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► **To cite this version:**

David Houben, Etienne Michel, Cécile Nobile, Hans Lambers, Ellen E. Kandeler, et al.. Response of phosphorus dynamics to sewage sludge application in an agroecosystem in northern France. *Applied Soil Ecology*, 2019, 137, pp.178-186. 10.1016/j.apsoil.2019.02.017 . hal-02176387

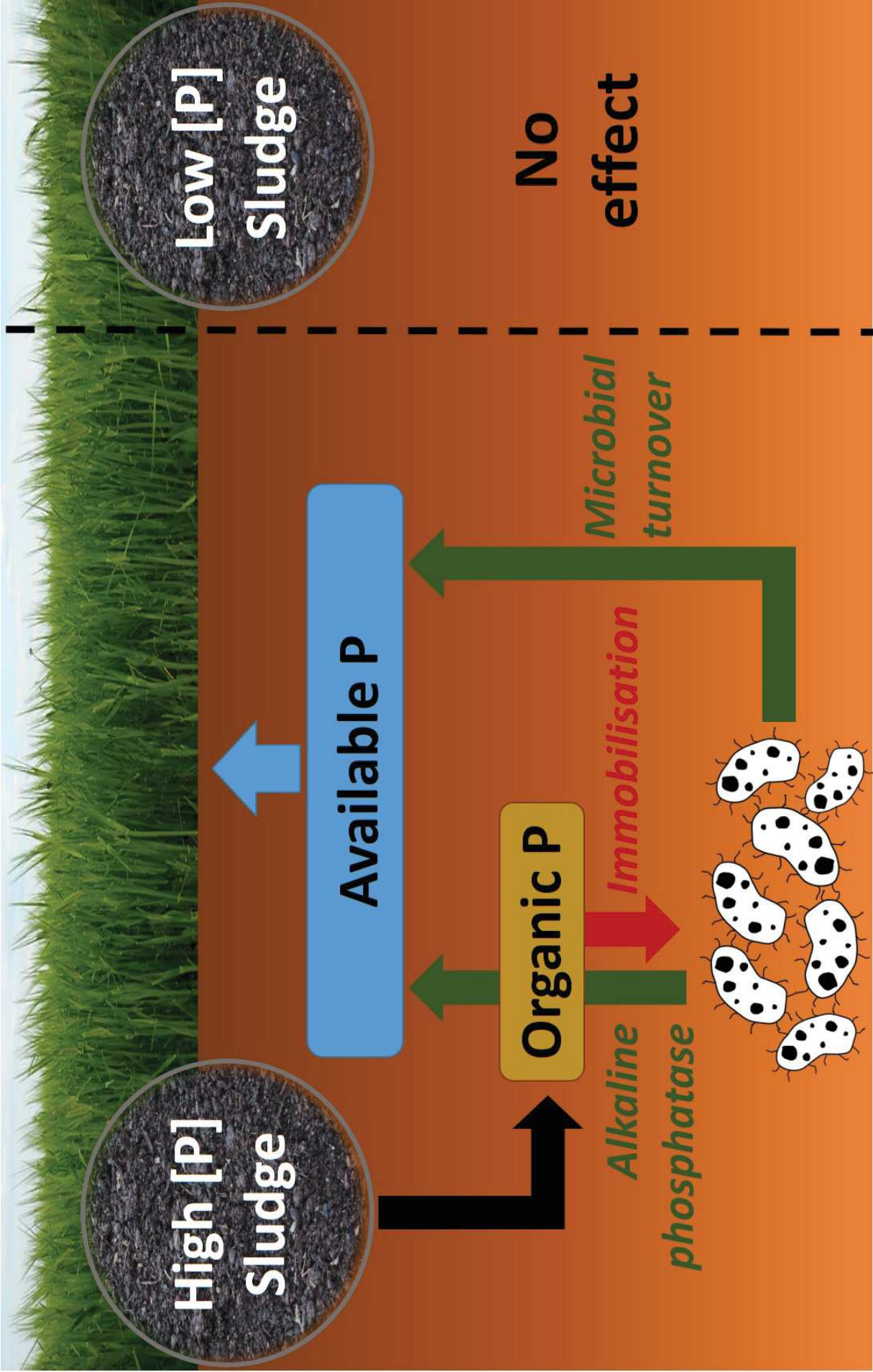
HAL Id: hal-02176387

<https://hal.science/hal-02176387>

Submitted on 8 Jul 2019

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High [P]
Sludge

Low [P]
Sludge

Available P

Organic P

Alkaline
phosphatase

Immobilisation

Microbial
turnover

No
effect

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
8 sludge, HS and composted sludge, CS) affect soil P pools and dynamics. A field experiment
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

