and negative controls (7.35 ± 0.05). The average pH of the soil for all treatments was 7.5 ± 0.02.

3.3 | Differences in Olsen P

Initial Olsen P at the first measurement date differed significantly between treatments (Figure 2 and Table 2). TSP was the form of P with the highest Olsen P. Across the poorly soluble P forms, P-Fe showed a significantly higher Olsen P. P-Ca, P-Org and P-Mix showed the same levels of Olsen P as the control treatments (Control and Amend, which were not significantly different from each other).

Changes in Olsen P over time were markedly different between treatments (Table 2 and Figure 3). Olsen P in the TSP treatment decreased over time exponentially showing an asymptote of 49.56 mg kg^{-1} (Figure 3). However, P levels significantly increased linearly over time in the P-Org treatment (P-Org slope 0.19 ± 0.056, $p = .027$) (Figure 3). The rest of the treatments, including the controls with no P addition showed no significant change in Olsen P over time ($p > .05$). The intercepts of TSP ($p < .005$) and P-Fe ($p < .005$) were greater than that of the control treatment, indicating higher initial Olsen P. The intercept of P-Org appeared marginally lower than that of the control ($p = .043$). No differences were found between Control and Amend.

The relative change in Olsen P, calculated by normalizing the values of Olsen P for each treatment, increased for all poorly soluble P treatments and the controls with no P addition (slope 0.006 ± 0.001, $p = .004$) (Figure 3 and Table 2). Moreover, relative Olsen P increased more for the treatments that received organic P, P-Org and P-Mix, than for the other treatments (P-Org slope 0.017 ± 0.003, $p < .001$ and P-Mix slope 0.013 ± 0.003, $p = .006$). Relative change in Olsen P was negative for TSP (TSP slope − 0.004 ± 0.003, $p < .001$).

3.4 | Multiple regression analysis of relative change in Olsen P

Different linear models in multiple combinations were ranked to determine the best explanatory model for the

TABLE 2 Analysis of covariance (ANCOVA) on the different soil chemical variables studied.

Variable		Control	Amend	TSP	P-Fe	P-Ca	P-Org	P-Mix
pH	I	7.47 **(a)**	7.5 **(a)**	7.35 **(b)**	7.53 **(a)**	7.44 **(a)**	7.53 **(a)**	7.53 **(a)**
	S	ns	ns	ns	ns	ns	ns	ns
Olsen P (mg P kg^{-1})	I	9.58 **(c)**	8.47 **(c)**	63.93 **(a)**	14.66 **(b)**	9.31 **(c)**	12.14 **(b)**	8.73 **(c)**
	S	ns **(b)**	ns **(b)**	−0.32 **(c)**	ns **(b)**	ns **(b)**	0.19 **(a)**	ns **(b)**
Relative Olsen P	I	0.88 **(b)**	0.75 **(b)**	0.82 **(b)**	0.86 **(b)**	0.76 **(b)**	1.08 **(a)**	0.82 **(b)**
	S	0.008 **(b)**	0.006 **(b)**	−0.004 **(c)**	0.003 **(b)**	0.006 **(b)**	0.014 **(a)**	0.013 **(a)**
K2SO4-extractable C (mg C kg-1)	I	19.07	21.34	19.43	18.2	18.88	20.83	22.75
	S	0.24	0.28	0.4	0.39	0.34	0.33	0.28
K_2SO_4-extractable N (mg N kg^{-1})	I	11.46 **(b)**	42.12 **(a)**	38.01 **(a)**	31.93 **(a)**	40.55 **(a)**	40.3 **(a)**	37.39 **(a)**
	S	0.87 **(a)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**
NO_3 (mg NO_3 kg^{-1})	I	40.17 **(b)**	145.51 **(a)**	142.03 **(a)**	134.26 **(a)**	149.2 **(a)**	156.08 **(a)**	152.2 **(a)**
	S	2.58 **(a)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**	ns **(b)**
Soil C:N (K_2SO_4-extractable)	I	2.49 **(a)**	0.49 **(b)**	0.89 **(b)**	0.95 **(b)**	0.5 **(b)**	0.66 **(b)**	0.74 **(b)**
	S	−0.04 **(b)**	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**	ns **(a)**

Note: Values represent the estimates for intercepts (I) and slopes (S) of all treatments. Letters in bold indicate significant differences between treatments ($p < .05$). When I or S estimates are not significantly different from 0, ns (non-significant) is indicated.

