

Earthworms change the distribution and availability of phosphorous in organic substrates

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Abstract

In laboratory controlled soil microcosms, the distribution and availability of phosphorous (P) were determined in the surface-casts and the burrows-linings of the anecic earthworm *L. terrestris* and were compared with non-ingested soil. To simulate more realistic earthworm community conditions, a combination of *L. terrestris* plus the endogeic *A. caliginosa* was tested. For a 2-month period, the earthworms were given two organic food substrates: rye-grass littered onto the soil surface and sewage sludge mixed with soil. The following treatments were designed: (i) soil alone (S), (ii) soil and sewage sludge (SS), soil and rye-grass litter (SL), and (iv) soil, litter and sludge (SSL). Analyses were performed for P contents (total, available and organic), organic matter content (organic carbon, C_{org} and total nitrogen, N_{tot}) and the two acid and alkaline phosphatase activities (AcPA and AkPA). Earthworms enhanced AcPA and were also responsible for additional AkPA in soil. The two AcPA and AkPA increased not only in surface-casts but also in burrows-linings that paralleled with the decrease of organic P in SL and SSL treatments. The stimulation of AcPA began quickly and declined rapidly in casts (from 19 to 8 $\mu\text{mol phenol g}^{-1} \text{ dry wt h}^{-1}$, respectively at week 2 and 8 in the SL treatment) but it was initiated later and maintained at a high level for longer in burrows (more than 10 $\mu\text{mol phenol g}^{-1} \text{ dry wt h}^{-1}$ at week 8 in the SL treatment). Significant positive correlations were found between the AkPA activities and N_{tot} contents ($r=0.95$, $p=0.001$) and to a lesser extend with C_{org} contents ($r=0.76$, $p=0.05$) in casts from the SL treatment, while AcPA significantly correlated with N_{tot} ($r=0.91$, $p=0.004$) but not with C_{org} ($r=0.72$, $p=0.06$). P availability was always highest in casts. However, the available P contents decreased sharply over time in casts and were still low in burrow-linings, suggesting that a large part of inorganic P produced was rapidly immobilized for the microbial growth. Total P content was unchanged except in the SL treatment in which it increased in casts and burrows (ca. 725 $\mu\text{g g}^{-1}$, at week 4). Organic P was first the highest in casts and then decreased over time (from 168 at week 1 to 140 $\mu\text{g g}^{-1}$ at week 8 in the SL treatment). This study illustrates that earthworms facilitate P transfer downward increasing a P patchy distribution in the soil, and significantly change the biogeochemical status of P (availability, organic phosphorous pool, AcPA activities) in certain hot spots such as casts and burrow-linings.

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1. Introduction

In many ecosystems, earthworms are keystone soil organisms in regulating nutrient cycling through: (i) their own metabolism that leads to high availability of carbon (C) and nitrogen (N) from metabolic wastes such as urine, mucus and tissue, (ii) the dispersal and the stimulation of

soil microorganism activity associated with passage through the intestinal tract and (iii) the distribution and the mixing of organic matter and soil mineral particles (Lee, 1985; Edwards and Bohlen, 1996; Lavelle and Spain, 2001). Many studies have examined impacts of earthworm on C and N fluxes in soils (Binet and Tréhen, 1992; Martin et al., 1992; Blair et al., 1997; Bohlen et al., 1997; Bouché et al., 1997; Lavelle et al., 1997; Whalen and Janzen, 2002); however, less attention has been paid to how and to the extend to which earthworms influence the dynamics of soil phosphorous (P). Earthworm casts collected directly from pasture were found to contain three times more water extractable P than the surrounding soil (Sharpley and Syers, 1976; 1977). In addition, the ingestion and thorough mixing of soil in the intestinal tract of *Lumbricus rubellus* and

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Aporrectodea caliginosa favor the dissolution of phosphate rock and thus the availability of the derived-P in the soil (Mackay et al., 1982). Lopez-Hernandez et al. (1993) also reported that water soluble P increased by 2.7 times in fresh casts of a tropical geophagous earthworm *Pontoscolex corethrurus*. The aim of the present study was to assess the mechanism (feeding, burrowing, and/or microbial stimulation) through which earthworms regulate P dynamics in soils. To explore the extent to which earthworm feeding may affect P cycling in agro-ecosystems, we compared two different sources of P (rye-grass litter and sewage sludge) under laboratory-controlled conditions. The anecic *Lumbricus terrestris* was used for this purpose. In order to simulate more realistic earthworm community conditions, a combination with the endogeic species, *Aporrectodea caliginosa*, was also tested. As far as we know, in contrast to worm casting activity, no information is available about how the burrowing activity affects the P distribution in the soil. In terms of P analyses, particular attention was, therefore, given to the earthworm burrow-linings, which were then compared with non-ingested and control soils. Organic matter (C and N) content was measured in addition to total and available P contents, and were then correlated to the phosphatase activity.

2. Materials and methods

2.1. Experimental design

Soil (a brunisol on Brioverian schists, 12% sand, 70% silt, 13% clay, 1.8% organic matter; pH 6.4) was collected from a corn agroecosystem (Brittany, France) from 5 to 30 cm depth of the soil profile. The soil was air-dried, sieved (2 mm) and remoistened until a water content of 20% w:w prior to fill microcosms (diameter: 20 cm; height: 35 cm) at a bulk density of 1.46 g cm^{-3} . The top 5 cm were left free of soil. Worms (*L. terrestris* and *A. caliginosa*) were collected in a clover field near the corn agroecosystem and kept for 3 weeks in large breeding pots at 12 °C. Before their introduction into the microcosms, worms were weighed after emptying their guts overnight on a wet filter paper.

To test the effects of earthworms on phosphorous cycle, two different P sources were used (rye-grass litter and sewage sludge) and four treatments were then defined: soil alone (S), soil and litter (SL), soil and sludge (SS) and soil, litter and sludge (SSL). The rye-grass litter (organic carbon C_{org} : 42.9%, total nitrogen N_{tot} : 2.1%, total phosphorous 2.58 mg g^{-1}) was hand-collected, air-dried and cut into 1–1.5 cm fragments, then remoistened with distilled water before added onto the soil surface (2 g per microcosm for SL and SSL treatments). During the experiment, earthworms were fed ad libitum. The sewage sludge had the following characteristics: water content of 40%, C_{org} 26.6%, N_{tot} 3.9%, total phosphorous 19.73 mg g^{-1} , available phosphorous (Olsen method): $1084 \text{ } \mu\text{g g}^{-1}$, organic

phosphorous: $450 \text{ } \mu\text{g g}^{-1}$. The sewage sludge was incorporated in the first 10 cm of the soil column as twice the NFU 44-041 AFNOR (the French national standards institute, member of the International Organization for Standardization) norm which is $3.5 \text{ ton ha}^{-1} \text{ yr}^{-1}$.

