Interactions between cover crops and soil microorganisms increase phosphorus availability in conservation agriculture

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Abstract

Aims An essential task of agricultural systems is to improve internal phosphorus (P) recycling. Cover crops and tillage reduction can increase sustainability, but it is not known whether stimulation of the soil microbial community can increase the availability of soil organic P pools.

Methods In a field experiment in southwest Germany, the effects of a winter cover crop mixture (vs. bare fallow) and no-till (vs. non-inversion tillage) on microbial P-cycling were assessed with soybean as the main crop. Microbial biomass, phospholipid fatty acids (PLFAs), P cycling enzymes, and carbon-substrate use capacity were linked for the first time with the lability of organic P pools measured by enzyme addition assays (using phosphodiesterase, non-phytase-phosphomonoesterase and fungal phytase).

Results Microbial phosphorus, phosphatase, and fatty acids increased under cover crops, indicating an enhanced potential for organic P cycling. Enzyme-stable organic P shifted towards enzyme-labile organic P pools. Effects of no-till were weaker, and a synergy with cover crops was not evident.

Conclusions In this experiment, cover crops were able to increase the microbially mediated internal P cycling in a non-P-limited, temperate agroecosystems.

Keywords Conservation agriculture • Metabolic diversity • Organic P fractions • Plant-microbial interactions • PLFA • P-mobilisation

Introduction

Crop production depends on a sufficient supply of major nutrients such as phosphorus (P). Improving the internal recycling of P in agroecosystems is needed and this is especially urgent in agroecosystems with a long history of P fertilisation, in order to reduce dependence on diminishing mineable P resources (Carpenter and Bennett 2011; Schröder et al. 2011), and to reduce detrimental effects that losses of excess P to other ecosystems can have (Ceulemans et al. 2014; Sharpley 2016). In soil, P is present either in inorganic (P$_i$) or organic (P$_{org}$) forms. Typically in agricultural temperate soils, only about 5% of total soil P is dissolved in soil solution and thereby available for plant uptake in the form of ortho-
phosphate (Stutter et al. 2015). Consequently, the soil solution has to be continuously replenished with orthophosphate, either by desorption processes from the soil mineral phase or by mineralization of organic P.

In industrialised countries, past organic and mineral fertilizer applications to agricultural soils have led to an accumulation of “residual P” or “legacy P”, which is composed of inorganic and organic P of limited availability (Stutter et al. 2015; Lemming et al. 2019). The residual P can be considered as a potential resource and its improved use could reduce dependence of modern agriculture on fertilizer inputs (Menezes-Blackburn et al. 2018). In recent years, management of soil organic P dynamics has received particular attention (George et al. 2018), since soil organic P can comprise between 30 and 80% of total soil P (Harrison 1987). A large proportion of organic P in soil is bound as monoesters in supramolecular structures (McLaren et al. 2015), phytates, non-phytate monoesters, and diesters (Turner et al. 2007). Plant-available orthophosphate can be released from P$_{org}$ in a process catalysed by different phosphatase enzymes produced by soil biota (Harrison 1987). Phosphomonoesters (e.g., inositol phosphates/phytates, sugar phosphates, and mononucleotides) are dephosphorylated by phosphomonoesterases, whereas for diesters (e.g., nucleic acids and phospholipids) an initial hydrolysisation by a phosphodiesterase is required. Phytases represent a specialized form of phosphomonoesterases additionally capable of initiating the cleavage of higher-order inositols (Konietzny and Greiner 2002). While some plants are capable of producing phosphomonoesterases, they do not release significant amounts of phosphodiesterases or phytases (Turner and Haygarth 2005), making soil microorganisms the main source of these enzymes and therefore the key drivers of mineralisation of organic P compounds (Bünemann et al. 2007; Richardson and Simpson 2011). The mobilisation of P$_i$ and P$_{org}$ is affected by the production and degradation of P-mobilising compounds by microbes (Jones and Oburger 2011). Additionally, soil microbes affect the P nutrition of plants via antagonistic effects on plant pathogens (Finckh et al. 2019), as well as production of phytohormones that modify both root growth and architecture (Hayat et al. 2010). Among these microbes, arbuscular mycorrhizal fungi (AMF) are the most studied, and their abundance can be directly related to improved P nutrition for plants, especially in P-limited agroecosystems (Jansa et al. 2011; Cozzolino et al. 2013).

Cropping systems that enhance soil microorganisms’ capacity to improve the efficient management of nutrients and the use of residual P by mobilising P$_i$ and mobilising and mineralising P$_{org}$ pools can be an option for a wide range of agroecosystems, from nutrient limited soils in the tropics to heavily fertilized temperate agroecosystems (Oberson et al. 2006; Wendling et al. 2016). Conservation agriculture, consisting of cover cropping in combination with tillage reduction, is such an option, providing multiple benefits to both soil fertility and to the environment (Hobbs et al. 2008; Büchi et al. 2018), as well as closing gaps in P cycling. Recently, Hallama et al. (2019) described three pathways of cover crop-derived P benefit for the main crop in a meta-analysis. First, nutrients are taken up from the soil and stored in the cover crop plant tissues, released after their mineralisation in spring. Second, cover crops interact with the soil microbial community, shaping its abundance, structure and functions, potentially increasing the P supply to the main crop (Deubel and Merbach 2005; Oberson et al. 2006). Finally, some cover crops, especially lupines, can modify the soil chemistry in their rhizosphere, mobilizing P sources that are otherwise limited (Lambers et al. 2013). Previous studies of P-cycling in agroecosystems focused either on chemical or microbiological soil properties, whereas the complex interactions between P-cycling microorganisms and the lability of different P fractions in soil have been less well studied (Frossard et al. 2000; George et al. 2018).

