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ABSTRACT

Anaerobic digestion is increasingly used in Europe to treat organic substrates and produce biogas as a renewable energy source. The residual matter (digestate) is used in agriculture as an organic fertilizer. The study aims at assessing the impact of digestate application in the field on earthworms from the short term (few hours) to the long term (two years), and at investigating under laboratory conditions the role of ammonia and earthworm behavior on digestate toxicity in the short term. First, we studied earthworm communities in fields fertilized with digestates, cattle effluents, or chemical fertilizers for two years. Earthworm abundance was assessed before and after the fertilization event of the third year. Earthworm mortality at the soil surface was also assessed immediately after fertilization. Next, the toxicity of digestate or ammonia solutions on *Aporrectodea caliginosa* and *Lumbricus terrestris* was measured in microcosms (110 cm^3) to better understand the short-term toxicity (two weeks). Finally, we spread digestate (40–80 t ha^{-1}) on soil columns (5300 cm^3) and used X-ray tomography after two weeks to assess the burrowing behavior of earthworms in the cores. Earthworm abundance was 150% higher in the fields treated for two years with digestates or cattle effluents compared to the field treated with chemical fertilizers. 0.5 to 2% of adult earthworms died at the soil surface a few hours after liquid digestate and cattle slurry spreading (18 to 24 t ha^{-1}). The digestate (10% to 20% (fresh digestate/dry soil)) and ammonia were also lethal to earthworms in the microcosms within two weeks. In contrast, no mortality occurred inside soil columns two weeks after digestate spreading; *A. caliginosa* avoided the soil surface with high digestate inputs. This case study highlighted the potential short-term toxicity of digestate (a few hours), which evolved towards a neutral to positive impact in the field in the longer term (from two weeks to two years). Further research is needed to understand the impact of diverse solid and liquid digestates on soil macrofauna in different soils.

1. Introduction

Anaerobic digestion is a waste treatment process in which organic materials (e.g., animal effluents, biowastes, wastes from agriculture and agroindustry) are digested under anaerobic conditions to produce biogas, which is considered a renewable energy source. Anaerobic digestion could thus contribute to climate change mitigation (Hijazi et al., 2016). After digestion, the residual organic material (digestate) is usually spread in the field as a valuable organic fertilizer and amendment to promote nutrient recycling (Bachmann et al., 2014; Coelho et al., 2020). For these reasons, anaerobic digestion has been promoted in recent years in France (Ministère de l'Écologie du Développement Durable et de l'Énergie, Ministère de l'Agriculture, de l'Agroalimentaire et de la Forêt, 2013; Pellerin et al., 2013) as well as in multiple regions in Europe and worldwide (Scarlat et al., 2018), leading to an increase in digestate use in agriculture. Digestates can originate from diverse inputs and be used in agriculture as-is, or after multiple post-treatments, including phase separation or composting. For these reasons, digestates have diverse physicochemical characteristics (Guilayn et al., 2019; Möller, 2015; Nkoa, 2014) and are thus applied for different purposes. Some digestates are liquid and used primarily as fertilizers for short-term N fertilization, in a similar fashion to animal waste slurries. Other digestates are solid and used as soil amendments to increase soil organic matter content and long-term nutrient release, like composts or

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solid manure (Houot et al., 2014). Many studies have confirmed the interest in digestates as fertilizers for crop production, but they have also highlighted the potential impacts of digestate application on greenhouse gas and ammonia emissions (Möller, 2015; Nkoa, 2014). However, their effects on living soil organisms still need to be better characterized.

Earthworms are key soil organisms that can be promoted by organic matter inputs in cropping systems (D'Hose et al., 2018). Thus, repeated applications of digestates could have a positive effect on earthworm populations. However, anaerobic digestion generally increases C stability (Béghin-Tanneau et al., 2019; de la Fuente et al., 2013; Thomsen et al., 2013) and modifies the nature of the spread organic matter, which may influence earthworm populations in soils. Indeed, the quality of organic matter applied to soils (energy content, particle sizes) influences earthworm growth (Sizmur et al., 2017). Only a few studies have focused on the effects of digestates on earthworm communities under field conditions after one or several applications (from a few weeks to a couple of years after spreading, i.e. in the mid- to long-term). Butt and Putwain (2017), Clements et al. (2012), and Koblenz et al. (2015) found higher earthworm biomass and abundance in treatments amended with solid or liquid digestate compared to unamended soils. The impacts of digestates and undigested animal effluents were generally similar. Digestate application was also shown to influence community composition (Koblenz et al., 2015). In contrast, other studies reported no change or a decrease in earthworm populations compared with unfertilized arable or grass fields from a few months to a couple of years after liquid or dry digestate applications (Bermejo et al., 2010; Frøseth et al., 2014; Rollett et al., 2020). In regards to the physico-chemical diversity of the digestates applied, as well as the different earthworm species involved, their contrasting impacts still need to be further investigated.

In contrast to these long-term neutral to beneficial effects of soil amendment on earthworm abundance, short-term negative impacts of digestates on earthworm communities were also reported. Indeed, during anaerobic digestion, organic substrates are degraded to produce biogas, leading to N mineralization and an associated increase in the ammonium concentration in the liquid digestates (Möller, 2015; Nkoa, 2014). Several studies highlighted short-term toxicity in earthworms after application of high slurry doses (Curry, 1976; Curry et al., 1980; Hansen, 1996; Van Vliet and de Goede, 2006). According to Curry (1976) and Hughes et al. (2008), urine degradation products, such as ammonia, benzoic acid, or sulphides, could be responsible for short-term toxicity to earthworms after slurry spreading. These compounds, including ammonia, can be present in liquid digestate (Ghidotti et al., 2018; Qiu et al., 2019). In Germany, Burmeister et al. (2015) observed dead earthworms at the surface immediately after liquid digestate or slurry were applied. A Norwegian study reported a similar observation after spreading of undigested and digested slurry (Johansen et al., 2015; Løes et al., 2014). However, earthworm populations were not affected after three years of repeated application. Such phenomena could depend on soil moisture (Van Vliet and de Goede, 2006). These results suggest that digestates influence earthworm behavior (surface foraging). The toxicity of solid or liquid digestate toxicity was assessed in the laboratory for diverse soil organisms (Tigini et al., 2016), including the epigeic earthworm *Eisenia fetida* (Krishnasamy et al., 2014; Pivato et al., 2016; Renaud et al., 2017) and the endogeic earthworm *Aporrectodea caliginosa* (Ross et al., 2017). Although numerous hypotheses have been proposed to explain digestate toxicity (ammonia, salinity, oxygen deficiency, heavy metals), none has been completely verified or rejected. Moreover, an insufficient number of studies has been carried out to distinguish the effects of solid or liquid digestates. The understanding and quantification of the short-term toxicity of digestates and slurries on earthworms remain incomplete.

The aim of this study was to investigate the short-term (a few hours to two weeks) and long-term (after two years in the field) effects of digestate application in the field on earthworms. We investigated four hypotheses. (H1) In the long term, we expect a positive effect of digestates because of organic matter inputs, despite possible short-term negative effects. (H2) Digestate is toxic to earthworms in the short term, with differences in toxicity at the surface or within the soil. (H3) Ammonia explains short-term digestate toxicity. (H4) Digestate affects earthworm behavior (e.g., surface foraging and/or avoidance) in the short term, which can influence exposure to organic products. To explore these hypotheses, we conducted a total of three experiments under field or controlled laboratory conditions. To assess the long-term effects on earthworm abundance, we performed a two-year field experiment with different fertilization strategies based on mineral fertilizer, undigested slurry and manure or digestates with or without phase separation (H1). The short-term toxicity related to liquid digestate and slurry spreading was assessed in the same field experiment after a fertilization event by sampling the earthworm population and counting dead earthworms at the soil surface after spreading (H2). The short-term ecotoxicity of the same organic products was further tested in two sets of microcosm experiments (110 cm^3) under laboratory conditions with a focus on the role of ammonia in short-term toxicity (H2,H3). In microcosm ecotoxicological tests, earthworms cannot avoid the digestate as it is possible in the field. Therefore, we explored the short-term impact of digestate and slurry application on earthworm behavior and survival under conditions closer to field conditions in soil columns (5300 cm^3) analyzed with X-ray tomography (H2, H4).