A total of 54 soil microcosms were set-up, three juvenile specimens of *L. terrestris* (biomass mean of 7–8 g microcosm⁻¹) introduced 24 of them (six replicates per treatment). To further test the inter-specific relationships between anecic and endogeic species, two juvenile specimens of both *L. terrestris* and *A. caliginosa*, (biomass mean of 7–8 g microcosm⁻¹) introduced 6 SSL microcosms. The remaining 24 soil microcosms without worms were used as a control. The soil microcosms were incubated at 12 °C with 12 h light day⁻¹ for 2, 4 and 8 weeks. At each date-incubation, two of the six replicates in each treatment were measured. The earthworms were collected and kept before weighing on humidified filter paper into Petri dish for 24 h. Soil microcosms were sampled as following: earthworm surface-casts, earthworm burrows-linings (the first two millimetres), non-ingested soil and control soil. For the last two, soil was collected only in the top 10 cm. Surface-casts were collected every day over the incubation time and kept at 12 °C to obtain seven age groups: 1, 2, 3, 4, 5, 6+7 and 8 weeks old. Six week- and 7 week-old casts were mixed together to have enough material for analyses. Only several millimetres of water leached at the bottom of the soil column did not allow to nutrient analysis.

2.2. Analytical procedures

2.2.1. Phosphatase activity

The procedure used was described by Tabatabai and Bremner (1969), reviewed by Eivazi and Tabatabai (1977). It consisted of a *p*-nitrophenol measure after soil incubation with *p*-nitrophenyl phosphate. Moist soil (1 g) treated with 0.25 ml of toluene, 4 ml of Modified Universal Buffer (MUB) at pH 3, 4, 5.5, 6.5, 9, 10 and 11. 1 ml of *p*-nitrophenyl phosphate solution made in the same buffer was added. After mixing and incubation for 1 h at 37 °C, 1 ml of CaCl_2 (0.5 N) and 4 ml of NaOH (0.5 N) were added and the soil solution was filtered through a Whatman 2V folded filter paper. The absorbance of the filtrate was measured at 400 nm.

2.2.2. Phosphorous analyses

2.2.2.1. Total, inorganic and organic phosphorous contents.

Total phosphorous was colorimetrically measured on mineralised soil with the molybdate acid procedure (Murphy and Riley, 1962). Soil extraction was conducted by adding H_2SO_4 36 N and $\text{K}_2\text{S}_2\text{O}_8$ to soil samples (aliquot of 25 mg d.w.) that were autoclaved at 120 °C for 2 h. Five aliquots per soil sample were made. Organic phosphorous (P_{org}) was typed by ignition of soil samples (1 g d.w.) at 550 °C (Anderson and Ingram, 1993). Both ignited and

unignited samples were placed into propylene tubes and 50 ml of H₂SO₄ 0.5 N were added. After shaking the tubes overnight, samples were filtered (Schleicher and Schuell, 512½) and P was determined as above. The difference in the acid extractable P of ignited and unignited samples gave a measure of the organic P. The difference between total P and P_{org} gave the amount of inorganic phosphorous (P_i).

2.2.2.2. Available phosphorous. Two methods were used to determine the amount of available phosphorous in casts, burrow-linings, non-ingested and control soil. The first was a P extraction with NaHCO₃ (Olsen-P=bicarbonate extractable phosphorous) while the second method was an isotopic exchange kinetics between ³²PO₄ and ³¹PO₄ orthophosphate ions, i.e. the ³²PO₄ that were added in the soil solution and the ³¹PO₄ ions located on the soil matrix (soil isotopically exchangeable phosphorous). The Olsen et al. (1954) procedure was performed by shaking 5 g of moist soil with 100 ml of 0.5 N NaHCO₃ (pH 8.5) over 30 min. Orthophosphate concentration in extracts was measured on neutralized filtrates by the colorimetric method of Murphy and Riley (1962). We used the isotopic exchange method described by Fardeau et al. (1985, 1991) when enough soil material was still available. This meant only *L. terrestris* treatments were carried out, unfortunately without any replicates. A 1:10 soil/water suspension was shaken overnight. At $t=0$, 1 ml of solution containing ³²PO₄ ions was introduced in 99 ml of suspension. Solution samples were made at 1, 10 and 40 min after the addition of radioactivity. Each sample was filtered and the concentration of radioactivity in solution was then measured by liquid scintillation counting (LSC). The r_1/R ratio gave information on the soil buffering capacity (Frossard and Sinaj, 1997). The concentration of orthophosphates (Cp), i.e. the water soluble P, was measured by the colorimetric method of Murphy and Riley (1962). The P content of the pool of free ions could be approximated in low P fixing soils by the amount of isotopically exchangeable P within 1 min (E_1) according to Salcedo et al. (1991, in Frossard and Sinaj, 1997).

2.2.3. Organic matter, carbon and nitrogen contents

Total nitrogen (N_{tot}, Kjeldahl method), organic carbon (C_{org}, Anne method) and organic matter contents in casts, burrow-linings, non-ingested and control soils were analysed by the INRA laboratories following the AFNOR norms.

2.3. Statistical analysis

Statistical analyses were performed using the software SPLUS 6.1 and R (Ihaka and Gentleman, 1996). Before analyses, data were tested for normality and homogeneity of variance (Kolmogorov–Smirnov tests). Each microcosm analyzed was independent from the others. According to Sokal and Rohlf (1995), a two- or three factors analysis of variance were performed to test the organic matter treatment, incubation time and earthworm burrowing on

the earthworm body mass and total P, P_{org} and Olsen-P, C_{org} and N_{tot} contents of soil samples. Pair-wise comparisons were made with Tukey–Kramer HSD tests. For experiments with small numbers of replicates, non-parametric Mann–Whitney *U*-tests were performed. Regarding the amount of isotopically exchangeable P, no statistical significance could be attributed to the data, only some tendencies could be seen. Relationships between enzymatic activities and C_{org} and N_{tot} contents in casts and burrows were investigated with correlation analysis (Pearson correlation).

3. Results

For a better understanding, this chapter is divided in two main sections, i.e. the biological activities and the distribution of nutrients. In each sub-section, explanations generally deal first with the S treatment, then the SS, SL, SSL and finally the SSL LT+AC treatments.

3.1. Biological activities

3.1.1. Earthworm biomass and activity

In the S and SS treatments without added litter, *L. terrestris* lost 20% weight on average (Fig. 1). In the S treatment, two individuals of *L. terrestris* died after 2 weeks of incubation and a third one after 4 weeks (data not showed). In the SS treatment, no worms died but all of them still lost body mass. In the litter treatments SL and SSL, the initial weight of earthworms was maintained during the first 2 weeks and then increased up to 50% after a 4- and 8-weeks incubation, for *A. caliginosa* and *L. terrestris*, respectively. *L. terrestris* biomass was significantly affected by the two organic matter treatments and incubation times (2-way ANOVA, $F=28.43$ (df 3), $p<0.001$ and $F=24.24$ (df 1), $p<0.001$ for treatment and incubation factors, respectively).