In order to test the validity of the pathways of cover crop-derived P-benefit mentioned in Hallama et al. (2019), the aim of the current study was to clarify whether conservation agriculture, with its component cover crops and no-till, stimulates microbial abundance and function and changes the lability of the P$_{org}$ fractions. Under conservation agriculture, an enhanced microbial community may lead to increased storage of P in living and dead biomass, resulting in a shift from P$_i$ and P$_{org}$ fractions with limited availability to more labile P$_{org}$ fractions. Thus, we hypothesize that under conservation agriculture (cover crop/no-till): (1) soil P shifts towards more available pools; (2) a stimulated microbial community with enhanced functions is associated with changed P pools, and; (3) cover crops and no-till may have synergistic effects on soil microbial biomass, microbial community structure, and P-cycling capacity.

To evaluate P dynamics under field conditions and to gain a more detailed understanding of the link between the function of P cycling microorganisms and the
potential lability of organic P compounds, an enzyme addition assay (EAA) was used. This biochemical method consists in the addition of enzymes targeting specific P classes and quantifies the hydrolysability of specific P$_{\text{org}}$ classes by substrate specific enzymes (Bünemann 2008). The relationship between enzyme activities and the soil microbial community was investigated by quantifying the total microbial P pool as well as the different microbial groups of soil organisms by analysis of neutral and phospholipid fatty acids.

Materials and methods

Site description

The field experiment was conducted at the Tachenhausen Experimental Farm near Stuttgart, Germany (48.649800 N, 9.387500 E, 330 m a.s.l.) and was established in autumn 2012. The soil is a Stagnic Cambisol (IUSS Working Group WRB 2015) with a very fine sandy loam texture. The field has an average pH$_{\text{H}_2\text{O}}$ of 6.5, a soil organic carbon content of 14 g kg$^{-1}$ soil and a rather high P$_{\text{CAL}}$, averaging 108 mg kg$^{-1}$ soil. The climate is temperate with a mean annual temperature of 8.8 °C and 809.3 mm precipitation (monitoring station Wetterstation Tachenhausen HiWU, 200 m from the site, 1961–1990). The field has a history of conventional agriculture, with a crop rotation consisting mainly of cereals and winter oilseed rape. The crop rotation for the experiment was winter wheat – cover crop mixture – soybean. An overview of climate and management is presented in Fig. 1; a detailed list with field observations and the agronomic management can be found in Table S1.

In the field trial, the effects of tillage and soil coverage on soil properties were compared in a full factorial design. Tillage consisted of either reduced (non-inversion) tillage (RT) or no-till/direct seeding (NT), while soil coverage included either a bare fallow or a cover crop mixture. The field trial was replicated with three complete blocks. To simplify handling of field operations, the experiment was set up in a split-plot design, with the levels of tillage randomly allocated to two main plots within each of the three blocks and the levels of cover crops randomized as two subplots (strips of 6 m by 100 m) within each main plot, resulting in a total of 12 plots. Conservation agriculture management consists of the simultaneous use of direct seeding and cover crops. Although tillage effects probably would have been greater with the extreme comparison of deep inversion tillage and no-till, the more modern non-inversion tillage approach was used as a control, as it is becoming standard in the region. In the cover crop treatments, a commercially available mixture (Terra Life Beta Maxx® 2014 provided by Deutsche Saatveredelung AG, Germany), containing *Trifolium alexandrinum*, *Pisum arvense*, *Vicia sativa*, *Lupinus angustifolius*, *Guizotia abyssinica* and *Phacelia tanacetifolia* was direct seeded at a rate of 45 kg ha$^{-1}$. This specific mixture including legumes was considered
a compromise between positive effects on soil structure, N supply, winter-killing and only a minor risk of pathogens for the main crops. At the end of the vegetation period in November 2014, the cover crop biomass of RT and NT was rather low with 1114 and 1689 kg dry mass ha^{-1}, respectively. The field emergence and biomass production of the cover crop species in the mixture can be found in Table S1. Despite repeated applications of herbicides, weed pressure was generally high. Rabbits, mice and snails constituted an additional problem for the cover crops.

Soil samples were taken in February 2015, after frost-death of the cover crops, and October, at soybean harvest, at 0–5 and 5–20 cm depths with an auger, from around eight locations inside each of the twelve plots and pooled per plot and depth. The samples were sieved at 5 mm and stored at −20 °C until analysis. For the chemical determination of calcium-acetate-lactate extractable P (P_{CAL}), a standard method to estimate soil P status for crops, soil samples were dried (60 °C for 72 h), milled and extracted with calcium-acetate-lactate (VDLUFA 2012).

Enzymatic availability of organic P pools

An enzyme addition assay was used to characterize different organic P forms in an alkaline soil extract, depending on their lability for enzymatic degradation (Bünemann 2008; Jarosch et al. 2015). In principle, substrate-specific enzymes are added to hydrolyse specific P_{org} compounds in soil NaOH/EDTA-extracts. The increase in molybdate-reactive P compared to an untreated control sample yields the quantity of the corresponding enzyme-labile P_{org} pool in the extract.

Monoesterase labile P_{org} = P_{org} hydrolysed by acid phosphatase

(1)

Phosphodiesterase-labile P_{org} was quantified by the addition of phosphodiesterase/nuclease (Sigma N8630) in combination with acid phosphatase (Formula 2), since in phosphodiesterase hydrolyses only the first of the two ester bonds in diesters, such that a phospho-monoesterase is also required to produce detectable phosphate.

Diesterase labile P_{org} = P_{org} hydrolysed by nuclease in combination with acid phosphatase–monoesterase labile P_{org}

(2)

Two phytases, a fungal (Peniophora lycii, Ronozyme NP, Novozyme, Denmark) and a commercial bacterial phytase (E. coli, Quantum blue, ABVista, USA), that target overlapping phytase-labile P_{org} pools, were used in order to reflect the activities of different microbial groups (Formula 3 and 4). The pool of monoesterase labile P_{org} must be subtracted from the phosphate released by the phytases, as the added phytases also mineralise non-phytate monoesters.
Fungal phytase labile P\(_{\text{org}}\) = P\(_{\text{org}}\) hydrolysed by fungal phytase–monooesterase labile P\(_{\text{org}}\) (3)

Bacterial phytase labile P\(_{\text{org}}\) = P\(_{\text{org}}\) hydrolysed by bacterial phytase–monooesterase labile P\(_{\text{org}}\) (4)

However, for the characterisation of the enzyme-labile and enzyme-stable P\(_{\text{org}}\) pools, only the fungal phytase was used (Formulas 5 and 6), as this specific enzyme has been employed in other studies (Annaheim et al. 2013; Jarosch et al. 2015).