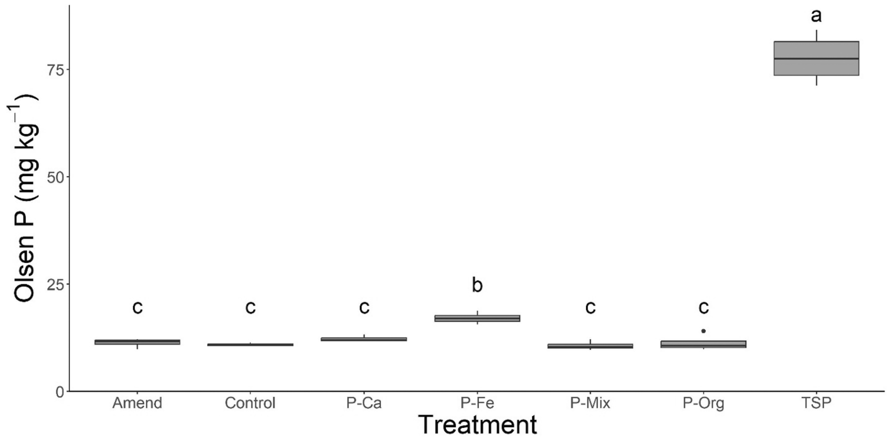

FIGURE 2 Boxplot of Olsen P at the first measurement date. Boxes represent the interquartile range of the data, line inside the box indicates the median value, whiskers show maximum and minimum values and dots indicate potential outliers. Control = no additions; Amend = addition of cellulose and KNO_3. P-Ca, Hydroxyapatite; P-Fe, Iron phosphate (III); P-Mix, 50/50 mixture of P-Ca and P-Org; P-Org, Phytic acid; TSP, Triple Super Phosphate. Letters indicate statistically different groups based on Games-Howell post hoc test.

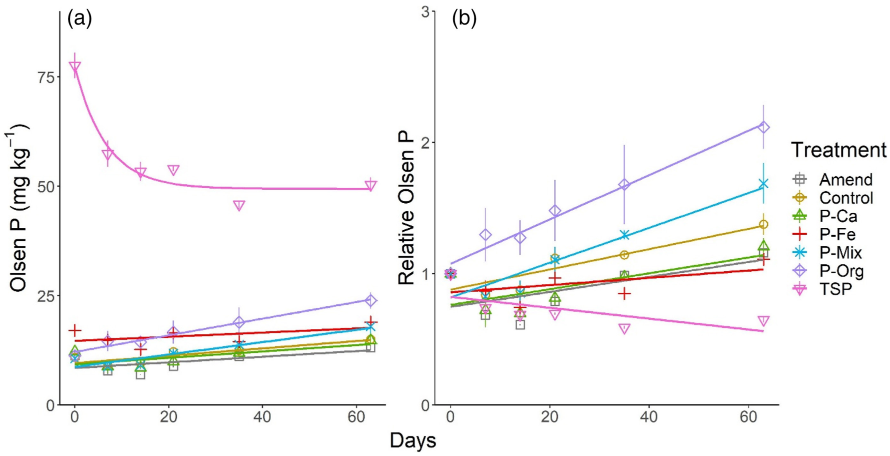

FIGURE 3 Change over time for absolute (a) and relative Olsen P (b). Relative Olsen P refers to changes in Olsen P with respect to their correspondent initial values. Control = no additions; Amend = addition of cellulose and KNO_3. P-Ca, Hydroxyapatite; P-Fe, Iron phosphate (III); P-Mix, 50/50 mixture of P-Ca and P-Org; P-Org, Phytic acid; TSP, Triple Super Phosphate.

TABLE 3 Summary of the first 5 top-ranked models to explain the change in the relative change in Olsen P.

