Manuscript Details

Manuscript number	APSOIL_2018_1123_R2
Title	Response of phosphorus dynamics to sewage sludge application in an agroecosystem in northern France
Article type	Research Paper

Abstract

Sewage sludge is a phosphorus (P) source alternative to P fertiliser derived from rock phosphate, but its impact on soil processes driving P cycling in agroecosystems requires further study. In order to optimise the use of sludge for sustainable P fertilisation, we need to elucidate the drivers of P dynamics. The present study aims at determining how different sludges (heated sludge, HS and composted sludge, CS) affect soil P pools and dynamics. A field experiment was established and soil was amended either with sludge or with inorganic P (triple superphosphate, TSP). Soil samples were collected five times during a vegetation period, and analysed for Hedley P fractions, microbial P and phosphatase activity. Phosphorus dynamics in soil was strongly influenced by P concentrations in sludge. About one year after application, sludge with the highest P concentration (HS) was as effective as TSP to improve soil P availability. The P source of TSP was immediately available for plant uptake, but the high phosphatase activity of the HS treatment evidenced that soil microorganisms released phosphatases which can hydrolyse HS-derived organic P compounds. In addition, the high content of microbial P in the HS treatment suggests that soil microorganisms assimilate P into their own biomass. By contrast, sludge with the lowest P concentration (CS) enriched primarily the weakly-soluble soil P fractions, resulting in lower P availability compared with that in the TSP treatment. Our findings suggest that both high P concentration and slow, but continuous microbial breakdown of organic P substrates derived from HS allow using this resource as an important source for plant mineral nutrition. This study stresses the need to both characterise P concentrations and P forms in sludge, prior to their application in the field.

Keywords	Hedley fractionation; Microbial phosphorus; Phosphatase; Phosphorus availability; Waste management
Taxonomy	Soil Chemistry, Soil Biology
Manuscript category	Microorganism-related Submissions
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Suggested reviewers	Eva Brod, Emmanuel Frossard, Olivier Duboc

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Data set

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a=a7ec45bc-0d75-4adb-94e4-a3ddaa01a594](https://data.mendeley.com/datasets/trtnx3syg4/draft?a=a7ec45bc-0d75-4adb-94e4-a3ddaa01a594)

Data for: Response of phosphorus dynamics to sewage sludge application in an agroecosystem in northern France

The file provides the data for Microbial P, phosphatase activity, Hedley fractions and pH measured in each replicate of the in situ experiment described in the paper "Response of phosphorus dynamics to sewage sludge application in an agroecosystem in northern France" by HOUBEN et al.

Beauvais, February 13th 2019

To the members of the editorial board of Applied Soil Ecology,

We thank you and the reviewer for the report on our manuscript entitled “**Response of phosphorus dynamics to sewage sludge application in an agroecosystem in northern France**” (previously entitled “Response of phosphorus dynamics to sewage sludge application in an agroecosystem”) by David Houben, Etienne Michel, Cécile Nobile, Hans Lambers, Ellen Kandeler and Michel-Pierre Faucon. The enthusiasms of the reviewer, advice and grammatical corrections have been extremely valuable to improve the manuscript.

Please find enclosed a revised version of the manuscript. Please find also enclosed our response along with reviewers’ comments. Revisions were performed in order to address all criticisms raised by the reviewer. The line numbers refer to the revised manuscript.

We thank you to take our revised version in consideration. We hope this version will be considered as responsive to the concerns of the reviewers and suitable for publication in Applied Soil Ecology.

Sincerely yours,

David Houben

We warmly thank the reviewer for the reports on our manuscript APSOIL_2018_1123 entitled "Response of phosphorus dynamics to sewage sludge application in an agroecosystem in northern France" (previously entitled "Response of phosphorus availability and lability to sewage sludge application in agroecosystem").

-Reviewer 1

1. The authors have done a fair revision work. Work can still be improved. For example, the title is now of a reasonable length but is too general. The authors must strive to find the strength of the work that deserves to occupy a place in the title: the type of experiment, the type of soil, the place, the most salient result, ...

> RESPONSE: The title was modified, as suggested by the reviewer, by emphasizing the place where the experiment took place. The new title is

"Response of phosphorus dynamics to sewage sludge application in an agroecosystem in northern France"

Instead of

"Response of phosphorus dynamics to sewage sludge application in an agroecosystem"

2. The article again does not adhere fully to appropriate reporting guidelines and community standards for data availability: although the richness of the supplementary information, the complete raw database is not fully made completely available in a public repository. The Authors declare that they their data have been made available in Mendeley Data. But, there is no indication within the manuscript. The full raw database must be available for further extrapolation by the whole scientific community.

> RESPONSE: As said in our previous revision, data were made available in Mendeley Data, which is a free-to-use, open access repository. This was also mentioned in the second page of the pdf (see the printscreen below) and the reviewer may access to the data by clicking on the link. This dataset will be listed and directly accessible to readers next to the published article online. In the revised paper, we have included (L467-468):

"Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version."

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Data for: Response of phosphorus dynamics to sewage sludge application in an agroecosystem

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3. Potentially, its potential impact upon the international scientific community of reference, now, is quite good.

> RESPONSE: thank you!

4. Conclusions show now more novelties and are more focused, although they are sometimes far from the reality. Really, the authors think it is realistic to ask a farmer to "measure both P concentration and P forms in sludge prior to their application in the field"?

> RESPONSE: We agree with the reviewer that measuring both P concentration and forms in sludge is an unrealistic task for farmers. However, this could be performed by the sludge provider as it is already done for nitrogen for example (i.e. NO₃⁻, NH₄⁺, organic N and inorganic N are routinely measured before sludge application). This was mentioned in the revised paper (L455-456).