Enzyme–labile P\(_{\text{org}}\) = fungal phytase labile P\(_{\text{org}}\) + diesterase labile P\(_{\text{org}}\) + monoesterase labile P\(_{\text{org}}\) (5)

Enzyme–stable P\(_{\text{org}}\)

= Total P\(_{\text{org}}\) – Enzyme–labile P\(_{\text{org}}\) (6)

Since the calculations are based on several subtractions of P concentrations in enzyme-treated and untreated extracts, as well as background concentrations in enzyme preparations, unrealistic values were sometimes obtained. When more than three of the five analytical replicates (i.e., wells of microtiter plates) had very low or even negative values, the entire pool was set to NA (data in S2). The individual analysis run (each of the three field replicates of each treatment was analysed in two separate runs) was included as a random effect in the statistical model.

Microbial biomass P

Phosphorus bound in the microbial biomass (P\(_{\text{mic}}\)) was determined on field-moist, unfrozen soil by hexanol fumigation and simultaneous extraction with anion exchange resin membranes (Kouno et al. 1995). For this, 2.5 g dry weight base frozen soil was extracted with 20 ml deionised H\(_2\)O and two resin strips that were charged with 0.5 M NaHCO\(_3\). Subsamples received either no treatment (P\(_{\text{resin}}\)), 1 ml of 1-hexanol (P\(_{\text{hex}}\)) or 1 ml of a solution with a known P spike (P\(_{\text{spike}}\)) equal to 25 mg P kg\(^{-1}\) soil. Samples were shaken horizontally for 16 h at 150 rpm. Thereafter, the resins were transferred to another vial, shaken for 1 h with 1 M HCl to desorb the phosphate from the resins, and the P concentration was measured colorimetrically according to Murphy-Riley at 610 nm (Murphy and Riley 1962). The difference between the fumigated and the unfumigated samples (Formula 7) was used as a proxy for microbial biomass P (P\(_{\text{mic}}\)), since the high recovery rate of P\(_{\text{spike}}\) revealed a very low sorption of released phosphate

\[
P_{\text{mic}} = P_{\text{hex}} - P_{\text{resin}}
\] (7)

A K\(_p\)-conversion factor to account for incomplete extraction of microbial P (Brookes et al. 1982) was not applied since it has not been determined for this specific soil (McLaughlin et al. 1986).

Microbial biomass carbon

Substrate-induced respiration (SIR) was determined to estimate microbial biomass C (C\(_{\text{mic}}\)) (Anderson and Domsch 1978) using automated electrolytic microrespirometry (Respiration Measurement System, ETS, Darmstadt, Germany) (Scheu 1992). Four grams of frozen soil were weighed in plastic cups and acclimatized over 48 h at room temperature. Four \(\mu\)g glucose g soil\(^{-1}\) were added in aqueous solution (100 \(\mu\)l g\(^{-1}\) soil fresh weight) and the samples were incubated for the respiration measurement at 22 °C. The initial respiration rate (average of the three lowest values within the first eight hours) was used to estimate C\(_{\text{mic}}\) using a conversion factor of 38 (Beck et al. 1997).
Potential activity of extracellular enzymes

Potential activities of acid phosphomonoesterase (EC 3.1.3.1), phosphodiesterase (EC 3.1.4.1), β-D-glucosidase (EC 3.2.1.21) and N-acetyl-glucosaminidase (EC 3.2.1.52) were determined using fluorescent 4-methylumbelliferone substrates based on Marx et al. (2001), modified by Poll et al. (2006). The substrates were obtained from Sigma–Aldrich, St. Louis, USA, except for the phosphodiesterase substrate, which was obtained from Carbosynth, Compton, UK.

For the analysis, 1 g of soil was ultra-sonicated at 50 J s\(^{-1}\) for 120 s in 50 ml of autoclaved H\(_2\)O. Fifty μl of soil suspension, 50 μl MES buffer (0.1 M MES-buffer, pH 6.1) and 100 μl substrate were pipetted onto microplates and incubated at 30 °C. The increase in fluorescence over time (slope) was measured at 5 intervals over 180 min at 360/460 nm on a Microplate Fluorescence Reader (FLX 800, Bio-Tek Instruments, USA) and converted into nmol substrate g soil\(^{-1}\) h\(^{-1}\) using a sample-specific standard curve with 4-methylumbelliferone added to the soil suspension.

Phospholipid fatty acids and neutral lipid acids

The structure of the soil microbial community was characterized by extraction and analysis of specific phospholipid fatty acids (PLFA) and neutral fatty acids (NLFA) (Frostegård et al. 1993, modified according to Kramer et al. 2013). Fatty acids were extracted from 2 g soil (Bardgett et al. 1996), based on the method of Bligh and Dyer (1959) and modified by White et al. (1979). Fatty acid methyl-esters were stored at −20 °C until identification by chromatographic retention time and comparison with a standard mixture of qualitatively defined fatty acid methyl-esters ranging from C11 to C20 (Sigma Aldrich, Germany). Specific biomarker fatty acids permit quantification of different microbial groups (Ruess and Chamberlain 2010; Willers et al. 2015). The PLFAs i15:0, a15:0, i16:0, and i17:0 were used as biomarkers for Gram-positive (Gram+) and cy17:0 and cy19:0 for Gram-negative (Gram−) bacteria. The sum of these fatty acids, together with 16:1ω7 and 15:0, can be used as general bacterial biomarkers. The PLFAs 18:2ω6,9 and 18:3ω6,9,12 were used as general markers for fungi (Frostegård and Bååth 1996). The sum of the bacterial and fungal markers, together with the general microbial PLFA 16:1ω5, was used as a proxy for microbial biomass. The neutral fatty acid (NLFA) 16:1ω5 was used as a marker for arbuscular mycorrhizal fungal abundance (Olsson 1999).