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

24

25 **Keywords**

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

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36 **1. Introduction**

37 Phosphorus (P) is a limiting nutrient for the productivity of many agroecosystems and
38 indispensable to feed an ever-increasing human population (Filippelli, 2008; Withers et al.,
39 2015). Currently, most of the P used in chemical fertilisers is derived from phosphate rocks that
40 are finite and located in only a few places on Earth (Edixhoven et al., 2013; Reijnders, 2014).
41 Developing sustainable fertilisation practices based on the use of renewable resources such as
42 P-rich waste is thus essential to ensure long-term food security (Dawson and Hilton, 2011;
43 Houben et al., 2017). Among P-rich wastes, sewage sludge appears an excellent candidate
44 because it represents the largest component of recycled P, and due to the world's population
45 growth, this reserve is expected to increase (Lwin et al., 2017). Its use in agriculture will likely
46 increase, requiring continued vigilance in assessing the impacts of the presence of potential
47 pollutants such as metals, organic contaminants (e.g., pharmaceuticals and personal care
48 products), emerging contaminants, viruses and other pathogens (Clarke and Smith, 2011). Since
49 1986, Council Directive No. 86/278/EEC has governed the use of sewage sludge in the
50 European Union by prescribing testing of sludge and soil for a number of potential pollutants.
51 This Directive has been implemented into the national legislation of member states, most often
52 with stricter limits than that prescribed in the Directive (Kirchmann et al., 2017).

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

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

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

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

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

100

101 **2. Materials and methods**

102 *2.1. Sewage sludge properties*

103 Two types of sewage sludge treated in different processes were provided by the Parisian
104 public sanitation service (Seine Aval wastewater treatment plant, SIAAP, Paris). The first
105 sludge (hereafter called heated sewage sludge, HS) was derived from a treatment process
106 comprising P precipitation using ferric chloride, an anaerobic digestion, followed by a
107 dewatering by thickening and thermal conditioning (heat exchange and heating at 195° C and
108 20 bars). The second sludge (hereafter called composted sewage sludge, CS) was treated by
109 anaerobic digestion followed by composting. Four biochemical fractions were determined using

110 a modified Van Soest method (AFNOR, 2009): soluble (SOL), hemicellulose-like (HCE),
111 cellulose-like (CEL) and lignin-like (LIG). The indicator of residual organic carbon (C), I_{ROC} ,
112 which represents the proportion of stable organic C was then calculated as (Lashermes et al.,
113 2009):

$$114 \quad I_{ROC} = 44.5 + 0.5 \times \text{SOL} - 0.2 \times \text{CEL} + 0.7 \times \text{LIG} - 2.3 \times \text{MinC3}$$

115 where I_{ROC} , SOL, CEL and LIG are expressed in % total organic C in sludge and MinC3 is the
116 proportion (%) of mineralised organic C during the first three days of incubation (AFNOR,
117 2009). Organic C concentration, C/N ratio (AFNOR, 2013, 2012) and water-soluble P (WSP)
118 concentrations (García-Albacete et al., 2012) were also determined. Phosphorus forms in
119 sewage sludge which were determined through the SMT (Standards, Measurements and Testing
120 programme) protocol. The SMT method is a harmonised protocol proposed by the European
121 Commission for sequential extraction of P initially in sediments which was then extended to
122 other materials, including sewage sludge (García-Albacete et al., 2012; Medeiros et al., 2005).
123 Briefly, inorganic P (IP) was extracted with 1 M HCl for 16 h and residues of this extraction
124 were calcined for 3 h at 450 °C and then again extracted with 1 M HCl for organic P (OP). Non-
125 apatite inorganic P (NAIP) associated with oxides and hydroxides of Fe, Al or Mn was extracted
126 with 1 M NaOH for 16 h, and then some part of this extract was treated with 3.5 M HCl. The
127 residues of this extraction were extracted for apatite P (AP) associated with Ca (Ca-P) with 1
128 M HCl for 16 h. Total P (TP) is the sum of OP and IP. As shown in Table 1, HS contains three
129 times more total P than CS, while AP, NAIP and OP concentrations are 2.6, 11.4 and 2.8 times
130 greater in HS than in CS.