Preference for rye-grass litter and avoidance of sewage sludge by *L. terrestris* were clear through examination of the burrow network. Without any litter supply (S and SS treatments), the whole soil column was burrowed, with burrows reaching the bottom of the microcosms; this indicated that earthworms searched for soil organic matter, avoiding the sewage sludge as the only food source. In contrast, with rye-grass litter supply (SL), burrow networks were localized in the first 20 cm top soil after 2 and 4 weeks, indicating that earthworms were concentrated close to the soil surface and fed mainly surface litter. An intermediate situation was found with the SSL treatment with a superficial burrow network (20 cm top-soil) at the beginning of the incubation (2 weeks) that extended to the bottom of the microcosms with time (4 and 8 weeks). As the exclusive food source, the sewage sludge reduced earthworm body mass losses (*L. terrestris*) slightly but the addition of litter and sludge did not result in a mass gain of the worms. In all treatments, except for S treatment in which mortality was

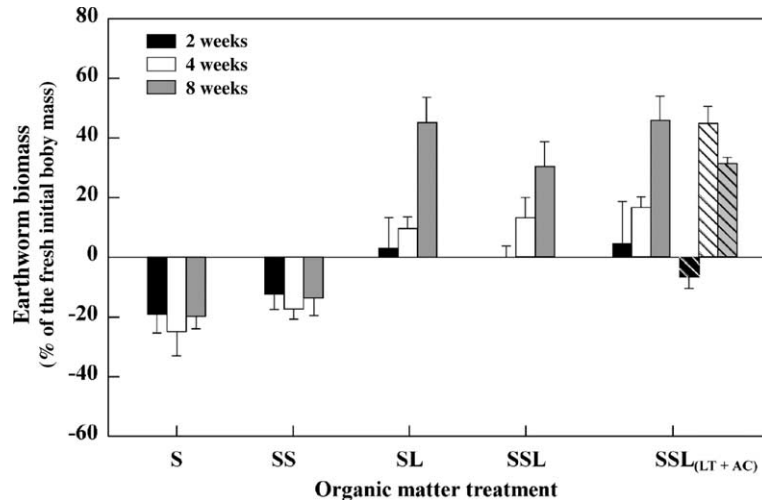


Fig. 1. Effects of organic matter treatment (S, SS, SL, SSL) and incubation time (2, 4 and 8 weeks) on the evolution of earthworm biomass (% of the initial fresh body mass, mean \pm SE). Full areas: *L. terrestris*; hatched areas: *A. caliginosa*.

observed, most worms reached sexual maturity after 4 or 8 weeks, although no cocoons were collected.

3.1.2. Phosphatase activity

Overall, in the control soil and irrespective of organic matter treatments, the acid phosphatase activity (AcPA) increased throughout the incubation (by 45–136% from week 2 to week 8), although it still remained low (less than $7 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$). This probably indicates a stimulation of the soil microbial activity due to both the soil rewetting and a medium temperature (12°C). Interesting results were seen at pH 4 (acid phosphatase activity, AcPA) and 11 (alkaline phosphatase activity, AkPA) (Fig. 2).

In the soil treatment (S), except at week 8, all soil samples, (casts, burrows, non-ingested and control soils) had a similar low basic acid phosphatase activity, which was 10 times lower than the sewage sludge sample (3.9 vs $45 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$ at pH 4, respectively, Fig. 2(a)). After 8 weeks of incubation, the basic AcPA increased significantly (Mann–Whitney U -test, 95%, $n=18$, $p=0.000$) in control soil (from 2.5 at week 2 to $5.9 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$ at week 8), non-ingested top soil (from 1.8 at week 2 to $13.4 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$ at week 8) and to a larger extent in burrows (from 3.4 at week 2 to $16.0 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$ at week 8). However, this trend was not observed in casts (from 3.2 at week 4 to $1.3 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$ at week 8). Only a few casts were collected in the S treatment. No obvious AkPA was observed in any soil samples.

The addition of sewage sludge (SS treatment, Fig. 2(b)) increased slightly the initial basic AcPA in all soil samples (4.0 – $6.4 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$ on average, Mann–Whitney U -test, 95%, $n=18$, $p=0.05$). Irrespective of the time of incubation, the increase of phosphatase activity (SS vs S) was of the same order of magnitude in casts, non-ingested and control soil, except in burrows in which

the activity was maximal after 8 weeks, as previously obtained in the S treatment. Note that in burrows AcPA was still high at pH 5.5 (more than $10 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$), suggesting that environmental conditions created by earthworms could extend the pH range of microbial enzymatic activity (data not shown).

By contrast, soil phosphatase activities were strongly affected by the supply of rye-grass litter (SL and SSL, Fig. 2(c) and (d)). Moreover, an additional AkPA was observed at pH 11 in casts and burrows but not so evident in non-ingested and control soil samples. At the beginning of incubation (first 4 weeks), AcPA at pH 5.5 increased by 2–4 times in casts and to a lesser extent in burrows; in the latter, it varied greatly but values were at least twice than in S treatment (19.4 vs 2.0 – $3.2 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$). These two AcPA and AkPA declined throughout the incubation time, particularly in casts when ageing (Fig. 2(f)). In burrows, phosphatase activities also declined with time but AcPA still remained high after 8 weeks ($8.8 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$ in Fig. 2(c), and $9 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$ in Fig. 2(d)).

In the SSL treatment with the two worm species, the burrows after a 4 week-incubation had an AcPA twice as high as with *L. terrestris* alone (7.7 vs $14.8 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$). In casts, AkPA activities were similar while AcPA tended to be lower in the worm-mixed community (e.g. after 2 weeks, with *L. terrestris* alone, $16 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$; with *L. terrestris* and *A. caliginosa*, around $12 \mu\text{mol phenol g}^{-1} \text{dry wt h}^{-1}$).

3.2. Distribution of nutrients

3.2.1. Contents and availability of phosphorous

As indicated by the three-way ANOVA (Table 1), the concentrations and the distribution of total phosphorous and organic phosphorous, as well as the fraction of available