5. This work is interesting and deserves to be published.

> RESPONSE: Thank you

In particular (page. row):

6. Highlights. Please, delete bullet #1

> RESPONSE: DONE

7. 7.143 Thank you for describing the soils according to WRB soil classification system. The WRB comprises two levels of categorical detail: the first one having 32 Reference Soil Groups (RSGs), Luvisol in this case, and the second one consisting of the name of the RSG combined with a set of principal

and supplementary qualifiers that allow a number of possible combinations. Please, add as more details as possible as it will help further extrapolations and meta-analyses by other authors.

> RESPONSE: DONE, the soil is Haplic Luvisol (L143)

8. 9.196 Murphy & Riley is a very well-known method, this details are unnecessary. I mentioned 882 nm as another wavelength was indicated. And, it is indicated at row 214. If a different wavelength was used an explanation is necessary.

> RESPONSE: We used 712 nm to reduce possible interference from traces of organic matter, as recommended by Tiessen and Moir (2008). This is now mentioned in the revised paper (L197-198)

9. 17.387 Independently on

> RESPONSE: DONE (L388)

10. 19.437 This sentence appears a quite important finding. It probably deserves being moved to the Conclusions section

> RESPONSE: DONE (L447-451)

11. Figure 1. Again, one graph only with all the lines (which, moreover, do not overlap, mostly) would be better

> RESPONSE: As answered in our previous revision, we prefer to keep two separated graphs in Fig. 1 in order to better highlight the comparison between sludge and their mineral control as well as to make clearer the statistical analyses.

12. Figure 2. Please, in colour. And, remove the% symbol from the axis of the ordinates and from the individual bars.

> RESPONSE: DONE

13. Figure 3. Full bar and empty bar is better than textured.

> RESPONSE: DONE

Highlights

- Sludge with a high P concentration increased labile soil P concentration
- Addition of organic P to the soil increased phosphatase activity and the microbial P pool
- Sludge with a low P concentration had no effect on soil P forms
- Sludge can be considered a slow-release fertilizer

1 **Response of phosphorus dynamics to sewage sludge application in an** 2 **agroecosystem in northern France**

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3 **Abstract**

4 Sewage sludge is a phosphorus (P) source alternative to P fertiliser derived from rock
5 phosphate, but its impact on soil processes driving P cycling in agroecosystems requires further
6 study. In order to optimise the use of sludge for sustainable P fertilisation, we need to elucidate
7 the drivers of P dynamics. The present study aims at determining how different sludges (heated
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9 was established and soil was amended either with sludge or with inorganic P (triple
10 superphosphate, TSP). Soil samples were collected five times during a vegetation period, and
11 analysed for Hedley P fractions, microbial P and phosphatase activity. Phosphorus dynamics in
12 soil was strongly influenced by P concentrations in sludge. About one year after application,
13 sludge with the highest P concentration (HS) was as effective as TSP to improve soil P
14 availability. The P source of TSP was immediately available for plant uptake, but the high

phosphatase activity of the HS treatment evidenced that soil microorganisms released phosphatases which can hydrolyse HS-derived organic P compounds. In addition, the high content of microbial P in the HS treatment suggests that soil microorganisms assimilate P into their own biomass. By contrast, sludge with the lowest P concentration (CS) enriched primarily the weakly-soluble soil P fractions, resulting in lower P availability compared with that in the TSP treatment. Our findings suggest that both high P concentration and slow, but continuous microbial breakdown of organic P substrates derived from HS allow using this resource as an important source for plant mineral nutrition. This study stresses the need to both characterise P concentrations and P forms in sludge, prior to their application in the field.

Keywords

Hedley fractionation; Microbial phosphorus; Phosphatase; Phosphorus availability; Waste management

1. Introduction

Phosphorus (P) is a limiting nutrient for the productivity of many agroecosystems and indispensable to feed an ever-increasing human population (Filippelli, 2008; Withers et al., 2015). Currently, most of the P used in chemical fertilisers is derived from phosphate rocks that are finite and located in only a few places on Earth (Edixhoven et al., 2013; Reijnders, 2014). Developing sustainable fertilisation practices based on the use of renewable resources such as P-rich waste is thus essential to ensure long-term food security (Dawson and Hilton, 2011; Houben et al., 2017). Among P-rich wastes, sewage sludge appears an excellent candidate because it represents the largest component of recycled P, and due to the world's population growth, this reserve is expected to increase (Lwin et al., 2017). Its use in agriculture will likely increase, requiring continued vigilance in assessing the impacts of the presence of potential pollutants such as metals, organic contaminants (e.g., pharmaceuticals and personal care products), emerging contaminants, viruses and other pathogens (Clarke and Smith, 2011). Since 1986, Council Directive No. 86/278/EEC has governed the use of sewage sludge in the European Union by prescribing testing of sludge and soil for a number of potential pollutants. This Directive has been implemented into the national legislation of member states, most often with stricter limits than that prescribed in the Directive (Kirchmann et al., 2017).