131

132

133 2.2. *Study site and experimental design*

134 A field experiment was established in September 2015 in Beauvais, North of France
135 (49°28'N; 2°4'W). The experimental site is included in a long-term (> 20 years) cropland field
136 with an oilseed rape – winter wheat – winter barley rotation and an organic and mineral
137 fertilisation based on soil tests, crop requirements, and timed to crop uptake. Cattle manure (25
138 Mg ha⁻¹) and lime amendments were applied, respectively, every three years, and six years
139 before this experiment. Reduced tillage (5 cm of soil deep rotary harrow) was practiced since
140 2010. The oceanic climate is characterised by an average precipitation of 669 mm year⁻¹.
141 Average minimum and maximum temperatures vary from 1 to 6.7°C in winter, 5 to 14.5°C in
142 spring, 12 to 23°C in summer and from 7.2 to 15.3°C in autumn. The studied soil was classified
143 as a Haplic Luvisol (IUSS Working Group WRB, 2015). The soil texture was a silt loam with
144 12% sand, 66% silt and 22% clay. The soil pH_{H2O} was 7.53, the organic carbon concentration
145 was 24 g kg⁻¹ and the cation exchange capacity was 15 cmol_c kg⁻¹. Given its soil properties and
146 long-term fertilisation and cropping history, the study site is considered representative of the
147 agricultural areas receiving the SIAAP sludge.

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

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

162

163 2.3. *Plant sampling and analysis*

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

176

177 2.4. *Soil sampling and analysis*

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

182 phosphatase activity (May and July) analyses or oven-dried (35°C, 48 h), crushed and sieved
183 through a 2-mm plastic sieve. Soil pH and electrical conductivity were measured in suspensions
184 by shaking 2 g of soil with water (1:5 ratio). The Hedley et al. (1982) sequential fractionation
185 method as modified by Tiessen and Moir (2008) was used to fractionate soil P. This method
186 uses a sequence of increasingly strong extractants that remove labile inorganic P (Pi) and
187 organic P (Po) forms first, then stable P forms. Briefly, 0.5 g of air-dried soil (ground to pass
188 through a 2 mm sieve) was successively extracted with resin strips in deionised water (plant-
189 available Pi), 0.5 M NaHCO₃ (pH 8.5) (Pi and Po adsorbed onto the soil surface), 0.1 M NaOH
190 (Pi and Po held more strongly by sorption to surfaces of Al and Fe oxides), 1 M HCl (P
191 associated to Ca, derived from primary mineral-apatite). The last fraction (residual-P; stable Po
192 forms and relatively insoluble Pi forms) was determined using a microwave digestion (Mars 5,
193 CEM Corporation, USA) with *aqua regia* and hydrofluoric acid, EN 13 656 (Gaudino et al.,
194 2007). Labile P was considered the sum of resin-P and NaHCO₃-extracted P (Cross and
195 Schlesinger, 1995; Tiessen and Moir, 2008). The Pi concentration in the extracts was
196 determined colourimetrically using the molybdate blue method according to Murphy and Riley
197 (1962) at 712 nm, as recommended by Tiessen and Moir (2008) to reduce possible interference
198 from traces of organic matter. Total P (P_{tot}) in the extracts was determined by ICP-MS and the
199 concentration of Po was estimated by subtracting Pi from P_{tot}. In addition to Hedley's
200 fractionation, Olsen-P concentration was determined (Olsen, 1954).

201

202 2.5. *Microbial phosphorus*

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

207 water and two anion-exchange membrane strips. One bottle received 1 mL of hexanol and the
208 samples were shaken for 16 h. The membranes were then removed and rinsed in deionised water
209 and the phosphate ($P_{\text{fumigated}}$) recovered by shaking for 1 h in 20 mL (HCl 0.5 M). Another bottle
210 only contained water and was treated the same way as previously ($P_{\text{non-fumigated}}$). To determine
211 the amount of P retained by soil particles and complexation after fumigation incubation, a
212 defined P concentration (KH_2PO_4), which was equal to the measured P concentrations in
213 fumigated subsamples, was added to additional non-fumigated, but otherwise identically treated
214 subsamples. Phosphorus concentrations were analysed colourimetrically (Murphy and Riley,
215 1962) using a spectrophotometer (712 nm). The ratio of recovered P to added P was used to
216 calculate the P_{mic} concentration as follows (Nassal et al., 2018):

$$217 \quad P_{\text{mic}} [\mu\text{g g}^{-1}] = \frac{(P_{\text{fumigated}} [\mu\text{g g}^{-1}] - P_{\text{non-fumigated}} [\mu\text{g g}^{-1}])}{(P_{\text{recovered}} [\mu\text{g g}^{-1}] / P_{\text{added}} [\mu\text{g g}^{-1}])}$$

218

219 2.6. Alkaline phosphatase activity

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

228

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 $\text{NaHCO}_3\text{-P}$ 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) ($\chi^2= 9.85$, $\text{df}=2$, $p \text{ value}<0.01$). However,

252 after 240 days, the labile P concentration in HS was as high as that in MHS ($\chi^2= 8.77$, $df=2$, p
253 $value < 0.05$). Unlike HS, labile P concentrations in the presence of CS were similar to that of C
254 ($p > 0.05$) and significantly lower ($p < 0.05$) than its mineral control (MCS) for the duration of
255 the experiment (Fig. 1). Despite differences in labile P concentrations among fertilisation
256 treatments, P concentrations and quantity in shoot (straw) and grain were not significantly
257 different among treatments (Supplementary Fig. 1, 2, 3).

