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The role of earthworms in the biological processes of the phosphorus cycle

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Rôle des vers de terre dans le recyclage biologique du phosphore organique.

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Soutenu à Rennes

le 12/09/2019

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Les analyses et les conclusions de ce travail d'étudiant n'engagent que la responsabilité de son auteur et non celle d'AGROCAMPUS OUEST

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1 State of the art

The scientific community is seriously concerned by the fate of phosphorus (P) supplies. Some studies regarding this so-called “P-Crisis” presented some extreme scenarios, in which we might run out of raw materials for the production of P-fertilizers within the current century (Cordell and White, 2011). If P-fertilizers happen to be more and more scarce the problem could be dramatic. Phosphorus is an essential element for plants, yet the soil solution’s concentration is often under the optimum for plant growth (Kruse et al., 2015). To tackle this situation, P fertilizers needs were estimated to 50 kg of P_2O_5 /ha in average in Britany (Tóth et al., 2014). This has led to the creation of new research platforms such as the European Sustainable Phosphorus Platform (Kruse et al., 2015). Research should focus on improving the understanding of P cycle in the soil and increasing the use efficiency of this resource.

1.1 The phosphorus cycle

1.1.1 The different pools of phosphorus in the soil

Phosphorus is, by far, less abundant than the other macronutrients in soils. The P content depends on soil texture and its geological history. Soils do not contain elemental phosphorus but orthophosphates which are “the salt of phosphoric acid where a small central P^{5+} - ion builds a tetraeder with four bigger O^{2-} ” (Weihrauch and Opp, 2018). Orthophosphate can be present in organic or inorganic forms. These two categories are themselves separated in different pools characterized by a different turn-over time (Helfenstein et al., 2018).

The organic phosphorus forms after plant or microorganisms uptake and returns to soil after their death (Weihrauch and Opp, 2018). The soil content of organic phosphorus is depending on biological productivity and mineralization and as a consequence on various factors such as pedogenesis, pH, climatic condition, land-use (Weihrauch and Opp, 2018). Organic phosphorous can be found in various forms of different solubility and bio-availability: phosphonates and P-mono and di-esters. Yet the major organic phosphorus pool in soils is constituted by monoesters which are slightly soluble.

Moreover, up to 24% of the organic P pool is contained in the microbial biomass. Microbial phosphorus is considered as a labile pool as it only takes 2 hours after the death of the microorganism for the P to be bio-available (Kruse et al., 2015; Weihrauch and Opp, 2018).

The inorganic phosphorus does not always derive from the action of an organism and the changes in this pool depend on chemical equilibria (Weihrauch and Opp, 2018). Inorganic phosphorus can be separated in different pools as well, often operationally-defined, i.e. by the kind for chemical extraction used to

quantify them. These different pools are represented by phosphorus present in Calcium-minerals as well as the phosphorus sorbed in the soil constituents (Weil and Brady, 2017). Inorganic phosphorus can be sorbed to iron, aluminum or manganese oxyhydroxides, clay, carbonate or metal cations (Weihrauch and Opp, 2018). Sorption and desorption cannot often reach an equilibrium state as some of these reactions can be really slow and because the equilibrium continuously changes due to non-stationary conditions. Sorption is influenced by the ionic competition in the soil solution, the texture, the accessibility of the materials that will sorb phosphorus as well as the climate (Weihrauch and Opp, 2018). Depending on the sorption and on the pH of the soil, sorbed-phosphorus is more or less available. The other pool of the inorganic phosphorus is found in Calcium-minerals as a structural element.

Improving the bio-availability of phosphorus means increasing the content of phosphorus in the soil solution or increasing the soil buffering capacity for P, i.e. the capability of replenishing the soil solution of P when depleted. The soil solution is composed of ionic orthophosphate forms and some soluble organic compounds (Weihrauch and Opp, 2018; Weil and Brady, 2017). The predominance of orthophosphate ions depends on the pH of the soil: HPO_4^{2-} in more basic soils and H_2PO_4^- in acid ones (Kruse et al., 2015). The portion of organic phosphorus contained in the soil solution is operationally defined by the particles smaller than $0,45\mu\text{m}$, which includes organic P in colloidal form. It is still unclear, if plants can take up organic molecules or colloidal P (Weil and Brady, 2017) and it is mainly assumed that only orthophosphate ions are a source of phosphorus for plants.

1.1.2 Processes responsible for exchanges between pools

Exchanges between these different P pools and the soil solution are constant and are possible through different processes such as sorption, desorption, or mineralization. Sorption and desorption depend on physico-chemical properties of the soil such as pH, concentration of organic carbon or calcium carbonate, soil texture or the presence of iron and aluminum oxides (Alamgir and Marschner, 2016). These factors are controlling the availability of phosphorus.

Another essential process replenishing soil solution is mineralization which allows the transformation of organic phosphorus into ionic bio-available orthophosphate. This reaction is catalyzed by phosphatase enzymes produced by animals, microorganisms or plants (Richardson and Simpson, 2011). These enzymes will, step by step, liberate the orthophosphate contained into phosphate esters. Phosphatase enzymes can have different pH optimums. According to that, they are classified as alkaline and acid, yet the alkaline phosphatase is only produced by microorganisms (Plassard et al., 2014). This means that in alkaline soils, only microbes are able to mineralize phosphate.

This biochemical process can be accelerated by different factors such as the pH, the C/P ratio of the organic substrate or the climate (Weihrauch and Opp, 2018; Weil and Brady, 2017). Some soil

organisms, such as earthworms can also accelerate mineralization as they accelerate the evolution of organic matter by stimulating the microbial activity (Scheu et al., 2002).

1.2 Earthworms: soil engineers

1.2.1 Functional groups of earthworms

Earthworms are among the most important organisms of soil macro-fauna in terms of abundance and they are usually classified in three functional groups: the Anecic, Endogeic and Epigeic. These groups are different in both their morphology and their behavior (Bouché et al., 1977):

- The Anecic earthworms live in permanent vertical burrows opened on soil surface from which they take their food (organic and mineral matter). They digest under the soil surface and then put their cast on the soil surface as well as in the burrows where it forms a coat of cast which is a hot spot for microbial activity. Anecic earthworms are themselves separated into two distinct groups that are the Anecic strict and Epi-Anecic earthworms based on their morphological traits (Briones and Álvarez-Otero, 2018), burrowing (Bastardie et al., 2005) and feeding behaviors (Eisenhauer et al., 2008).
- The Endogeic earthworms live in horizontal and temporary burrows that are refilled with their cast. They never go up to soil surface so they eat organic matter from the soil.
- The Epigeic earthworms live close to the soil surface or in the first centimeters of the soil surface and are very active in the organic matter evolution.

The Anecic and Endogeic earthworms are the two functional groups more present in agricultural land as Epigeic only represent 2 to 3% of the earthworms density in conventional and organic fields (Pelosi et al., 2009). Moreover it is important to notice that under both crops management, endogeic earthworms represented more than 50% of the earthworms density (Pelosi et al., 2009). The dominance is mainly due to a lower exposure of both functional groups to pesticides compared to Epigeic earthworms. However they are both highly sensitive to ploughing due to the destruction of their burrows with a higher sensibility reported for the Anecic group (as reviewed in Bertrand et al., 2015).

1.2.2 Soil parameters under the influence of earthworms

Anecic and Endogeic earthworms both produce casts that will then be incorporated in the bulk soil. This cast presents many differences compared to the bulk soil (Barois and Lavelle, 1986). Regarding the chemical properties, casts are more basic than the bulk soil (Trigo and Lavelle, 1993; Vos et al., 2014) and increase the availability of most of the elements (Vos et al., 2014). There are also some changes

regarding the biological characteristics such as the microbial biomass which is more important in the cast than in bulk soil (Scheu, 1987). But this increase of microbial biomass is not as important for casts situated in the burrows, compared to casts on the soil surface (Scheu, 1987). This increase of microbial biomass is accompanied by a boost of the activity as there is an increase of microbial respiration (Scheu, 1987). These increases can be explained by an optimal pH for microbes as well as by the presence of more available nutrients, such as labile carbon. However, the microbial biomass present in cast goes back to its initial value within a few days. Scheu, 1987 also showed that within weeks, the microbial biomass is even lower than the one in the bulk soil.

Moreover, even in bulk soil, the presence of earthworms leads to modification of some properties. First the physical parameters are modified by the burrowing activity of earthworms that creates macro, meso and micro porosity (see reviews of Bertrand et al., 2015; Blouin et al., 2013). The chemical parameters are modified too by an increase of the mean turn-over time of the soil organic matter and nutrients, which increases their availability (reviewed in Bertrand et al., 2015). The biological parameters are also impacted as the microbial biomass is overall increased by the presence of earthworms (Burtelow et al., 1998). This microbial biomass increase is the consequence of the creation of favorable conditions for microorganisms: more accessible organic matter, more assimilable mucus, good habitat conditions.

For this reason, earthworms are known as soil engineers. The modification of the soil properties by earthworms leads to the fulfilment of many ecosystems services such as soil structure, water regulation, climate regulation, pollution remediation, plant production as well as nutrients cycling (Bertrand et al., 2015; Blouin et al., 2013 and references therein). Several studies were done on nutrients cycling in cast which is considered a hot spot for nutrients. The concentration of most minerals such as magnesium and calcium was found to be higher (Pommeresche et al., 2009), as well as for macro elements such as nitrogen, carbon and phosphorus (Buck et al., 1999; Pommeresche et al., 2009).

1.3 Current knowledge regarding the influence of earthworms on phosphorus

1.3.1 Modification of the pools of phosphorus

Many studies showed that casts are richer in total phosphorus (Chapuis-Lardy et al., 2011). This was proved in many different conditions: laboratory and field experiments (Jiménez et al., 2003) as well as under different land-uses (Jiménez et al., 2003; Kuczak et al., 2006; Scheu et al., 2002). This increase of total phosphorous can reach up to nearly 250% compared to the bulk soil (Kuczak et al., 2006).

More than just a global increase, an augmentation of the more labile pool of phosphorus has been observed in bulk soil as well as in casts. Devliegher and Verstraete, 1996 noticed an increase of the

phosphorus availability due to the presence of earthworms. Kuczak et al., 2006 showed that casts are richer in available phosphorus as well as in moderately available one. These two pools were defined as sums of the different fraction of Hedley fractionation (i.e. a sequential P extraction, Tiessen and Moir, 1993): the available phosphorous was defined by the resin-, and bicarbonate- extractable fractions and the moderately available phosphorous was defined as the sum of the inorganic and organic sodium hydroxide- and the dilute acid- extractable fractions.

More precisely, an increase in the plant-available pool of phosphorus in the cast was visible in the field by a concentration of root biomass under earthworm's casts (Decaëns et al., 2003). Moreover, it has been reported an increase of the dissolved organic and inorganic phosphorus in casts for both Anecic and Epigeic earthworms (Ros et al., 2017; Vos et al., 2014). This means that soil solution in casts is richer in phosphorus.

The presence of earthworms can also affect the other pools of phosphorus in casts or in bulk soil. The organic phosphorus is globally higher in cast (Chapuis-Lardy et al., 2011) and more precisely the more stable forms of phosphorus contained in the sodium hydroxide-extractable organic phosphorous fraction. The increase of the microbial phosphorus was also reported as a part of the augmentation of the organic P pool (Guggenberger et al., 1996; Jiménez et al., 2003). The stable inorganic pools are also affected, notably P sorbed to iron or aluminum oxi-hydroxides or in calcium minerals (Kuczak et al., 2006).

1.3.2 Earthworms influences in the pool-changes

The processes allowing these changes are still unclear but the biogeochemical state of phosphorus is changed in the soil in presence of earthworms as well as in their guts (Lavelle, 1997)

Studies regarding phosphatase showed different results as some showed that casts had a higher phosphatase activity (Hoang et al., 2016; Satchell and Martin, 1984), but some studies also showed that phosphatases were more present in bulk soil than in casts (Jiménez et al., 2003; Zhang et al., 2000). The decrease of the phosphatase activity in cast reached up to 44% in (Jiménez et al., 2003). The decrease in casts would mean that mineralization happens rather in earthworms gut rather than in casts as it was proven that earthworms were able to produce alkaline phosphatase (Satchell and Martin, 1984).

This could be explained by different characteristics of earthworms gut such as the higher pH (nearly + 2.1 (Barois and Lavelle, 1986)) and an increase of the microbial activity during digestion (Chapuis-Lardy et al. 2011). Another explanation for the gut mineralization could be linked to P release from the sorption complexes due to competition of phosphate with carboxyl groups produced by the microbial

mineralization of the soil eaten by the earthworms in the first part of the gut (Lopez-Hernandez et al., 1993) or with dissolved organic carbon (Ros et al., 2017).

More than earthworm's guts properties, some soil properties influenced by earthworms can also explain some changes in the pools. These soil properties could explain the variability that we can find in the different studies regarding the increase of phosphorous. This variability could be explained, for instance, by the initial phosphorus quantities in the soil (Kuczak et al. 2006) or by the selective ingestion of food by earthworms (Ros et al., 2017). This means that there is a high variability in the effects on P availability that could be soil-dependent or species-dependent (Kuczak et al., 2006).

1.4 Objectives of our study

This study will try to improve the knowledge of the influence of earthworms on the phosphorus availability under close-to-field conditions. Its aims are to answer to these questions:

- What is the impact of earthworms on the repartition of phosphorous in its different pools?
- How can we explain the changes observed between pools?