2. Material and methods

2.1. Field and soil description

The field study was carried out at Nouzilly, Centre-Val de Loire, France (47°32′ N, 0°48′ E) (Pasquier et al., 2019). It is a temperate oceanic climate, with an average annual mean temperature of 11.9 °C and total annual rainfall of 650 mm. Prior to this experiment, the field has been homogeneously managed for more than ten years under conventional arable cropping systems, including wheat, barley, maize, sunflower and fertilization with mineral N with occasional inputs of cattle manure (c.a. once every five years). The soil was sampled in 2017 at the beginning of the experiment (45 samples), and the following properties/characteristic were analyzed by a specialized laboratory (US Laboratoire d'Analyses des Sols - INRAE, Arras, France): sedimentation to measure texture (NF X 31-107), total C and N content by dry combustion, gas chromatography, and thermal conductivity detector (NF ISO 10694 and NF ISO13878), pH in a water suspension (NF ISO 10390), $CaCO_3$ content by acidification and measurement of released CO_2 volume (NF ISO 10693), mass loss after combustion for SOM (1100 °C), corrected from $CaCO_3$ content. Bulk density of the upper layer was measured on fifteen locations with the core method (NF X31-501). The soil was a silty loam with the following characteristics in the ploughed layer (0–20 cm depth): 20% clay, 64% silt, 16% sand, pH 6.7, SOM content 23.3 g kg^{-1}, C:N ratio 10.1, $CaCO_3$ content 1.1 g kg^{-1}, bulk density 1.37 g cm^{-3}. The water holding capacity (WHC) was estimated in the field to be 30% (gravimetric humidity), which corresponded to the maximal soil moisture measured in the field (44 measurement dates in three years, five replicates per treatments).

The soil was sampled once in winter to conduct the laboratory experiments. The soil was sieved fresh to a size of 4 mm and stored in a closed container at 5 °C before being used. The WHC of the sieved soil was measured by saturating 25 g of dry sieved soil with water in elevated cylinders and waiting 48 h at 5 °C for the excess water to runoff. The WHC of sieved soil was 40% (gravimetric humidity). When needed, the gravimetric soil moisture was measured by mass loss after 48 h of drying at 105 °C. When needed, soil pH was measured in a water suspension (1:5 volume ratio, i.e., 5 g of dry soil in 24 mL): after addition of the reverse osmosis-purified water, the pH was measured with an electrode after agitation for 1 h.

2.2. Fertilizers and chemical solutions used as treatments

All experiments used digestates from the same anaerobic digester located in the Centre-Val de Loire region of France, and referred to as 'territorial digestate' here. In the digester, a continuous wet mesophilic process is performed, with one main digester and one postdigester. The digester treats up to 12,000 t of waste per year, consisting of cattle slurry, cattle and horse manure, sewage sludge, grease, agroindustrial wastes, cereal middlings, and grass silage. The retention time is 100 days. The digestate can be applied raw or after phase separation using a screw press. In the latter case, the liquid digestate is stored in an open lagoon and the solid digestate is stored in dedicated outdoor platform. The experiments mainly used the liquid territorial digestate, but also the raw and solid ones. We also used aerated liquid territorial digestate. Aeration was performed in a 2 L container with an aquarium oxygenizer (1.5 L_{air} min^{-1}) under agitation for 24 h. Aeration promoted the volatilization of diverse volatile compounds and decreased ammonia concentrations. Finally, we used the undigested cattle effluents. We used both cattle slurry and cattle manure, which came from a dairy farm.

A second digestate resulting from the digestion of cover crops was studied for comparison. This digestate from cover crops was sampled in 2020 (Île-de-France, France). This second digester uses a continuous wet mesophilic process to treat 28,000 t of waste per year. Animal manure is excluded from the feedstock, which mainly includes silage maize or other cereals, completed with soy wastes and agroindustrial wastes. The retention time is 90 days. The digestate is not post-treated and is stored in covered lagoons. Only the raw digestate was used in the experiments for this second digestate.

Ammonia solutions were used as control treatments in the laboratory experiments. They were prepared by diluting a commercial ammonia solution to reach the target ammonia concentration. An ammonium chloride solution was also used as another control and was produced from the ammonia solution by the addition of concentrated hydrochloric acid to reach a target pH value. Ammonia and hydrochloric acid were obtained from Carlo-Erba (Val-de-Rieu, France).

Before each laboratory experiment, organic products were sampled and stored in closed containers at 5 °C for less than one month before use. Aeration of liquid territorial digestate was performed one day before the laboratory experiment. Ammonia and ammonium solutions were prepared less than one hour before the experiment to avoid volatilization.

The dry matter (DM) content of all organic products was determined after 48 h of drying at 105 °C. The volatile solid (VS) content (mass loss after combustion, NF EN 13039), total Kjedhal N content (NF EN 13654-1), and pH and conductivity (measured in a water suspension NF EN 13037 and NF EN 13038) were analyzed in fresh products by specialized laboratories (AUREA, Ardon, France, and AUREA, La Rochelle, France). Their ammoniacal nitrogen (N_{amm}) contents were determined after the extraction of 25 g of a fresh sample with 100 mL of 1 mol L^{-1} KCl for 1 h, after which the liquid phase was analyzed by colorimetry on a continuous flow analyzer (Skalar, The Netherlands). The concentration accounted for both NH_3 and NH_4^+ forms. In the microcosm experiments, the liquid digestate and slurry applied to soils contained large amounts of water, which was taken into account to achieve common soil moisture levels in all treatments. Because of the large amount of water associated with organic matter in the organic products, the DM content of the organic products is not relevant for this experiment. Thus, we defined the "moistening power" of an organic product as the quantity of water that significantly humidified soils in the microcosm experiments. It was determined by weighing free water after centrifugation of the organic products for 10 min at 614 ×g (three replicates). The physico-chemical characteristics of all organic products and chemical solutions are summarized in Table 1, which also includes their use in the different experiments.

Heavy metal (Cd, Cr, Cu, Ni, Pb, Zn) contents were analyzed in one replicate in organic products at the CIRAD Laboratory of Water, Soil and Plant Analysis (US Analyses, CIRAD, Montpellier, France). The organic products were mineralized (NF ISO 14869-1) and heavy metal contents were measured using inductively coupled plasma mass spectrometry (ICP-MS) (Table 2). Le Bars et al. (2018) included some of these heavy metals analyses in their study and gave more details in the methods. Seven polychlorinated biphenyls (PCB), including PCB 028, PCB 052, PCB 101, PCB 118, PCB 138, PCB 153 and PCB 180, were measured by an external laboratory (AUREA, Ardon, France) (Table 2). The method consisted in an accelerated solvent extraction (ASE) followed by a dosage with Gas Chromatography - Tandem Mass Spectrometry (GC–MS-MS) (XP X 33-012). Thirteen polycyclic aromatic hydrocarbons (PAH) were measured (Table 2). Sertillanges et al. (2020) included these PAH analyses in their study and gave more details in the methods.

2.3. Earthworms

In the laboratory experiment, we considered two earthworm species, *L. terrestris* and *A. caliginosa*. *L. terrestris* were bought in a fishery store (Decathlon®). *A. caliginosa* were sampled from an orchard, that was pesticide free for more than five years, located close to Avignon, France. All earthworm individuals were acclimated in the experimental soil for two weeks before the experiments. Before and after all laboratory experiments, the earthworms were weighed after a fasting period (Fründ et al., 2010). For this purpose, they were left in Petri dishes with moistened filter paper, which was changed every day. After three days, the earthworms with an empty gut were weighed. *L. terrestris* is an epianecic earthworm (Bouché, 1972). *A. caliginosa* is an endogeic species and a good model for studying agricultural practices (Bart et al., 2018). *L. terrestris* and *A. caliginosa* were chosen because they are both common in agricultural fields of temperate soils.

2.4. Experiment setup

2.4.1. Field experiment setup

The field experiment started in 2017. Four treatments are tested in plots of 24 × 75 m under conventional management (wheat - rapeseed - wheat succession, export of wheat straw, ploughing). The treatments consisted of (A) fertilization with only mineral N solution in winter. It did not receive any organic amendment; (B) fertilization with solid bovine farmyard manure in summer and liquid cattle slurry in spring; (C) application of raw territorial digestate in both summer and spring (no phase separation); and (D) application of solid territorial digestate in summer and liquid territorial digestate in spring (Table 3). Prior to the experiment, we assumed similar earthworm populations in all plots due to similar management practices in the long-term and plot proximity. Treatment (A) was assumed to be representative of the existing initial earthworm populations.

In February 2019, after two years of the different fertilization schemes, we assessed their long-term effects. On the 12th of February 2019, seven days before applying the fertilizer treatments, we excavated the soil to assess earthworm populations. Fertilizers were first applied on the 19th of February 2019, and one hour later, earthworms that came up to the surface and died were counted. A second evaluation of the earthworm population was carried out two weeks later, on the 5th of March 2019, to assess the short-term effect of fertilization with liquid mineral N or the organic products. Fertilizers were applied for a second time on the 12th of March 2019. The number of earthworms that came to the surface and died was assessed after 1 h and 24 h (Table 3).

Earthworm populations were assessed by chemical extraction, soil excavation (three replicates per treatment, 40 × 40 × 20 cm), hand sorting and formaldehyde conservation, as described in Pelosi et al. (2009). The number of earthworms at the surface after fertilizer application was counted in six 1 m^2 subplots. Earthworm morbidity was tested according to their reaction to physical stimuli (earthworms that did not react were considered dead; Langdon et al., 1999). At the same date, soil moisture was measured (0–20 cm, five replicates per

Table 1
Physico-chemical characteristics of the organic products and chemical solutions. Their use in the different experiments is also summarized. Missing values are indicated by N.A. (not available).