258

259 3.3. *Phosphorus forms and microbial-P in soil*

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

268

269 3.4. *Soil pH*

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

273

274

275

276 3.5. *Alkaline phosphatase activity*

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

279

280 **4. Discussion**

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

286

287 4.1. *Effect of sludge on labile P concentration over time*

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

298 the course of a growing season, because it cycles readily through the microbial community and
299 is made available to plant roots through a variety of chemical and biological processes. Our
300 results reveal that HS significantly increased the labile P concentration over time (by 100%
301 after 124 days). After 240 days, the HS treatment showed the same concentration of labile P as
302 the mineral control (MHS). According to Huang et al. (2012), who observed an increase in P
303 availability in sludge-amended soil after 100 days of incubation, the strong increase of labile P
304 concentration in the presence of HS likely results from the continuous mineralisation of its large
305 organic P pool (9.49 g kg⁻¹). This increase in P availability with time suggests that sludge with
306 a high organic P concentration must be regarded as a slow-release P fertiliser. This slow-release
307 P fertiliser behaviour has also been reported by Lemming et al. (2017). The authors showed that
308 when applying a sludge similar to HS to a soil with pH 7.8, the efficiency in terms of available
309 P relative to TSP increased from 20% to 46% after 84 days. Since many crops have high P
310 demands in the early stage of their life cycle (Fageria, 2016), for example wheat plants take up
311 50-60% of their P in the first six weeks of growth (Römer and Schilling, 1986), there is a risk
312 that the slow release of P from HS negatively impacts early plant growth. This might, however,
313 be compensated by adding fertiliser mixtures of soluble P (e.g., TSP) and HS. In contrast with
314 HS, CS did not influence the available P concentration. Since both sludges did not differ in their
315 P fractions, it is likely that the much lower total P concentration in CS was the predominant
316 driver of the lack of increase in P availability after addition to the soil (Welch et al., 2002). Due
317 to its low total P concentration, the potential of CS to be used as a P fertiliser is thus limited, at
318 least during the first year after its application, in such cropping systems. One possibility to
319 mobilise the sparingly available CS-derived P might be to develop cropping systems with
320 species having highly efficient P-acquisition strategy such as *Lupinus albus* L. (e.g., by
321 intercropping it with cereals) (Hallama et al., 2019; Lambers et al., 2013). Further researches
322 are however needed to determine to what extent this would counteract the limited P fertiliser

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

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

332

333 4.2. *Phosphorus forms and concentrations in sludge influence P forms in soil*

334 Interactions between P forms in sludge and P forms in soil are not fully understood so far,
335 most studies focusing only on total and water-extractable P concentrations. As shown by other
336 researchers using pot experiments (Frossard et al., 1996; Meyer et al., 2018; Nanzer et al.,
337 2014), our results indicate that coupling the determination of P concentration and forms in
338 sludge is, however, essential. Indeed, depending on total P concentration, P forms in sludge
339 drive P distribution in various soil pools, which, in turn, impact P availability. By contrast to
340 HS, CS had no effect on P distribution in soil pools compared with its mineral control (MCS).
341 Since CS and HS had a relatively similar P composition (OP is ca 20% while insoluble
342 AP+NAIP is ca 80% in both sludges), the lack of any effect of CS is thus most likely related to
343 its much lower total P concentration.

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

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

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

371

372 4.3. *Phosphorus in microbial biomass*

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

397 P (Trap et al., 2016). Consequently, the importance of food webs in agroecosystems for biotic
398 release of P derived from organic resources (like sludge) needs further study (Richardson and
399 Simpson, 2011). Additional work including the characterisation of microbial biomass and
400 structure of microbial community using phospholipid fatty acid analysis, sequencing and
401 quantification of genes involved in P dynamic in soil (i.e. *phoB* and *phoR*) (Anderson et al.,
402 2011) might be of help to better understand this microbial P increase in the presence of sludge.