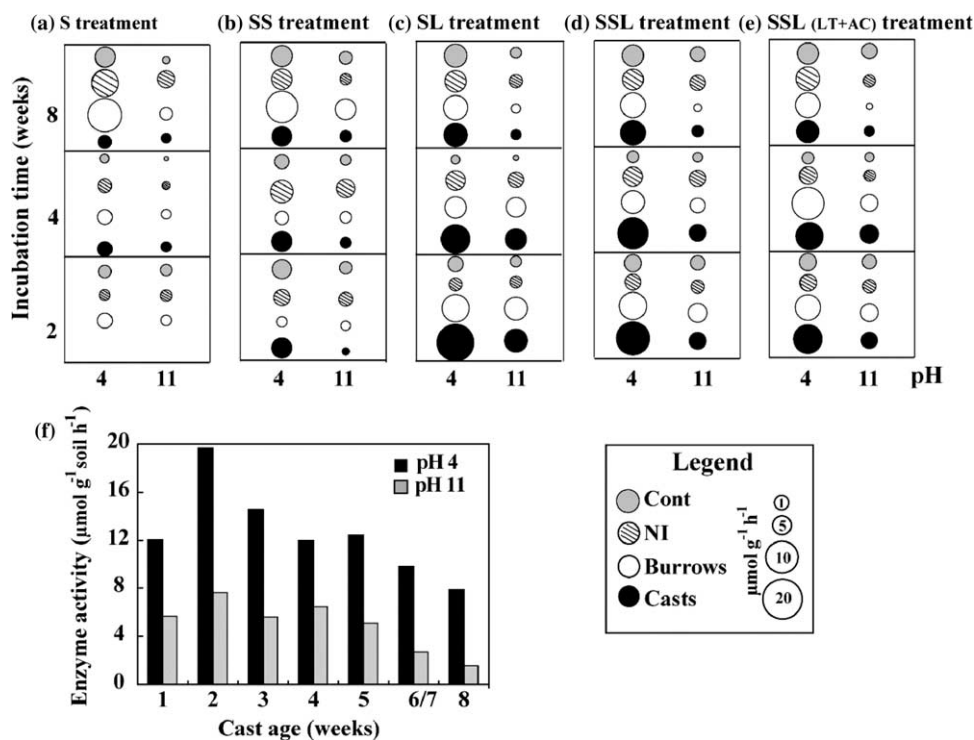


Fig. 2. Phosphatase activity (phosphomonoesterase, $\mu\text{mol } p\text{-nitrophenol g}^{-1} \text{ soil h}^{-1}$) at different pH in casts, burrows, non-ingested (NI) and control (Cont) soils in all treatments: (a) S, (b) SS, (c) SL, (d) SSL for *L. terrestris*, and (e) SSL(LT+AC), combination of *L. terrestris* and *A. caliginosa*. Graph (f) represents the enzyme activity over casts ageing from 1 to 8 weeks for SL treatment only. SE are not represented because replicates vary little from 0.05 to 0.1 $\mu\text{mol g}^{-1} \text{ soil h}^{-1}$.

phosphorous in soil were affected by the properties of the organic matter supply (treatment effect) although this influence varied significantly with earthworm soil burrowing (soil microsite effect) and with increasing incubation time (time effect).

3.2.1.1. Total and organic phosphorous. The total P contents were significantly increased by the organic supply, especially when the sewage sludge was incorporated in the soil (control soil, SS vs S, SS vs SL, Tukey test, $p < 0.05$). Compared to non-ingested soil, no significant enrichment or decrease in total phosphorous was observed in casts and burrows of *L. terrestris*, except

in the SL treatment. In this case, total P contents were significantly higher in burrows at 2 and 4 weeks (mean of 828 and 728 $\mu\text{g g}^{-1}$) and in casts at 4 weeks (724 $\mu\text{g g}^{-1}$) than in other soil microsites (577 and 530 $\mu\text{g g}^{-1}$ after 2 weeks, 588 and 529 $\mu\text{g g}^{-1}$ after 4 weeks of incubation, for non-ingested and control soil, respectively), that reflected the incorporation and the selective feeding on rye-grass litter by the anecic *L. terrestris* (Table 2). In SSL(LT+AC) treatment, the total P contents in casts did not vary (Mann–Whitney *U*-test, 95%, $n=6$, $p=0.125$), but were significantly higher in burrows in the presence of the two worm species (Mann–Whitney *U*-test, 95%, $n=6$, $p=0.013$).

Table 1

Three-way ANOVA analysis of the effects of organic matter treatment (S, SS, SL, SSL), incubation time (2, 4, 8 weeks) and soil microsites (control and non-ingested soil, burrows, casts), on the concentrations of total phosphorous, organic phosphorous and bicarbonate extractable phosphorous in soil

Source of variation	Total phosphorous		Organic phosphorous		Extractable phosphorous	
	df	<i>F</i> -value	df	<i>F</i> -value	df	<i>F</i> -value
Soil microsite	3	10.07 ***	3	52.18 ***	3	329.04 ***
Treatment	3	112.53 ***	2	12.60 ***	3	50.85 ***
Incubation time	2	8.1852 ***	2	58.18 ***	2	105.82 ***
Microsite × treatment	9	20.71 ***	6	5.42 ***	9	8.66 ***
Microsite × incubation	6	21.32 ***	6	17.04 ***	6	20.92 ***
Treatment × incubation	6	7.3513 ***	4	3.04 *	6	8.62 ***
Microsite × treatment × incubation	18	19.25 ***	12	1.56 ns	18	3.02 **
Residuals	48		36		48	

df: Degrees of freedom. Codes: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns: not significant.

Table 2

Concentration of phosphorous, organic phosphorous, carbon and nitrogen in casts and burrows, as well as in non-ingested soil (NI) and soil control (Cont) in all treatments (S, SS, SL, SSL, SSL (LT+AC)) over incubation time. For clarity SE are not represented for C and N

Time (weeks)	Treatment	Total P ($\mu\text{g g}^{-1}$) and organic P (% of total P, in italic)							
		Casts		Burrows		NI		Cont	
2	S	661.0 \pm 7.5	21.7	587.2 \pm 14.3	18.4	625.2 \pm 4.9	26.6	576.8 \pm 6.4	14.3
	SS	786.8 \pm 6.8	16.6	681.5 \pm 3.9	12.5	824.5 \pm 9.7	14.3	680.3 \pm 20.5	10.8
	SL	578.9 \pm 7.1	27.6	827.6 \pm 14.7	14.7	576.6 \pm 16.0	22.0	529.8 \pm 3.5	13.4
	SSL	752.7 \pm 8.2	22.4	709.7 \pm 19.3	10.7	777.0 \pm 42.0	19.7	683.1 \pm 10.9	13.8
	SSL (LT+AC)	739.9 \pm 28.0	19.4	913.6 \pm 27.4	9.1	632.6 \pm 58.6	12.8	683.1 \pm 10.9	13.8
4	S	623.5 \pm 7.0	–	735.8 \pm 21.8	16.7	705.5 \pm 17.1	24.4	573.4 \pm 22.7	14.5
	SS	687.9 \pm 7.3	19.4	730.9 \pm 15.7	12.3	778.1 \pm 1.0	15.2	750.0 \pm 6.3	7.5
	SL	724.0 \pm 7.8	21.4	727.5 \pm 20.8	16.2	588.3 \pm 9.6	25.5	529.3 \pm 12.9	18.0
	SSL	591.7 \pm 6.9	23.3	686.1 \pm 5.8	11.7	703.6 \pm 14.3	24.0	791.1 \pm 0.5	11.2
	SSL (LT+AC)	608.1 \pm 5.8	20.4	749.6 \pm 8.3	9.7	704.4 \pm 5.1	13.7	791.1 \pm 0.5	11.2
8	S	626.9 \pm 6.8	–	551.8 \pm 6.4	25.1	568.6 \pm 29.6	23.1	541.7 \pm 16.5	25.5
	SS	565.4 \pm 7.1	24.2	828.4 \pm 7.1	18.0	661.9 \pm 3.1	24.4	999.1 \pm 12.1	14.8
	SL	647.7 \pm 7.2	21.7	571.8 \pm 6.8	29.5	572.4 \pm 16.3	24.9	661.8 \pm 3.3	23.5
	SSL	588.9 \pm 7.5	22.9	716.7 \pm 7.1	21.4	712.1 \pm 19.4	24.6	701.6 \pm 7.7	19.2
	SSL (LT+AC)	853.7 \pm 6.9	21.0	727.9 \pm 10.4	19.2	478.9 \pm 27.5	26.0	701.6 \pm 7.7	19.2
		Organic C %				Total N %			
		Casts	Burrows	NI	Cont	Casts	Burrows	NI	Cont
2	S	1.03	1.10	1.16	1.03	1.14	1.17	1.14	1.15
	SS	1.15	1.13	1.14	1.26	1.25	1.19	1.35	1.49
	SL	1.56	1.21	1.13	1.07	1.62	1.20	1.11	1.12
	SSL	1.49	1.27	1.19	1.17	1.67	1.33	1.35	1.37
	SSL (LT+AC)	1.48	1.26	1.20	1.17	1.66	1.32	1.47	1.37
4	S	1.06	1.04	1.04	1.04	1.17	1.19	1.09	1.14
	SS	1.08	1.09	1.19	1.25	1.31	1.13	1.35	1.35
	SL	1.36	1.34	1.08	1.09	1.52	1.32	1.12	1.14
	SSL	1.40	1.31	1.21	1.24	1.54	1.36	1.32	1.38
	SSL (LT+AC)	1.44	1.24	1.18	1.24	1.57	1.28	1.35	1.38
8	S	1.20	1.05	1.04	1.05	1.20	1.11	1.18	1.15
	SS	1.17	1.09	1.18	1.25	1.31	1.21	1.39	1.33
	SL	1.09	1.35	1.04	1.06	1.18	1.44	1.18	1.17
	SSL	1.14	1.23	1.23	1.21	1.35	1.35	1.45	1.34
	SSL (LT+AC)	1.15	1.28	1.18	1.21	1.31	1.40	1.38	1.34