Substrate use capacity expressed as metabolic potential diversity

The capacity of microbial communities to mineralise different substrates characterises functional diversity. In this study, Biolog EcoPlates (Biolog Inc., Hayward, CA) were used, in which soil suspensions are added to commercially available microplates containing a standardised set of carboxylic acids, carbohydrates, polymers, amines/amides and amino acid substrates and a colouring agent in the wells (Insam 1997; Insam and Gobena 2004). Colour development is observed when microorganisms inoculated into the wells utilise the substrates (Frač et al. 2012).

Soil suspensions were prepared from 1 g frozen soil in 99 ml of sterile saline peptone water, shaken for 20 min at 20 °C and incubated at 4 °C for 30 min for the sedimentation of soil particles. Each well of the EcoPlates was inoculated with 120 μl of soil solution. The EcoPlates, covered by lids, were incubated at 25 °C in the dark (Gryta et al. 2014). Absorbance was measured at 590 nm at time intervals of 24 h for 9 days in a Biolog Microstation (Biolog Inc., USA). The microbial response in each well of microplates, regarded as substrate utilization, was expressed as the average well colour development (AWCD). Shannon-Weaver’s diversity index (H) was calculated from the number of oxidized C substrates at the threshold of 0.25 (Gomez et al. 2006). For calculations, the average of the measurements after 72, 96 and 120 h of incubation was used.

Statistical analysis

To account for the split-plot design (three field replicates per treatment), linear mixed models with block and the interactions with depth and date as fixed effects and the interaction of mainplot and subplot with depth and date as random effects (Piepho et al. 2003) were fitted using the package lme4 v1.1–19 (Bates et al. 2015), in R v3.5.0 (R-Core Team 2013) and R-Studio v1.1.453 (RStudio 2013). Interactions with random factors were considered random according to Piepho et al. (2003). The complex structure of the models was reduced by elimination of the random effects with a standard deviation of 0, afterwards applying the step function in R to reduce the fixed effects but keeping the block effects. The residuals were checked.
using Q-Q-plots and histograms (Schützenmeister et al. 2012; Kozak and Piepho 2018). The structure of the fitted models and the F-tests are provided in Online Resource S3, R code in Online Resource S4. The following packages were employed: readxl (Wickham and Bryan 2018), openxlsx (Schauerberger and Walker 2019), dplyr (Wickham et al. 2019b), stringi (Gagolewski 2018), tidyverse (Wickham et al. 2019a), pbkrtest (Halekoh and Højsgaard 2014) and LmerTest (Kuznetsova et al. 2017). The figures were produced with estimated means and 95% confidence intervals using emmeans (Lenth 2018) and multcomp (Hothorn et al. 2008) with ggplot2 (Wickham 2009), cowplot (Wilke 2017) as well as RColorBrewer (Neuwirth 2014). The radar chart was elaborated using the package fmsb (Nakazawa 2018). The figures were produced with the estimated means of the full models in order to be able to show also non-significant factors, while the F-tests of the significant effects were calculated with the respective reduced models.

To simultaneously visualise and test the responses of multiple properties that characterise microbial community composition and function to the treatments, linear discriminant analysis (LDA) was used. In this dimensionality reduction technique, multiple microbial properties are loaded on the linear discriminant axes that maximise the separation between the four groups (treatments). For microbial community structure, abundances of single fatty acid biomarkers were used, while for microbial activity, enzyme activities and carbon substrate group utilisation data were used (R code in supplementary material S4).

**Results**

Treatment effects were more pronounced in the topsoil (0–5 cm) than in the deeper soil layers (5–20 cm). Consequently, the presentation of the results was focused on the upper 0–5 cm of the soil. Data on soil properties of 5–20 cm can be found in Online Resource S2.

Cover crops increase enzymatic availability of organic P pools

Total P in the NaOH-EDTA extracts ranged from 690 to 780 μg g⁻¹ soil, of which around 60% were Pᵢ and the remaining 40% Pᵋ (Fig. 2a). Of the Pᵋ pool, on average 98 μg Pᵋ g⁻¹ (around 40% of total Pᵋ) were enzyme-labile, with cover crops increasing the amount of enzyme-labile Pᵋ in October in comparison to bare fallow treatments (Fig. 2a and Table 1, Fig. 2b)

**Fig. 2** Soil P pools at Tachenhausen field site in 0–5 cm. a In the left figure, the top, middle and bottom bars correspond to inorganic P (Pᵢ), enzyme-stable organic P (Pᵋ) and enzyme-labile Pᵋ, respectively; b The enzyme-labile P pool can be further subdivided into Pᵋ hydrolysable for phosphodiesterase, non-phytase-phosphomonoesterase and fungal phytase (bare = without cover crops, RT = reduced tillage, NT = no-till). The bars represent the estimated marginal means of the three field replicates; error bars show the modelled 95% CI. The corresponding models and F-tests can be found in Table 1 and Online Resource S3.
The largest proportion of enzyme-labile \( \text{P}_{\text{org}} \) was available for phytase. Fungal phytase-labile \( \text{P}_{\text{org}} \) was highest under cover crops and RT (Fig. 2b and Table 1, Cover crop x Tillage \( p = 0.015 \)). A bacterial phytase hydrolysed slightly greater quantities of phytate than the fungal phytase and was highest under cover crops in October (Fig. S5). Phosphomonoesterase-labile \( \text{P} \) increased under cover crops in October (Fig. 2b and Table 1, Cover crop x Date \( p = 0.079 \)). The pool of phosphodiesterase-labile \( \text{P} \) was the lowest and most variable of the pools, and showed no treatment effects (Fig. 2b and Table 1).