403

404 4.4. *Effect of sludge on alkaline phosphatase activity*

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

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

431

432 4.5. *Implications*

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

444

445 5. **Concluding Remarks**

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

460

461 **Acknowledgments**

462 We thank the SIAAP wastewater treatment for funding and Thomas Pucheu from SEDE
463 Environnement for technical discussion. We also thank Vincent Hervé, Adeline Dubos, Sabine
464 Rudolph, Petru Jitaru and Philippe Jacolot for technical assistance. Olivier Pourret, Sabine
465 Houot, Frédéric Gérard and Isabelle Trinsoutrot-Gattin are acknowledged for their valuable
466 comments to this research.

467 **Appendix A. Supplementary data**

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

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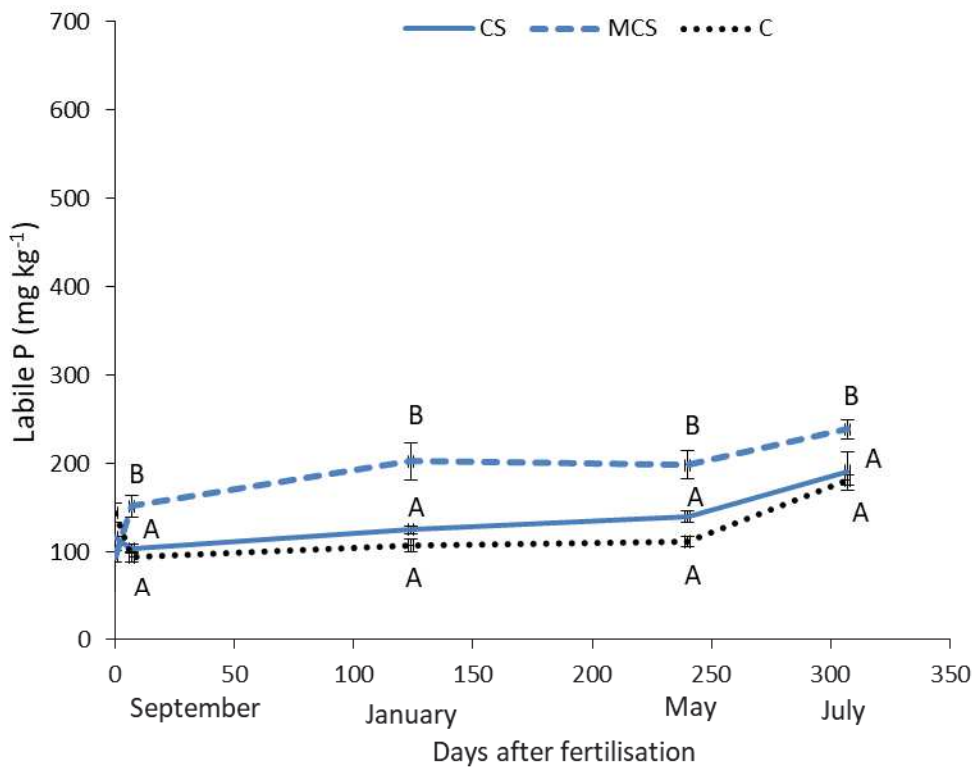
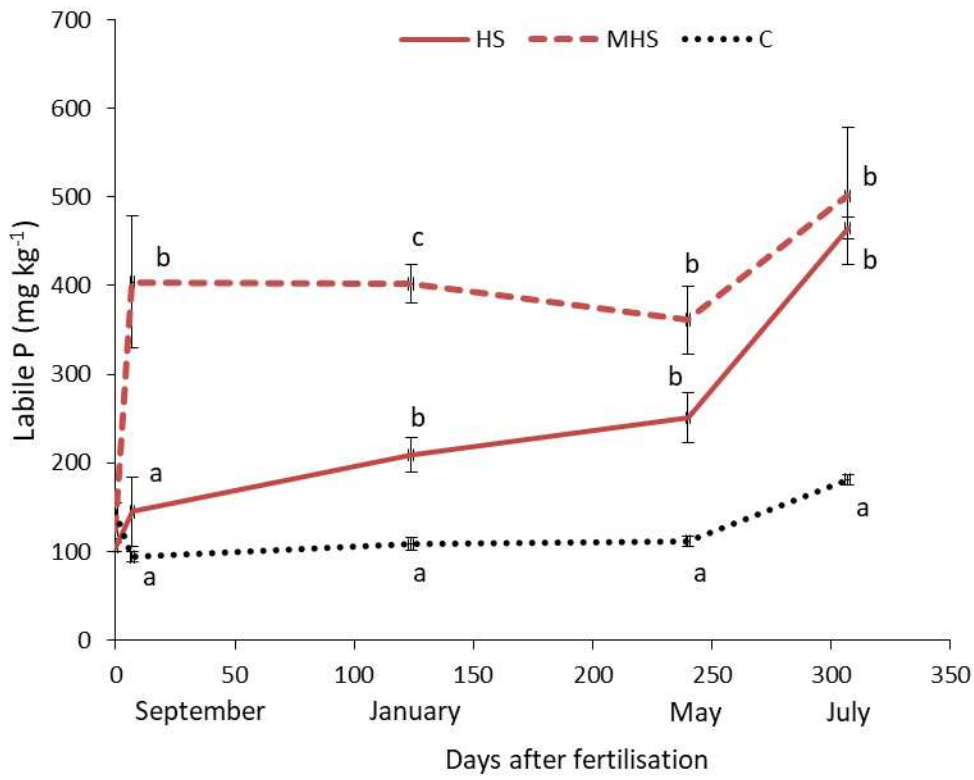
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Figures