Organic products			Physico-chemical characteristics							Use					
Type	Origin	Sampling date	DM (% FW)	[N_{amm}] (g $kgFW^{-1}$)	[N_{tot}] (g $kgFW^{-1}$)	pH	Moistened power (% FW)	VS (% FW)	Conductivity (mS cm^{-1})	Long-term field experiment (2017-2018)	First short-term field experiment (February 2019)	Second short-term field experiment (March 2019)	Microcosm experiment, series #1 (2019)	Microcosm experiment, series #2 (2020)	Column experiment
Cattle slurry	Nouzilly, Centre-Val de Loire	2017-2018	3.3	0.9	1.8	7.3	N.A.	2.4	2.0	X					
		February 2019	4.0	2.0	2.7	7.6	59	2.8	3.2		X		X		X
		March 2019	3.2	1.1	2.2	7.9	N.A.	2.2	2.4			X			
		2020	4.9	1.0	2.0	6.9	46	3.5	2.7					X	
Cattle manure	Nouzilly, Centre-Val de Loire	2017-2018	32.5	0.7	8.3	9.7	N.A.	25.3	6.1	X					
Territorial digestate, liquid	Nouzilly, Centre-Val de Loire	2017-2018	4.8	2.2	4.4	7.9	N.A.	2.8	3.9	X					
		February 2019	5.1	3.2	4.3	8.2	81	3.0	4.3		X		X		X
		March 2019	4.8	2.2	4.2	8.1	N.A.	2.9	4.4			X			
		2020	5.5	2.4	4.1	8.0	58	3.8	4.7					X	
Territorial digestate, liquid, aerated	Nouzilly, Centre-Val de Loire	2019	5.0	1.9	N.A.	9.3	81	N.A.	N.A.				X		X
		2020	5.3	1.9	N.A.	8.9	58	N.A.	N.A.					X	
Territorial digestate, raw	Nouzilly, Centre-Val de Loire	2017-2018	6.8	2.4	4.7	8.0	N.A.	4.6	4.3	X					
		February 2019	6.1	3.9	4.6	7.9	N.A.	4.3	4.5		X				
		March 2019	6.0	2.2	4.2	7.9	N.A.	4.3	4.2			X			
Territorial digestate, solid	Nouzilly, Centre-Val de Loire	2017-2018	27.2	1.6	6.6	9.3	N.A.	23.2	3.1	X					
Digestate from cover crop, raw	Île-de-France	2020	6.9	4.2	4.9	8.3	51	5.2	5.3					X	
Ammonia (NH_3) solution			0.0	10.0	10.0	11.8	100	0	N.A.					X	
Ammonium (NH_4Cl) solution			0.0	10.0	10.0	8.0	100	0	N.A.					X	

Table 2
Heavy metals, polycyclic aromatic hydrocarbons (PAH), and polychlorinated biphenyls (PCB) content in the organic fertilizer and amendments spread in the field experiment. Results are expressed in mg $kgDM^{-1}$. N.D.: not detected (below the threshold detection content). For 13 PAH, results are indicated as mean ± standard deviation (n = 4). Heavy metals were analyzed only on one sample; when it is available, the uncertainty of analysis (inter laboratory comparison) is indicated as mean ± uncertainty.

	Cd	Cr	Cu	Ni	Pb	Zn	7 PCB	13 PAH
Cattle slurry	N. D.	2	36 ± 3	2	2	180 ± 22	N.D.	191 ± 75
Territorial liquid digestate	1.0	59 ± 3	249 ± 20	21	22 ± 0.8	663 ± 79	N.D.	741 ± 27
Territorial raw digestate	1.0	34 ± 2	266 ± 22	16	23 ± 0.8	701 ± 84	N.D.	762 ± 125
Cattle solid manure	N. D.	3	22	2	1	83 ± 10	N.D.	89 ± 33
Territorial solid digestate	0.1	14	64 ± 5	5	8 ± 0.3	178 ± 21	N.D.	508 ± 49

treatment).

2.4.2. Laboratory ecotoxicity tests in microcosms

Ecotoxicity tests were carried out in microcosms to evaluate the mortality and weight change of earthworms living in a mixture of soil and organic products. For each treatment, sieved soil and the organic product were mixed and then the soil moisture was adjusted to reach 90% of the sieved soil-WHC considering the "moistening power" of the organic products (see Section 2.2). Seven replicates were used for each treatment. For each replicate, one fasted and weighed (as in Section 2.3) *L. terrestris* or *A. caliginosa* adult was placed in a Petri dish (diameter: 14 cm) with 150 g of the mixture. During the experiment, microcosms were placed in a dark chamber at 15 °C (±2 °C) for 2 weeks. We assessed mortality every day during the first five days and then every two or three days (earthworms that did not react to physical stimuli were considered dead). At the end of the incubation, living earthworms were fasted and weighed. Treatments with fewer than three living earthworms were excluded from the data analysis because of the low sample size. These methods were adapted from Leveque et al. (2013) and Olvera-Velona et al. (2008).

We conducted two series of ecotoxicity microcosm experiments. Series #1 (2019) was designed to compare L. *terrestris* and *A. caliginosa* mortality in the presence of liquid digestate and slurry. In each species, we tested the eight following treatments (Supplementary Table S1): four doses of liquid territorial digestate (2.5%, 5%, 10%, 20%, in g of fresh organic product per 100 g of dry soil); two doses of cattle slurry (10%, 20%); one control without organic products; and one dose of aerated liquid territorial digestate (20%). Series #2 (2020) was designed to explore the hypothesis that ammonia was responsible for the toxicity of the organic products in an experiment conducted only in L. *terrestris*, which was the species on which digestate had the greatest impact in the field. We tested the following treatments (Supplementary Table S2): one control without any organic products; four doses of liquid territorial digestate (5%, 10%, 15%, 20%); four doses of aerated liquid territorial digestate (5%, 10%, 15%, 20%); five doses of digestate from cover crops (2.5%, 5%, 10%, 15%, 20%); and 3 doses of cattle slurry (5%, 10%, 20%). We tested five doses of ammonia (NH_3) solution (5% D_{eq}, 9% D_{eq}, 18% D_{eq}, 27% D_{eq}, 36% D_{eq}) and four doses of ammonium chloride (NH_4Cl) solution (5% D_{eq}, 9% D_{eq}, 18% D_{eq}, 27% D_{eq}, 36% D_{eq}). The X% D_{eq} doses correspond to an amount of ammoniacal N similar to a dose of X% of the liquid territorial digestate. In both series, aerated liquid territorial digestate was used to test whether a decrease in the ammonia concentration would decrease mortality. Digestate from cover crops was used to test whether the inputs could influence digestate toxicity. NH_3 and NH_4Cl solutions were used to test the toxicity of ammonia according to its chemical form. We calculated the theoretical contents of ammoniacal N in the mixture at the beginning of the experiment, expressed in mg of ammoniacal N per g of dry soil ($mgN_{amm}\ kgDS^{-1}$), on the basis of the ammoniacal N concentration in organic products. These contents are presented in Supplementary Tables S1 and S2. In Series #2, because of its impact on $NH_3 \rightleftarrows NH_4^+$ equilibrium, we analyzed the pH of each mixture: one part of the mixtures was left for 24 h without earthworms,

Table 3
Field assessment of the impact of the use of organic products on earthworms: summary of the 4 treatments and of the experimental procedure. The described experiment is a 3-year field experiment with 4 fertilization treatments that started in October 2016 (undifferentiated fields).

Date	Event	Treatments			
		Mineral N	Slurry and manure	Raw territorial digestate	Liquid and solid territorial digestates
		A	B	C	D
January 2017 to April 2017 (wheat)	Fertilization (2 events)	Mineral fertilization 86 + 43 kgN ha^{-1}	Cattle slurry 37 + 60 t ha^{-1}	Raw digestate 38 + 31 t ha^{-1}	Liquid digestate 36 + 32 t ha^{-1}
August 2017	Amendment	/	Cattle manure 35 t ha^{-1}	Raw digestate 32 t ha^{-1}	Solid digestate 33 t ha^{-1}
March 2018 (rapeseed)	Fertilization	Mineral fertilization 99 kgN ha^{-1}	Cattle slurry 27 t ha^{-1}	Raw digestate 32 t ha^{-1}	Liquid digestate 36 t ha^{-1}
September 2018	Amendment	/	Cattle manure 12 t ha^{-1}	Raw digestate 16 t ha^{-1}	Solid digestate 16 t ha^{-1}
February 12, 2019 (wheat)	Earthworm population evaluation				
February 19, 2019	Fertilization	Mineral fertilization 40 kgN ha^{-1}	Cattle slurry 24 t ha^{-1}	Raw digestate 12 t ha^{-1}	Liquid digestate 23 t ha^{-1}
1 h after spreading	Surface mortality evaluation				
March 5, 2019	Earthworm population evaluation				
March 12, 2019	Fertilization	Mineral fertilization 80 kgN ha^{-1}	Cattle slurry 18 t ha^{-1}	Raw digestate 20 t ha^{-1}	Liquid digestate 18 t ha^{-1}
1 h after spreading	Surface mortality evaluation				
24 h after spreading	Surface mortality evaluation				

after which pH was analyzed as described in Section 2.1. Ammoniacal N together with pH was a relevant proxy for NH_3 concentration in soil mixture, as it was difficult to discriminate between NH_3 and NH_4^+ forms in soil mixtures.