Earthworm effects were particularly marked on the distribution of organic phosphorous over incubation (3-way ANOVA, Table 1). After 2 weeks of incubation, *L. terrestris* casts showed higher significant concentrations of organic P (from 130 to 168 $\mu\text{g g}^{-1}$, i.e. 16–28% of the total P) than burrows (from 76 to 122 $\mu\text{g g}^{-1}$, i.e. 9–18% of the total P), non-ingested soil (from 81 to 166 $\mu\text{g g}^{-1}$, i.e. 13–26% of the total P) and control soil (from 71 to 94 $\mu\text{g g}^{-1}$, i.e. 10–14% of the total P), except for S treatment (Table 2). This ‘cast’ organic P significantly declined with age especially in litter treatments (from 168 to 140 $\mu\text{g g}^{-1}$ and from 180 to 135 $\mu\text{g g}^{-1}$ in the SL and the SSL treatments, respectively) while it stabilized in the sludge treatment (Fig. 3). This is in accordance with the highest and the lowest phosphatase activities within the first 4 weeks of incubation respectively found in litter and sludge treatments (Fig. 2). Hence conversely, after 8 weeks-incubation and in the SS, SL and SSL treatments, concentrations of organic phosphorous were higher in burrows than in casts (Tukey test, $p < 0.05$) suggesting an accumulation of organic matter in burrows

with increasing of time incubation relative to the anecic behaviour of *L. terrestris*.

3.2.1.2. Available phosphorous. Concentrations of the bicarbonate extractable P (Olsen-P) were significantly increased by the organic matter supply, especially with the addition of sludge (3-way ANOVA, $F = 50.85$ (df 3), $p < 0.001$, $S \ll SS \cong SL \cong SSL$). In *L. terrestris* casts, Olsen-P contents were the highest irrespective of the treatment and time of incubation (Fig. 4); these concentrations increased slightly (by 10%) in the sludge treatment and markedly (by 35%) in litter treatments.

For the S treatment without an organic matter supply, Olsen-P in the control soil was low (a mean of 64 $\mu\text{g g}^{-1}$ over the incubation time) but it doubled after passage through the gut of worms (a mean of 130 $\mu\text{g g}^{-1}$ in casts). However, Olsen-P in casts decreased with age and this was particularly marked in the litter treatments SL and SSL (Fig. 4). In the SS treatment, Olsen-P varied but finally did not change significantly throughout cast ageing. In burrows,

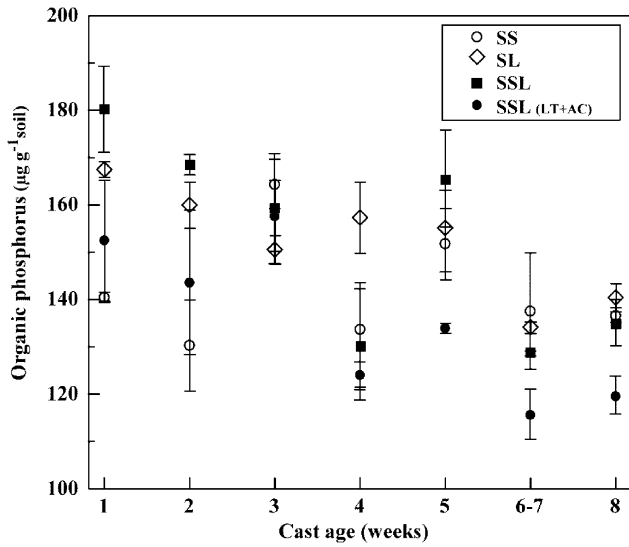


Fig. 3. Organic phosphorus content ($\mu\text{g g}^{-1}$ soil, mean \pm SE) in casts of different age groups. The treatments SS, SL and SSL are indicated. LT+AC: *L. terrestris* and *A. caliginosa*.

Olsen-P contents were strongly lower than in casts (Tukey test, $p < 0.001$), while values in non-ingested and control soils were quite similar (non-ingested soil \approx control soil \ll casts, Tukey test, $p < 0.001$). Indeed, at the beginning of the incubation, non-ingested and control soils showed high inorganic P contents in all treatments with organic matter supply. In the SS and SSL treatments, this was because of the initial high inorganic P concentration of the sludge which was 15-fold higher than those of the control soil (Olsen-P of $1084 \mu\text{g g}^{-1}$ vs a mean of $70 \mu\text{g g}^{-1}$). Inorganic P input in the litter treatment (SL) probably indicated water soluble P that had leached from the ryegrass surface litter during decomposition. Comparing SSL treatments at 2 and 4 weeks of incubation, the combination of the two species *L. terrestris* and *A. caliginosa* resulted in higher available-P contents in casts than with *L. terrestris* alone (Mann–Whitney *U*-test, 95%, $n = 4$, $p = 0.03$) (Fig. 4).