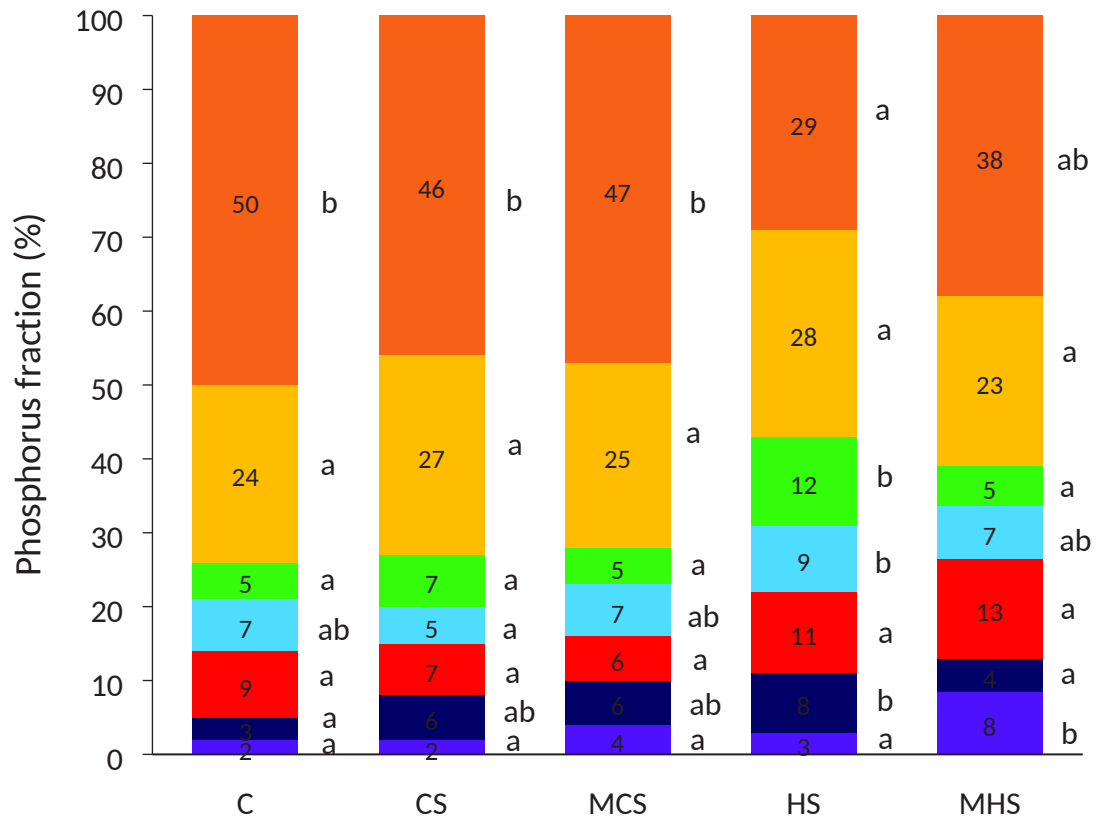
Figure 1. Release of labile phosphorus (resin-P + NaHCO₃-extractable P) over time. C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge. Different letters indicate significant differences between treatments ($P < 0.05$). 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.