2.4.3. Laboratory ecotoxicity tests in soil columns

We compared earthworm biomass and burrowing behavior in soil columns before and after spreading organic products on the top of the column. The soil columns consisted of PVC cylinders (40 cm in height and 15 cm in diameter) filled with sieved soil to a height of 30 cm. Columns were iteratively filled with sieved soil in 2 cm-high layers, which were individually moistened and compressed to control the homogeneous bulk density at 1.3 g cm^{-3} following the methodology of Capowiez et al. (2015). Prior to the experiment, the earthworms were weighed. At t = 0 days, five *A. caliginosa* and two L. *terrestris* were added to each column. We covered the columns with plastic grids to prevent the earthworms from escaping. The columns were placed in a chamber under controlled conditions with a 12 h light and 12 h dark cycle to reproduce the diurnal cycle. The temperature was controlled to ensure a constant soil temperature of 15 °C (±1 °C). The soil moisture content in the columns was adjusted every three days by weighing them and adding water at the top of the columns.

The experimental setup first consisted of 14 days of incubation, after which the macrocosms were imaged by X-ray tomography to assess the 3D burrowing systems. Then, at t = 15 days, we spread the organic products corresponding to each treatment on the top of each column. The columns were constantly observed for 4 h and were then regularly observed for the next two days to report the occurrence of any earthworms at the soil surface. Columns were observed regularly and the presence of casts recorded. Two weeks later, at t = 28 days, the macrocosms were imaged again by X-ray tomography. The soil columns were opened at t = 32 days to retrieve the earthworms, and all living earthworms were fasted and weighed. For both imagery sessions, the X-ray tomography (CT Siemens Somatom® Definition AS 128, Siemens, Germany) used the same parameters: 120 kV, 50 mA, resolution of 0.7 mm × 0.7 mm × 0.7 mm. Tomography images were analyzed using ImageJ software (Schindelin et al., 2012). We first binarized the images to separate soils and burrows using a single manually fitted threshold. Macropores with a section less than to 5.0 mm^2 were not considered (noise). Then, we used the 3D diameter of the burrows to discriminate the burrows of L. *terrestris* and *A. caliginosa* using the Local Thickness plugin (the threshold for the diameter was set to 7.57 mm). For each macrocosm and each species, we computed the volume of macropores at t = 14 days, the difference of macropores volume between t = 28 days and t = 14 days, and the mean burrow diameter at t = 28 days. We computed the volume of macropores on four soil layers of 7.5 cm each per macrocosm (0 - 7.5 cm, 7.5–15 cm, 15–22.5 cm, 22.5–30 cm).

We compared five treatments (with five replicates each) run at the same soil humidity of 70% WHC as measured for sieved soil: (C) control treatment, in which 40 t ha^{-1} of water was spread; (LD40) 40 t ha^{-1} of liquid territorial digestate; (S40) 40 t ha^{-1} of slurry; (LD80) 80 t ha^{-1} of liquid territorial digestate; (ALD40) 40 t ha^{-1} of aerated liquid territorial digestate. A supplementary treatment was added (LD40-H+) with 40 t ha^{-1} of liquid territorial digestate, and a higher soil moisture content of 80% WHC. We also included two control columns without earthworms (70% and 80% WHC), reaching a total of 32 columns. The (ALD40) treatment was used to test whether a decrease in the ammonia concentration affects earthworm behavior. The (LD40-H+) treatment was used to test whether soil moisture can change the impact of digestate according to worm locations.

2.5. Statistical analyses

Statistical analyses were performed with R software (v.4.0.2) (R Core Team, 2020). The statistical significance among treatments of all variables was tested using a significance level of 0.05. In the field experiment, the difference in the abundance and biomass of earthworms within each sampling date among treatments was assessed using Kruskal-Wallis (kruskal.test function from stats package, R Core Team, 2020) and Dunn's tests (dunn.test function from the *dunn.test* package, Dinno, 2017). The evolution of earthworm abundance and biomass within each treatment between both sampling dates was tested using one-sided Wilcoxon-Mann-Whiney tests (wilcox.test function from *stats* package, R Core Team, 2020). We also simultaneously examined the effects of the treatment and date on earthworm abundance in the field (grouped data of both sampling dates) with the Sheirer-Ray-Hare test. The Sheirer-Ray-Hare test is a nonparametric test conducted in lieu of two-way ANOVA and an extension of the Kruskal-Wallis test (Sokal and Rohlf, 1995), followed by a post hoc Dunn's test. We used the scheirerRayHare function from the *rcompanion* package (Mangiafico, 2020). In the microcosm experiments, the difference in earthworm weight change among treatments was tested using Kruskal-Wallis with Dunn's tests. The difference in earthworm mortality among treatments was tested with a Fischer exact test followed by a pairwise Fischer exact test as a post hoc test (fisher.bintest function from *RVAideMemoire* package, Hervé, 2020). We examined the correlation between mixture pH and earthworm mortality using the cor.test function (*stats* package, R Core Team, 2020), with the "pearson" method. In the column experiment, the difference in earthworm weight change and burrow system characteristics among treatments were tested using Kruskal-Wallis with Dunn's tests. Fischer exact tests and post hoc pairwise Fischer exact tests were used to test a difference in the presence of casts at the surface of the columns among treatments.

3. Results

3.1. Long-term and short-term impacts on earthworms of applying organic products in the field

In February 2019, after two years of the different amendments, the main species found in the soil in the field were L. *terrestris*, *A. caliginosa*, *Aporrectodea rosea* and *Allolobophora chlorotica* in all treatments.

Following both spreading events (19th of February 2019 and 12th of March 2019), we observed earthworm mortality at the soil surface immediately after applying the liquid digestate and raw digestate. The observed mortality rates were 0.1 to 2.7 earthworms m^{-2} (Table 4). Mortality appeared in the first 20 min after spreading, the only exception was the first application of slurry after which mortality was reported after 24 h (data not recorded) (Table 4). In all treatments, most dead earthworms were adult L. *terrestris*. Some large endogeic earthworms, such as *A. caliginosa*, also died after the second application (12th of March 2019). Juveniles as well as smaller endogeic earthworms, such as *A. chlorotica* or *A. rosea,* were not observed at the soil surface. Soil moisture was 26% (gravimetric, i.e. 85% of WHC) on 19th of February 2019, and 27% (gravimetric, i.e. 89% of WHC) on 12th of March 2019.

Table 4
Evaluation of short-term mortality in earthworms in the field experiment: the numbers of earthworms found dead at the soil surface after the spreading of the organic products (earthworms m^{-2}) are indicated as the mean ± standard error. Adult earthworm abundance is expressed in earthworms m^{-2} (± standard error).

Date	Liquid digestate	Raw digestate	Cattle slurry	Mineral fertilization
First spreading event, 1 h after spreading	0.6 ± 0.3	0.1 ± 0.2	0	0
Second spreading event, 1 h after spreading	2.7 ± 1	1.5 ± 0.6	1.7 ± 0.7	0
Second spreading event, one day after the spreading	1.8 ± 1.2	0.7 ± 0.3	1.0 ± 0.6	0
Mean adult abundance (both sampling dates)	138 ± 21	82 ± 9	126 ± 15	68 ± 17

Adult earthworm abundance did not differ before and after the first application (19th of February 2019) of any treatments ($p > 0.05$). During the same period, total earthworm abundance only increased in the mineral N treatment ($p = 0.05$) (Fig. 1). Considering biomass, the only change was an increase in total biomass in the slurry and manure treatment ($p < 0.05$) (Supplementary Fig. S1).

For the two years of the different fertilization treatments, before the first spreading in 2019, the inputs of organic matter were 0 t VS ha^{-1}, 13.7 t VS ha^{-1}, 6.7 t VS ha^{-1}, and 15.7 t VS ha^{-1} for mineral N, slurry and manure treatment, raw digestate treatment, and liquid and solid digestate treatment, respectively. At the first sampling date, the total abundance of earthworms in the slurry and manure treatment was significantly higher than under the mineral N treatment without organic products ($p < 0.05$) (Fig. 1). Treatment did not have a significant effect on the abundance of adult earthworms ($p > 0.05$). None of the treatments had a significant effect on biomass ($p > 0.05$) (Supplementary Fig. S1). At the second sampling date, the treatments had no effect on total or adult abundance ($p > 0.05$). Thus, because earthworm abundance did not change before and after fertilization, the data were grouped. Considering both dates, total and adult abundance were significantly higher under the slurry and manure treatment (+161% and +87%) and in the territorial solid and liquid digestates treatment (+147% and + 109%) than under the mineral N treatment ($p < 0.05$). Total and adult abundances were not influenced by the sampling date ($p > 0.05$). Earthworm biomass was not impacted by treatment ($p > 0.05$).