Because sewage sludge has a much lower P concentration than mineral P fertilisers, large amounts of sludge need to be transported to and applied on farms which may result in higher costs compared with mineral P fertilisers (Mackay et al., 2017a). Moreover, in contrast to conventional P fertilisers in which P is predominantly in a soluble form readily available to plants, sewage sludge contains a range of P forms with varying availability to plants (Kahiluoto et al., 2015). Phosphorus is present as a mixture of inorganic P forms including calcium (Ca) phosphates and amorphous aluminium (Al)- or iron (Fe)-bound P, while organic P generally represents a small fraction (Xie et al., 2011a). We already have solid evidence from different

laboratory studies that the concentration of P in the soil solution, which is the form of P for plant uptake, after adding sewage sludge is controlled by the forms of P in sludge which depend on the origin and the treatment process of sludge (Frossard et al., 1996; Maguire et al., 2001). However, very little is known on changes in other soil P forms, and processes that are often driven by functional traits of plants and soil microorganisms (e.g., mycorrhizal colonisation, release of extracellular enzymes involved in P cycling) (Mackay et al., 2017b; Requejo and Eichler-Löbermann, 2014).

In addition to supplying P to the plant-available soil P pool through dissolution of inorganic P and mineralisation of organic P, sludge application can also alter P dynamics in soil by modifying abiotic and biotic soil properties (Faucon et al., 2015). Like other organic amendments, the presence of sludge in soil may enhance the activity of phosphatases (Bastida et al., 2008). Phosphatases are a broad group of enzymes that catalyse the hydrolysis of organic P, leading to the release of available ortho-phosphate. Recent findings have shown that organic P, rather than available P, is the most important P fraction in regulating phosphatase activity in soil (Margalef et al., 2017). Transformation of organic P through enzymatic reactions and its subsequent potential immobilisation by microbial biomass play a fundamental role in P dynamics, and is likely affected by the source of P (Saha et al., 2008). However, although studies about the effects of sewage sludge addition on soil biological properties have been numerous, they generally focused on one type of sludge (Criquet et al., 2007) and were carried out under controlled conditions, which do not fully mimic field scenarios. As a result, the extent of biological processes involved *in situ* in P cycling (i.e. organic P hydrolysis, P immobilisation) under different sludge sources has not been clearly identified, and it appears difficult to generalise previous results to different types of sewage sludge applied under field conditions.

Given the interest in using sewage sludge for P fertilisation, it is crucial to find out how its properties mediate both chemical and microbial processes affecting P cycling in

agroecosystems. In particular, unravelling the role of sludge application in changes in soil P forms over time by characterising both chemical and microbial soil properties involved in P dynamics may shed light on the pathway of P release in sludge-amended soils. Ultimately, this knowledge helps predict the prospective P availability and establish the dose, frequency, and timing of sludge application. Therefore, this study aimed at gaining a better insight into the effects of sludge application on P lability and availability under field conditions, taking into account the impacts on soil microbial activities. We monitored shifts in soil P fractions over time (Hedley fractionation method; Tiessen and Moir, 2008) and determined phosphatase activity and microbial P in a field experiment using soil amended with two types of sewage sludge with different P concentrations. In order to estimate the potential of sludge to supply plant-available P relative to that by conventional P fertilisers, we also performed treatments with triple superphosphate (TSP). We hypothesised that i) changes in soil P availability would be driven by P composition of the sludge, and ii) sludge would increase microbial alkaline phosphatase activity, possibly resulting in higher P availability over time.

2. Materials and methods

2.1. Sewage sludge properties

Two types of sewage sludge treated in different processes were provided by the Parisian public sanitation service (Seine Aval wastewater treatment plant, SIAAP, Paris). The first sludge (hereafter called heated sewage sludge, HS) was derived from a treatment process comprising P precipitation using ferric chloride, an anaerobic digestion, followed by a dewatering by thickening and thermal conditioning (heat exchange and heating at 195° C and 20 bars). The second sludge (hereafter called composted sewage sludge, CS) was treated by anaerobic digestion followed by composting. Four biochemical fractions were determined using

a modified Van Soest method (AFNOR, 2009): soluble (SOL), hemicellulose-like (HCE), cellulose-like (CEL) and lignin-like (LIG). The indicator of residual organic carbon (C), I_{ROC} , which represents the proportion of stable organic C was then calculated as (Lashermes et al., 2009):

$$I_{ROC} = 44.5 + 0.5 \times \text{SOL} - 0.2 \times \text{CEL} + 0.7 \times \text{LIG} - 2.3 \times \text{MinC3}$$

where I_{ROC} , SOL, CEL and LIG are expressed in % total organic C in sludge and MinC3 is the proportion (%) of mineralised organic C during the first three days of incubation (AFNOR, 2009). Organic C concentration, C/N ratio (AFNOR, 2013, 2012) and water-soluble P (WSP) concentrations (García-Albacete et al., 2012) were also determined. Phosphorus forms in sewage sludge which were determined through the SMT (Standards, Measurements and Testing programme) protocol. The SMT method is a harmonised protocol proposed by the European Commission for sequential extraction of P initially in sediments which was then extended to other materials, including sewage sludge (García-Albacete et al., 2012; Medeiros et al., 2005). Briefly, inorganic P (IP) was extracted with 1 M HCl for 16 h and residues of this extraction were calcined for 3 h at 450 °C and then again extracted with 1 M HCl for organic P (OP). Non-apatite inorganic P (NAIP) associated with oxides and hydroxides of Fe, Al or Mn was extracted with 1 M NaOH for 16 h, and then some part of this extract was treated with 3.5 M HCl. The residues of this extraction were extracted for apatite P (AP) associated with Ca (Ca-P) with 1 M HCl for 16 h. Total P (TP) is the sum of OP and IP. As shown in Table 1, HS contains three times more total P than CS, while AP, NAIP and OP concentrations are 2.6, 11.4 and 2.8 times greater in HS than in CS.