The standard soil P test \( \text{P}_{\text{CAL}} \) tended to be highest in bare+NT (Fig. 3), whereas resin-P did not show any treatment effects (Fig. S6). The high values, generally above 100 mg \( \text{P}_{\text{CAL}} \) kg\(^{-1}\) soil, suggest an excess availability of \( \text{P} \) for crops.

Microbial carbon, microbial phosphorus and total PLFAs

Microbial carbon (\( \text{C}_{\text{mic}} \)) and total PLFA concentrations were used as proxies for microbial biomass. Cover cropping enhanced microbial biomass in the topsoil (Fig. 4a and b) by around 12%. After the growing season of soybean in October, microbial biomass increased compared to February. The measured \( \text{P}_{\text{mic}} \) in February was highest in the cover crop treatment with NT, but in October the plots with RT had higher \( \text{P}_{\text{mic}} \), regardless of cover cropping (Fig. 4c).

### Table 1

\( P \)-values for main effects and interactions of the fitted models of different \( \text{P} \) pools presented in Fig. 2. The factor levels were: cover crops (bare and cover crops), tillage (no-till and reduced tillage), date (February and October) and depth (0–5 and 5–20 cm). The corresponding raw data can be found in Online Resource S2, models and full ANOVA tables in Online Resource S3, and the corresponding R code in Online Resource S4. Interactions where no significance was detected were omitted.

| Variable | Cover crops (CC) | Depth | Date | Tillage | CC x Date | CC x Depth | Date x Depth | CC x Tillage | CC x Date x Depth |
|----------|------------------|-------|------|---------|-----------|------------|-------------|-------------|------------------|
| P\(_i\) | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| enzyme-stable P\(_{\text{org}}\) | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| enzyme-labile P\(_{\text{org}}\) | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| fungal phytase-labile P\(_{\text{org}}\) | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| monoesterase-labile P\(_{\text{org}}\) | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| diesterase-labile P\(_{\text{org}}\) | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

![](image-url)
Microbial community structure (PLFA pattern)

Fatty acid biomarkers for Gram+ bacteria increased under cover crops (Fig. 5a), whereas Gram- bacteria also increased in October under no-till (Fig. 5b). Cover crops increased the abundance of fungal biomarkers, while reduced tillage showed a tendency toward further increase in comparison to no-till (Fig. 5c). The abundance of AMF, based on the NLFA marker 16:1ω5, tended to increase under cover crops in the topsoil (Fig. 5d). In the rooting zone (5–20 cm, Fig. S7) cover crops+NT had the highest content of 16:1ω5 NLFA in February. In general, the content of the mycorrhizal biomarker was higher at 5–20 cm and was more variable than in the topsoil, especially in October, after soybean growth.

Potential C- and P-cycling enzyme activities and metabolic diversity

Cover cropping increased the activities of phosphomonoesterases (Fig. 6a), phosphodiesterases (Fig. 6b), and β-glucosidases (Online Resource S3), especially in February and in combination with no-till. N-actyl-glucosaminidase activity was highly variable and did not exhibit any treatment effects (Online Resource S3). Cover crops also increased metabolic diversity, determined using a variety of C-substrates calculated as average well colour development and Shannon-Weaver’s diversity index from the carbon source utilisation data (Figs. S7 and S8). The use of Glucose-1-Phosphate and DL-α-Glycerol Phosphate as carbon sources was increased by cover crops above average compared to the other C substrates (Figs. S9 and S10).

When relating P-cycling enzymes with P pools in soils, the relation between enzymatic activity and enzyme-labile P pools was affected by the treatment (Fig. 7). Phosphomonoesterase activity, composed of phytases and other phosphomonoesterases, correlate negatively with the sum of the pools monoesterase- plus phytase-labile Porg in the topsoil in the no-till treatments (R² = 0.36, p = 0.038, Fig. 7a), whereas with non-inversion tillage or in the lower 5–20 cm there was no visible relation at all. Conversely, the relation of phosphodiesterase activity with phosphodiesterase-labile Porg was not influenced by depth, but interacted with cover cropping and date, with a significant negative correlation with cover crops in February (R² = 0.43, p = 0.041, Fig. 7b), but not later in the year in October.
Multivariate analyses of microbiological data

Linear discriminant analysis (LDA) was used to assess whether the treatments resulted in distinct microbial community structures and activity and to obtain an overview of the properties that dominated the dissociation (coefficients are reported in Online Resource S3).

Overall, the treatments resulted in differentiation of the soil microbial community structure and activity (Fig. 8). The effect of cover crops on community composition was most visible in October, indicated mainly by Gram+ and AMF biomarkers (Online Resource S3). Cover crops affected microbial activity already in February and the differentiation was dominated by enzymatic activities. Tillage had its greatest overall effect on microbial community structure and activity in October.

In this experiment, both phosphomonoesterase and -diesterase activity showed a positive correlation with the abundance of Gram+ bacteria (Pearson’s R = 0.5 and 0.36; p = 0.0002 and 0.012, Online Resource S3), Gram- bacteria (R = 0.8 and 0.62; both p < 0.0001), as well as fungi (R = 0.4 and 0.37; p = 0.003 and 0.008).

Discussion

Cover crops influence P-cycling within soil-plant systems (Eichler-Löbermann et al. 2009; Honvaul et al. 2020). In this study, combined chemical, biochemical, and microbiological methods were used to elucidate whether the growth of cover crops in combination with no-till might change microbial abundance and functions...
and lead to modifications in plant available P pools in soil. To interpret the data, first the impact of the treatments on soil P pools was characterised. Further, the role of the soil microbial community as a likely driver for these changes is described and the mechanistic relationship of phosphatases to enzyme-available P org pools are discussed. Then, the multivariate response of microbial activity and microbial community structure is outlined. Finally, the potential synergies between cover crops and no-till and the effects of the treatments on soil phosphorus dynamics are summarised.

Cover crops increase the enzymatic availability of organic P pools

The cultivation of cover crops induced a shift in P dynamics in the soil that could help to explain the commonly observed P benefit with cover crops (Hallama et al. 2019). The enzymatic availability of the P org pools was sensitive to management practices (Fig. 2b), despite the abundant P i and P CAL (Figs. 2a and 3) that dominated the P availability of the soil.