3.2. *Short-term ecotoxicity of organic products in microcosms under controlled laboratory conditions*

In the microcosm ecotoxicity experiment, the mortality rate was more than 75% when territorial digestate was mixed in soils at doses of 10% (series #1) and 15% (series #2) for L. *terrestris*, and 20% for *A. caliginosa* (Fig. 2). As a comparison, slurry caused a mortality rate of more than 75% at a 20% dose only for series #1 (both species), but not for series #2. A 15% dose of digestate from cover crops was also lethal to 75% of L. *terrestris*. Mortality occurred rapidly: in all treatments in both series, 64% and 94% of deaths occurred within the first 24 h then within the first four days for L. *terrestris* (48% and 83% for *A. caliginosa*,

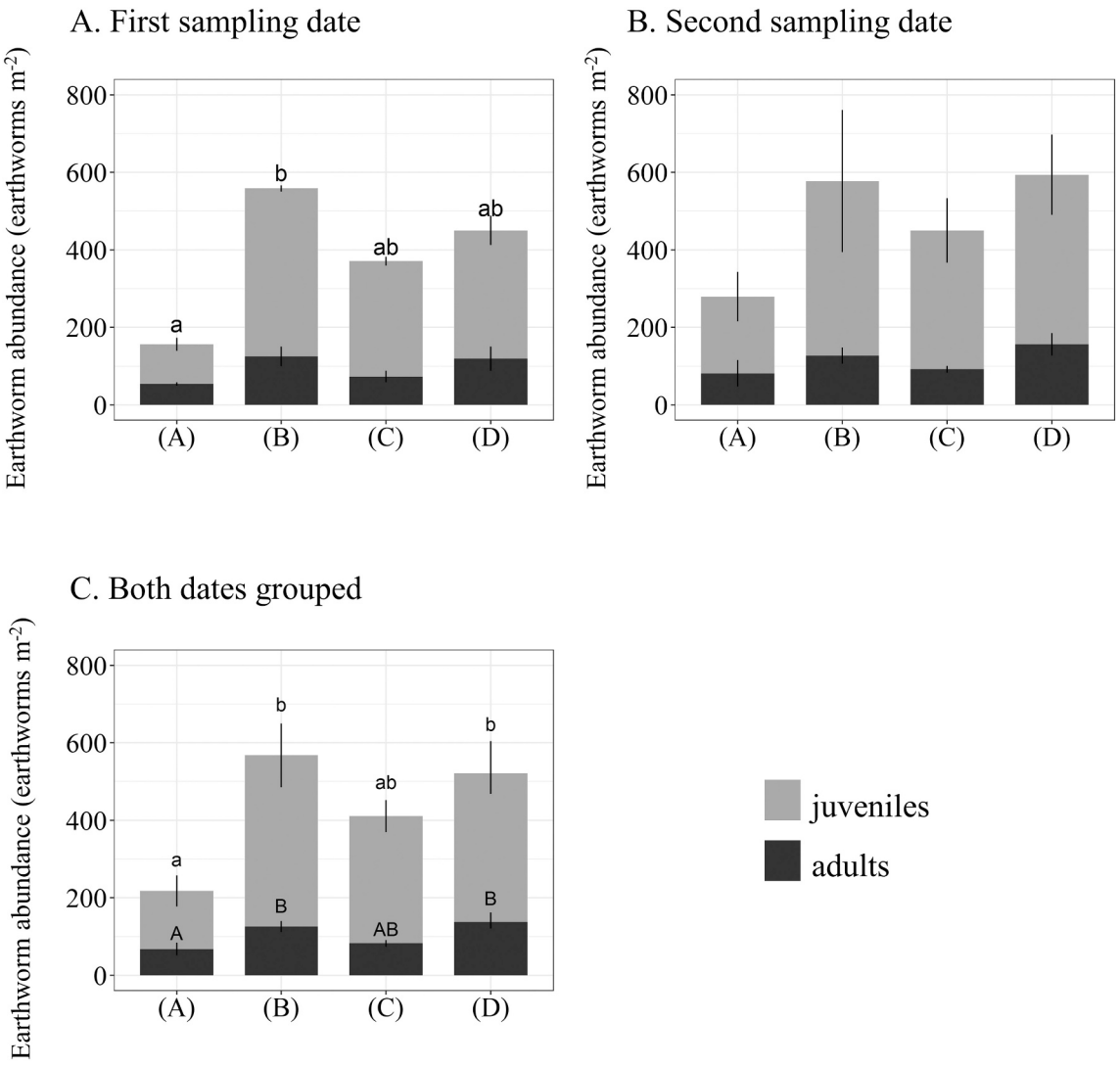

Fig. 1. Abundance of earthworms in fields before and after application of organic products. Error bars indicate standard errors. Two treatments with different lower case (or capital) letters indicate that the total (or adult, respectively) earthworm abundance in those treatments was significantly different ($p < 0.05$). No letter means that the treatment did not have a significant impact on adult or total abundance ($p > 0.05$). Each treatment is a fertilization system, using (A) chemical N fertilizers, (B) slurry and manure, (C) raw territorial digestate, (D) liquid and solid territorial digestates. A. Long-term effect of repeated application of organic products: first sampling date, in plots which received the different amendments for two years, seven days before spreading. B. Second sampling date, 14 days after spreading. Comparison with subfigure 1.A shows the impact of fertilization after two weeks on soil earthworm populations. C. Two sampling dates grouped together.

respectively).

Considering only the treatments with at least three living earthworms, the mean weight change of *L. terrestris* was +6% and +4% in the control treatments of series #1 and #2, respectively. It ranged from −1% (slurry 10%) to +6% (liquid territorial digestate 2.5%) in series #1 (Supplementary Table S3), from +8% (cattle slurry at 20%) to −9% (aerated liquid territorial digestate at 15%) in series #2 (Supplementary Table S4). It was not affected by the treatments (series #1: $p > 0.05$; series #2: Kruskal-Wallis test $p < 0.01$, but Dunn's test $p > 0.05$). The mean weight change of *A. caliginosa* was −14% in the control treatment, ranging from −34% (10% slurry treatment, which was lethal to 42% of earthworms) to −7% (5% territorial digestate). The weight change was significantly different only between this 10% slurry treatment and the 5% territorial digestate treatment ($p < 0.05$). The experimental procedure was valid because earthworm weight loss in the control treatment was less than 20% (Fründ et al., 2010).

3.3. Microcosm experiment to investigate the causes of short-term ecotoxicity to L. terrestris

Three observations from our experiments were consistent with the hypothesis that ammonia is partly responsible for digestate toxicity. First, the ammonia solution was toxic to more than 75% earthworms at a dose of 723 mg N_{amm} kg DS^{-1} (0.9 g NH_3 kg DS^{-1}), which corresponded to the ammoniacal N content of the soil mixture containing 27% of territorial digestate (Fig. 2B). Moreover, the lethal doses of the digestate from cover crops (15% and 20%) corresponded to 630 mg N_{amm} kg DS^{-1} (0.8 g NH_3 kg DS^{-1}) and 840 mg N_{amm} kg DS^{-1} (1.1 g NH_3 kg DS^{-1}), which were similar to lethal ammoniacal N concentrations in ammonia solutions (Fig. 2B). Finally, as a partial negative control, aeration of the