With regards to the isotopically exchangeable phosphorous, the ^{32}P remaining in solution (r_1/R) fluctuated between 40 and 60% which corresponds to a low P-sorbing capacity of the samples for phosphate (Table 3a). This low P sorption was increased in earthworm casts with consequently a low

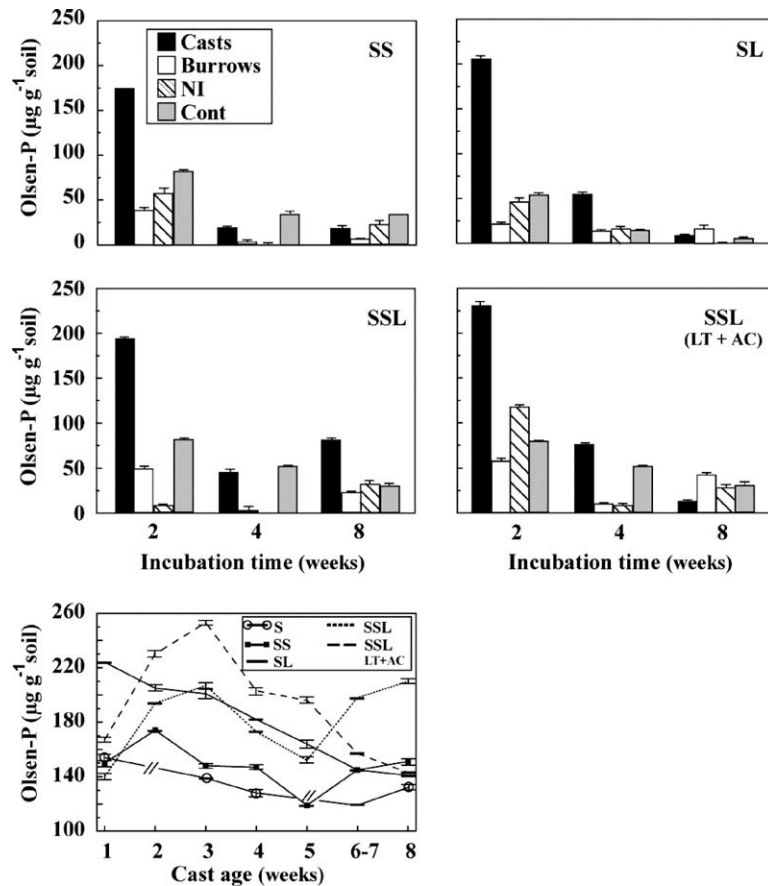


Fig. 4. Evolution of bicarbonate extractable phosphorous (Olsen-P, mean \pm SE) for casts (curves and histograms) and burrows, non-ingested soil and soil control (histograms only) after organic matter treatment over the incubation time. For the histograms, values of the control treatment S were subtracted from SS, SL and SSL treatments ($\mu\text{g g}^{-1}$ soil). LT+AC: *L. terrestris* and *A. caliginosa*.

Table 3a

Isotopically exchangeable phosphorous in 1 min (E_1 , $\mu\text{g g}^{-1}$ soil) in all treatments (S, SS, SL, SSL) for casts, burrows (Bur), non-ingested soil (NI) and control soil (Cont) at 2, 4 and 8 weeks of incubation

Incubation (weeks)	4							8													
	S		SS		SL			SSL			S			SS			SL			SSL	
Microsite	Bur	Bur	Bur	Bur	Bur	NI	Cont	Bur	NI	Cont	Bur	NI	Cont	Bur	NI	Cont					
% Water	18.9	19.2	21.5	19.9	19.9	19.6	17.8	19.9	19.1	21.6	19.9	19.2	20.8	19.9	20.6	21.2					
r_1/R	0.48	0.43	0.50	0.48	0.56	0.50	0.53	0.50	0.45	0.56	0.59	0.45	0.56	0.48	0.42	0.50					
Cp (mg l^{-1})	1.15	1.06	1.38	1.28	1.06	1.10	0.94	1.05	1.18	1.12	1.35	0.99	1.17	1.31	1.21	1.13					
$E_{1\text{min}}$ ($\mu\text{g g}^{-1}$)	24	24	28	27	19	23	18	21	26	20	23	22	21	28	29	22					

Results only for *L. terrestris*. The humidity is expressed. Cp: water soluble phosphorous (mg l^{-1}); R: quantity of radioactivity added; r_1 : radioactivity remaining in solution after 1 min.

Table 3b

Isotopically exchangeable phosphorous (E_1 , $\mu\text{g g}^{-1}$ soil) in age groups of casts of *L. terrestris* in treatments SL and SSL

Treatment	SL							SSL					
	1	2	3	4	5	8	1	2	3	4	5	8	
% Water	4.4	1.6	1.1	1.4	0.9	0.7	8.1	1.2	1.1	1.2	–	0.9	
r_1/R	0.83	0.77	0.77	0.77	0.91	0.71	0.59	0.67	0.67	0.77	–	0.59	
Cp (mg l^{-1})	2.2	3.27	2.90	3.29	2.36	1.96	2.02	3.50	3.61	3.33	–	1.80	
$E_{1\text{min}}$ ($\mu\text{g g}^{-1}$)	25.9	42	42	42	25	27	34	51	52	43	–	32	

The humidity is expressed. Cp: water soluble phosphorous (mg l^{-1}); R: quantity of radioactivity added; r_1 : radioactivity remaining in the system after 1 min.

buffer capacity and higher water soluble P contents (Table 3b). These results were moreover confirmed by our previous Olsen measurements. The amount of isotopically exchangeable P in 1 min (E_1) was also higher in casts compared with burrows, non-ingested or control soils after 8 weeks for SL (27 vs 21–23 $\mu\text{g g}^{-1}$) and SSL (32 vs 22–29 $\mu\text{g g}^{-1}$) treatments. Casts ageing (Table 3b) in SL and SSL, inorganic P and available-P contents increased by 50% from week 1 to week 2, then stabilized at a maximum (38 vs 52 $\mu\text{g g}^{-1}$ available-P after 3 weeks of incubation, in SL and SSL, respectively). They then slowed down to the initial concentrations (26–27 vs 32–34 $\mu\text{g g}^{-1}$ available-P at week 1 and 8 in SL and SSL, respectively). In conclusion, inorganic P in casts was more rapidly available than in other soil samples. No particular trend was found for inorganic P contents and availability in burrows comparing SL and SSL treatments.

3.2.2. Carbon and nitrogen contents

As expected, the incorporation of sewage sludge in the 10 cm-soil top layer (S vs SS and SL vs SSL) increased the carbon content by 10–17% (3-way ANOVA, $F=15.51 \text{ e}+31$ (df 3), $p<0.001$) and the nitrogen content by 15–17% (3-way ANOVA, $F=3.00 \text{ e}+30$ (df 3), $p<0.001$) (Table 4). In contrast, the addition of rye-grass litter at the soil surface did not significantly change the C_{org} and N_{tot} contents of non-ingested and the control soil (Table 2).

In the S treatment, the C_{org} content of *L. terrestris* casts was quite similar after 2 and 4 weeks (1.03 and 1.06%, respectively) but increased significantly after 8 weeks

(1.20%). N_{tot} increased gradually from 1.14 to 1.20‰ (Table 2). These increases might be related to the re-use of C and N lost from dead worms. In the SS treatment, casts had no significant concentration of organic matter that confirmed our previous results with phosphatase activity, i.e. earthworms seemed to avoid sewage sludge.