We suggest that the decrease in P CAL (Fig. 3) was a result of both the uptake of P by cover crops, and by the immobilisation of P in the microbial biomass (Fig. 4c). This supports the concept that cover crops quickly take up labile P (Hallama et al. 2019) and that microbially-immobilised P contributes to the build-up of organic P in soil (Bünemann et al. 2008). Overall, our results indicate that the increased availability of enzyme-labile P in soil with cover crops (Fig. 2, Table 1) represents a relative shift from inorganic phosphate towards organic P sources, confirming our first hypothesis.

Conservation agriculture enhances the P-cycling capacity of the soil microbial community

As microbes are the main drivers of soil organic P dynamics (Richardson and Simpson 2011), the role of soil microorganisms underlying the observed shifts in labile P org pools was investigated. The detected increases in enzyme-labile P org pools with cover crops (Fig. 2) are concurrent with increases in microbial abundance (Figs. 4 and 5, Online Resource S3) and activity (Fig. 6, Online Resource S3). The cover crop effect was, in most cases, greater than that of no-till, and treatment differences were more visible in the topsoil (0–5 cm) compared to the deeper soil layers (5–20 cm, data in Online Resource S2). Considerable treatment effects on microbial properties were already visible in February (Figs. 4, 5 and 6), whereas total enzyme-labile organic P increased in October. This delayed response of the pools is logical if changes in the P pools are attributed to microbial activity. The observed cover crop effects may have resulted from the following processes: in February, the microbial community reflected mostly the direct effects of a living plant cover in the off-season (Kumar et al. 2013), although limited mineralisation of shoots and roots occurs during cold months (Kramer et al. 2013). In October, mineralisation of the cover crop biomass provided nutrients (Damon
et al. 2014). Additionally, the rhizosphere of the soybean crop probably shaped the soil microbial community by rhizodeposition, altering the nutrient dynamics, as shown by Manna et al. (2007).

Not only changes in available P pools were of interest, but also in microbial drivers of these processes. The abundances of both Gram+ and Gram- bacteria increased under cover crops (Figs. 5a and b), probably due to above- and belowground litter inputs and rhizodeposits from cover crops. Tillage had no effect on Gram+, but NT tended to increase abundance of Gram-. This effect could be related to organic matter inputs from cover crops that favoured predominantly Gram- bacteria (e.g., copiotrophic Proteobacteria). The finding that Gram+ bacteria were less enriched in the conservation agriculture treatments could be explained by the fact that members of the biggest group of Gram+ bacteria in bulk soil, Actinobacteria, utilize predominantly more oligotrophic life strategies (Uksa et al. 2015; Ho et al. 2017). The finding that fungi benefited

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**Fig. 7** Relation between enzymatic activity and the respective enzyme-available organic P pools for (a) phosphomonoesterase and (b) phosphodiesterase. The trend lines, $R^2$ and $p$-values were calculated using a simple linear model. As the relation of enzymatic activity and $P_{org}$ pools interacted with depth and tillage as well as date and cover crops in the case of phosphomonoesterase and phosphodiesterase, respectively, the trendlines were fitted to the corresponding subsets. Coefficients and R-code can be found in Online Resource S3 and S4, respectively.
most from cover crops in combination with RT instead of no-till was unexpected, as fungi are commonly considered to be more sensitive to tillage than bacteria due to the disruption of their hyphal networks with soil movement (Jansa et al. 2003). We suggest that non-inversion tillage resulted in an increase in the abundance of saprotrophic fungi with RT (Fig. 5c), because of the availability of substrate due to the mixture of cover crop litter with the soil.

Increases in the activities of P cycling enzymes in cover crops+NT compared to the other treatments in February, were detected both in absolute values (Fig. 6) and per unit Cmic. The contributions of the different microbial groups to this increase were presumably unequal. Relating activities of P cycling enzymes to different groups of microorganisms showed that phosphomono- and -diesterase activities correlated positively with abundances of bacteria and fungi. The genetic potential for the production of acid and alkaline phosphatases is widespread in soil microorganisms (Bergkemper et al. 2016), but there are no detailed studies of the abundance of single bacterial and/or fungal species’ connections to in-situ activity of phosphatases. In our experiment, mainly bacteria might have increased the release of phosphatases to cover their demand for phosphate, while increasing microbial P immobilisation.

In order to evaluate enzymes from the same family but produced by different groups of soil microorganisms (Menezes-Blackburn et al. 2013), a commercial bacterial phytase was included in addition to the fungally-derived phytase in the enzyme addition assay. The bacteria-derived phytase mineralised around 20% more Porg than the phytase derived from fungi. However, the addition of the bacterial phytase had more variable results (Fig. S5). The different amounts of phosphate released by bacterial and fungal phytases indicate that the two enzyme families act on different but overlapping subpools of Porg (Hill and Richardson 2007). Apparently, the differences in terms of enzyme activity between the two phytases produced by these organisms may reside more in the environmental conditions (i.e., pH) of their location (Wyss et al. 1999) than on substrate specificity. Fungal phytase-labile P was especially abundant in cover crops with reduced tillage (Fig. 2b), corresponding to the greatest fungal abundance (Fig. 5c). Therefore, it seems reasonable that phytate produced by fungal microorganisms (Turner 2007) contributed to the pool of fungal phytase-available Porg, representing a substrate that is located in micro-environments with favourable conditions for the activity of fungal phytases.

Arbuscular mycorrhizal fungi are of particular interest in plant production due to their role in P nutrition for many crops, and enhanced AMF abundance after cover crops is positively related to phosphorus uptake (White and Weil 2010). In our experiment, the abundance of AMF biomarker NLFA 16:1ω5 tended to be greater under cover crops (Fig. 5d), but tillage had apparently no effect on AMF. Possible explanations for the lack of an AMF abundance response to no-till could be that the dominant AMF species were resistant to tillage effects (Jansa et al. 2003) or to antagonistic relationships between different soil microorganisms (Li et al. 2020). Overall, our results add to the emerging body of literature that has shown the evident and positive effects of cover crops on microbial properties (Kim et al. 2020) and relate these changes in microbial properties with soil P dynamics, potentially increasing labile organic P pools. The effects of cover crops were more evident than those of no-till.