Rank	Model	AICc	ΔAICc	Aw	Cumulative aw	LogLik
1	Microbial C:P × Treatment + 18S × Treatment	−35.34	0	0.94	0.94	43.39
2	Microbial P × Treatment + Microbial C × Treatment + 18S × Treatment	−29.92	5.42	0.06	1	50.76
3	Microbial C:P × Treatment	−10	25.34	0	1	21.63
4	Microbial C × Treatment + Microbial P × Treatment + Microbial N × Treatment	−4.92	30.42	0	1	39.99
5	Microbial C × Treatment + Microbial P × Treatment	−2.69	32.65	0	1	26.99

Note: ΔAICc = AICc − $AICc_{min}$ for best model.
Abbreviations: AICc, Akaike's information criterion; Aw, Akaike's weight; LogLik, Log likelihood ratio.

changes in relative Olsen P (Table 3). Models were ranked based on their Akaike's information criterion (AICc) (Moinet et al., 2016).

The first model in Table 3 (Microbial C:P × Treatment + 18S × Treatment) is the best at explaining the change in relative Olsen P. The ΔAICc with the second

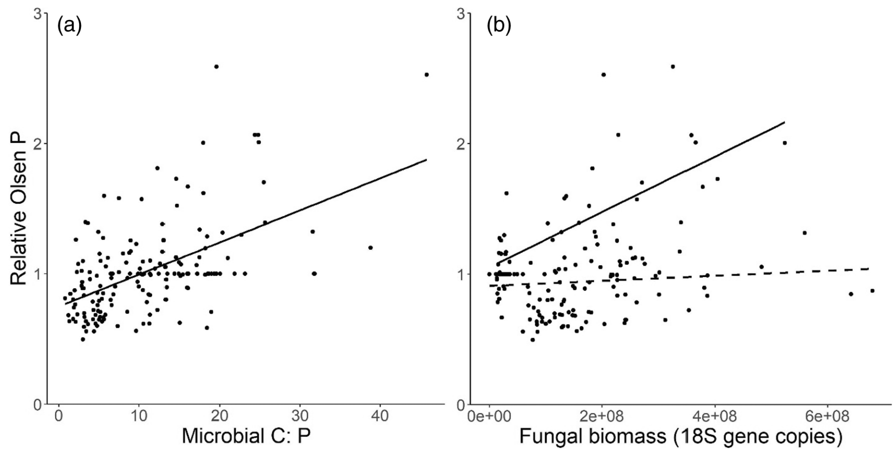

FIGURE 4 Correlation between relative change in Olsen P and microbial C:P (panel a), and fungal biomass (18S gene copies) (panel b) as explained by the first ranked model: Relative Olsen P = Microbial C:P × Treatment + 18S × Treatment. In panel a, the plain line represents the average fit of the positive correlation for all treatments ($p < .05$). In panel b, the plain line represents the fit of the significant positive correlation for the P-Org treatment only and the dashed line represents the average fit for the rest of the treatments ($p > .05$). The adjusted R^2 were .69, .61 and .5 for the first ranked model, panel a and panel b, respectively.

model was 5.42, showing a considerably better explanatory power of the first model. The Aw of the first model was 0.94, which means a 94% chance of being the most robust model. Model 1 contained biological variables (C:P in microbial biomass and 18S gene copies) as well as their interaction with the P forms treatments. Models 2 to 5 were also related to biological values. The adjusted R^2 of the first model was 0.693.

In the first ranked model, C:P in microbial biomass was positively correlated with a relative change in Olsen P for all treatments ($p = .0015$) and no statistical difference was found in slopes among treatments nor intercepts (Figure 4, panel a). The model relating changes in relative Olsen P to microbial C:P biomass and treatment explained a substantial amount of the variation in the data, with an adjusted R^2 of 0.61, indicating that 61% of the variability in the changes in relative Olsen P was accounted by the model. Similarly, in the first ranked model, fungal biomass (18S gene copies) was only positively correlated with the relative change in Olsen P for the treatments that received organic P, P-Org treatment ($p < .001$) and P-Mix treatment with marginal significance ($p = .051$) (Figure 4, panel b). No significant correlation was found for 18S gene copies for the other treatments. The adjusted R^2 of this model was .5.

4 | DISCUSSION

4.1 | Olsen P from different poorly soluble P forms

Olsen P in the TSP positive control was found to be the highest across all treatments (Figures 2 and 3). As expected, Olsen P levels in the poorly soluble P forms were much lower than that of TSP. Olsen P in the TSP treatment decreased over time, indicating a fixation of the most readily soluble P molecules onto soil particles and soil organic matter.