To answer to these questions a collaboration with the CNRS-Ecotron was established. Ecotron is a facility put in place by the CNRS in order to “allow precise conditioning to better understand states and activities at various scales”. The experiment we contributed to began in January 2017. Its aim is to measure the effect of Anecic and Endogeic earthworms on the emission of greenhouse gases under close-to-field conditions. In addition, we combined established techniques such as sequential P extraction or High Pressure Ionic Chromatography (HPIC) with innovative ones such as the analysis of oxygen isotopes in phosphates.

We will first see how the experiment was carried out and different analyses we performed. Then we will present the results that will be discussed in the next part to understand the mechanisms responsible for the P pool changes.

2 Material and Methods

2.1 Experimental setting and design

The experiment at the Ecotron site is carried out on 12 separated macrocosms. In each macrocosm, the soil was taken from the experimental site EFELE of the agricultural high school Théodore Monod (Le Rheu, France) at 3 different depths. The soil profile was then recreated at the Ecotron site. Soil and atmosphere moisture and temperature are controlled and reproduce the same field conditions as in

Rennes. All macrocosms have the same crop rotation and practices as the experimental site in Le Rheu. This means that there is a wheat, mustard and maize rotation that started in October 2017 under minimum-tillage. The initial fertilization of the macrocosm was done with organic residues (corn, hey and wheat straw) in July 2017 (660g/m²). Some digestate was also added twice in April and June 2018 (equivalent of 90kgN/ha). The earthworms were added in the beginning of 2017 to reach a density of 100 g/m² compared to a mean of 91g/m² in Brittany (Bellido et al., 2010). They were collected in Rennes as well as in Avignon. The species added were for Anecic earthworms: *Lumbricus terrestris*, *Apporectodea longa* and *Scherotheca sp*, and for the Endogeic: *Apporectodea chlorotica*, *Apporectodea caliginosa* and *Apporectodea icterica*.

The experiment design included 3 different treatments with 4 replicates each:

- The first treatment is the control, where there was almost no earthworm (nearly 3g/m²). The absence of earthworms was assured by periodical electrical shocks that induce all the earthworms to reach the soil surface and allows removing them.
- The two other treatments are the Endogeic and Anecic treatment. The soils were cleared of existing earthworms and earthworms from one specific functional group were successively introduced.

In October 2018, samples were collected by research staff for the realization of our study. For each macrocosm, a composite sample of bulk soil from 5 subsamples was collected. Additionally, in the Anecic treatment, casts from soil surface were collected without distinction of the species. Moreover, some bulk soil was also sampled to be incubated for 7 days at 16°C with a high biomass of earthworms (around 700g of soil for 25 to 80g of earthworms). The incubation was done with at constant temperature and in darkness, yet the humidity was not controlled and it could vary. This treatment allowed us to have soil that was totally digested by earthworms. Overall our samples set includes 12 bulk soil samples (from the three treatments), 4 casts samples from soil surface (Anecic treatment) and 8 samples of freshly digested soil (Anecic and Endogeic treatment).

Samples were stored fresh at 4°C before analysis.

2.2 Laboratory analysis

2.2.1 Soil parameters and phosphorus pools

Some general analyses were done by a student on all our samples to be able to detect if earthworms modified the chemical or biological properties of the soil. The parameters analyzed were:

- Microbial Nitrogen
- Microbial Carbon

- Dissolved Organic Carbon
- Soluble Nitrogen
- Inorganic Carbon
- pH

To quantify the phosphorus pools in the bulk soil and in the casts and assess whether the presence or the digestion of earthworms modified the repartition of P in these pools, an Hedley sequential fractionation as modified by Tamburini et al., 2018 was done by the same student. A sequential extraction aims to use progressively stronger chemical reactants to extract by each new step a more stable P pool. Five different pools were then quantified:

- Extraction with anionic resins (BDH #55164, 6 cm × 2 cm) targeting the plant-available phosphorus
- Extraction with anionic resins and hexanol that targets the microbial phosphorus.
- Extraction with NaOH-EDTA targeting P sorbed to Fe and Al (oxy)hydroxides and the non-refractory organic phosphorus.
- Extraction with HCl that targets the less available phosphorus bound in calcium minerals.

P concentration was measured in these pools using the Malachite green method (Van Veldhoven and Mannaerts, 1987). For total P, we mineralized our soils with concentrated nitric acid by a microwave cycle of 32 minutes that reach up to 220°C.

2.2.2 HPIC analysis

A quantification and speciation of low molecular weight organic molecules through High Pressure Ionic Chromatography (HPIC) was realized. This technique allowed us to identify and quantify with high degree of sensitivity organic P molecules and verify if their concentrations changed under the influence of earthworms or after digestion. To do so, the soils were extracted with 0.5M NaOH and subsequently humic acids in soil extracts were eliminated by precipitation with HCl. Chlorides, which could interfere with the detection of some species, were removed from the mobile phase thanks to cartridges containing AgNO₃, according to the protocol given in (Waithaisong et al., 2015).

The HPIC separates at different times diverse molecules depending on their negative charge, which are then detected through conductimetry. The area contained under the conductivity peak depends on the concentration. Standard curves allowed us to calculate the sample concentrations and were obtained by spiking a sample with three different concentrations of standards of the organic compounds. Standards we included were: sodium nitrate (Riedel-de-Haën), malonic acid (MERCK-Schuchardt), oxalic acid dihydrate (Fluka), malic acid (Sigma), potassium phosphate monobasic (Sigma), citric acid (Sigma), sodium pyrophosphate dibasic (Sigma), D-fructose 1,6-biphosphate sodium salt (Sigma), Adenosine 5'

triphosphate disodium salt hydrate (Sigma), Adenosine 5' monophosphate sodium salt (Sigma), D-glucose-6 phosphate disodium salt (Sigma) and sodium phytate (Sigma).

Phytate was separated from the other anions to avoid contamination that would biased the other standard curves as phytate standard presented other spikes. The concentration of glucose-6-phosphate was instead measured with a SIGMA kit (MAK014), due to an interference with sulfate in the HPIC. For our statistical analysis, we only applied a partial dilution correction and did not take into account the dilution effect that the cartridge can have.

2.2.3 Mineralization of organic phosphorus

In order to measure the isotopic ratio of oxygen in phosphates, three of these pools (plant available, microbial and mineral phosphorus) were purified to transform all phosphorus into silver-phosphate for isotopic analyses according to the protocol of Pistocchi et al., 2014, adapted from Tamburini et al., 2010. This sample preparation is a succession of precipitation and dissolution that aims at eliminating all compounds that could interfere on the isotopic ratio. The samples were sent to Zurich (Laboratory of the Group of Plant Nutrition, ETH Zurich) to analyze their oxygen isotopic ratio by an elemental analyzer coupled to an isotopic ratio mass spectrometer (Isoprime 100, Elementar GmbH).

This isotopic ratio allows to know if the phosphate underwent biological reactions via enzymes (Tamburini et al., 2014). This ratio is indeed preserved in the absence of biological activity and in case of sorption/desorption of phosphorus onto minerals or precipitation/dissolution (Jaisi and Blake, 2010). The activity of living organisms and enzymes instead alter the oxygen isotope ratio. First, an enrichment of the heavier isotopes in the residual phosphate in the environment can be an effect of microbial uptake, as they are more likely to uptake the lighter isotopologues. Additionally, the isotopic ratio is altered as a phosphate molecule enters a living cell, as it turned over to reach a temperature-dependent isotopic equilibrium under the effect of intracellular enzymes such as pyrophosphatases. Finally, the phosphate produced by the hydrolysis of phosphoesters by extracellular phosphatase enzymes has a depleted isotopic ratio compared to the original compound, as these enzymes induce a negative fractionation: -10 to -30‰ according to the type of enzyme, e.g. acid or alkaline phosphatases.

Results will be presented in the conventional delta notation ($\delta^{18}\text{O-P}$) (Tamburini et al., 2014). Our results were interpreted according to:

- the equilibrium between water and dissolved phosphate driven by the intracellular pyrophosphatase activity, which was calculated with an equation adapted from (Chang and Blake, 2015) where the isotopic ratio of water was the mean value for the Occitanie region (Genty et al., 2014):

$$\text{Equilibrium } \delta^{18}\text{O-P} = -0.18 \times T + 26,3 - \delta^{18}\text{O-H}_2\text{O}$$

T : Temperature

$\delta^{18}\text{O-H}_2\text{O}$: Water isotopic ratio

- the acid and alkaline phosphatase fractionation values (Liang and Blake, 2006; von Sperber et al., 2014).

Measured isotopic values similar to the expected isotopic equilibrium indicate the imprint of intracellular P turnover, while depleted values indicate the effect of extracellular phosphatases and therefore the imprint of mineralization of organic P. The measured isotopic values were additionally compared with the reference value for P in the soil constituents approximated by the mineral phosphorus (P extracted by 1M HCl).

Finally, we analyzed the enzymatic activity of the phosphatase in our soil and cast samples. Our dosage relied on the previous extraction of the enzymes in the soil suspension. The activity was measured by the fluorometric determination of 4-MUB. The protocol was adapted from (Bell et al., 2013).

2.3 Calculation and statistical analysis

Statistical analyses were done with the R software (the R Project for Statistical Computing). To analyze all our data, we used the ANOVA analysis from the FactoMineR package that was completed by a Tuckey test on means with a limit of significance for the p value of 0.05. The ANOVA were only accepted if they were negative for the Levene and Shapiro tests. For all data except the HPIC data, we completed our analysis with orthogonal contrasts using the “contrasts” function in R (package stats) Orthogonal contrasts for analysis of variance are independent linear comparisons between the groups of a factor. Hence, they allow to highlight differences between a particular set of levels of a factor.

As the sampling design gave us 6 independent treatments (control, bulk soil from Anecic or Endogeic treatment, soil surface casts from the Anecic treatment and fresh casts produced by Endogeic and Anecic earthworms), 5 contrasts were possible. Contrasts were imposed by our treatments as following: Control compared to the mean of all other treatments; Mean of bulk soil with Endogeic and Anecic earthworms compared to the mean of all casts; Soil surface cast compared to the mean of fresh casts produced by Anecic and Endogeic earthworms; Bulk soil under Anecic treatment compared to bulk soil under Endogeic treatment and fresh cast produced by Anecic compared to fresh casts produced by Endogeic.

To explain our results regarding phosphorus pools size and HPIC, two distinct PCA were realized thanks to the FactoMineR and factoextra packages. Regarding HPIC, clusters were built thanks to hierarchical clustering (HCPC function) and confirmed with a permutation test (randtest function) in the Ade4 package.

Missing data were added using the missMDA package.

**Table 1: Influence of earthworms on soils properties.
(DOC, Inorganic C, Soluble N, Microbial C and Microbial N are in mg/kg of soil)**

Mean and P value according to orthogonal contrasts.

Control: Bulk soil without earthworms. *Bulk A or E*: Bulk soil with Anecic earthworms (A) or Endogeic earthworms (E). *Bulk*: Mean of bulk soils A and E. *Fresh cast A and E*: Soil incubated with either Anecic earthworms (A) or Endogeic earthworms (E) *Fresh Cast*: Mean of fresh cast A and E. *Old cast*: Cast sampled on soil surface (only for Anecic treatment). *Cast*: Mean of both fresh casts and old casts. *All*: mean of all treatments except the control.

| | GLOBAL EFFECT OF EARTHWORMS | | | EFFECT OF DIGESTION | | | EFFECT OF CAST-AGE | | |
|--------------------------|-----------------------------|--------|--------------|---------------------|--------|--------------|--------------------|----------|--------------|
| | Control | All | p value | Bulk | Casts | p value | Fresh Cast | Old Cast | p value |
| pH | 6.4 | 6.5 | 0.509 | 6.3 | 6.6 | 0.080 | 6.5 | 6.9 | 0.121 |
| Dissolved Organic Carbon | 30.45 | 104.32 | 0.003 ** | 22.18 | 159.08 | 1.47E-08 *** | 160.30 | 156.63 | 0.934 |
| Inorganic Carbon | 0.50 | 7.94 | 0.022 * | 2.00 | 11.90 | 0.001 *** | 15.15 | 5.40 | 0.003 ** |
| Soluble Nitrogen | 5.48 | 112.63 | 2.73E-07 *** | 4.40 | 184.78 | 6.93E-13 *** | 245.48 | 63.38 | 2.25E-04 *** |
| C/Nmic | 7.98 | 13.44 | 0.660 | 9.85 | 15.84 | 0.383 | 11.77 | 23.98 | 0.331 |
| C/Pmic | 25.34 | 23.21 | 0.857 | 15.46 | 28.38 | 0.091 | 35.95 | 13.23 | 0.013 * |
| Microbial C | 81.38 | 201.70 | 1.31E-05 *** | 68.98 | 290.18 | 3.48E-11 *** | 357.74 | 155.05 | 6.15E-06 *** |
| Microbial N | 11.68 | 24.63 | 0.191 | 8.63 | 35.29 | 1.04E-04 *** | 45.25 | 15.38 | 0.001 *** |

| | EFFECT OF THE FUNCTIONAL GROUP (FRESH CAST) | | | EFFECT OF THE FUNCTIONAL GROUP (BULK SOIL) | | |
|--------------------------|--|--------------|---------|---|--------|---------|
| | Fresh Cast A | Fresh cast E | p value | Bulk A | Bulk E | p value |
| pH | 6.5 | 6.6 | 0.597 | 6.3 | 6.4 | 0.894 |
| Dissolved Organic Carbon | 131.75 | 188.85 | 0.292 | 16.20 | 28.15 | 0.220 |
| Inorganic Carbon | 5.13 | 25.18 | 0.053 | 1.90 | 2.10 | 0.968 |
| Soluble Nitrogen | 228.06 | 262.90 | 0.988 | 4.8 | 4.00 | 0.436 |
| C/Nmic | 12.68 | 10.86 | 1.000 | 11.67 | 8.02 | 0.672 |
| C/Pmic | 36.18 | 35.73 | 0.216 | 11.29 | 19.63 | 0.380 |
| Microbial C | 312.13 | 403.35 | 0.146 | 74.00 | 63.95 | 0.334 |
| Microbial N | 36.00 | 54.50 | 0.245 | 7.63 | 9.63 | 0.513 |

3 Results

3.1 Influence of earthworms on soil properties

3.1.1 Microbial communities

The microbial communities were analyzed through 4 indicators (microbial carbon and nitrogen, microbial C/N and C/P ratios). The size of the microbial community was globally impacted by the presence of earthworms as the microbial carbon was higher for the global soil (mean of the treatments: bulk soil and casts) than in the control treatment (Table 1). This difference is mainly due to the high microbial C present in casts. Indeed, the digestion of the soil by earthworms increased the size of the community as both microbial carbon and nitrogen were higher in casts compared to bulk soils (Table 1). However, this value was highly age-dependent as there was a decrease of both N and C in the aged-cast, although without attaining the value of bulk soil (Table 1).