Organic P compounds and phosphatase enzymes

The approach of quantifying soil Porg pools according to their potential hydrolysability by adding substrate specific enzymes (phytase, phosphomonoesterase and phosphodiesterase) together with the assessment of soil enzymatic activity (phosphomonoesterase and phosphodiesterase activity) provides deeper insights into the dynamics of Porg cycling than have before been seen. The EAA method uses excess enzyme concentrations to measure the potential availability of different native Porg pools for enzymatic mineralisation, while methods analysing enzyme activities optimise the concentrations of Porg substrates to assess the amount of enzymes in the soil, i.e., the mineralisation potential of organic compounds.

The association of monoesters and diesters, two of the most abundant chemical forms of Porg, with their respective enzymes, appeared to be influenced by the treatment. To interpret these findings, we must keep in mind the different processes that control the substrate-enzyme relation, as they affect each other mutually (Bünemann et al. 2011). The production and release of phosphatases by roots and microorganisms in soils is assumed to be controlled mainly by the requirements of the organisms and the concentration of available

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substrate (Quiquampoix and Mousain 2005). However, other factors, such as stabilisation and turnover times of P-cycling enzymes, as well as complexation of substrates, seem to be important for enzymatic turnover in-situ (Rao et al. 2000). The other side is the size of available substrate pools. Here, monoesters (including inositol-P) constitute most (in our case, around 80%, Fig. 2b) of the enzyme-labile $P_{\text{org}}$, although chemical stability and sorption on particle surfaces limit their availability for mineralisation (Gerke 2015). Diesters, on the other hand, interact less with the soil matrix, but persist to a certain degree because of the low stability of the enzymes that degrade them (Lang et al. 2017; Jarosch et al. 2019; Müller et al. 2020). Counter-intuitively, phosphodiesterase activity may constitute a rate-limiting step for mineralisation in a soil with $P_{\text{org}}$ pools formed by abundant but enzymatically unavailable monoesters and less abundant, but more available diesters (Turner and Haygarth 2005). The absence of a clear main effect of enzymatic activity as a covariate for enzyme-labile $P_{\text{org}}$ may indicate that the soil was not in a steady-state, where enzymatic activity and organic P control each other mutually. Both enzymatic activity and organic P pools varied over time and depth and were affected by the addition and availability of fresh organic matter and microbial activity.

The detected increases in phosphomonoesterase activity with cover crops are accompanied by an increased capacity of the microbial community to use specific phosphate-bearing substrates, such as glycerol-phosphate and glucose-1-phosphate (Figs. S9 and S10). Besides a general increase in organic compounds and microbial mineralisation under cover crops, one explanation for this specific increase in the capacity to degrade phosphate-bearing substrates could be the presence of phosphate compounds in root exudates of cover crops and the adaptation of microbes to use these substrates effectively. Sugar phosphates are involved in intracellular carbohydrate metabolism and participate in co-transportation of plastid-localized sugar-phosphate in several species of plants (Flügge et al. 2011). Although import and export mechanisms of sugar phosphates into and from root cells are not characterized, these compounds are detected in plant exudates (Sasse et al. 2018). In addition, cover crops induce priming effects in the rhizosphere by influencing the turnover of soil organic matter (Dijkstra et al. 2013), hence altering soil nutrient content, including phosphorus. However, higher turnover of glycerol-phosphate and glucose-1-phosphate could alternatively reflect the higher demand for P when microbial biomass is increased under cover crop treatment. Therefore, stimulation of phosphomonoesterase activity becomes plausible. Unfortunately, the biolog plates used in this study did not contain any substrates with phosphodiesters.

The enhancement of enzyme activity under cover cropping can be explained by the increase in the availability of organic P substrates (Quiquampoix and Mousain 2005), the reduction of the concentration of $P_i$ (i.e., product-inhibition) (Burns and Dick 2002), and the increase in microbial abundance, as well as microbial production of phosphatases. With the detection of an association between the increase in abundance of various microbial groups, increased enzymatic activity and increased enzyme-available $P_{\text{org}}$ pools, we confirm our second hypothesis, which assumed that a stimulated microbial community with enhanced functions would be associated with changed P pools. With our current understanding of soil organic P dynamics, the changes in $P_{\text{org}}$ pools can be expected to be driven by the stimulation of the microbial community (Richardson and Simpson 2011). However, simultaneous substrate-driven processes, e.g., increases in microbial activity due to greater availability of $P_{\text{org}}$ from cover crops residues, may also take place.

Multivariate response of microbial functions and microbial community composition to conservation agriculture

The soil microbial community was affected by the conservation agriculture treatments, resulting in a differentiated community structure and activity (Fig. 8). In February, the treatments, especially cover cropping, affected microbial activity more than community structure, which is in line with other studies that have found microbial activity to be more sensitive than community composition to management changes (Bier et al. 2015). By October, both tillage and cover crops had resulted in distinct community compositions, though for microbial activity more than community structure, which is in line with other studies that have found microbial activity to be more sensitive than community composition to management changes (Bier et al. 2015). By October, both tillage and cover crops had resulted in distinct community compositions, though for microbial activity more than community structure, which is in line with other studies that have found microbial activity to be more sensitive than community composition to management changes (Bier et al. 2015). By October, both tillage and cover crops had resulted in distinct community compositions, though for microbial activity more than community structure, which is in line with other studies that have found microbial activity to be more sensitive than community composition to management changes (Bier et al. 2015). The tillage operations in the RT treatments that were done after the sampling in spring likely were the reasons for the greater tillage effect in October.