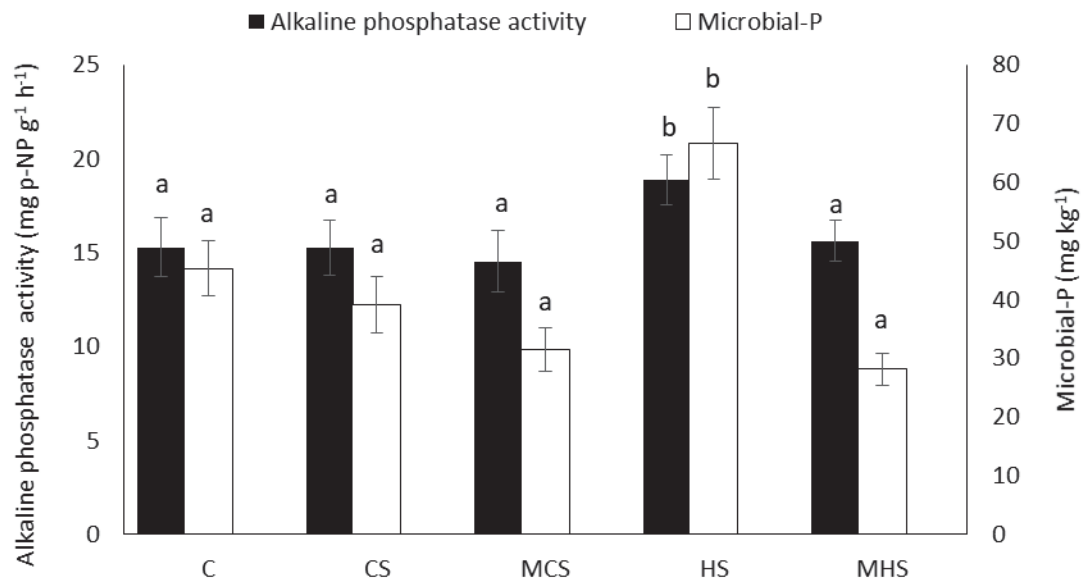
Figure 2. Effect of sewage sludge application on phosphorus (P) fractionation (Hedley scheme) after 307 days. C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge. The means with the same letters are not significantly 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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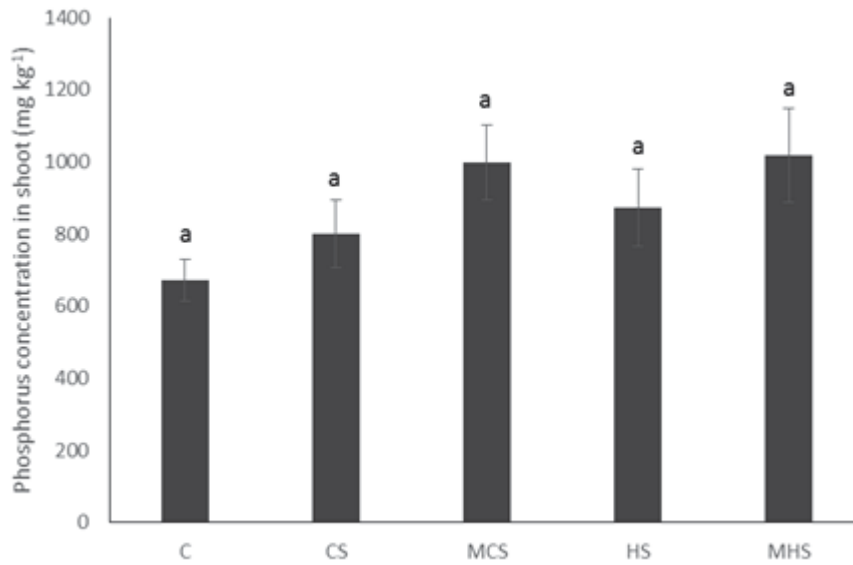
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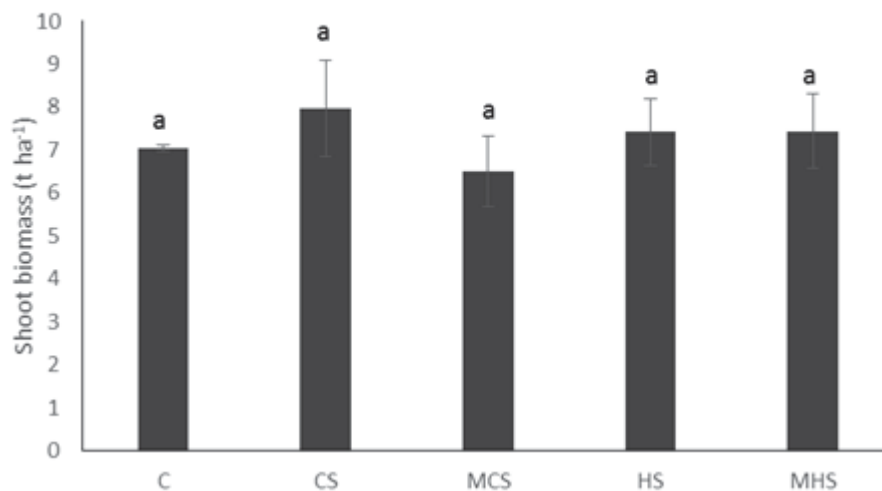
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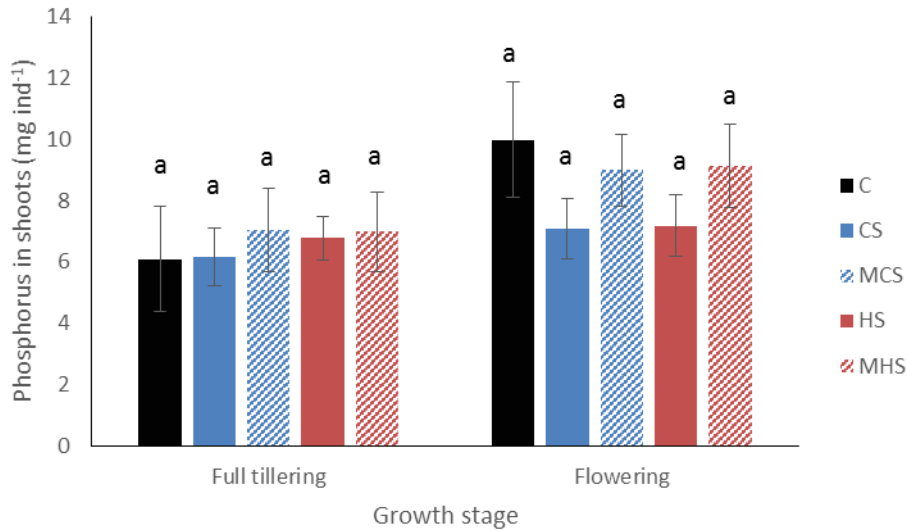
Email: david.houben@unilasalle.fr; michel-pierre.faucon@unilasalle.fr