The stoichiometry of the microbial community was also analyzed through the C/N and C/P ratios in the microbial biomass. These ratios were not significantly different between the global soil (bulk soils and casts) and the control as well as between fresh casts and bulk soils (Table 1). However, when comparing fresh to old casts, we noticed a significant difference concerning the C/P ratio with higher values for the fresh casts (Table 1).

Moreover, it is important to notice that the functional group of earthworms did not affect the microbial communities on neither its size nor its stoichiometry.

3.1.2 Chemical indicators of the microbial activity

Four chemical parameters were analyzed: pH, soluble nitrogen, labile organic and inorganic carbon. These last three indicators are indicators of the microbial activity (Xing et al., 2010). The presence of earthworms increased the soluble nitrogen as well as the labile and inorganic carbon present in global soil (Table 1). This means that the microbial activity was more important with the presence of earthworms and it led to an increase of available nutrients in the soil. Yet the presence of earthworms did not affect the pH of the soil in any of the orthogonal contrasts (Table 1).

When comparing bulk soils to casts, the soluble nitrogen, the labile and inorganic carbon were all significantly higher in the casts (Table 1). This means that the digestion of the soil by earthworms either concentrated the nutrients in casts, or made them more available due to the microbial activity. The soil was also found to be slightly more alkaline in casts (Table 1), yet the difference was not significant.

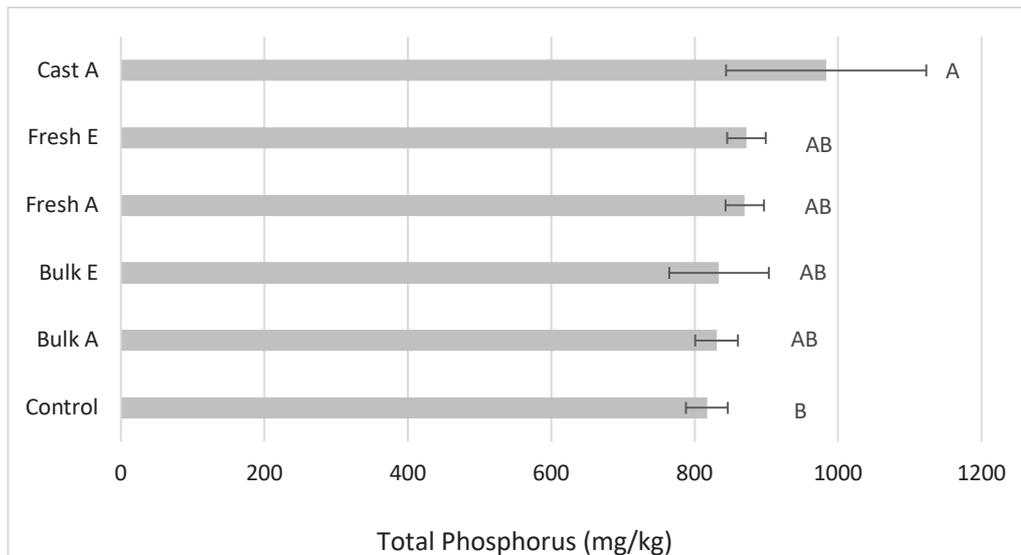


Figure 1: Total phosphorus.

Control: Bulk soil without earthworms. Bulk A or E: Bulk soil under the influence of Anecic (A) or Endogeic (E) earthworms. Fresh cast A or E: Soil digested by Anecic (A) or Endogeic (E) earthworms. Cast A: Casts sampled on soil surface only for Anecic treatment. Distinct capital letters indicate statistical significance ($p < 0.05$) according to ANOVA.

Table 2: Influence of earthworms on pools of phosphorus. (in mg/kg of soil)

Mean and P value according to orthogonal contrasts.

Control: Bulk soil without earthworms. Bulk A or E: Bulk soil with Anecic earthworms (A) or Endogeic earthworms (E). Bulk: Mean of bulk soils A and E. Fresh cast A and E: Soil incubated with either Anecic earthworms (A) or Endogeic earthworms (E) Fresh Cast: Mean of fresh cast A and E. Old cast: Cast sampled on soil surface (only for Anecic treatment). Cast: Mean of both fresh casts and old casts. All: mean of all treatments except the control.

| | GLOBAL EFFECT OF EARTHWORMS | | | EFFECT OF DIGESTION | | | EFFECT OF CAST-AGE | | |
|------------------------------|-----------------------------|--------|----------|---------------------|--------|--------------|--------------------|----------|--------------|
| | Control | All | p value | Bulk | Casts | p value | Fresh Cast | Old Cast | p value |
| Resin Extractable P | 22.24 | 28.03 | 0.029 * | 20.66 | 32.94 | 2.94E-06 *** | 26.86 | 45.08 | 8.08E-06 *** |
| Microbial P | 4.01 | 7.11 | 0.076 . | 4.38 | 8.93 | 0.003 ** | 8.26 | 10.26 | 0.283 |
| NaOH Extractable Inorganic P | 328.00 | 321.65 | 0.694 | 312.25 | 327.92 | 0.252 | 335.00 | 313.75 | 0.247 |
| NaOH Extractable Organic P | 91.26 | 166.64 | 0.007 ** | 108.11 | 205.66 | 0.002 *** | 269.45 | 78.10 | 2.07E-06 *** |
| HCl Extractable P | 103.60 | 118.25 | 0.057 . | 102.59 | 128.70 | 3.83E-04 *** | 140.63 | 104.83 | 3.09E-04 *** |
| Residual P | 267.90 | 236.30 | 0.542 | 284.25 | 204.33 | 0.076 . | 90.83 | 431.32 | 1.16E-05 * |
| Total P | 817.01 | 877.98 | 0.118 | 832.24 | 908.47 | 0.024 * | 871.04 | 983.34 | 0.014 * |

| | EFFECT OF THE FUNCTIONAL GROUP (FRESH CAST) | | | EFFECT OF THE FUNCTIONAL GROUP (BULK SOIL) | | |
|------------------------------|---|--------------|---------|--|--------|---------|
| | Fresh Cast A | Fresh cast E | p value | Bulk A | Bulk E | p value |
| Resin Extractable P | 29.83 | 23.90 | 0.043 * | 20.79 | 20.54 | 0.894 |
| Microbial P | 7.40 | 9.13 | 0.419 | 5.05 | 3.71 | 0.530 |
| NaOH Extractable Inorganic P | 350.55 | 319.45 | 0.147 | 321.25 | 303.25 | 0.392 |
| NaOH Extractable Organic P | 277.37 | 261.52 | 0.629 | 125.39 | 90.82 | 0.298 |
| HCl Extractable P | 131.96 | 149.3 | 0.078 . | 109.85 | 95.33 | 0.135 |
| Residual P | 72.74 | 108.92 | 0.589 | 248.16 | 320.34 | 0.286 |
| Total P | 869.86 | 872.22 | 0.961 | 830.49 | 833.99 | 0.943 |

The nutrient availability in casts depended on the age of the cast except for the labile carbon. Indeed, for inorganic carbon and soluble nitrogen we could see a significant decrease in aged-casts (Table 1). No differences were found between Anecic or Endogeic treatments for neither bulk soil nor fresh cast (Table 1).

3.2 Influence of earthworms on the different pools of phosphorus and its availability

3.2.1 Impact of earthworms on the total phosphorus

Total phosphorus was analyzed in all our samples. A trend of increasing total phosphorus from bulk to fresh cast and from fresh cast to old cast can be observed (Fig 1). Yet the increase was only significant between the control bulk soil and the old Anecic cast.

On the global soil (mean of the treatments: bulk soil and casts), earthworms did not increase the total phosphorus (Table 2). However, it seems that casts present a higher quantity of phosphorus than bulk soils (Table 2). When comparing the casts regarding their age we can see that the older casts contain more phosphorus than the young ones (Table 2). This means that due to earthworms, there is a concentration of phosphorus in the casts that is more important on the casts present on the soil surface.

3.2.2 Impact of earthworms on the repartition of pools of phosphorus

The repartition of the phosphorus in different pools was analyzed to see the impact of earthworms on the availability of phosphorus through an ANOVA on relative and absolute values that gave same results due to the similar amount of total phosphorus in all our samples.

3.2.2.1 Global effect of earthworms on phosphorus pools

The three treatments of bulk soil, whether with or without earthworms, did not present any significant difference regarding the relative size of their different P pools (Fig 2). This means that the earthworms did not have an impact on the bulk soil regarding phosphorus availability. Yet when comparing soil without any earthworms to all different treatments (global soil), we can see that the presence of earthworms increased both the directly-plant-available and the organic phosphorus (Table 2). This suggests that, globally, the soil was impacted by the presence of earthworms when considering both bulk soil and the hotspots represented by the casts.

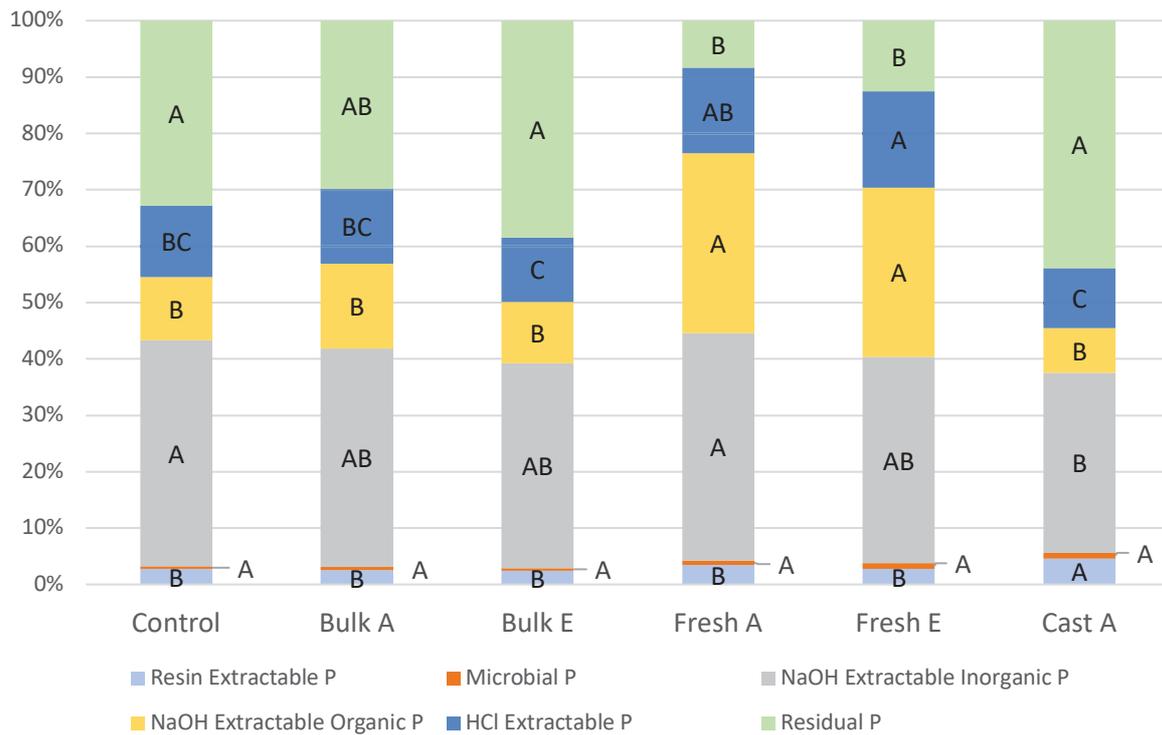


Figure 2: Repartition of phosphorus into the different pools.

Control: Bulk soil without earthworms. Bulk A or E: Bulk soil under the influence of Anecic (A) or Endogeic (E) earthworms. Fresh A or E: Soil digested by Anecic (A) or Endogeic (E) earthworms. Cast A: Casts sampled on soil surface only for Anecic treatment. Distinct capital letters indicate statistical significance ($p < 0.05$) according to ANOVA.

3.2.2.2 Effect of earthworms digestion on phosphorus pools

The ingestion and digestion of soil by the earthworms seems to have a clear impact on the phosphorus availability as the repartition of the phosphorus is clearly different in casts compared to bulk soil (Fig 2). Almost all the pools of phosphorus were impacted by the soil digestion. There was an increase of the more available pools of phosphorus, namely plant-available (resin extractable) and microbial phosphorus, but the organic phosphorus was also increased as well as the phosphorus bound in calcium minerals (HCl extractable P, Table 2).