2.2. *Study site and experimental design*

A field experiment was established in September 2015 in Beauvais, North of France (49°28'N; 2°4'W). The experimental site is included in a long-term (> 20 years) cropland field with an oilseed rape – winter wheat – winter barley rotation and an organic and mineral fertilisation based on soil tests, crop requirements, and timed to crop uptake. Cattle manure (25 Mg ha⁻¹) and lime amendments were applied, respectively, every three years, and six years before this experiment. Reduced tillage (5 cm of soil deep rotary harrow) was practiced since 2010. The oceanic climate is characterised by an average precipitation of 669 mm year⁻¹. Average minimum and maximum temperatures vary from 1 to 6.7°C in winter, 5 to 14.5°C in spring, 12 to 23°C in summer and from 7.2 to 15.3°C in autumn. The studied soil was classified as a Haplic Luvisol (IUSS Working Group WRB, 2015). The soil texture was a silt loam with 12% sand, 66% silt and 22% clay. The soil pH_{H2O} was 7.53, the organic carbon concentration was 24 g kg⁻¹ and the cation exchange capacity was 15 cmol_c kg⁻¹. Given its soil properties and long-term fertilisation and cropping history, the study site is considered representative of the agricultural areas receiving the SIAAP sludge.

Five treatments were tested: two sludge amendments (HS and CS) were applied at a rate of 4.78 Mg dry matter ha⁻¹ (i.e. 7.5 Mg fresh matter ha⁻¹), which is based on common practices of farmers who use SIAAP sludge. Two mineral controls (triple superphosphate) adding the same quantity of P as in each sludge were also included (MHS for HS and MCS for CS) as well as a control without P fertilisation (C). A randomised complete block with four replicates per treatment was used. Each plot had an area of 40 m² (10 x 4 m) as used in Gallet et al. (2003). The experimental field had an area of 1000 m². The homogeneity of soil properties within the study area was previously assessed by a topsoil (0-5 cm) sampling (20 samples collected) and chemical characterisation. Before spreading sludge, the soil was tilled with a stubble cultivator in order to make the spreading homogeneous (5 cm depth). All the treatments were carried out

on the 15th of September 2015. Then, the soil was again tilled with a harrow to incorporate the sludge following the French legislation. Winter barley (Viva, two-rowed cultivar) was sown on the 9th October 2015. Nitrogen application of 130 kg ha⁻¹ was based on a nitrogen balance calculation.

2.3. *Plant sampling and analysis*

Plants were harvested on 26th June 2016 and the crop was weighed on board of a harvester. After harvest, plants were dried at 70°C for 72 h, weighed and then ground to 250 µm prior to analysis. Phosphorus concentrations in straw and grain were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Scientific XSERIES2) after digesting the dried biomass in *aqua regia*. Briefly, 0.2 g of plant powder was mixed with 8 mL of concentrated HNO₃ and 2 mL of concentrated HCl directly in a microwave Teflon vessel (Lange et al., 2014) with a control without plant. The vessels (Easyrep 432175A, CEM µWaves) were placed in the microwave system (Mars 5, CEM Corporation, Charlotte, USA). The digestion programme consisted of a ramp time of 5 min to reach 180 °C and digestion was performed for 15 min. The power was set at 1600 W (Lange et al., 2014). The digest was then diluted to approximately 30 g (accurately weighed) and then stored until being analysed with ICP-MS.

2.4. *Soil sampling and analysis*

Two composite soil samples were collected at the surface (0 – 10 cm) within each plot in September (before and one week after fertilisation), January (during winter), May (during spring) and July (a few days after harvesting) to characterise P fractions, Olsen-P and soil chemical properties. Subsamples were used either fresh for microbial P and alkaline

phosphatase activity (May and July) analyses or oven-dried (35°C, 48 h), crushed and sieved through a 2-mm plastic sieve. Soil pH and electrical conductivity were measured in suspensions by shaking 2 g of soil with water (1:5 ratio). The Hedley et al. (1982) sequential fractionation method as modified by Tiessen and Moir (2008) was used to fractionate soil P. This method uses a sequence of increasingly strong extractants that remove labile inorganic P (Pi) and organic P (Po) forms first, then stable P forms. Briefly, 0.5 g of air-dried soil (ground to pass through a 2 mm sieve) was successively extracted with resin strips in deionised water (plant-available Pi), 0.5 M NaHCO₃ (pH 8.5) (Pi and Po adsorbed onto the soil surface), 0.1 M NaOH (Pi and Po held more strongly by sorption to surfaces of Al and Fe oxides), 1 M HCl (P associated to Ca, derived from primary mineral-apatite). The last fraction (residual-P; stable Po forms and relatively insoluble Pi forms) was determined using a microwave digestion (Mars 5, CEM Corporation, USA) with *aqua regia* and hydrofluoric acid, EN 13 656 (Gaudino et al., 2007). Labile P was considered the sum of resin-P and NaHCO₃-extracted P (Cross and Schlesinger, 1995; Tiessen and Moir, 2008). The Pi concentration in the extracts was determined colourimetrically using the molybdate blue method according to Murphy and Riley (1962) at 712 nm, as recommended by Tiessen and Moir (2008) to reduce possible interference from traces of organic matter. Total P (P_{tot}) in the extracts was determined by ICP-MS and the concentration of Po was estimated by subtracting Pi from P_{tot}. In addition to Hedley's fractionation, Olsen-P concentration was determined (Olsen, 1954).