Functional diversity, calculated as Shannon-Weaver’s $H$ from carbon substrate group utilisation, increased under cover crops and NT (Figs. S7 and S8). It is commonly reported that cover crop mixtures
increase microbial diversity (Kim et al. 2020). In addition, tillage reduction may increase or preserve spatial heterogeneity that would be destroyed due to homogenisation by tillage. Besides potential pathogen suppressing effects (Weller et al. 2002), a diverse community with a variety of nutrient acquisition strategies may have an advantage for the utilisation of different nutrient pools, leading to their increased availability to the community as a whole. This theory of resource partitioning also applies to organic P pools (Turner 2008). The characterisation of cover crops according to plant traits provides a promising approach to understand the cover crop effects on soil microbes and hence P availability (Wendling et al. 2016; Boeddinghaus et al. 2019). This perspective, applied to plant communities in the form of community mean traits (Garnier et al. 2007), could also help to predict the complex action of cover crop mixtures.

Conservation agriculture techniques: Synergy between cover crops and tillage reduction?

Cover crops and no-till are two techniques in agricultural management that are often used with the expectation of enhancing microbial abundance and activity, and consequently crop nutrition. Substrate inputs and protection by the living and dead cover crops sustain the soil biota (Mukumbareza et al. 2015). No-till increases soil heterogeneity both in the soil profile and at the aggregate scale, with profound impacts on the soil microbial community (Young and Ritz 2000). This in turn provides greater more opportunity for soil to rest and a concentration of nutrients and soil organic matter (SOM) at the surface (Kabiri et al. 2016). A synergy between both management techniques is often assumed and frequently found (Wittwer et al. 2017; Boselli et al. 2020), but there are also reports of a greater relative
improvement of microbial properties in under tillage treatments (Balota et al. 2014).

Particularly to make the comparison between the no-till and reduced tillage, soil samples were taken at two different soil depths. The treatment effects in the deeper soil layer (5–20 cm) were generally rather weak; this was the case both for cover crops and for tillage. One factor could be the chosen sampling depth: the tillage operations in RT were conducted only up to 10 cm soil depth, in some cases even less (Online Resource S1). Thus, cores taken at the 5–20 cm depth included some soil that was not affected directly by the tillage treatments. However, the concentration of the cover crop effects at the surface corresponds to litter placement of aboveground plant biomass from crops and cover crops, and we had expected also effects of cover crop roots and their exudates at the 5–20 cm depth (Austin et al. 2017; Schmidt et al. 2018).

In our experiment, judging only by the results of the plots without cover crops, the positive effects of no-till on soil properties were rather limited. However, when comparing reduced tillage and no-till in the plots with cover crops, the picture gets more complicated. Fungal phytase-labile P (\(P_{\text{org}}\)) (Fig. 2b) was greatest with cover crops and reduced tillage, while other properties, such as abundance of Gram- bacteria or phosphomonoesterase and phosphodiesterase activity in February (Figs. 3 and 6) showed synergistic effects of the combination of cover crops with NT. Despite observed shifts in both microbial community composition and activity (Fig. 8), it is not possible to judge these differences in terms of agronomical relevance easily. We are still missing some of the causal relationships between the different soil and plant P pools, microbial community structure, and their potential functions (George et al. 2018). Therefore, our third hypothesis about synergistic effects of cover crops and no-till on soil microbial properties and P-cycling capacity can be only partially confirmed. Further experiments, taking into account the influence of conventional management (Romdhane et al. 2019) and alternative management systems (Mulvaney et al. 2017) are necessary.

In summary, assessment of the treatment combinations revealed a clear enhancement in microbial abundance and activity under cover crops compared to bare fallow (Fig. 9). This potential for (micro-) biological P cycling came with an increase in organic P pools. However, available inorganic P (here measured as \(P_{\text{CAL}}\)) was greatest in the bare fallow treatments.

Conclusions

This study demonstrated that a cover crop mixture and no-till, as components of conservation
agriculture, could enhance soil microbial abundance and activity and change the phosphorus dynamics in a temperate agricultural soil by stimulating organic P cycling. Cover cropping in particular shifted organic P towards pools of higher potential availability for enzymatic hydrolysis. Soil microbial abundance and activity were related to changes in P pools, highlighting the importance of soil microbes for nutrient cycling. More research is needed to study the drivers of the relation between enzymatic activity and organic P pools.

Despite the fact that this experiment was conducted in a field where P availability was not a limiting factor, the system responded after only two seasons of cover cropping. In the bare fallow treatments, representing more conventional systems without cover crops, P dynamics appear to have been dominated by the abundant available inorganic P. Although this study represent only one site and has to be repeated for more sites, we elucidated these two distinct patterns that might explain why both systems work in practice on many farms in central Europe: On the one hand, the conventional input-based, yield-optimised approach with a lower complexity; and on the other hand, the concept of sustainable intensification, making use of biological functions and internal nutrient cycling.

Cover crops are an important tool to mine P from the soil and hence to reduce the necessity to apply P as a mineral fertilizer. Tillage reduction also appears to have an impact, but the agroecosystem might need a longer time for a new measurable equilibrium to be achieved. These two components of conservation agriculture can help to reduce the current high consumption of P fertilizers and to decrease the environmental impact of agriculture. Cover crops constitute a promising, multifunctional tool for sustainable intensification of agriculture the agricultural goals. Scientific efforts and agricultural policies should be directed to overcoming barriers to the widespread adoption of these soil improving cropping systems.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-021-04897-x.

Abbreviations AMF, Arbuscular Mycorrhizal Fungi.; Cmic, Microbial biomass C.; EAA, Enzyme Addition Assay.; LDA, Linear Discriminant Analysis.; NLFA, Neutral Lipid Fatty Acids.; NT, No-till/direct seeding.; P, Phosphorus.; Pn, Inorganic P.; PLFA, Phospholipid Fatty Acids.; Pmic, Microbial biomass P.; Porg, Organic P.; Pcalc, Calcium-acetate-lactate extractable P.; RT, Reduced/non-inversion tillage.; SOM, Soil Organic Matter.

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