However, these differences were mainly due to fresh cast, as the older one had a repartition of P pools comparable to bulk soil (Fig 2). First, the organic pool of phosphorus was more important in fresh casts (twice as in the bulk soil). The phosphorus bound in calcium minerals was also increased by the digestion but only for the Endogeic treatment. Interestingly, the residual phosphorus, i.e. the fraction not recovered through the sequential extraction, decreased nearly 3 times in the fresh cast compared to bulk soil.

3.2.2.3 Effect of cast-age on phosphorus pools

The effect of earthworms on the repartition of the different pools does not seem to last in time as we can see by comparing fresh casts to old casts (Fig 2). There was a significant difference of the repartition for all pools except the P sorbed onto Fe and Al hydroxides and microbial phosphorus (Table 2). It seems that with time, the soil ingested by the earthworms takes back the same properties as bulk soil under the influence of earthworms (Fig 2). It is worth noticing that the old casts present a higher proportion of plant-available phosphorus than both bulk soils and fresh casts (Fig 2).

3.2.2.4 Effect of the functional group on phosphorus pools

Functional groups of earthworms did not seem to have an influence on the repartition of the phosphorus into the different pools (Fig 2). However, we can notice that the absolute value of plant-available phosphorus was higher in the cast of Anecic earthworms compared to Endogeic ones (Table 2). This is the only significant difference for both fresh cast and bulk soil when comparing them according to the earthworms functional group. This suggests earthworms of different functional groups have the same impact on phosphorus.

Table 3: Concentration (mg/kg of soil) of organic acids obtained by HPIC analysis.
Control: Bulk soil without earthworms. Bulk A or E: Bulk soil under the influence of Anecic (A) or Endogeic (E) earthworms. Fresh cast A or E: Soil digested by Anecic (A) or Endogeic (E) earthworms. Tur A: Casts sampled on soil surface only for Anecic treatment. Distinct letters indicate significant differences ($p < 0.05$) according to ANOVA

| | Control | Bulk A | Bulk E | Fresh A | Fresh E | Tur A |
|----------|----------|--------|----------|----------|---------|---------|
| Malate | 6.15 b | 6.09 b | 5.55 b | 4.4 b | 4.55 b | 10.33 a |
| Malonate | 14.5 b | 13 b | 15.7 b | 12.9 b | 13.9 b | 32.9 a |
| Oxalate | 12.51 ab | 9.94 b | 13.15 ab | 12.47 ab | 12.6 ab | 29.7 a |
| Citrate | 2.51 b | 2.27 b | 2.45 b | 6.39 a | 6.29 a | 3.58 b |

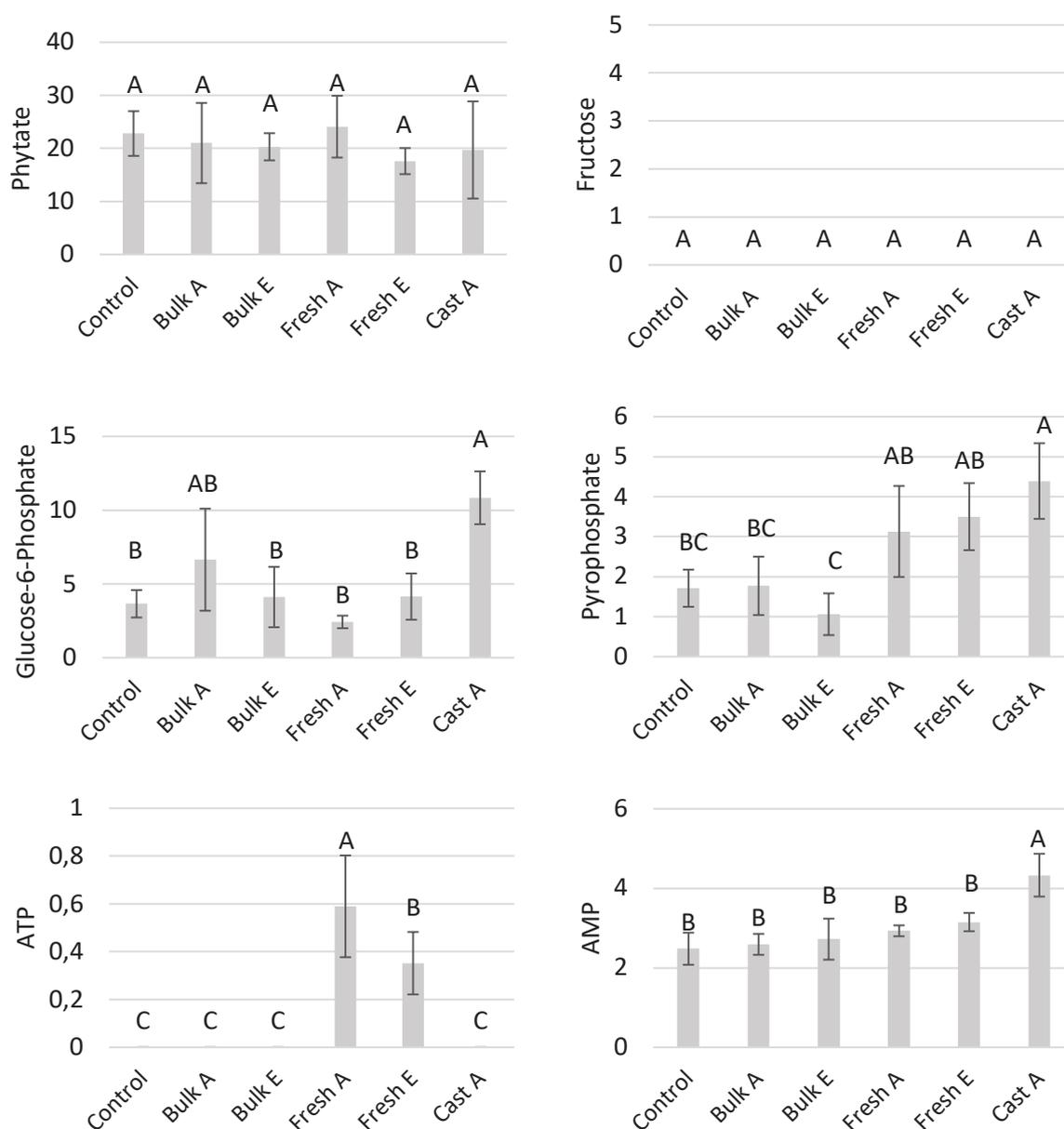


Figure 3: Concentration (mg of P/kg of soil) of organic phosphorus molecules tested by HPIC
Control: Bulk soil without earthworms. Bulk A or E: Bulk soil under the influence of Anecic (A) or Endogeic (E) earthworms. Fresh cast A or E: Soil digested by Anecic (A) or Endogeic (E) earthworms. Cast A: Casts sampled on soil surface only for Anecic treatment. Capital letters indicate statistical significance ($p < 0.05$) according to ANOVA.

3.3 Influence of earthworms on the speciation of the organic phosphorus pools and on organic anions

Even though the efficiency of the method applied to our samples was not high as around 15% of the total organic P was analyzed, we were able to compare the different treatments as the efficiency did not differ between them. However, we were able to analyze only a part of the NaOH-extractable components, the one constituted by low molecular weight organic compounds.

3.3.1 Organic acids analyzed by HPIC

Different elements have to be noticed. First, we can notice that malate and malonate significantly increased (by a factor of 2) in the casts collected on the soil surface (Table 3). Secondly, oxalate did not present any significant change among all treatments except for bulk soil with Anecic earthworms and casts produced by the same functional group and sampled on the soil surface (Table 3). The increase from bulk to cast was of a factor 3. Even though there was a slight increase from bulk soil to fresh cast this increase was not significant. Finally, citrate presented a totally different pattern (Table 3). There was no difference between bulk soils and old casts sampled on soil surface. However, there was a significant increase from these four treatments compared to fresh casts.

3.3.2 Speciation of the organic phosphorus pool

Six organic compounds containing phosphorus were analyzed by HPIC. None of them was found at the same concentration among the different treatments. There were two exceptions: phytate presented no difference of concentration between the five treatments and the control bulk soil (Fig 3), meaning that phytate was not affected by the presence of earthworms and fructose-bis-P, which was rarely detected.

Glucose-6-phosphate was affected by the presence of earthworms. It was more concentrated into soil surface cast than in any of the other treatments (Fig 3). But the difference was not significant compared to bulk soil under the presence of Anecic earthworms. Casts on soil surface were also more concentrated in pyrophosphate (Fig 3) than the bulk soil. This increase of the concentration seems to start when soil is ingested, as fresh casts seemed to present a slightly higher concentration, although not significant.

ATP and AMP were also analyzed and presented different patterns of concentration. ATP was found neither in bulk soil nor in old casts. (Fig 3) However it was found in fresh cast. Additionally, there was a higher concentration of ATP in fresh casts produced by Anecic earthworms than by the ones produced by Endogeic earthworms. Unlike ATP, AMP was present in all treatments (Fig 3). Its concentration was

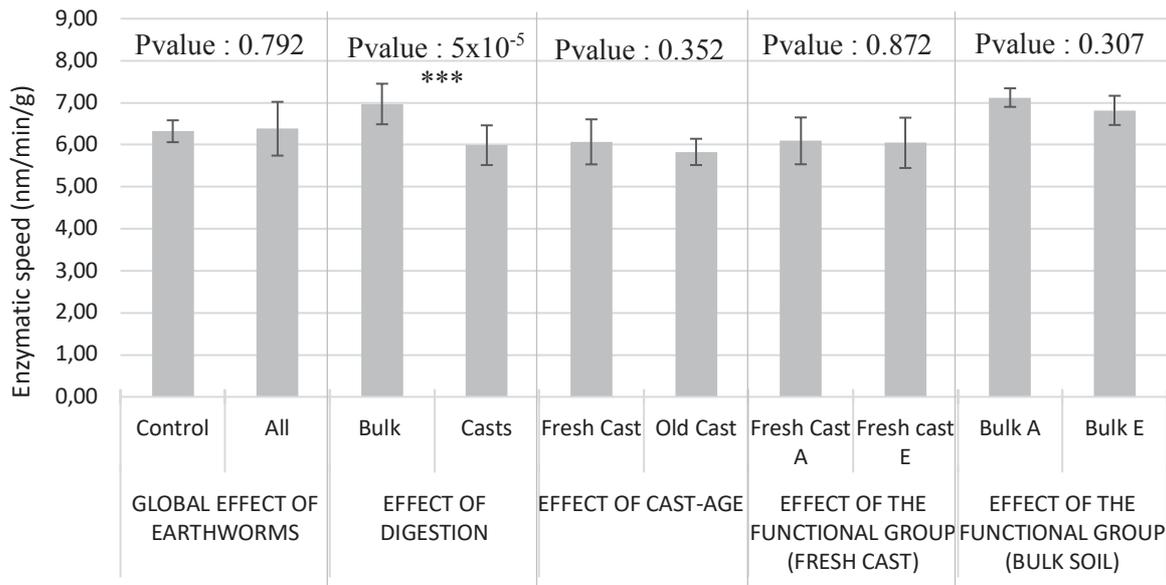


Figure 4: Influence of earthworms on the phosphatase activity.

Mean and P value according to orthogonal contrasts.

Control: Bulk soil without earthworms. Bulk A or E: Bulk soil with Anecic earthworms (A) or Endogeic earthworms (E). Bulk: Mean of bulk soils A and E. Fresh cast A and E: Soil incubated with either Anecic earthworms (A) or Endogeic earthworms (E) Fresh Cast: Mean of fresh cast A and E. Old cast: Cast sampled on soil surface (only for Anecic treatment). Cast: Mean of both fresh casts and old casts. All: mean of all treatments except the control.

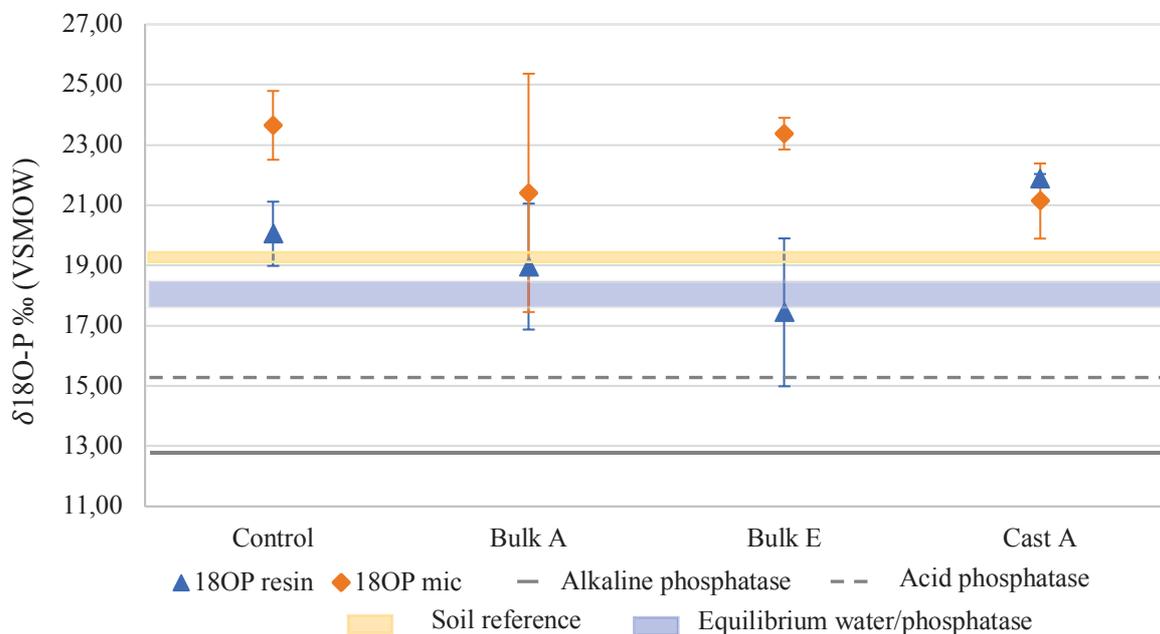


Figure 5: Representation of the isotopic ratio of $^{18}\text{O-P}$ of bulk soils and old cast.