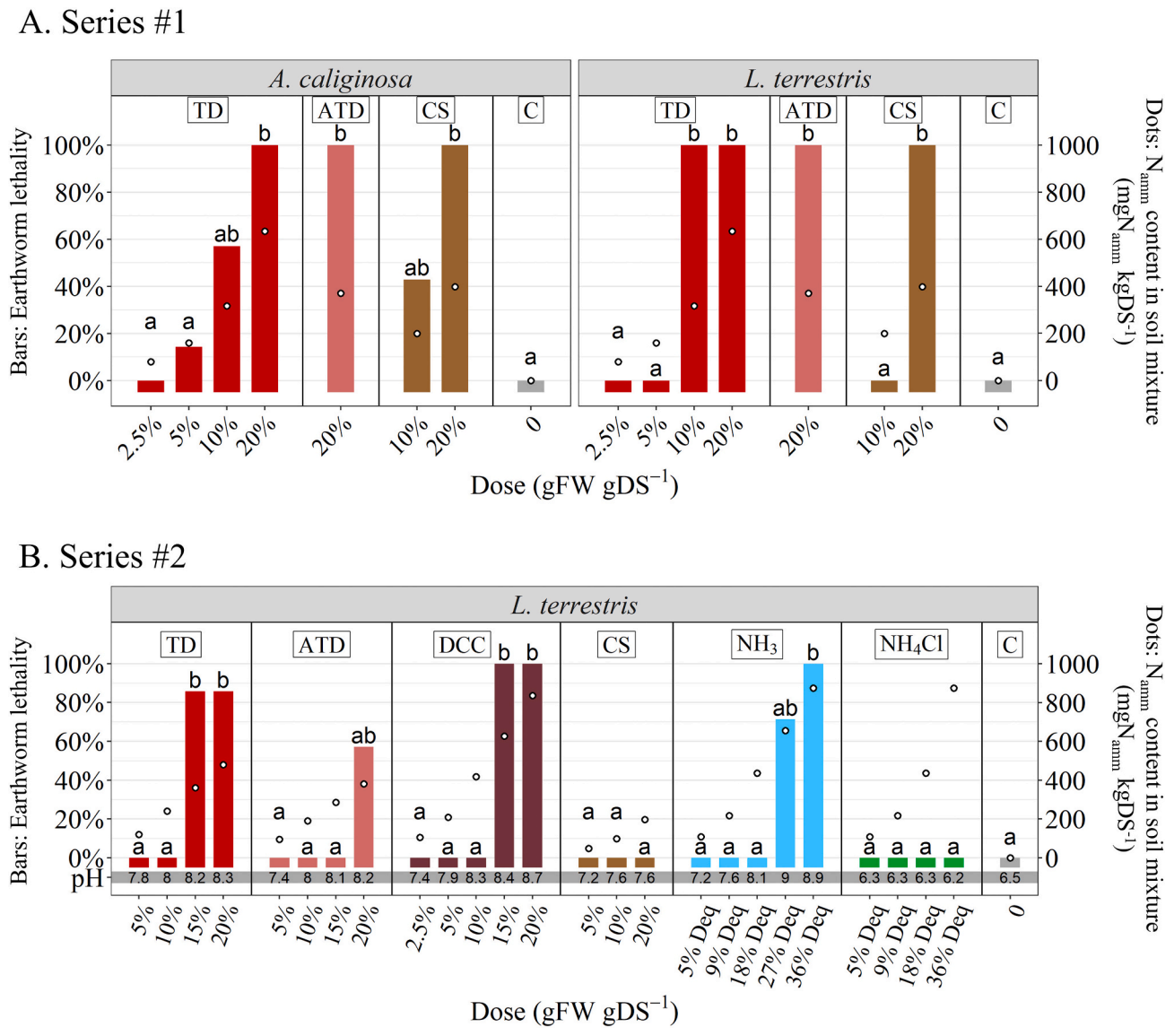

Fig. 2. Ecotoxicity tests of organic products in microcosms, series #1 (A) and series #2 (B). Bars represent the proportion of dead earthworms within the seven replicates for each treatment. Bars are still presented in the figure for treatments with no mortality. Dots indicate the ammoniacal N content in the (soil + organic products) mixtures, expressed in mg of ammoniacal N per g of dry soil (mgN_{amm} $kgDS^{-1}$). The organic product mixed with soil is indicated in boxes: (TD) liquid territorial digestate, (ATD) aerated liquid territorial digestate, (DCC) digestate from cover crops, (CS) cattle slurry, (NH_3) ammonia solution, (NH_4Cl) NH_4Cl solution, (C) control. The dose of organic products in the mixture is expressed in g of fresh weight of organic product per g of dry soil (gFW gDS^{-1}). The doses of the ammonia (NH_3) and ammonium (NH_4Cl) treatments are expressed in the territorial digestate equivalent dose (D_{eq}): a dose of 5% D_{eq} corresponds to an amount of ammoniacal N similar to a dose of 5% of the territorial digestate (gFW gDS^{-1}). Within each series and species, different letters indicate that two treatments showed a significant difference ($p < 0.05$).

territorial digestate, reduced the ammoniacal N concentration (Table 1) and decreased toxicity in series #2. Aerated territorial digestate caused the same mortality rate at similar doses as territorial digestate in series #1 (both species), and lower mortality rates in series #2, with 0% at the 15% dose and 60% at the 20% dose (Fig. 2B). Regarding the hypothesis that ammonia drove toxicity, soil mixed with the aerated territorial digestate (20%) had a similar soil N_{amm} content to the soil mixed with unaerated territorial digestate (10%), which was lethal to *L. terrestris* (Fig. 2A). However, the ammonia content alone could not explain territorial digestate toxicity. The ammoniacal N content of lethal mixtures containing territorial digestate (15% and 20%) were equal or lower than 380 mg N_{amm} kg DS^{-1}. A similar ammoniacal N concentration in the NH_3 treatment was not lethal (Fig. 2B). The territorial digestate was lethal at the same dose as the digestate from cover crops (15%), which had a similar pH but lower ammoniacal N concentration (Fig. 2B).

The pH of the soil + organic product mixtures correlated with mortality (Pearson's $r = 0.65$, $p < 0.001$). All lethal treatments showed a mixture pH greater than 8.2 (Fig. 2B). NH_4^+ was never lethal, demonstrating the importance of the ammoniacal N form (NH_4^+/NH_3) in toxicity.

3.4. Short-term impact of organic products on earthworm behavior and survival in soil columns

In the soil columns, despite spreading the organic products at high (40 t ha^{-1}) and very high (80 t ha^{-1}) doses, we did not observe mortality at the surface or inside the soil. The mortality recorded at the end of the experiment was low and was not significantly different between treatments (3% for L. *terrestris* and 6% for *A. caliginosa* on average). The treatments did not significantly affect the changes in earthworm weight observed over the course of the experiment ($p > 0.05$ for both L. *terrestris* and *A. caliginosa*) (Supplementary Table S5). In the control treatment, earthworm weight decreased by 18% and 16% for *L. terrestris* and *A. caliginosa*, respectively. This validated the experimental procedure (Fründ et al., 2010).

L. terrestris casts were found at the surface in all columns in the S40 treatment, in two columns for ALD40, LD80, and LD40-H+, and in no columns in the LD40 and control treatments. We found that a treatment had a significant effect on the presence of casts ($p < 0.01$). The addition of slurry resulted in some solids, including straw, remaining on the soil surface, which was burrowed into the soil by the earthworms within the week after spreading. In contrast, the digestate infiltrated the soil during the first day following spreading. Traces of digestate were found in large L. *terrestris* burrows when the columns were destroyed at the end of the

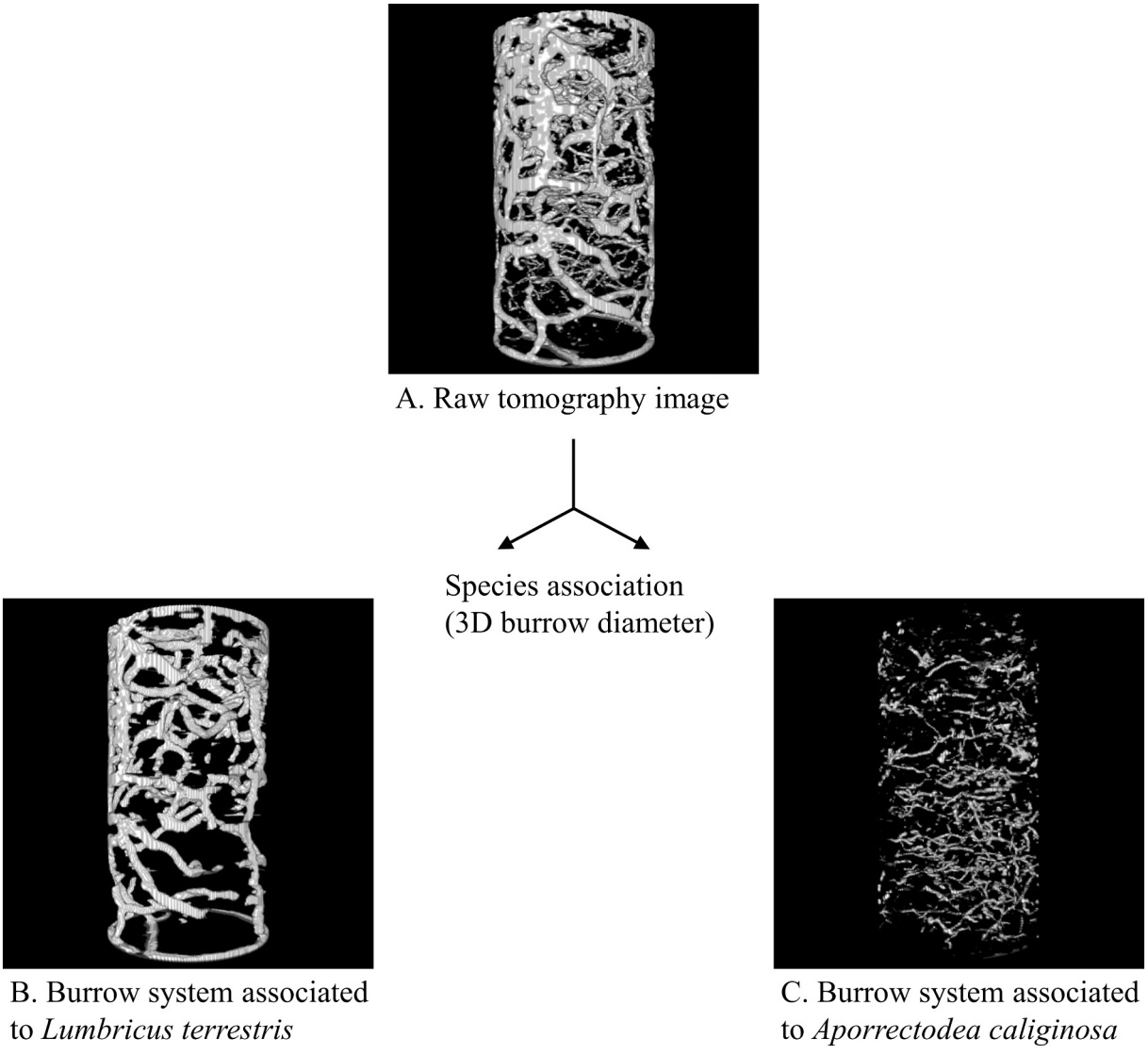

Fig. 3. Example tomography images before (A) and after the separation of the different burrow systems built by L. *terrestris* (B) or *A. caliginosa* (C).

experiment, suggesting the quick preferential infiltration of digestate.