2.5. *Microbial phosphorus*

Microbial P was determined by hexanol fumigation (Bergkemper et al., 2016) and extraction with anion-exchange membranes (VWR, 551642S). Anion-exchange membrane strips were prepared by initially shaking in 0.5 M NaHCO₃. For each sample, three portions of fresh soil (2 g on a dry-weight basis) were weighed into 50 mL bottles with 30 mL deionised

water and two anion-exchange membrane strips. One bottle received 1 mL of hexanol and the samples were shaken for 16 h. The membranes were then removed and rinsed in deionised water and the phosphate ($P_{\text{fumigated}}$) recovered by shaking for 1 h in 20 mL (HCl 0.5 M). Another bottle only contained water and was treated the same way as previously ($P_{\text{non-fumigated}}$). To determine the amount of P retained by soil particles and complexation after fumigation incubation, a defined P concentration (KH_2PO_4), which was equal to the measured P concentrations in fumigated subsamples, was added to additional non-fumigated, but otherwise identically treated subsamples. Phosphorus concentrations were analysed colourimetrically (Murphy and Riley, 1962) using a spectrophotometer (712 nm). The ratio of recovered P to added P was used to calculate the P_{mic} concentration as follows (Nassal et al., 2018):

$$P_{\text{mic}} [\mu\text{g g}^{-1}] = \frac{(P_{\text{fumigated}} [\mu\text{g g}^{-1}] - P_{\text{non-fumigated}} [\mu\text{g g}^{-1}])}{(P_{\text{recovered}} [\mu\text{g g}^{-1}] / P_{\text{added}} [\mu\text{g g}^{-1}])}$$

2.6. Alkaline phosphatase activity

According to the pH of the soil (7.53), alkaline phosphomonoesterase (alkaline phosphatase) activity was assayed as an enzyme involved in P cycling. Alkaline phosphatase activity was assayed by the method of Tabatabai and Bremner (1969), which involves the determination of p-nitrophenol released by incubation at 37°C for 1 h of 1 g soil with 0.2 ml toluene, 4 ml universal buffer and 1 ml substrate. Sodium p-nitrophenyl phosphate was used as substrate for assay of phosphatase activities (Eivazi and Tabatabai, 1977). The quantity of p-nitrophenol produced by alkaline phosphatase was measured using a spectrophotometer (410 nm).

229

230 2.7. *Statistical analyses*

231 All recorded data were analysed using descriptive statistics (mean $\pm$ standard error) and
232 normality was determined using the Shapiro-Wilk test. The data were subjected to one-way
233 ANOVA and Tukey's post-hoc test to compare treatments, which had a normal distribution.
234 Data without normal distribution were subjected to the Kruskal-Wallis test and Mann-Whitney
235 post-hoc test Two-way ANOVAs were performed considering time as a fixed factor and
236 fertilisation type as variable. All statistical analyses were performed using R software version
237 3.5.0. (R Core Team, 2017) and the package Rcmdr (Fox, 2005).

238

239 **3. Results**

240 *3.1. Sewage sludge properties*

241 Table 1 indicates that CS and HS had a similar I_{ROC} and C/N ratio, while HS was slightly
242 more alkaline than CS, and had a higher C concentration. For each sludge, NAIP was the
243 predominant P form. The proportion of AP was slightly higher in HS, while the proportions of
244 WSP and OP were similar between both sludges. More importantly, the total P concentration
245 in HS was three times as high as that in CS, suggesting that the main difference between both
246 sludges is not P fraction, but total P concentration.

247 *3.2. Changes in labile P concentration over time*

248 The effect of sludge addition on the labile P concentration (i.e. the sum of resin-P and
249 $NaHCO_3$ -P concentration) was strongly affected by the type of sludge applied (Fig. 1). After
250 124 days, the labile P concentration in the presence of HS was greater than that of the no-P
251 control (C), but lower than the mineral control (MHS) ($\chi^2=9.85$, $df=2$, p value <0.01). However,

after 240 days, the labile P concentration in HS was as high as that in MHS ($\chi^2= 8.77$, $df=2$, p value <0.05). Unlike HS, labile P concentrations in the presence of CS were similar to that of C ($p > 0.05$) and significantly lower ($p < 0.05$) than its mineral control (MCS) for the duration of the experiment (Fig. 1). Despite differences in labile P concentrations among fertilisation treatments, P concentrations and quantity in shoot (straw) and grain were not significantly different among treatments (Supplementary Fig. 1, 2, 3).

3.3. *Phosphorus forms and microbial-P in soil*

Among the studied forms, only HCl-P was unaffected by fertilisation treatment, while only Resin-P was unaffected by time (Table 2). NaHCO_3 -P, NaOH-P and residual P fractions were significantly affected by fertilisation treatment and also by time (Table 2). After 307 days (Fig. 2), HS significantly increased the NaHCO_3 -Pi fraction and decreased the residual-P fraction compared with MHS and C. The NaOH-Po fraction was also significantly greater in the presence of HS compared with that in the other treatments. Moreover, only HS significantly increased the concentration of microbial-P in the soil (Fig. 3). In contrast with HS, CS had no effect on P forms in soil compared with both C and its mineral control (MCS).

3.4. *Soil pH*

Soil pH was influenced by fertilisation treatment and time (Table 2). The soil amended with MHS was significantly ($p < 0.05$) more acidic over the entire experimental period, while the pH did not differ significantly among the other treatments (Supplementary Fig. 4).

3.5. *Alkaline phosphatase activity*

Alkaline phosphatase activity was significantly increased by the application of HS while no significant difference was observed for the other treatments (Fig. 3).

4. Discussion

Most of previous studies investigating the P fertiliser potential of waste products such as sewage sludge have only focused on change in available P concentration after their application, disregarding how other soil P forms are affected. Here, our findings suggest, however, that P availability may change with time due to chemically and biologically controlled shifts in soil P fractions, which are themselves mediated by P concentrations in sludge.