Soils amended with P-Fe, however, showed a slightly higher Olsen P than the other non-soluble treatments at the beginning of the incubation (Figure 2). It has been previously shown that the solubility of poorly soluble P-Fe minerals increases with alkaline pH (Lindsay & De Ment, 1961; Penn & Camberato, 2019), which could explain the larger difference in Olsen P between P-Fe and the other treatments. Iron phosphates can supply significant amounts of P for crops. The use of soluble sources of P from iron phosphates, such as vivianite, is of great potential in agriculture (Wu et al., 2019). In our study, we selected a crystalline form of Fe-P that may be formed during the production of biofertilizers from wastewaters which have been treated with Fe salts. Studies which have tested and compared the solubility from crystalline Fe sources are scarce. Strengite, a crystalline P-Fe mineral, has been compared with fluorapatite (a poorly soluble calcium phosphate) showing slightly higher P solubility values in alkaline pH (Lindsay & De Ment, 1961). Strengite has also been shown to be used by plants in greenhouse studies (Armstrong et al., 1993). P from strengite can also be solubilized by soil microorganisms (Bolan et al., 1987). However, in other field and greenhouse studies in which biosolids with different concentrations of P-Fe and P-Ca forms, no large differences were found and their low plant availability was confirmed (Ashekuzzaman et al., 2021; Maguire et al., 2001). It should be noted that different degrees of crystallinity in the poorly soluble forms that were used in this study can also affect largely their extractability in $NaHCO_3$. For example, different types of P-Fe salts

(Bolan et al., 1987; Lindsay & De Ment, 1961), P-Ca (Tosun et al., 2021) and organic P forms (Amadou et al., 2021) are known to lead to different levels of available P.

The estimated Olsen P of the studied forms is also affected by the extraction method used. In our study, we chose extraction in $NaHCO_3$ (Olsen) because of its widespread use as an indicator of potential plant-available P. Olsen P is also suitable for this specific soil as its pH is slightly alkaline. Yet, other methods are used for other types of soils and may show different levels of P (Wuenscher et al., 2015). For instance, Ashekuzzaman et al. (2021) reported that P-Ca forms were overestimated when using Morgan's P test. In our study, we observed also an overestimation of soluble P when using ammonium oxalate (Joret-Hébert) as a P extractant (data not shown).

4.2 | Change in Olsen P: the role of soil microbes

The absolute change in Olsen P depends on the initial levels of Olsen P, as the P values in the TSP treatments were considerably higher than in the poorly soluble ones. We, therefore, normalized the values of Olsen P and analysed the relative change over time.

Relative Olsen P was found to significantly increase over time for all the poorly soluble treatments including the controls (Figure 3, panel b). Soil microorganisms have been found to solubilize P from a wide range of P forms and different soils (Brucker et al., 2020; Houben et al., 2019; Pastore, Kaiser, et al., 2020; Pastore, Kernchen, & Spohn, 2020; Pistocchi et al., 2018). As shown by the model in Table 3, the changes in Olsen P in our incubation were correlated with microbial C:P and, in the case of the P-Org treatment, fungal biomass (18S gene copies). These results can be interpreted as a dependence of microbes on C to access P. Microbes might start mobilizing poorly soluble P when their C:P is over their homeostatic value (Spohn, 2016). The correlation between microbial C:P and changes in Olsen P were observed also in our control treatment in which no cellulose nor KNO_3 was added. This could mean that the change in Olsen P does not depend uniquely on the amount of C in the soil, but also on the ability of soil microorganisms to incorporate C into their biomass. In a previous experiment (Figures S1–S3), we did not observe microbial-induced changes in Olsen P. Nevertheless, in the rhizosphere, the constant release of different C sources by roots or the decomposition of crop residues could improve the acquisition of C by soil microorganisms (Shahbaz et al., 2017; Zhang et al., 2019). We attempted to replicate this higher exchange of C and N with the addition of cellulose and nitrogen.