Using image analysis, we could satisfactorily distinguish between *A. caliginosa* and *L. terrestris* burrows (Fig. 3). Before applying organic matter, there was no difference in burrow excavation at any depth for the two species in any of the standard humidity conditions ($p > 0.05$; Fig. 4). In the LD40-H+ treatment, in the presence of high soil moisture content, *A. caliginosa* burrowed significantly more in the first 15 cm and less below 15 cm compared to in the other treatments ($p < 0.05$). While *A. caliginosa* burrowed homogenously within the first 22.5 cm before inputs, these endogeic earthworms burrowed less in the first and fourth soil layers after application of the digestates (LD80, LD40). In contrast, in the S40 and C treatments, endogeic earthworms still burrowed homogeneously at all depths (Fig. 4). Considering the entire soil core, *A. caliginosa* burrowed significantly less after spreading in the LD80 treatment than in the slurry and control treatments ($p < 0.05$). Except for LD40-H+, *L. terrestris* burrowing activity was not affected by treatment at any depth. None of the treatments had a significant impact on burrow mean diameter for either species ($p > 0.05$).

4. Discussion

4.1. Long-term impact of digestates on earthworm populations

Here, we confirmed that (H1) digestates can have a positive effect on earthworms in the long term, despite short-term negative effects. This is in accordance with previous studies. In organic grassland, Clements et al. (2012) observed a similar earthworm biomass but higher earthworm abundance in plots fertilized with slurry (+125%) or liquid digestate (+53%) six weeks after application compared with unamended plots. Four months after spreading slurry or liquid digestate, Koblenz et al. (2015) found similar positive effects on earthworm abundance (+33% to +126% and +34% to +100%, respectively) and biomass (+49% to +208%, and +42% to +143%, respectively) compared with unamended treatments. In a restored colliery soil, Butt and Putwain (2017) observed a higher abundance (+150% to +232%) and biomass (+120% to +195%) of earthworms in plots amended with solid digestate than in the untreated control plots after two years of application.

This positive effect of digestate and animal effluents was likely due to the input of organic matter, which can serve as a food source for earthworms (D'Hose et al., 2018; Ernst et al., 2008; Sizmur et al., 2017). After two years, the lower observed earthworm abundance in plots treated with raw digestate was most likely due to the overall lower input of organic matter contained in the raw digestate compared to liquid and solid digestate or slurry and manure treatments (see Section 3.1). Solid organic amendments (manure or solid digestate) and liquid organic fertilizers (slurry or liquid digestate) could promote earthworm populations differently, we cannot determine their relative impact from the field experiment described in this study. The different organic products may have different feeding properties due to their C and N availability and calorific value (Ernst et al., 2008; Sizmur et al., 2017) and thus benefit earthworm species differently (Ernst et al., 2008; Onrust and Piersma, 2019). Replacing animal effluents with digestate may affect earthworm populations, depending on primarily the quantity but also the quality of organic matter contained in the products (Abail and Whalen, 2018).

Another hypothesis to explain the evolution of earthworm populations in the long-term was a change in the soil C:N ratio due to the organic product inputs. Earthworm abundance appeared to increase in soil with a low C:N ratio (De Wandeler et al., 2016; Nieminen et al., 2011). Although the C:N ratio was determined only at the beginning of the field experiment, several other studies showed no change in C:N ratios in long-term field experiments with repeated digestate inputs similar to ours (Barlóg et al., 2020; Glowacka et al., 2020; Pastorelli et al., 2021; Persson et al., 2020; Zicker et al., 2020). Thus, this scenario appears to be less probable than one where the amount of organic matter brought to field explains the observed differentiation in earthworm populations.

4.2. Comparison of short-term effects under field and laboratory conditions

Consistent with our findings, Burmeister et al. (2015) found that digestate toxicity had a low effect on earthworm populations in the field, recording less than one dead earthworm per m^2 after liquid digestate spreading (0.3% of the population). A Norwegian study (Johansen et al., 2015; Løes et al., 2014) observed a higher mortality of L. *terrestris*, *Lumbricus rubellus*, *A. caliginosa*, and *A. rosea* (between two and 19 dead earthworms m^{-2}, respectively, corresponding to 1.3% and 17% of the population) after undigested and digested slurry was applied at different doses in a grass-clover field.

Several studies focused on solid digestates (Pivato et al., 2016; Renaud et al., 2017; Ross et al., 2017; Sizmur et al., 2017) that were later buried in the soil, so these authors did not assess earthworm surface foraging immediately after spreading. To our knowledge, this is only the second study (after Natalio et al. (2021)) to propose a microcosm experiment for assessing liquid digestate toxicity. We found lower lethal concentrations of digestate compared to that reported by Pivato et al. (2016) (40% solid digestate on a dry matter basis, corresponding to 145% on a fresh matter basis) and Krishnasamy et al. (2014) (80% fresh solid digestate together with 20% sawdust) for *E. fetida*. This can be explained by the relatively lower sensitivity of this species (Pelosi et al., 2013) and the nature of the digestates applied (solid versus liquid). In contrast to the findings from microcosm experiments, we detected no mortality within the soil two weeks after slurry and digestate was spread in either the field or in the soil columns. This can be explained by avoidance leading to a lower probability of contact between the earthworms and the products. Only earthworms that came to the surface immediately after spreading were in direct contact with digestate concentrations close to or higher than the lethal concentration observed in our laboratory experiments.

We thus confirmed that the (H2) digestate and slurry can be lethal to earthworms in the short term and that this mortality appears to be limited to the soil surface.

4.3. Probable causes of liquid organic product toxicity in the short term

The presence of ammonia in the liquid organic products likely explains some of their ecotoxicity to earthworms. Hughes et al. (2008) measured an LC_{50} of 1.5 g NH_3 per kg of soil for *E. fetida*, which was slightly higher than the ammonia concentration that was lethal to *L. terrestris* in our microcosm experiment. Once more, the difference may be explained by the different sensitivity of the two species (Pelosi et al., 2013). Krishnasamy et al. (2014) found that concentrations of a solid digestate that were toxic to *E. fetida* in soils corresponded to ammonium concentrations between 0.9 and 1.2 g NH_3 kg Dry $Soil^{-1}$ (g NH_3 $kgDS^{-1}$), which is consistent with the lethal dose of digestate from cover crops. Furthermore, aeration of the digestate decreased its toxicity in our experiment, suggesting that at least one toxic compound in the digestate is volatile, which is a well-established characteristic of ammonia. Ammonia toxicity can also explain why we observed differences in the toxicity of slurry in the microcosms between series #1 and series #2. In the first series, the slurry was toxic and had a higher N_{amm} concentration than in the second series where no toxicity was observed. Consistent with our results, Hughes et al. (2008) also found that the form of ammoniacal N (NH_3 or NH_4^+) influenced its toxicity. The $NH_4^+ \rightleftarrows NH_3 + H^+$ acid-base equilibrium depends on the pH. A high pH promotes a high NH_3 concentration, with a pKa of 9.25 being observed at 25 °C in ideal aqueous solution. Both digestates and soil pH may thus influence ammoniacal N toxicity.