Control: Bulk soil without earthworms. Bulk A or E: Bulk soil under the influence of Anecic (A) or Endogeic (E) earthworms. Tur A: Casts sampled on soil surface only for Anecic treatment. Soil reference: mean of $\delta^{18}\text{O-P}$ values obtained for HCl-extractable phosphorus. Water/phosphatase equilibrium: calculated with the equation of Chang and Blake, 2015. Alkaline and acid phosphatase: calculated with the equations of Liang and Blake, 2006 and von Sperber et al., 2014.

almost identical in all treatments with the exception of casts present on soil surface (old cast) where it was higher.

3.4 Influence of earthworms on processes involved in the mineralization of organic phosphorus

3.4.1 Phosphatase activity

The enzymatic activity of the phosphatase was not impacted by the presence of earthworms as no difference was observed between the control treatment compared to the mean of all other treatments corresponding to a real soil with both bulk soil and casts (Fig 4). However, we can see a significant decrease of the activity from the bulk soil to casts. This suggests that the digestion of the soil by earthworms decrease the enzymatic activity. Yet, as there is no significant difference between old and fresh cast, the enzymatic activity in casts appeared to persist with time. The Anecic and Endogeic functional groups had the same influence on the enzymatic activity in both bulk soil and fresh casts.

3.4.2 Isotopic ratios of oxygen in phosphate ($\delta^{18}\text{O-P}$)

For all bulk soils, the resin-extractable phosphorus pool was at the expected isotopic equilibrium between dissolved phosphate and water and was also close to the soil reference defined by the mean of the $\delta^{18}\text{O-P}$ values obtained for the HCl-extractable phosphorus of bulk soils (Fig 5). However, the $\delta^{18}\text{O-P}$ of microbial phosphorus in bulk soils were all higher than the equilibrium (Fig 5) meaning that there was a biological activity that affected this pool different from intracellular turnover (Tamburini et al., 2014). However, these values were not influenced by the presence of earthworms (Fig 5) nor by the functional group of earthworms, as no significant differences were found between these treatments (Table 4). The isotopic values of both resin and microbial P in the bulk soils was not close to the expected alkaline or acid phosphatase signatures, i.e. phosphate resulting from mineralization of phosphomonoesters, respectively at 12.5‰, and 15‰ (Fig 5).

When focusing on old casts, we noticed that both resin-extractable phosphorus and microbial phosphorus had a $\delta^{18}\text{O-P}$ higher than the equilibrium and the soil's reference (Fig 5). As for the bulk soils, these values appear not influenced by phosphatases activity. Interestingly, $\delta^{18}\text{O-P}$ values of resin-extractable and microbial phosphorus were identical in these samples.

Fresh cast were plotted separately as the equilibrium between water and dissolved phosphate was different from the other samples due the different temperature during the incubation (Fig 6). The

Table 4: Influence of earthworms on the $\delta^{18}\text{O-P}$ (‰)

Mean and P value according to orthogonal contrasts.

Control: Bulk soil without earthworms. *Bulk A or E*: Bulk soil with Anecic earthworms (A) or Endogeic earthworms (E). *Bulk*: Mean of bulk soils A and E. *Fresh cast A and E*: Soil incubated with either Anecic earthworms (A) or Endogeic earthworms (E) *Fresh Cast*: Mean of fresh cast A and E. *Old cast*: Cast sampled on soil surface (only for Anecic treatment). *Cast*: Mean of both fresh casts and old casts. *All*: mean of all treatments except the control.

| | GLOBAL EFFECT OF EARTHWORMS | | | EFFECT OF DIGESTION | | | EFFECT OF CAST-AGE | | |
|---|-----------------------------|-------|---------|---------------------|-------|---------|--------------------|----------|---------|
| | Control | All | p value | Bulk | Casts | p value | Fresh Cast | Old Cast | p value |
| Resin-Extractable $\delta^{18}\text{O-P}$ ‰ | 20.05 | 18.37 | 0.17 | 18.20 | 18.47 | 0.77 | 16.78 | 21.87 | 0.00 ** |
| Microbial $\delta^{18}\text{O-P}$ ‰ | 23.65 | 21.71 | 0.22 | 22.39 | 21.25 | 0.42 | 21.31 | 21.14 | 0.92 |

| | EFFECT OF THE FUNCTIONAL GROUP (FRESH CAST) | | | EFFECT OF THE FUNCTIONAL GROUP (BULK SOIL) | | |
|---|---|--------------|---------|--|--------|---------|
| | Fresh Cast A | Fresh cast E | p value | Bulk A | Bulk E | p value |
| Resin-Extractable $\delta^{18}\text{O-P}$ ‰ | 18.62 | 14.93 | 0.06 | 18.96 | 17.44 | 0.23 |
| Microbial $\delta^{18}\text{O-P}$ ‰ | 22.26 | 20.36 | 0.43 | 21.41 | 23.37 | 0.37 |

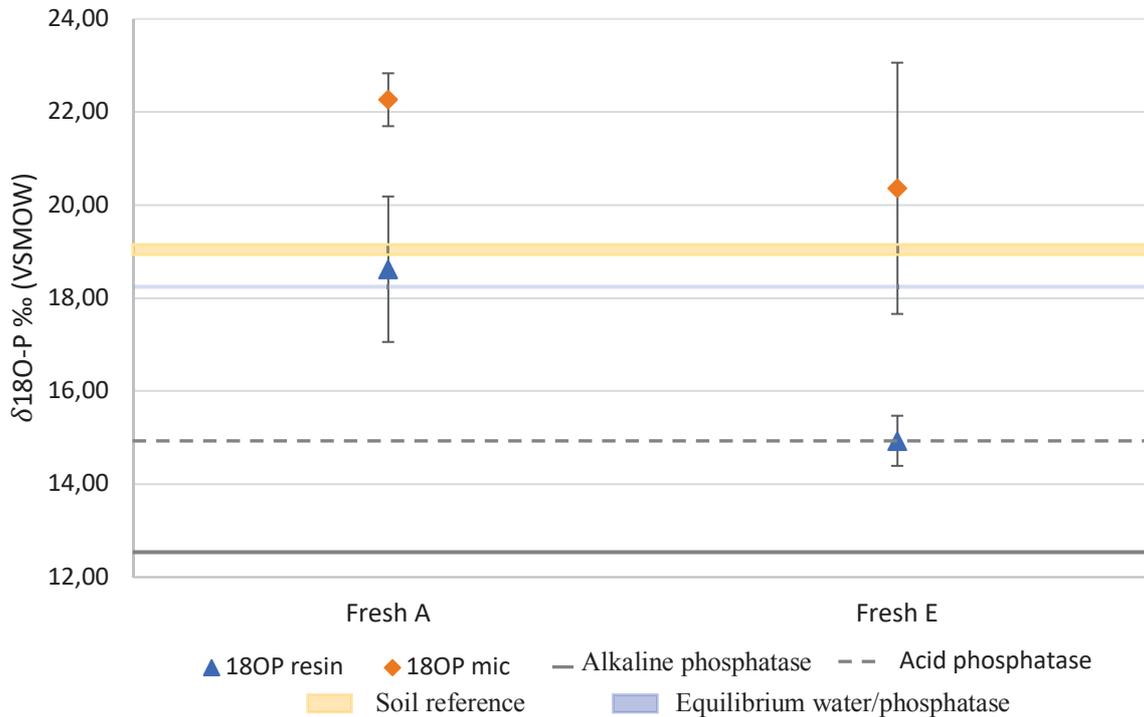


Figure 6: Representation of the isotopic ratio of $^{18}\text{O-P}$ of fresh casts.

Fresh cast A and E: Soil incubated with either Anecic earthworms (A) or Endogeic earthworms (E). *Soil reference*: mean of $\delta^{18}\text{O-P}$ values obtained for HCl-extractable phosphorus. *Water/phosphatase equilibrium*: calculated thanks to Chang and Blake, 2015. *Alkaline and acid phosphatase*: calculated with the equations of Liang and Blake, 2006 and von Sperber et al., 2014.

microbial phosphorus presented no significant difference according to the functional group of earthworms (Fig 6; Table 4), however the Endogeic value was close to the equilibrium between water and dissolved phosphate, while the Anecic one was not. Moreover, there was no difference between fresh and old cast regarding this P-pool (Table 4) suggesting $\delta^{18}\text{O-P}$ was not impacted by cast aging. Resin-extractable phosphorus pool in fresh casts presented a different pattern. The $\delta^{18}\text{O-P}$ value was slightly different according to the functional group studied (Fig 6), even though this difference was not significant. In the Anecic fresh casts, the resin-extractable phosphorus was similar to the equilibrium value and to the soil reference $\delta^{18}\text{O-P}$ (Fig 6), as for the bulk soils. In fresh casts produced by Endogeic earthworms, instead, the resin-extractable phosphorus pool was depleted compared to the equilibrium value and the soil reference (Fig 6). Moreover, the $\delta^{18}\text{O-P}$ value for old casts was significantly higher than the mean of $\delta^{18}\text{O-P}$ fresh casts values (Table 4).

The differences in the casts were not significant enough to induce a significant modification in the global soil (average of bulk soils and casts) (Table 4).

4 Discussion

Soil ingestion and digestion by earthworms impacted the repartition of the organic phosphorus species and pool sizes of both available and stable pools except for the NaOH-extractable inorganic phosphorus. Three hypothesis can be proposed to explain the observed differences: (1) soil chemical parameters and microbial activity were modified by the presence of earthworms and led to a modification of the repartition in the pools of phosphorus or (2) biochemical reactions inside the earthworms' guts that are responsible of the pool changes or (3) a selective ingestion of the soil explaining these differences for the phosphorus pools.

4.1 Explaining changes on P pools thanks to changes of other soil parameters

4.1.1 Pool size

We tried to explain the mechanisms that caused changes in the different pools with a PCA analysis representing the pools of phosphorus and the soil parameters. The PCA analysis (Fig 7) showed that NaOH extractable organic phosphorus and HCl extractable phosphorus were correlated to the microbial biomass (C_{mic} and N_{mic}) and to the nutrient availability linked to the microbial community (inorganic and dissolved organic carbon and soluble nitrogen). Surprisingly there was no correlation between

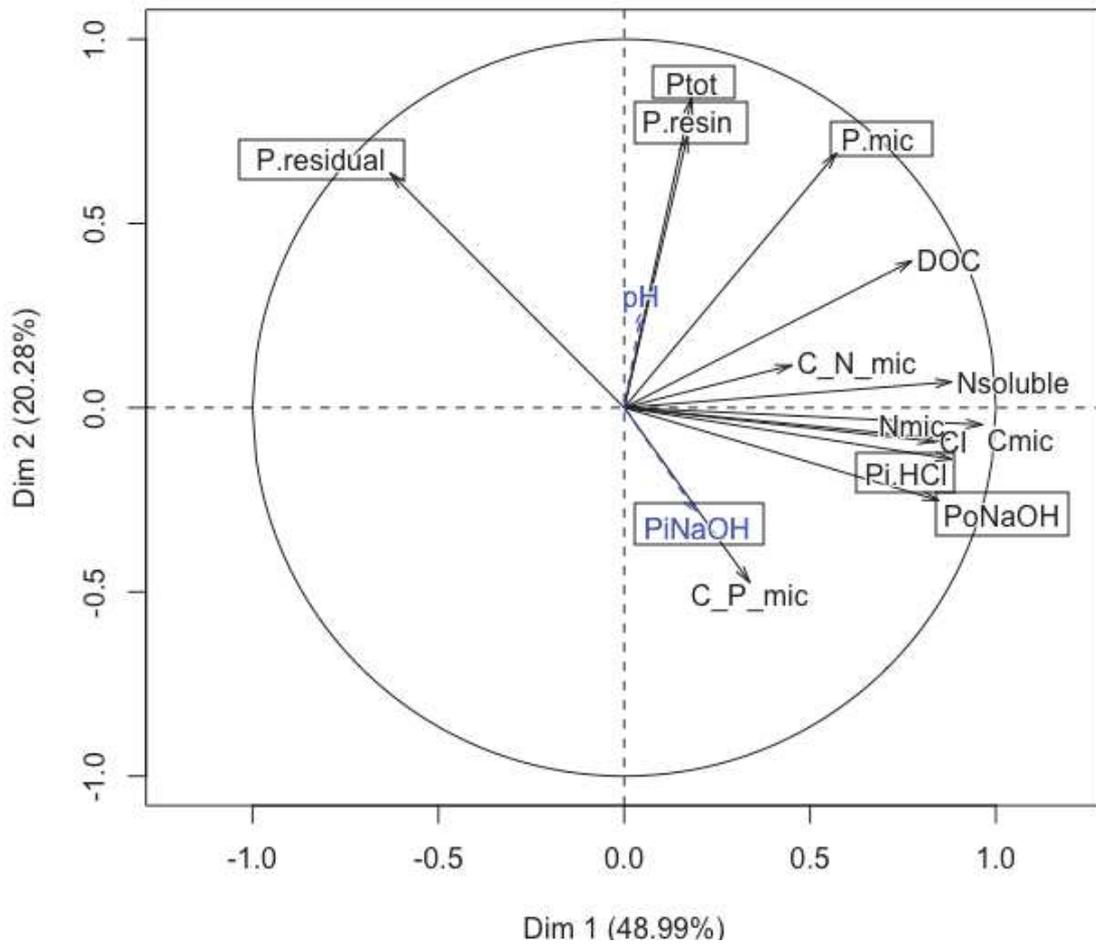


Figure 7: Correlation circle of a PCA representing the different pools of phosphorus and different soil parameters. (pH and NaOH extractable inorganic P are illustrative variables)
Framed variables are phosphorus pools

theses microbial and nutrient parameters and the more available P pools that are the resin-extractable and microbial phosphorus.