4.1. *Effect of sludge on labile P concentration over time*

Monitoring labile P concentration over time is necessary to ensure that P supplies will meet crop demand. Labile P can be continuously supplied to the soil solution, for instance, by organic P mineralisation through microbial activity and by mineral P desorption through chemical reactions (Chen et al., 2004; Sanyal and Datta, 1991). Chemical extractions have been extensively used to evaluate P lability in soils (Sharpley, 2009), even though they may be inappropriate in detecting changes produced by natural environmental soil modification such as cycles of alternating oxidation and reduction conditions (Scalenghe et al., 2014). Here, as recommended by Cross and Schlesinger (1995), we consider labile P to be the sum of resin-extractable P + NaHCO₃-extractable P. According to these authors, P extracted by ion exchange resins and NaHCO₃ solutions represents the most likely contributors to plant-available P over

the course of a growing season, because it cycles readily through the microbial community and is made available to plant roots through a variety of chemical and biological processes. Our results reveal that HS significantly increased the labile P concentration over time (by 100% after 124 days). After 240 days, the HS treatment showed the same concentration of labile P as the mineral control (MHS). According to Huang et al. (2012), who observed an increase in P availability in sludge-amended soil after 100 days of incubation, the strong increase of labile P concentration in the presence of HS likely results from the continuous mineralisation of its large organic P pool (9.49 g kg⁻¹). This increase in P availability with time suggests that sludge with a high organic P concentration must be regarded as a slow-release P fertiliser. This slow-release P fertiliser behaviour has also been reported by Lemming et al. (2017). The authors showed that when applying a sludge similar to HS to a soil with pH 7.8, the efficiency in terms of available P relative to TSP increased from 20% to 46% after 84 days. Since many crops have high P demands in the early stage of their life cycle (Fageria, 2016), for example wheat plants take up 50-60% of their P in the first six weeks of growth (Römer and Schilling, 1986), there is a risk that the slow release of P from HS negatively impacts early plant growth. This might, however, be compensated by adding fertiliser mixtures of soluble P (e.g., TSP) and HS. In contrast with HS, CS did not influence the available P concentration. Since both sludges did not differ in their P fractions, it is likely that the much lower total P concentration in CS was the predominant driver of the lack of increase in P availability after addition to the soil (Welch et al., 2002). Due to its low total P concentration, the potential of CS to be used as a P fertiliser is thus limited, at least during the first year after its application, in such cropping systems. One possibility to mobilise the sparingly available CS-derived P might be to develop cropping systems with species having highly efficient P-acquisition strategy such as *Lupinus albus* L. (e.g., by intercropping it with cereals) (Hallama et al., 2019; Lambers et al., 2013). Further researches are however needed to determine to what extent this would counteract the limited P fertiliser

potential of CS. Taken together, our results indicate that the efficacy of sludge to increase P availability in soils would depend on the concentration and the forms of P added and the cropping system used.

It is important to note that the lack of any improvement of P concentration and quantity in plants in spite of the increase in available P concentration in soil after the addition of HS likely results from the already high P concentrations in these soils as a result of the long-term addition of P fertiliser (Gallet et al., 2003). According to Glæsner et al. (2019), a significant increase in P uptake might be expected in the long run, as P applied with sludge remained highly plant available in the soil after long-term application.

4.2. *Phosphorus forms and concentrations in sludge influence P forms in soil*

Interactions between P forms in sludge and P forms in soil are not fully understood so far, most studies focusing only on total and water-extractable P concentrations. As shown by other researchers using pot experiments (Frossard et al., 1996; Meyer et al., 2018; Nanzer et al., 2014), our results indicate that coupling the determination of P concentration and forms in sludge is, however, essential. Indeed, depending on total P concentration, P forms in sludge drive P distribution in various soil pools, which, in turn, impact P availability. By contrast to HS, CS had no effect on P distribution in soil pools compared with its mineral control (MCS). Since CS and HS had a relatively similar P composition (OP is ca 20% while insoluble AP+NAIP is ca 80% in both sludges), the lack of any effect of CS is thus most likely related to its much lower total P concentration.

The application of HS increased the $\text{NaHCO}_3\text{-P}$ pool, which is considered readily available to plants and is strongly related to P uptake by most crops (Saleque et al., 2004). However, some crops with highly efficient P-acquisition strategies (e.g., white lupine, canola)

can access additional P fractions (Gardner et al., 1983; Hoffland et al., 1989). These results confirm the high potential of HS as a P fertiliser.

The application of HS also strongly increased the NaOH-P (Pi +Po) concentration. The NaOH-Pi pool is considered P sorbed to Al and Fe (hydr)oxides while NaOH-Po has been found to be predominantly associated with organic matter, namely fulvic and humic acids (Cassagne et al., 2000; Schroeder and Kovar, 2006; Tiessen and Moir, 2008). Although Al and Fe oxides are important P-sorbing components in soil (Hinsinger, 2001; Houben et al., 2011), it is unlikely that they act as a significant sink for P added by HS, since, even in the mineral controls, the fraction of P in the NaOH-Pi was not significantly increased compared with the control. The higher proportion of NaOH-Pi in HS-amended soil might be due to the high NAIP content in HS which results from the pre-treatment of this sludge (i.e. iron chloride precipitation) (Kahiluoto et al., 2015). On the other hand, the significantly higher NaOH-Po fraction following the application of HS probably results in part from the high concentration of organic P in this sludge (Malik et al., 2013; Smith et al., 2006). As a slowly exchangeable P pool (Frossard et al., 2000), the NaOH-P pool can be mobilised when P in soil solution is depleted from the soil by plant uptake (Guo et al., 2000; Saleque et al., 2004). Beck and Sanchez (1994) reported that NaOH-P is the dominant pool related to availability of P to plants in an 18-year continuously-cultivated and fertilised cropping system because it maintains the levels of plant-available P through P mineralization. According to Crews and Brookes (2014), the NaOH-P pool holds P for years to decades before crop demand shifts the equilibrium in the soil solution and causes P to be released. In addition to increasing the readily-available P pool in soil, our findings suggest that the addition of HS might also supply available P in the long run. Further investigations will however, be necessary to determine the contribution of the NaOH-P pool to long-term P release and thus better predict the dynamics of P in sewage sludge-amended soils.