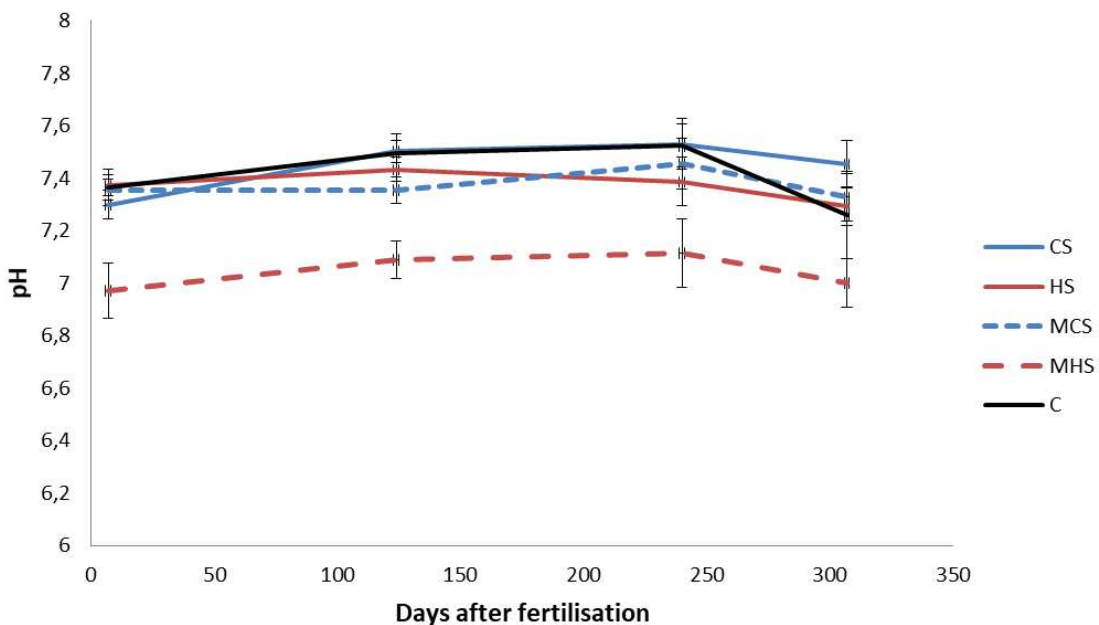
Supplementary Figure 1. Phosphorus concentration in winter barley at crop maturity. C: No-P control; CS: Composted Sludge; MCS: Mineral control of Composted Sludge; HS: Heated Sludge; MHS: Mineral control of Heated Sludge. Values are average ($n = 4$) \pm standard error. Columns with the same letter 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) ± 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.