Resin-extractable phosphorus is correlated to total phosphorus (Fig 7). This could mean that the increase of total phosphorus lead to an increase of the resin-extractable phosphorus as it would modify the equilibrium with the soil solid phase. This is coherent with our results because the only treatment showing an increase of total phosphorus which is old casts (Fig 1) is also the only one presenting an increase of the resin-extractable phosphorus (Fig 2). But resin-extractable phosphorus is not correlated to any other parameter (Fig 7). An hypothesis to explain the increase of resin-available phosphorus from bulk soil to casts and from fresh to old casts (Table 2) could be that some labile P was liberated from microorganisms after their death (Oberson et al., 2001; Seeling and Zasoski, 1993). This hypothesis is supported by the decrease of the microbial carbon from fresh to casts but not from bulk soil to casts where it increased (Table 1). However, we did not observe a corresponding decrease of microbial P from fresh to old casts. In addition, an increase of the dissolved organic carbon is also noticed from bulk soils to casts (Table 1). This increase of dissolved organic carbon could explain the increase of available phosphorus as it can compete with orthophosphate for sorption sites on metal-hydroxides (Ros et al., 2017) and as a consequence it would liberate some adsorbed orthophosphate.

The absence of correlation between microbial phosphorus and microbial nitrogen and carbon (Fig 7) leads us to three hypotheses. Changes in microbial C/N and C/P ratios might indicate shifts in the microbial community (Heuck et al., 2015). However, no evidence of differences were found when analyzing the C/P and C/N microbial ratios except between old and fresh casts (Table 2). Secondly, the metabolic state of the microbial community was different in casts compared to bulk soil with a higher microbial activity in casts compared to bulk soil (Scheu, 1987). This higher microbial activity in cast was still visible in old casts, even if the activity was lower than for fresh casts (Scheu, 1987). This hypothesis is supported by our results as we found more microbial phosphorus in fresh cast and cast compared to bulk soils (Table 2). A third hypothesis is that the increase of microbial phosphorus from bulk soils to casts (Table 2) could come from the NaOH-extractable inorganic phosphorus pool as bacteria are able to mobilize phosphorus adsorbed to aluminum hydroxides (Illmer et al., 1995). Moreover, Illmer et al., 1995 confirmed a correlation between this phenomenon and the concentration of citrate. Our results supported this hypothesis as there was an increase of citrate in fresh casts compared to bulk soils but not in old casts (Table 3). However, there was no decrease of NaOH-extractable inorganic phosphorus from bulk soil to casts (Table 2) so we consider this hypothesis as unlikely.

NaOH-extractable organic phosphorus and HCl extractable phosphorus were related to the size of the microbial community (C_{mic} and N_{mic}) as well as with the inorganic carbon and soluble nitrogen (Fig 7). This suggests that microbes affected also the more stable pools of phosphorus. Our results also suggest

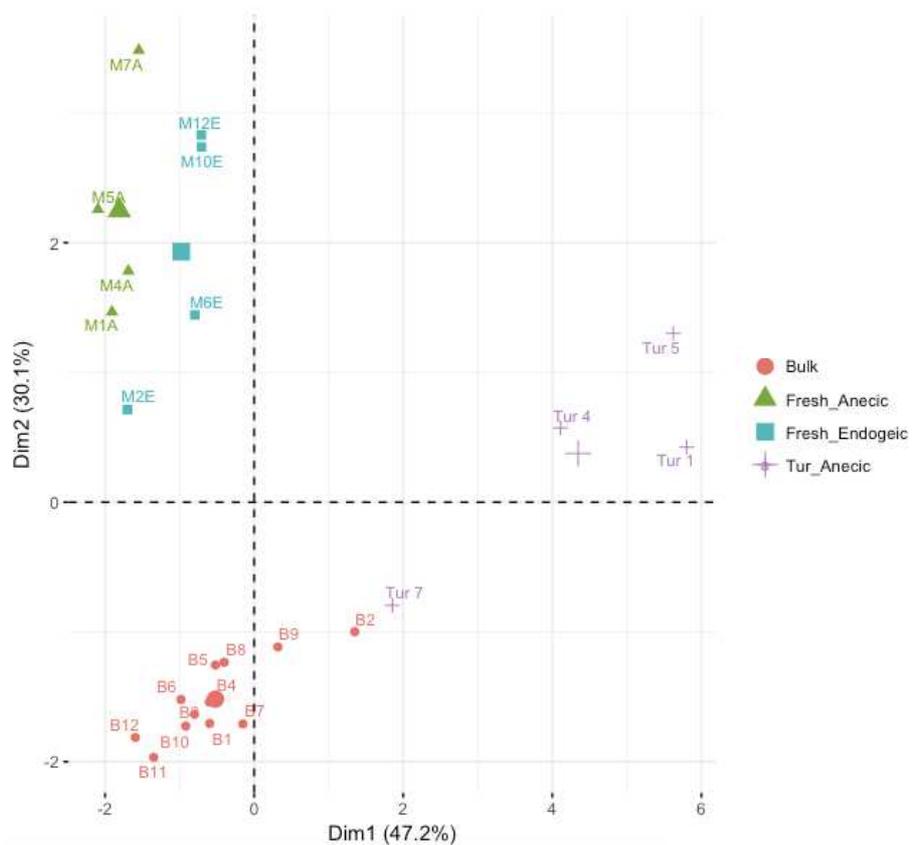


Figure 8: Individuals map of a PCA presenting organic acids, organic phosphorus compounds and microbial carbon. (Organic acids and organic phosphorus compounds were analyzed by HPIC)

that the size of the microbial community or its metabolic status changed with the aging of the casts, this is indicated by the C/P microbial ratio, which decreased with the aging as well as the microbial C and N (Table 1). However, the stoichiometry of the microbial community, given by the ratios of microbial carbon, nitrogen and phosphorus, was not well represented in the PCA first and second axes and does not impact the size of the more stable pools of phosphorus (Annex I).

It seems that the increase of the organic phosphorus in the casts (Fig 2) was closely related to the microbial biomass and to the nutrient availability linked to the microbial activity (Fig7). The link between the increase of organic phosphorus and the microbial community and activity is still unclear. Some studies show that the microbial community contributes to the increase of organic phosphorus by synthesizing organic P compounds and then releasing them upon their death or predation (Seeling and Zasoski, 1993), while others show that the organic pool of phosphorus is not impacted by the microbial community and activity (Lee et al., 1990).

Regarding the HCl-extractable phosphorus, the PCA suggest that it was also influenced by the microbial biomass and the labile C and N (Fig 7). This means that the more the microbial activity or biomass was important, the more phosphorus bound to calcium there would be. This is contrary to what was found by Lee et al, 1990 who has shown that either the microbial activity did not change the quantity of Ca-bound-phosphorus, or that it decreased it. The increase of Ca-bound-phosphorus could be a consequence of the re-precipitation of the residual phosphorus into secondary calcium phosphate. This hypothesis is supported by the decrease of residual phosphorus and increase of HCl-extractable phosphorus in fresh casts (Table 2).

The relation of labile and inorganic carbon with the size of the different pools of phosphorus could be explained by the fact that the phosphorus mineralization is often linked to the microbial carbon needs (Spohn and Kuzyakov, 2013). However, this should affect all pools of phosphorus and especially the plant-available one which is not the case in our study.

4.1.2 Speciation of the organic phosphorus

When analyzing the HPIC results we can notice that the yield of the analysis in terms of organic P recovered and analyzed versus organic P originally extracted was low (15%). This could be due to the fact that the method was initially adapted to tropical soils and that temperate soils don't have the same organic phosphorus pool constitution. Nevertheless, the compounds we analyzed with the HPIC are the low molecular weight compounds, which represent the more labile organic P pool and therefore likely subject to faster turnover.

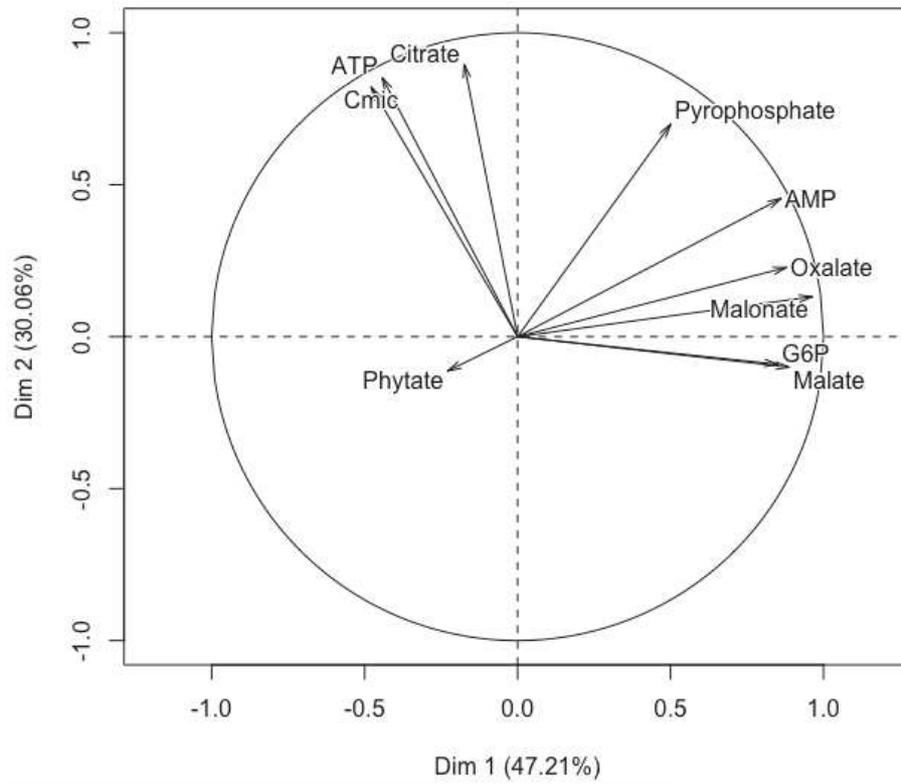


Figure 9: Correlation circle of a PCA presenting organic acids, organic phosphorus compounds and microbial carbon. (Organic acids and organic phosphorus compounds were analyzed by HPIC)

As for the chemical parameters, we performed a PCA analysis. According to the PCA, (Fig 8) four groups of individuals can be identified. All old casts seem to present similar patterns regarding organic phosphorus speciation. The same is true for bulk soil samples as well as for fresh casts. Moreover, for this last group, there seems to be two distinct groups made by the functional group of the earthworms. A hierarchical clustering confirmed our choice to put all bulk samples into a single group as there is no difference between the control, Anecic and Endogeic treatment (Annex II). Our choice to separate our individuals into four distinct clusters was confirmed by a Monte-Carlo test (p value = 0,001). This means that bulk soil, old cast and Anecic and Endogeic fresh cast present significant differences for their organic phosphorus speciation. This suggests that soil digestion by earthworms modify the concentration of the organic P compounds and that there is an effect of the functional group.

Then we analyzed through a PCA the links that can exist between all the different organic compounds and the soil parameters. ATP was only present in fresh casts (Fig 3) and was correlated to microbial carbon (Fig 9). ATP could have various sources: it could be sorbed to soil particles and made unavailable to be mineralized and therefore persist in the soil, it could also be extracted from living cells during the NaOH extraction as it was suggested by various authors (Cade-Menun et al., 2010; Turner et al., 2005). Its correlation with microbial carbon, which is an indicator of the size of the microbial community, supports this last hypothesis which means that ATP comes likely from living cells. This is another proof that the size and activity of the microbial community was more important in fresh cast as the concentration of ATP is correlated with the number of microbial cells (Hammes et al., 2010).

AMP was correlated to pyrophosphate (Fig 9). This correlation could be explained by the origin of AMP and pyrophosphate that would be the products of the hydrolysis of ATP. However AMP and ATP were independent (Fig 9) and not inversely correlated, as observed for example by Waithaisong, 2015 who related this inverse correlation to the degradation of ATP to AMP. Therefore, we cannot conclude about the origin of AMP.

Citrate was also correlated to microbial carbon (Fig 9). This can be explained by a possible secretion of citrate by the microbial community. It was shown that citrate can be produced by bacteria (Machuca et al., 2007). This would explain the fact that citrate is found in higher concentration in fresh casts (Table 3) as we showed the microbial community is bigger and likely more active.