Relative Olsen P increased to a greater extent in the organic P treatments (P-Org and P-Mix) (Figure 3) compared to the rest of poorly soluble P treatments and controls. Concurrently, the number of 18S gene copies was a significant factor in the best explanatory model for P-Org and (marginally) for P-Mix (Figure 4). This would suggest that fungi were particularly adapted to access P from organic recalcitrant sources such as phytate. Organic sources of P have been shown to be solubilized by fungi such as *Aspergillus* and *Penicillium* genera (Efthymiou et al., 2018; Gaind & Nain, 2015). Moreover, fungi have been shown to solubilize P from phytate at a greater extent than bacteria by their greater production of phytase (Gaind & Nain, 2015; Singh & Satyanarayana, 2015). In our experiment, the addition of cellulose significantly reduced 16S:18S ratio and, on average, increased fungal biomass (yet non-significant) (Table 1). This higher proportion of fungal biomass might have contributed to a higher production of phytase that ultimately could have increased Olsen P in the P-Org treatment.

On the contrary, the lack of differences in relative Olsen P between the controls with no P application and the P-Ca and P-Fe treatments would suggest that the newly soluble P does not necessarily come from the added P (hydroxyapatite or iron phosphate (III)), but potentially from the native soil P pool. These results suggest that P-Ca and P-Fe were very recalcitrant for the soil microorganisms under our experimental conditions. This contrasts with other studies in which soil microorganisms were able to solubilize P from apatite (Brucker et al., 2020) and goethite (Fe-phosphate) (Pastore, Kaiser, et al., 2020).

For the TSP positive control, relative Olsen P decreased over time (Figure 3). In this case, levels of soluble P likely exceeded the requirements of soil microorganisms. The application of large concentrations of soluble P had also a negligible impact on soil microbial activities (Figure 1 and Tables 1 and 2), P microbial biomass and the relative abundances of bacteria and fungi (16S:18S). These results contrast with literature in which phosphatase activities decreased (Mori, 2022), soil respiration increased (Ozlu & Kumar, 2018), P microbial biomass increased (Fontana et al., 2021) and microbial community composition was altered (Wang & Huang, 2021) after P application. We assume that these effects are dependent on long-term and repeated P fertilization practices and not observable in shorter experiments like ours and that the effect of P fertilizer is generally small on soil microorganisms (Bünemann et al., 2004).

Strategies to solubilize P by soil microorganisms are numerous. In our study, we could not associate any strategy with changes in Olsen P as we did not detect a significant difference among treatments for pH (as a proxy of soil acidification by LMWOAs production) or in phosphatase activities. Nevertheless, the production of LMWOAs is expected to be small and could not directly change soil pH (Evangelou et al., 2008; Shi et al., 2011). Likewise, enzymatic activity results indicate potential activity and not the actual enzymatic activity. Therefore, our results would indicate that there is no significant difference in the potential of soil microorganisms in producing phosphatase (Nannipieri et al., 2018). Solubilization of P-Org though, might be more related to the activity of other P-related enzymes such as phytase (Amy et al., 2022).

5 | CONCLUSION

In this study, we compared Olsen P of different poorly soluble P forms and assessed the role of microbes in the changes found over time in the top horizon of a Cambisol. To our knowledge, this is the first time these forms are compared to one another in a soil incubation. We found that Olsen P values were dependent on the interaction between the nature of the P form applied and the soil microbial community. Our findings show that the activity of soil micro-organisms has the potential to alter the solubility of P from different common recalcitrant P molecules in recycled fertilizers.

Our results suggest that rather than a static pool, soil microbes have the potential to mobilize poorly soluble organic sources such as phytate. This is supported by our most strongly supported model, which involved both the Microbial C:P and Fungal biomass as explanatory variables and which, combined, explained 69% of the variation in the temporal dynamics of Olsen P. Our findings suggest that improving fungal biomass and available C in soils could result in increasing the amount of plant-available P. This may have an impact on the valorization of new recycled biofertilizers that contain organic P forms (e.g. hydrochars, biochars, digestates, etc.), as these forms might become available for crops by the action of soil microorganisms.

Further research should investigate which chemical and biological soil factors trigger the different dynamics of P when it comes to compare poorly soluble forms of P. Different soil properties (e.g. pH, soil organic matter, texture, microbial composition, etc.) may lead to different trends. It remains unknown how soil microorganisms would contribute to the release of P from different P molecules in soils with contrasting pH. Similarly, different initial ratios of bacteria to fungi or land uses could also result in the solubilization of different poorly soluble forms of P.

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DATA AVAILABILITY STATEMENT

All data files are present in the Zenodo online repository: Velasco-Sánchez et al. (2022). https://doi.org/10.5281/ZENODO.6901821.

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SUPPORTING INFORMATION

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