In SL and SSL treatments, an accumulation of organic matter was found in casts and burrows (Tukey tests, $p<0.05$). In these litter treatments, C_{org} and N_{tot} enrichment in casts

Table 4

Three-way ANOVA analysis of the effects of organic matter treatment (S, SS, SL, SSL), incubation time (2, 4, 8 weeks) and soil microsites (control and non-ingested soil, burrows, casts) on the organic carbon and total nitrogen contents in soil

Source of variation	Organic carbon		Total nitrogen	
	df	F-value	df	F-value
Soil microsite	3	2.48 e+30 ***	3	4.98 e+29 ***
Treatment	3	15.51 e+31 ***	3	3.00 e+30 ***
Incubation	2	7.60 e+29 ***	2	1.80 e+29 ***
Microsite × treatment	9	4.04 e+30 ***	9	5.71 e+29 ***
Microsite × incubation	6	9.98 e+29 ***	6	1.58 e+29 ***
Treatment × incubation	6	1.11 e+30 ***	6	1.77 e+29 ***
Microsite × treatment × incubation	18	5.98 e+29 ***	18	5.71 e+28 ***
Residuals	48		48	

df: Degrees of freedom. Codes: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns : not significant.

reflected the rye-grass litter consumption by *L. terrestris*, especially at the beginning of the incubation (C_{org} at week 2: 1.56 and 1.49% for SL and SSL vs 1.03 and 1.15% for S and SS; N_{tot} at week 2: 1.62 and 1.67% for SL and SSL vs 1.14 and 1.25% for S and SS). However, over the incubation period (not all data shown), these C_{org} and N_{tot} contents declined in casts (SL: from 1.56 to 1.09% for C_{org} and 1.62–1.18‰ for N_{tot}) while they increased in burrows (SL: from 1.21 to 1.35% for C_{org} and 1.20–1.44‰ for N_{tot}).

In the SS treatment, C_{org} and N_{tot} contents were even lower in burrows and casts than in the non-ingested and control soil but slightly higher than the initial parent soil (control from the S treatment). This intermediate position of C_{org} content of casts indicated that *L. terrestris* fed mainly on soil organic matter and partly on sludge as a minimum for survival. The values of C_{org} and N_{tot} in casts were generally constant throughout the incubation period (1.09–1.15% for C_{org} and 1.25–1.31‰ for N_{tot}). Paradoxically there was no more C_{org} and N_{tot} enrichment in 8-week old casts and even more slight C_{org} and N_{tot} losses, especially comparing SL to SS treatments. These observations suggest differences in the mineralization of organic matter over time probably due to the quality of the organic matter that differed between rye-grass litter and sewage sludge.

In the SSL treatment where earthworms were given the choice between rye-grass litter and sludge, there was no additional enrichment in casts compared to the SL treatment. Moreover, C_{org} and N_{tot} contents in casts and burrows were not different in the presence of both the endogeic *A. caliginosa* and the anecic *L. terrestris*, than *L. terrestris* alone (data not showed, Mann–Whitney *U*-test, 95%, $n=6$, $p=0.25$). This is probably due to the predominant role of the anecic species in the formation of the burrow network and middens, as well as on casting.

Significant positive correlations were found between the AkPA activities and N_{tot} contents ($r=0.95$, $p=0.001$) and to a lesser extend with C_{org} contents ($r=0.76$, $p=0.05$) in casts collected in the litter treatment (SL), while AcPA was significantly correlated with N_{tot} ($r=0.91$, $p=0.004$) but not with C_{org} ($r=0.72$, $p=0.06$) Fig. 5. In SL treated burrows, no significant results were obtained. AcPA values were positively correlated with N_{tot} ($r=0.42$, $p=0.72$) at the opposite from alkaline activities ($r=-0.97$, $p=0.161$). For C_{org} , no correlation was found at pH 3–4 ($r=-0.022$, $p=0.99$). A negative correlation was observed at pH 10–11 ($r=-0.76$, $p=0.45$).

4. Discussion

4.1. Earthworm activity

This study shows that anecic earthworms markedly affected both the distribution and the availability of P in soil. This is governed by the ecophysiological constraint of worm

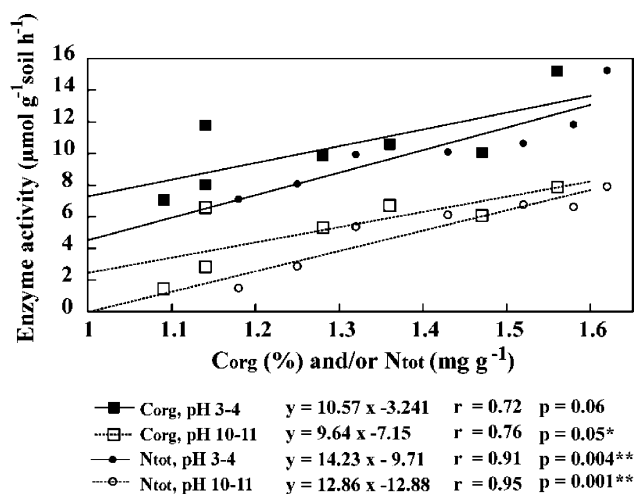


Fig. 5. Pearson correlations between the acid (pH 3–4, line) and the alkaline phosphatase (pH 10–11, dotted line) activities (phosphomonoesterase, $\mu\text{mol } p\text{-nitrophenol } \text{g}^{-1} \text{ soil } \text{h}^{-1}$) and the organic carbon content, C_{org} , and the total nitrogen content, N_{tot} , in casts over ageing (1 to 8 weeks old) for the litter treatment (SL).

species as their specific behaviour in soil (vertical distribution, burrowing activity, food preferences). In our study, the earthworms *L. terrestris* and *A. caliginosa* were given two organic food substrates (rye-grass litter and sludge) as two different sources of P. The deep-burrower *L. terrestris* creates deep vertical burrows in which it incorporates rye-grass, while *A. caliginosa*, as a geophagous worm, lives within the soil profile and feeds preferentially on organic matter well mixed into the soil matrix. In soil with low organic matter content as in the S treatment, *A. caliginosa* increased their burrowing activity to satisfy their energy requirements as has also been demonstrated for *Octolasion cyaneum* (Buck et al., 2000). Two worms died in the S treatment but none in the sludge treatments. However, the significant losses of earthworm body mass in the SS treatment contrasted greatly with the body mass gain in litter treatment (SL and SSL). Despite not having monocultures of *A. caliginosa* that could have enforced our assumptions, their increased body mass in the SSL treatment and the presence of endogeic faeces in the anecic burrows suggests that *A. caliginosa* fed organic molecules and/or microbial compounds derived from rye-grass litter that was pulled by *L. terrestris* in their own burrows. As a matter of fact, even the sewage sludge was not toxic by itself in a medium term of 8 weeks, it did not appear to be as suitable a food substrate as litter did. In fact, as the worms increased their weight in the presence of sludge with additional rye-grass supply, we conclude that the sludge ecotoxicity was probably low and could be easier compensated with the rye-grass litter.