The other organic acids (oxalate, malonate and malate) were independent to the microbial indicators but were correlated to G6P (Fig 9). It has been shown that oxalate, malonate and malate can be secreted by fungal communities (Khan et al., 2007; Machuca et al., 2007; Plassard and Fransson, 2009). This could explain the correlation between these organic acids with G6P as fungi are able to transform glucose into G6P. This interpretation associated with the individuals' map (Fig 8), leads us to the conclusion that

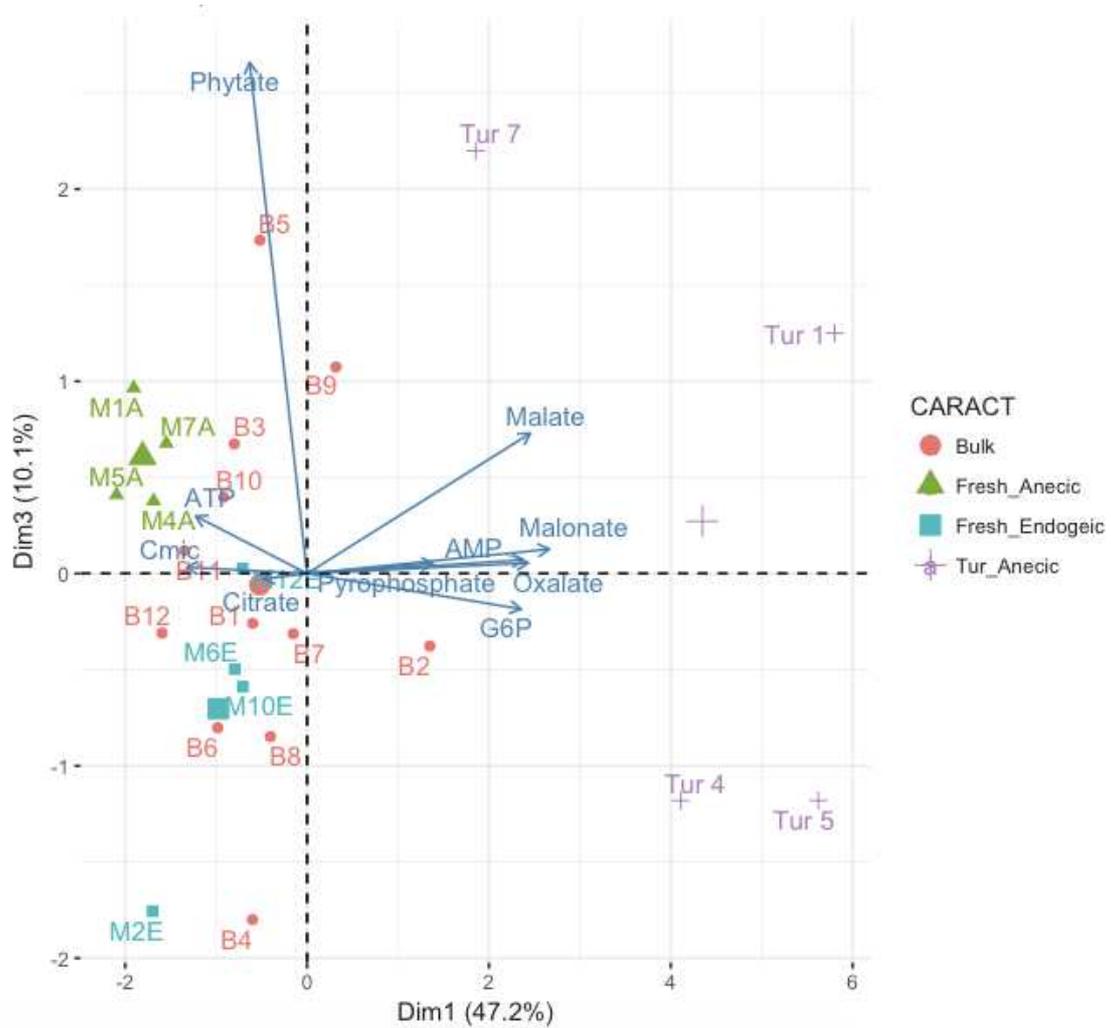


Figure 10: Individuals and variables map of a PCA presenting organic acids, phosphorus organic compounds and microbial carbon. (Organic acids and phosphorus components were analyzed by HPIC)

there could be a colonization of fungi of the old casts present on soil surface, while bacteria are more abundant on fresh casts. This hypothesis is also supported by the significant difference in the microbial C/P ratio between fresh cast and old cast possibly indicating a shift in the microbial community (Heuck et al., 2015).

Phytate was not well represented in the two first axis of the PCA, however it was well represented in the third one (Fig 10). This shows that phytate's concentration does not depend on the earthworms impacts on soil. Indeed, phytate is mostly of plants origin as it a storage molecule present in grains (Gerke, 2015 and references therein).

4.2 The enzymatic activity of phosphatases does not explain the pools changes

The increase of resin-extractable phosphorus in old and fresh cast made us hypothesized that the phosphatase activity would be higher in casts than in bulk soil. However, our results showed the opposite, with a higher enzymatic activity in bulk soils than in casts, which was similar to the results found in Zhang et al., 2000 and Jiménez et al., 2003. Jiménez et al., 2003 explains this difference as being an artefact of the laboratory experiment as this study did not find similar results in the field experiment. Zhang et al., 2000 gave two possible explanation to these results: either there was a degradation of the phosphatase in the guts or there was an inhibition of its activity by the inorganic phosphorus. This suggests that the mineralization does not occur in casts, but more likely in the earthworms' guts as suggested by Devliegher and Verstraete, 1996.

Most of the phosphatase enzymes are secreted by microbes, therefore the increase of phosphatase activity is often correlated with the size of the microbial community. In our results, however, we see an increase of the microbial community which does not go in the same direction as the enzymatic activities. Regarding the microbial community, not only the size but also the composition of the microbial pool can explain changes in phosphatase activity (Margalef et al., 2017). Our communities seem to change between bulk soils and casts as the C/P ratio and the speciation of small molecular weight organic compounds vary between these treatments (Table 1).

Some other parameters related to nutrients cycling are controlling the phosphatase activity such as organic phosphorus, total carbon and nitrogen (Margalef et al., 2017). The higher these values are, the higher the phosphatase activity will be. Organic phosphorus, carbon and nitrogen concentrations are all more important in casts compared to bulk soil (Table 1 and 2). This is in contrast with the findings of Margalef et al., 2017).

Another explanation could be linked to the regulation of the extracellular phosphatase secretion by the availability of phosphorus. As it was shown in Olander and Vitousek, 2000, the phosphatase production by microbes is inversely correlated to the phosphorus available. As there is more phosphorus available in the casts than in the bulk soils, this hypothesis is supported by our results.

4.3 Biological processes that can explain the more available pool changes

It has been pointed out that phosphatase activities which are measured under conditions of substrate saturation indicate potential rather than actual rates, as the rates derived from these assays are often one to two orders of magnitude greater than organic P mineralization measured with isotopic dilution techniques (Bünemann, 2015).

Only isotopic tracers such as $\delta^{18}\text{O-P}$ can give a hint on actual mineralization activity. Indeed, in our study, it seems that in fresh cast produced by Endogeic earthworms the available P extracted by anionic exchange resins was partially coming from the mineralization of organic P by alkaline/acid phosphatases as it presented a lower $\delta^{18}\text{O-P}$ value than all other treatments. This depleted value was close to the expected signature imprinted by phosphomonoesterase enzymes (Fig 6), which in fact induce a negative fractionation. This means that the digestion of soil by Endogeic earthworms stimulated organic phosphorus mineralization by extracellular phosphatases. When considering the pH of our soils (around 6.5 (Table 1)) and the pH of the earthworms guts (around 6 according to Barois and Lavelle, 1986), the acid phosphatase need to be privileged in our study compared to the alkaline one as the optimal pH for the acid phosphatase is 5.8 compared to 9 for the alkaline one (Mobley et al., 1984). An explanation is that the mineralization effect induced by the Anecic functional group was lower than the Endogeic and consequently not detectable on the isotopic ratios. This lower mineralization could be explained by the regulation of phosphatase activity by the availability of phosphorus (Olander and Vitousek, 2000). This is confirmed by our results showing that the quantity of resin-extractable phosphorus is more important in Anecic fresh casts than Endogeic ones (Table 2).

Almost all our microbial phosphorus isotopic ratio were above the calculated equilibrium values (Chang and Blake, 2015) and were all very similar (Fig 6 and Fig 7). This could be explained by the fact that the $\delta^{18}\text{O-P}$ observed values (around 22.03‰) in microbial P are the result of the equilibration of phosphate with the water in the living cells (Tamburini et al., 2014). An estimated value of this equilibrium can be obtained by using the metabolic $\delta^{18}\text{O-H}_2\text{O}$ (Li et al., 2016) and it gives a value around 20%. This value is higher than the equilibrium between phosphate and water in the soil (max 18.3‰), however not high enough to be the only explanation. An alternative explanation would be the difference of status of the different microbial communities that are present in our soil (Achat et al., 2010). Active communities and communities in dormancy won't have the same equilibrium signature because they

would be active at different times under different temperatures and water isotopic composition (Chang and Blake, 2015).

In the old casts, the $\delta^{18}\text{O-P}$ values obtained for microbial and resin-extractable phosphorus were almost identical, while this was not the case in bulk soil. This suggests, as hypothesized before, that at least part of the resin-extractable phosphorus in the old cast treatment could originate from microbial P after the microorganisms' death (Oberson et al., 2001; Seeling and Zasoski, 1993a). The increase of the resin-extractable phosphorus in old casts (Table 2) could be therefore explained by the decrease of the microbial biomass due to the death of the microorganisms. This hypothesis is supported by the decrease of the microbial carbon and nitrogen in old casts (Table 1). However, the concentration of microbial phosphorus did not decrease between fresh and old casts (Table 2). It has to be pointed out that casts collected in the macrocosms and casts from the incubation were not produced under the same conditions (e.g. greater earthworm's density in the incubation), so any comparison has to be made with a certain caution.

All bulk soils samples showed similar $\delta^{18}\text{O-P}$ values in the resin pool, which were not close to the mineralization signature as they were close the equilibrium state (Fig 5). We can therefore conclude that mineralization it is not much affecting the available P pool in the bulk soil. It is, however, difficult to disentangle the equilibrium effect, which is induced by intracellular P turnover, from the abiotic processes such as the sorption/desorption or dissolution precipitation as the signatures of these two processes overlap (Fig. 5).

In summary, we can conclude that: (1) mineralization of organic P by extracellular phosphatase was likely occurring in fresh casts and affected the available P, as highlighted by the $\delta^{18}\text{O-P}$ depleted signatures, (2) these depleted signature disappeared in old casts, thus suggesting that the mineralization effect was transient, (3) release of microbial phosphorus in old casts could explain at least partially the increase of the resin-extractable phosphorus, as the $\delta^{18}\text{O-P}$ of resin-extractable P was identical to the one measured in microbial P, while it was not the case in all the other treatments, and (4) no signs of mineralization were visible in bulk soil samples and their isotopic signatures suggest either the intracellular P re-cycling effect or the effect of exchanges with the soil-matrix. However, considering the relative size of the available, microbial and mineral pool the last hypothesis is more likely.

5 Conclusion and perspectives

5.1 General conclusion of our study

The presence of earthworms strongly modified the repartition of the phosphorus inorganic and organic pools assessed with a sequential extraction and HPIC. There was an increase of the directly-plant-available phosphorus and of the organic phosphorus in the global soil (mean of casts and bulk soils values) compared to a soil with no earthworms. Most of this increase was due to increases detected in casts as no difference was noticed between control and bulk soils in the presence of earthworms. Moreover, almost every pool of phosphorus (except the NaOH-extractable inorganic P) was increased in casts at the expenses of the residual pool, which was strongly reduced in fresh casts. This effect evolved with time causing a modification of the pools which tended to go back to the original values of bulk soil.

This increase of the phosphorus availability in casts enforces the statement made by most studies: earthworms' casts are hotspots for nutrients availability and this even when considering aged-casts on soil surface. The increase of the availability of phosphorus in casts could not be highlighted by a measurement of potential phosphatase activity. However, the isotopic composition of oxygen in phosphate did show an effect of the mineralization of organic P on the available P. This effect was transient and detectable only on fresh casts.

The increase of the more available pools of phosphorus (plant-available and microbial phosphorus) in casts was linked to microorganism's activity. Our results show that different mechanisms are involved. In addition to mineralization, we could highlight that on old casts available P is probably affected by P released after their death. Additionally, we could show that sorbed phosphorus might be released by secretion of dissolved organic carbon or citrate.

The organic pool was increased by earthworm digestion due to a selective soil ingestion or due to a microbial synthesis of organic phosphorus. This increase is only temporary as aging of casts decreases the organic phosphorus pool likely because of a diminution and a change of the microbial community composition due to a colonization by fungi.

We analyzed the speciation of the low molecular weight organic acids and P compounds. This allowed us to see that the composition of the organic pool was different among bulk soil, fresh cast and old cast, meaning that there is an impact of earthworms digestion on the composition of the organic pool. It would be interesting to analyze other phosphorus compounds to get a deeper insight on these transformations.

Our comparison between fresh and old casts need to be taken with caution as they were not sampled under the same conditions. A study of casts sampled on the day of their apparition would be more precise.

No difference appeared between Anecic and Endogeic earthworms regarding the different pools of phosphorus for both fresh casts and bulk soils. It could be interesting to remodel the experimental design to add another treatment which would be with the two functional groups in the same proportion as we can find in agricultural lands (around 80% of Endogeic earthworms and 15% of Anecic earthworms according to (Pelosi et al., 2009)). This could help us to understand if these two functional groups are transforming phosphorus from the same source or if the combination of functional groups would have a summative effect on phosphorus availability. This hypothesis is relevant knowing that different functional groups of earthworms live in different depth of soil and so have access to different resources of phosphorus.