At the lethal doses of the territorial digestate in the microcosms, the ammoniacal N soil content was too low to completely explain the

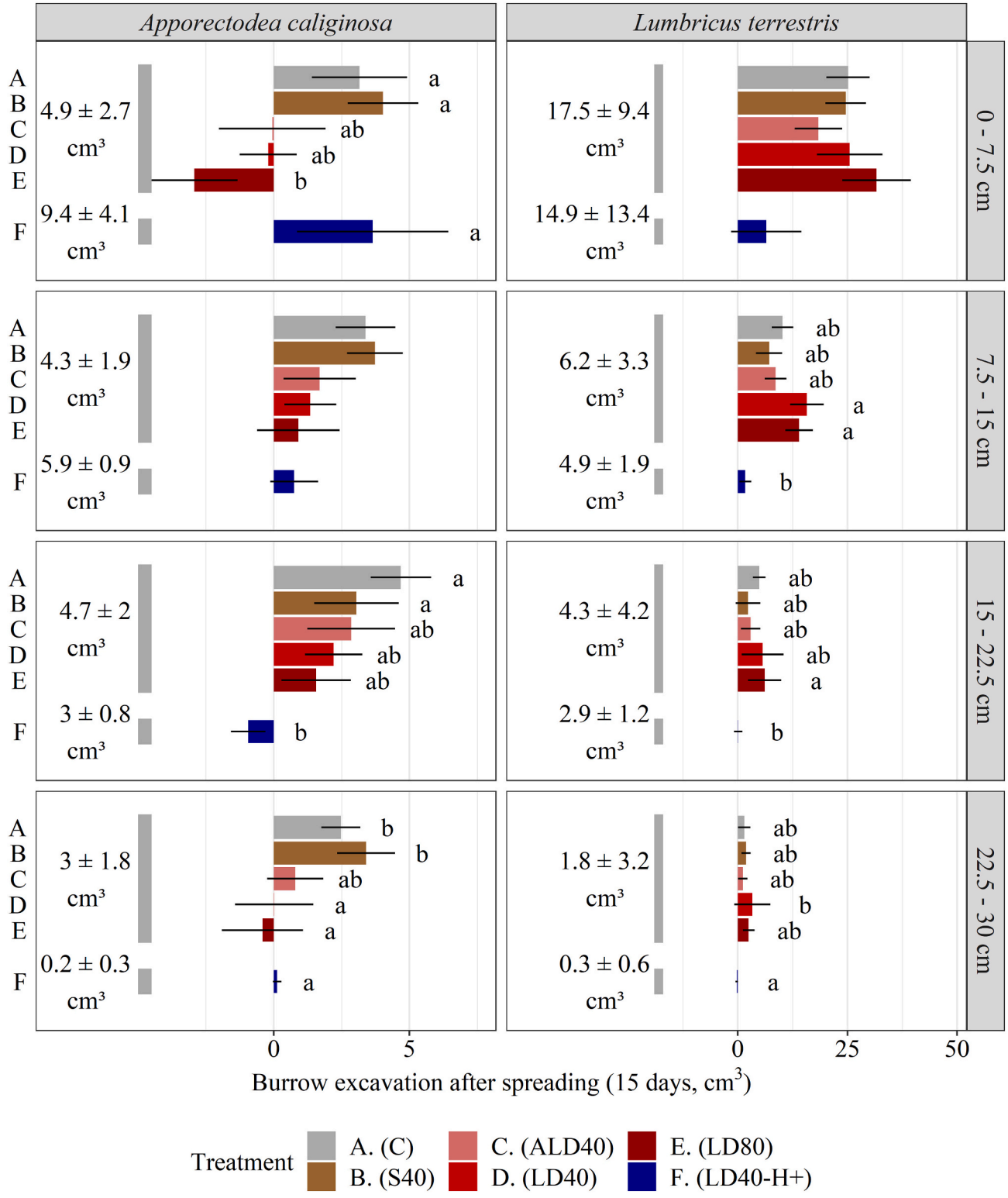

Fig. 4. Difference in burrow volume after the application of organic products between day 14 and day 28 (15 days) for both earthworm species and for all four soil layers. For each species and soil layer, different letters indicate that the treatments showed significant differences ($p < 0.05$). The absence of letters indicates that there was no significant difference between the treatments ($p > 0.05$). In the left part of each subgraph, we indicate the burrow volume associated with each soil moisture content during the first 14 days of the experiment (day 0 to day 14) before spreading (±standard deviation). Treatments are: (C) control, (S40) cattle slurry (40 t ha^{-1}), (ALD40) aerated liquid territorial digestate (40 t ha^{-1}), (LD40) liquid territorial digestate (40 t ha^{-1}), (LD80) liquid territorial digestate (80 t ha^{-1}), (LD40-H+) liquid territorial digestate (40 t ha^{-1}), high SWC.

observed toxicity: this suggests that other compounds originating from the inputs may also affect digestate toxicity to earthworms. Urine degradation products such as benzoic acids or sulphides (Curry, 1976) could also be toxic, as could diverse ionic compounds through salinity or CEC (Natalio et al., 2021; Pivato et al., 2016; Tigini et al., 2016). However, it is unlikely that heavy metal contamination was involved in the observed short-term toxicity. In the lethal 20% dose of the territorial digestate, the concentrations of heavy metals were: 2.5 mg Cu kg DS^{-1}, 6.6 mg Zn kg DS^{-1}, and 0.2 mg Pb kg DS^{-1} (Table 2). This is much lower than the estimated LC_{50} values for *Aporrectodea tuberculata* found by Lukkari et al. (2005) (134 mg Cu kg DS^{-1} and 234 mg Zn kg DS^{-1}) or the concentration of heavy metals in soils that was not lethal soils to *L. terrestris* (Kennette et al., 2002). Similarly, the PAH content in this treatment was 7.4 mg kg DS^{-1}, much lower than the LC_{50} for PAH towards *E. fetida* estimated in Eom et al. (2007).

In summary, we confirmed that (H3) ammonia is partly responsible for digestate toxicity, but further studies are required to understand other potential toxicity mechanisms.

4.4. Earthworm behavior in the short term after organic product spreading

When liquid organic products, i.e. slurry and digestates, were spread on the soil, earthworms came up to the surface. This is a key mechanism for understanding field ecotoxicity. Mortality was only observed 24 h after the first application of slurry in the field. As the solid phase of slurry could not infiltrate the soils very rapidly, we assume that anecic earthworms came to the surface at night, as commonly observed in epi-anecic species such as *L. terrestris* (Bastardie et al., 2005), and then came in contact with pools of slurry. Similar to our experiment, Brauckmann and Broll (2007) spread a digestate within soil cores (30 cm high, 6800 cm^3) and did not observe surface mortality. Because the phenomenon affected only a small percentage of earthworms in the field, it makes sense that it was not detected in the column experiments where the total number of earthworms involved was only 35 per treatment.

In contrast to the slurry treatments, no *L. terrestris* surface activity (casts) was observed in columns amended with digestate. This suggests that the digestate and thus organic matter infiltrated efficiently within the column and that *L. terrestris* did not need to come to the surface to feed. *A. caliginosa* burrowed less in the presence of digestate than slurry, particularly in the surface soil layer. A first hypothesis is that *A. caliginosa* avoided the first soil layer and decreased its activity due to possible digestate toxicity. A second hypothesis is that the infiltration of the digestate within the soil increased the presence of accessible food, allowing these endogeic earthworms to decrease their activity (Frazão et al., 2019; Hughes et al., 1996). The lack of significant differences in weight change between the treatments as well as the toxic effect shown in the microcosm experiments tends to favor the first hypothesis of a toxic effect. Indeed, Ross et al. (2017) observed that *A. caliginosa* avoided soil layers containing digestate and Ernst et al. (2008) found that *A. caliginosa* could not feed on raw digestate (weight loss was observed in these 1500 mm^3 mesocosms). This avoidance of digestate could explain why some *A. caliginosa* came to the surface in the field after digestate spreading, which is not a common behavior for endogeic species. The negative burrowing activity of *A. caliginosa* seen in some treatments (e.g., LD80) was due to large quantities of digestate or the worms own casts filling burrows, or L. *terrestris* activity destroying the endogeic burrows. We confirmed that the (H4) digestate has an impact on earthworm behavior in the short term, with different possible outcomes, to either limit toxicity (avoidance) or trigger mortality (surface foraging).

According to Burmeister et al. (2015) and Van Vliet and de Goede (2006), digestate toxicity only occurs under humid soil conditions, which was the case when we observed earthworm mortality in the field. When the soil is moist, earthworms stay closer to the surface and may be more exposed to the applied organic products. However, we did not observe surface mortality after liquid digestate spreading in soil columns with high soil humidity (LD40-H+), where the earthworms were close to the surface; therefore, we could not reach a conclusion regarding this point.

5. Conclusions

To promote sustainable agricultural practices, the impacts of using digestates and slurry need to be better assessed in the soil and on the associated living organisms. This case study provided new insights regarding the short- and long-term effects of anaerobic digestates on earthworms. In the short term, liquid organic products may be toxic to earthworms mainly when direct contact occurs. This toxicity depends on the ammonia concentration, which is promoted by high pH, and other elements that could not be clearly identified here and will require further research. In the field, mortality likely occurred shortly after application because earthworms foraged at the soil surface, where the products were highly concentrated. In the longer term, we found that earthworm populations increased after two years of regular input of organic products. Further research is needed to better identify the causes of the toxicity of digestates from different substrates, to highlight the parameters explaining why earthworms come to the surface after spreading, to compute the frequency of lethal events in the field, and to estimate the feeding properties of digestates in the long term. The impact of different solid and liquid digestates on earthworm ecology (e.g., species, behavior, functions) still needs to be investigated in different soils.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2021.104149.

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