4.3. *Phosphorus in microbial biomass*

Microbial biomass plays a key role in P availability in agroecosystems. It acts as a buffer by immobilising P from the soil solution and potentially preventing it from bonding with soil particles (Crews and Brookes, 2014). Microbial P is released when cells are disrupted, e.g., in response to sudden changes in soil temperature, water content, and carbon availability (Turner et al., 2003) or due to predation (Bonkowski, 2004), which results in an increase in available P (Oehl et al., 2001). Our results indicate that sludge application may have a strong impact on the microbial-P pool. By contrast to CS, which showed a similar microbial-P pool to its mineral control (MCS), HS application significantly increased the microbial-P pool compared with its mineral control (MHS). Andriamananjara et al., (2016) also observed a significant increase in microbial-P concentration after sewage sludge application compared with TSP. Consistently with Crews and Brookes (2014), this can be related to the increase of the NaOH-P_o fraction brought about by this treatment, resulting in a stronger assimilation of P into soil microorganisms. Two different mechanisms might lead to the enrichment of P in soil microorganisms: 1. Soil microorganisms might grow faster due to the accelerated decomposition of organic P compounds, or 2. Soil microorganisms might change the stoichiometry of their cells towards a lower C/P ratio. Independently on the mechanism of P accumulation of soil microorganisms, the mean residence time of P in soil microorganisms is only 18 to 39 days and depends on soil P availability (Spohn and Widdig, 2017). Therefore, soil microorganisms represent a short-term storage of easily-available P fractions in soil, and store P somewhat longer in the case of restricted P access from soils. The P fraction stored in soil microorganisms is not only released by death of soil microorganisms, but also by the feeding behaviour of soil animals that require carbon resource to a higher extent than nutrients like P and N. This phenomenon, which was first described by Clarholm (1985) for protozoa, is now extended to a wider group of soil animals (protists, nematodes) and to other nutrients like

P (Trap et al., 2016). Consequently, the importance of food webs in agroecosystems for biotic release of P derived from organic resources (like sludge) needs further study (Richardson and Simpson, 2011). Additional work including the characterisation of microbial biomass and structure of microbial community using phospholipid fatty acid analysis, sequencing and quantification of genes involved in P dynamic in soil (i.e. *phoB* and *phoR*) (Anderson et al., 2011) might be of help to better understand this microbial P increase in the presence of sludge.

4.4. *Effect of sludge on alkaline phosphatase activity*

The relationship between phosphatase activities and P in soil amended with sludge is still poorly understood, despite extensive investigations (Xie et al., 2011b). Phosphatase activities are expected to increase after application of organic matter, resulting in higher P availability in soil (Garg and Bahl, 2008). However, the response of phosphatase activities to applied organic waste also depends upon its initial constituents, amount and size, and must be investigated prior its application (Criquet et al., 2007; Saha et al., 2008; Tejada et al., 2008; Xie et al., 2011b) (Criquet et al., 2007; Saha et al., 2008; Tejada et al., 2008). Increases of alkaline phosphatase activity after the application of organic amendments have been primarily attributed to both the supply of easily decomposable organic compounds and change in soil pH (Dick et al., 2000; Garg and Bahl, 2008). In our study, application of HS did not affect soil pH. For this treatment, it can therefore be inferred that the supply of large amounts of substrates, including substrates for phosphatases (Bachmann et al., 2014), was the main process responsible for the stimulation of enzyme production by soil microorganisms (including mycorrhizal fungi). The stimulation of phosphatase activity is largely due to increased microbial numbers in the soil which, with time, cause a build-up of enzymes (Feder, 1973). Therefore, the higher alkaline phosphatase activity in the presence of HS is consistent with the higher NaOH-P_o fraction and the subsequent increase in microbial-P concentration measured under this treatment. Unlike HS,

CS application did not increase alkaline phosphatase activity, most likely because it did not increase the soil organic P pool due to its low total P concentration, as discussed above (Margalef et al., 2017; Requejo and Eichler-Löbermann, 2014). In line with the recent efforts directed toward increasing P availability by mobilising recalcitrant soil P (Menezes-Blackburn et al., 2017), gaining insight into the factors responsible for the higher phosphatase activity in the presence of HS might help develop strategy to transform organic P to inorganic P available for plants. More generally, our data are consistent with the literature showing a positive effect of sewage sludge on the soil enzyme activities, with a positive correlation between rate of sludge application and enzyme activity (Fernandes et al., 2005; Siebielec et al., 2018).

4.5. *Implications*

Moving toward more sustainable sources for managing the P nutrition in agroecosystems, it is increasingly suggested to replace mineral fertilizers by P-rich materials originating from waste materials (Dawson and Hilton, 2011). In addition to studying the release of available P from these soil amendments, it is however essential to gain further insights into their indirect effects on the soil P pools, especially by paying attention to modifications of soil biota (Faucon et al., 2015). Here, our results showed that changes in soil biological properties after the addition of sewage sludge with high total P concentration (and 20% as organic P) contributed to increase the labile P concentration to a level as high as that of conventional fertiliser. It is necessary, however, to conduct further studies to identify the relative contribution of soil-derived P and sludge-derived P to this P lability increase, especially through the use of isotopic labelling techniques.