5.2 Agroecological perspectives

The main conclusion regarding the agroecological aspect is that the presence of earthworms increases the plant-available phosphorus. This is one of the eco-systemic services fulfilled by earthworms that is nutrient availability. This increase is induced by several mechanisms, in which the interaction of earthworms and microorganism plays a role. Mechanisms we highlighted involve the acceleration of the turnover of P pools especially the living and dead organic P.

We could not, however, assess the global effect on the soil as we did not have the mass proportion of casts compared to the bulk soil. The results regarding casts, confirmed that middens as well as cast mantles are hotspots for available nutrients and more specifically for phosphorus. However, this effect is transient and it has to be established whether the plants are able to take advantage of these hotspots. As burrows are an optimal place for the growth of roots (Springett and Gray, 1997), the increase of available phosphorus in casts is supposed to be directly positive for plants. The data from the Ecotron experiment showed no significant difference on crop yield, however some differences were visible regarding the root biomass but still need to be analyzed. Indications will be provided by the data on nutrients uptake by the crop, which are not available yet.

This study provides more evidences to encourage farmers to adopt practices that could be beneficial for earthworms and generally stimulate biological activity in the soil. In our study, we highlighted that: a) almost all P pools, included the most refractory ones, were affected by ingestion by earthworms and b) this effect was detectable on a relatively P rich soil (80-90 mg/kg Olsen P), which is the case of many soils in France and Europe. By favoring earthworms' activity, P pools, which are not or little available

to crops, could be mobilized, thus helping to cope with the P accumulation deriving from decades of over-fertilization of European soils (P legacy).

Suitable practices include a reduction of the pesticides inputs that have negative impacts on the earthworms abundance (reviewed in Pelosi et al., 2014) or the application of organic matter such as manure or vegetable compost that have a positive effect on the earthworm abundance (Leroy et al., 2008) and on microbial biomass and activity. Another practice that affects earthworms is tillage. Tillage can be highly negative on the earthworm abundance as it can kill them and it destroys their habitats (Bertrand et al., 2015). The adoption of reduced tillage or no-tillage can be positive for Anecic earthworms that are very sensitive to tillage due to the destruction of their burrows. Tillage can be positive also for Endogeic earthworms as it will increase food supplies (Chan, 2001). Trade-offs need to be found in all systems to improve earthworms' habitat conditions in order to increase their abundance.

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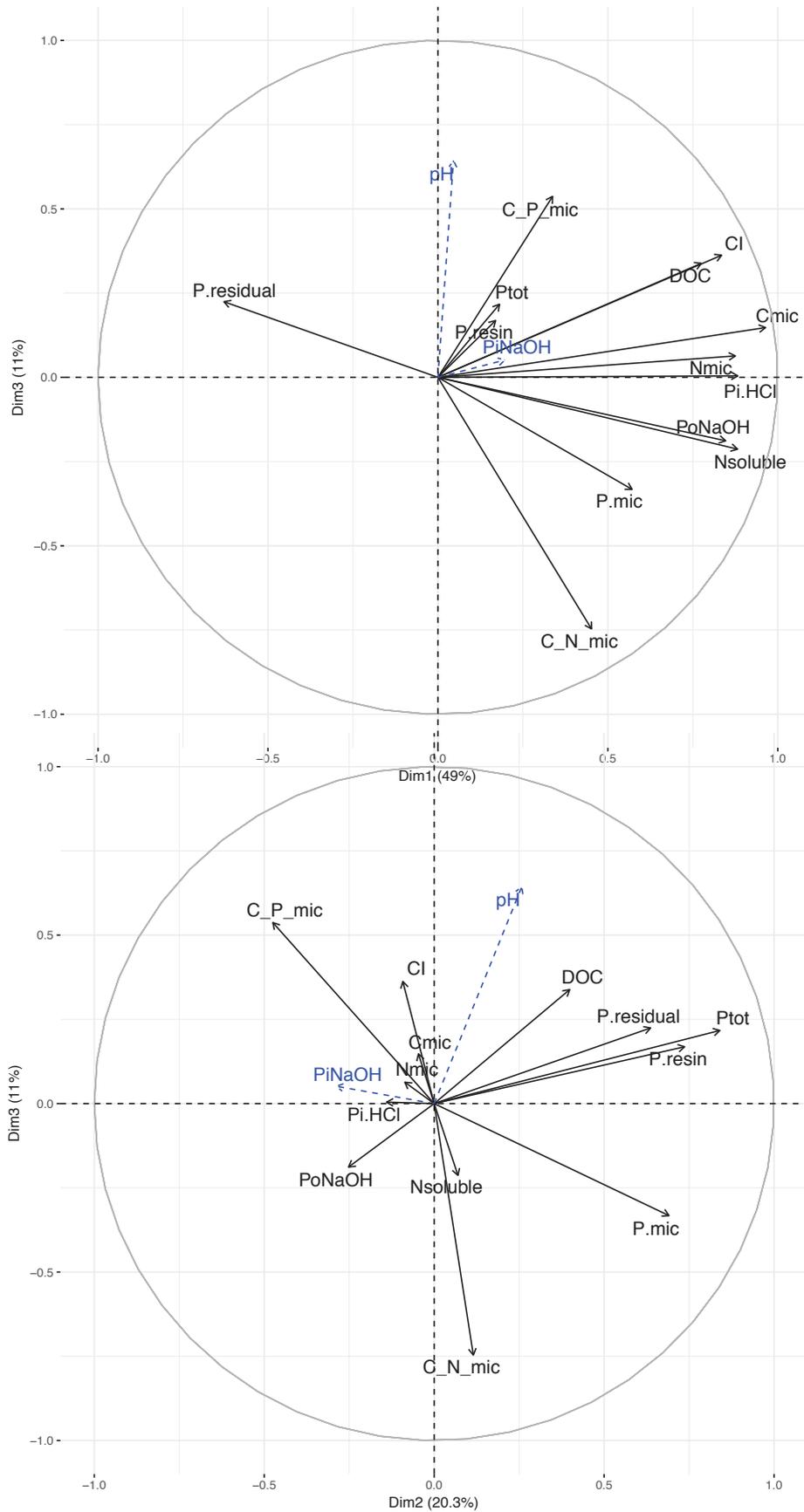
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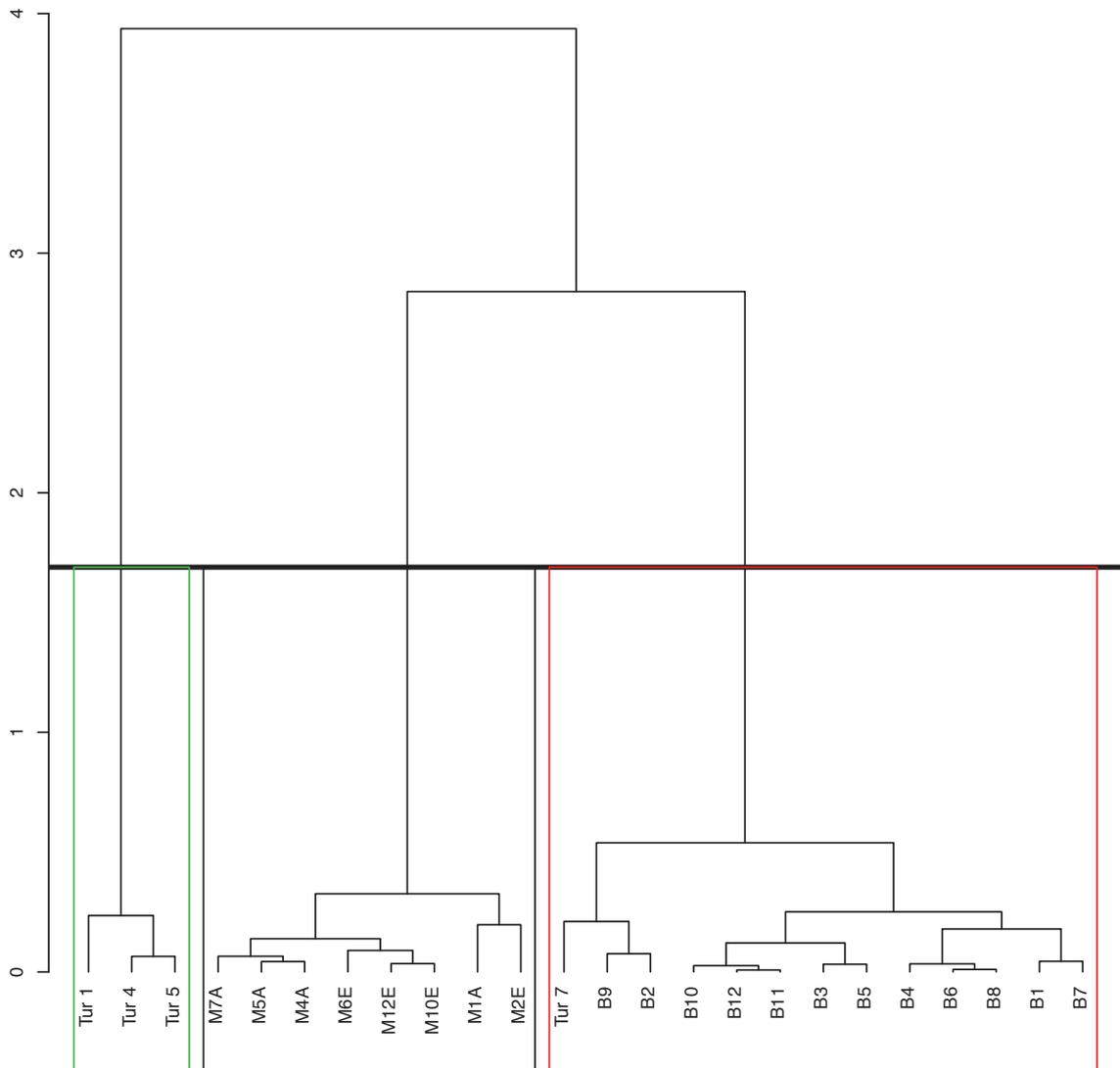
Annex I : Correlation circles of a PCA representing the different pools of phosphorus and different soil parameters. (pH and NaOH extractable inorganic P are illustrative variables)



Annex II : Hierarchical clustering of individuals regarding the concentration of organic acids.

Tur: Old Cast. *B*: Bulk soil. *M*: Fresh cast.

A is for Anecic earthworms. *E* is for Endogeic earthworms.





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Titre français : Le rôle des vers de terre dans le recyclage biologique du phosphore organique.

Titre anglais : The role of earthworms in the biological processes of the phosphorus cycle.

Résumé (1600 caractères maximum) :

Les vers de terre, ingénieurs du sol, réalisent de nombreux services écosystémiques notamment par leur participation aux cycles géochimiques. Notre étude a pour but de comprendre l'impact des vers de terre sur les pools de phosphore (P) et les processus impliqués. Pour cela, un partenariat a été mis en place avec l'Ecotron (CNRS) organisé en macrocosmes sans vers de terre ou avec des endogés ou anéciques. La taille des différents pools a été étudiée (par extraction séquentielle) ainsi que la spéciation du P organique (par HPIC). Les mécanismes impliqués ont été analysés par le dosage des phosphatases, de paramètres chimiques et biologiques du sol et par le ratio isotopique $^{18}\text{O-P}$. Nos résultats montrent que la digestion du sol et le vieillissement des turicules impactent à la fois les pools disponibles et les stables. Sur le sol global, ces changements ne sont visibles que sur le P extractible aux résines et sur le P organique. Le P extractible aux résines augmente dans les turicules frais suite à la minéralisation par les phosphatases. Les turicules secs montrent aussi une augmentation de ce pool due à sa libération après la mort des microorganismes. L'augmentation du P organique peut être expliquée soit par une ingestion sélective par les vers de terre soit par la synthèse et libération par les microorganismes. L'étude de la spéciation du P nous suggère que les microorganismes sont plus actifs dans les turicules frais (ATP) mais pas dans les turicules sec (ATP → AMP + Pyrophosphate) qui ont été colonisés par les champignons (augmentation de l'oxalate, malonate, malate, G6P).

Abstract (1600 caractères maximum) :

Earthworms are soil engineers that are responsible for many ecosystem services such as nutrient availability. Our study will focus on the role of earthworms in the phosphorus (P) cycle to understand their impact on P pools and the processes involved. To do so, a partnership with the Ecotron (CNRS) was put in place. It was organized in 3 different macrocosms treatments: no earthworms, endogeic or anecic earthworms (100g/m²). Bulk soil and casts composites were sampled. Fresh casts were obtained by incubation. The size of P pools was analyzed (by a sequential extraction) as well as the speciation of organic P (by HPIC). The mechanisms were analyzed by a dosage of phosphatase, chemical and biological parameters as well as with the $^{18}\text{O-P}$ ratio. Our results showed that soil digestion increased both available and stable pools and that pool-changes were not stable with cast-aging. Our global approach showed that increases were only visible for resin-extractable P and organic P. Resin-extractable P was increased in fresh cast due to a phosphatase activity visible by $^{18}\text{O}_\text{P}$ ratios. It was even higher in old casts due to its liberation from microorganisms after their death. Organic P was either increased by selective ingestion by earthworms or by the synthesis and release by microorganisms. The speciation of the organic P suggested that microbes were more active in fresh casts (more ATP and citrate) but not in old casts (no ATP, more AMP and pyrophosphate) that were colonized by fungi (more oxalate, malate, malonate, G6P). There was no difference for phytate and fructose.

Mots-clés : Vers de terre, Phosphore, Pools, Minéralisation, Spéciation.

Key Words: Earthworms, Phosphorus, Pools, Mineralization, Speciation

* Élément qui permet d'enregistrer les notices auteurs dans le catalogue des bibliothèques universitaires