4.2. Phosphatase activity, carbon and nitrogen contents

Neither the rye-grass litter supply nor the sludge input increased soil phosphatase activities. Water-soluble P when

leached during litter decay was not sufficient to prime higher microbial activities. Litter incorporation by worms led to significantly enhanced phosphatase activities in *L. terrestris*' burrows, especially obvious after 8 weeks. This corresponded to C_{org} and N_{tot} enrichments of burrow-linings (Table 2). By contrast, the sewage sludge application did not change soil phosphatase activities. Some studies support the idea that phosphatase production and activity (i) are linked to the biotic demand for P (plants or microflora) and, (ii) are regulated by the supply of the nutrient (inorganic P) that the enzyme produces through the mineralization of organic P (Olander and Vitousek, 2000). So, in our case, it could be expected that the high initial amount of available inorganic P in the sludge ($1084 \mu\text{g g}^{-1}$) acted as a negative feedback mechanism that repressed the production of phosphatases and indirectly their activities. This possibility was also raised by Spiers and McGill (1979).

In our study, the enhancement of phosphatase activities in soil was earthworm-mediated. Earthworms were responsible for additional alkaline phosphatases, some of them being produced in the worm gut and are then excreted through cast deposition (Satchell and Martin, 1984; Ranganathan and Vinotha, 1998 in Vinotha et al., 2000). Therefore in our study, the AkPA was only observed in casts and less often in burrows while the AcPA was detected in the whole soil. As the parent soil used was slightly acidic (pH 6.4), this is in accordance with the observations of Eivazi and Tabatabai (1977) who showed a greater AcPA in acid soils and a greater AkPA in alkaline soils. The increased basic AcPA we observed in casts extended earlier observations made with casts of temperate epigeic worms, such as *Eisenia fetida*, *Dendrobaena veneta*, *L. rubellus* (Satchell and Martin, 1984) and *D. octaedra*, (Flegel and Schrader, 2000), of temperate endogeic worms such as *A. caliginosa* (Satchell and Martin, 1984), *O. cyaneum* (Buck et al., 1999), and of tropical endogeic worms (*Eudrilus eugeniae*, Parthasarathi and Ranganathan, 1999). On the contrary, casts of the epigeic worm *E. fetida* and the Chinese anecic worm *Metaphire guillelmi* had lower AcPA and AkPA activities (Zhang et al., 2000), probably explained by an inhibition of the expression of enzymatic activities due to the concentration of inorganic P in the control soil.

The findings of that higher enzyme activity is novel due to earthworms was not restricted to their casts but also occurred to their burrows-linings. The main difference is that the stimulation of AcPA began quickly and declined rapidly in casts while it was initiated later and at higher levels in burrows. In casts, our results are in line with those from Satchell and Martin (1984) who found a rapid development of phosphatase activity at 20 °C in casts from 14 days to 21 days. In burrows, the enhanced AcPA due to the soil microbial activity was initiated later because the priming effect was less effective than during thorough mixing of soil and litter in the worm gut.

In the S treatment, the higher AcPA in burrows should follow from the intense soil bioturbation by the worms searching for food. This resulted in (i) a high deposition of C- and N- compounds derived from the earthworm metabolism (mucus, urea) during successive passages, (ii) a release of some faeces also, and (iii) an airflow input through the burrow network. Dependence of phosphatase activity on C amounts or on N amounts in soil is still under debate. Buck et al. (1999) found a positive correlation between C/N values and phosphatase activity in earthworm casts and suggested a C-dependence, while a N-dependence was underlined by Flegel et al. (1998). Olander and Vitousek (2000) also showed a stimulation of soil phosphatase activity by inorganic N soil fertilization. Our study underlines N-dependence more so than a C-dependence in casts while no clear relation was found in burrows, although a clear demonstration would need to quantify the fraction of mineral N which was easily bioavailable. With ageing, the phosphatase activity in casts decreased over time in parallel to air-drying which was found to decrease the microbial biomass in earthworm faeces (Scheu, 1987; Pedersen and Hendriksen, 1993 in Parthasarathi and Ranganathan, 1999). In addition, the rapid depletion in casts of C_{org} (consumption by bacteria) and N_{tot} (denitrification (Lavelle et al., 1992) or leaching (Knight et al., 1990)) through ageing would cause the decline of microbial activity thereby limiting the phosphatase activity. Compared to casts, soil desiccation in burrows was limited and their high soil moisture combined with their high C_{org} content probably sustained longer phosphatase activities.

The combination of the anecic *L. terrestris* and the endogeic *A. caliginosa* worms did not change phosphatase activities in burrows while in casts, AcPA tended to be lower in the mixed-community than with *L. terrestris* alone. However, further experiments with *A. caliginosa* alone would help to a better understanding of this inter-specific relationships.

4.3. Phosphorous availability

As a result of enhanced phosphatase activity, organic phosphorous content decreased in casts while inorganic phosphorous content increased, the total phosphorous content being unchanged in the most cases. Chapuis-Lardy et al. (1998) observed no change in the total P content between control, non-ingested soil and surface-casts for a tropical geophageous worm *Pontoscolex corethrurus*. Our study generalizes the enhance of available P in worm casts, increment ascribed to changes in sorption complexes induced by competition for sorbing sites between orthophosphates and carboxyl groups of a glycoprotein as the mucus produced in gut (Lopez-Hernandez et al., 1993). However, available P in casts decreased rapidly with age: 4 days in casts of *P. corethrurus* (Lopez-Hernandez et al., 1993) and 3 weeks in casts of *L. terrestris* (this study). This progressive P decrease paralleled both those of carbon and

nitrogen. Moreover, the high microbial activity in fresh young casts led to a high request of inorganic P subsequently immobilized and trapped by microorganisms. Inversely, enhanced phosphatase activity in burrows did not lead to higher inorganic phosphorous content. As the growth of soil microflora is limited by P (Scheu, 1987), we suggest that the whole inorganic P pool produced in earthworm burrows was directly used by microorganisms.

In conclusion, the impact produced by earthworms on P biogeochemical transformations in the soil depends on the close relationships between the properties of the organic P source and the specific burrowing behaviour and food preferences of worms. We showed an active and extended distribution of P derived from rye-grass litter incorporated into the burrow network (ingestion and defecation), while P from unsuitable food substrate as sewage sludge was slightly redistributed within the soil profile. Not only by casting but also by soil burrowing, earthworms facilitate P transfer downward increasing a P patchy distribution in the soil. As a consequence, earthworms markedly change the biogeochemical status of P (availability, organic phosphorous pool, AcPA activities) in certain hot spots of the soil, as casts and burrow-linings. With the widespread use of sewage sludge as a fertilizer in agriculture, greater consideration should be given to the role of earthworm communities in the P cycle in soil agrosystem.

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