5. **Concluding Remarks**

This study showed that the impact of sewage sludge with similar P fractions on P dynamics in agroecosystem is predominantly driven by total P concentration in sludge. Our findings suggest that, despite a relatively low water-soluble P concentration, the addition of sewage sludge with high total P concentration and 20% of total P as organic P has a great potential to improve sustainable P fertilisation, since it increased alkaline phosphatase activity and microbial-P, temporarily protecting P from fixation by soil particles. The positive effect of sludge with high total P concentration (and 20% as organic P) on the microbial-P pool and phosphatase activity is a pivotal result to understand the effects of organic waste on agroecosystem functioning, and, ultimately, to improve sustainable P fertilisation. Overall, our results stress the need that sludge provider measure both P concentration and P forms in sludge prior to their application in the field. The perspective is to elucidate the sludge's long-term effects on P pools and availability over the whole crop rotation in several soil types to define new sustainable practices of P fertilisation by taking into account both microbial and plant functional traits involved in P mobilisation/acquisition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

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Figures

Figure 1. Release of labile phosphorus (resin-P + NaHCO₃-extractable P) over time. C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge. Different letters indicate significant differences between treatments ($P < 0.05$). Capital letter indicate differences between composted-sludge/Mineral control of CS/ No-P control, lower case letter between heated sludge/ Mineral control of HS/ No-P control. Values are the mean of four replicates $\pm$ standard error. Significant differences have been tested using Kruskal Wallis test for the comparison HS-MHS-C and One Way ANOVA for the comparison CS-MCS-C.

Figure 2. Effect of sewage sludge application on phosphorus (P) fractionation (Hedley scheme) after 307 days. C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge. The means with the same letters are not significantly different. Values are the mean of four replicates $\pm$ standard error. Significant differences has been revealed by a Kruskal-Wallis test with a Mann-Whitney post hoc test (Resin-P and NaOH-P) or One Way ANOVA with Tukey post hoc test. Phosphorus fractions are ordered by their availability (highest for Resin-P and lowest for Residual-P). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Figure 3. Alkaline phosphatase activity (left y-axis) and microbial-phosphorus (P) (right y-axis) after 307 days. C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge. Significant differences has been revealed using a Kruskal Wallis test with a Mann Withney post hoc test (for alkaline phosphatase) and One Way ANOVA followed by Tukey post hoc test. Values are the mean of four replicates $\pm$ standard error. The means with the same letters are not significantly different at the 5 % level.

■ Resin-P
 ■ NaHCO₃-Pi
 ■ NaHCO₃-Po
 ■ NaOH-Pi
 ■ NaOH-Po
 ■ HCl-P
 ■ Residual-P

Tables

Table 1. Chemical properties of sludge. I_{ROC} : proportion of stable organic matter; Corg: organic carbon; WSP: Water-soluble phosphorus; AP: Apatite phosphorus; NAIP: Non-apatite inorganic phosphorus; OP: Organic phosphorus. Carbon and P concentrations are expressed in dry matter of sludge ($\text{g kg}^{-1}\text{MS}$). Values in parentheses represent the percentage of P in each fraction (%).

Sludge	I_{ROC}	pH	Corg (g kg^{-1})	C/N ratio	WSP (g kg^{-1})	AP (g kg^{-1})	NAIP (g kg^{-1})	OP (g kg^{-1})	Total P (g kg^{-1})
CS	79.9%	7.4	185	9.5	0.03 (0.2)	12.46 (74.9)	0.83 (5.0)	3.32 (19.9)	16.63
HS	80.8%	8.4	251	12.3	0.08 (0.2)	32.18 (63.2)	9.49 (18.6)	9.17 (18.0)	50.92

Table 2. Summary of Two Way ANOVA analysis.

	Treatment				Time				Treatment x Time			
	Df	MS	F	P	Df	MS	F	P	Df	MS	F	P
pH	4	0.7383	14.346	***	4	0.493	9.581	***	16	0.0479	0.931	NS
Olsen-P	4	128613	104.216	***	4	52 886	42.854	***	16	9489	7.689	***
Resin-P	4	296.34	56.393	***	3	3.33	0.634	NS	12	5.44	1.035	NS
$\text{NaHCO}_3\text{-P}$	4	91.25	27.183	***	3	199.67	59.478	***	12	5.37	1.599	NS
NaOH-P	4	89.77	29.82	***	3	23.41	7.776	***	12	5.64	1.874	NS
HCl-P	4	56.92	1.898	NS	3	153.35	5.114	**	12	18.57	0.619	NS
Residual-P	4	658.5	18.343	***	3	577	16.073	***	12	47.3	1.316	NS

NS: non significant; **: $P < 0.01$; ***: $P < 0.001$

Supplementary Materials

Response of phosphorus dynamics to sewage sludge application in an agroecosystem in northern France

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Supplementary Figure 1. Phosphorus concentration in winter barley at crop maturity.

C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge. Values are average ($n = 4$) $\pm$ standard error. Columns with the same letter do not differ significantly at the 5% level.

Supplementary Figure 2. Dry biomass at crop maturity. C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge. Values are average ($n = 4$) $\pm$ standard error. Columns with the same letter do not differ significantly at the 5% level.

Supplementary Figure 3. Phosphorus exported by shoot at full tillering and flowering stages. C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge. Values are average ($n = 4$) $\pm$ standard error. Columns with the same letter do not differ significantly at the 5% level.

Supplementary Figure 4. Soil pH. C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge.