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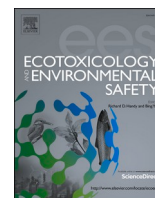
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## Assessing the chronic toxicity of spreading organic amendments on agricultural soil: Tests on earthworms and plants

Olivier Roques<sup>a,b</sup>, Rémy Bayard<sup>b</sup>, Julia Le Maux<sup>b</sup>, Dominique Patureau<sup>c</sup>, Sylvie Néliou<sup>d</sup>, Isabelle Lamy<sup>d</sup>, Jean-Philippe Bedell<sup>a,\*</sup>

<sup>a</sup> Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA, F-69518 Vaulx-en-Velin, France

<sup>b</sup> Univ Lyon, INSA Lyon, DEEP, EA7429, 69621 Villeurbanne, France

<sup>c</sup> INRAE, Université de Montpellier, LBE, 102 Avenue des étangs, 11100 Narbonne, France

<sup>d</sup> Université Paris-Saclay, INRAE, AgroParisTech, UMR EcoSys, 91120 Palaiseau, France

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### ABSTRACT

Recycling organic wastes on agricultural soils improves the soil quality, but the environmental and health impact of these organic amendments closely depends on their origins, their bio-physicochemical characteristics and the considered organisms potentially affected. The aim of this study was to assess the potential chronic ecotoxicity of spreading organic amendments on agricultural soils. To do this, we characterized three different organic amendments: sewage sludge from an urban wastewater treatment plant, cow manure and liquid dairy manure. Their chronic ecotoxicity was studied through assays exposing earthworms of the species *Eisenia fetida* and two plants: *Medicago sativa* and *Sinapis alba*. Of the three amendments, the sewage sludge presented the highest concentrations of micropollutants and a considerable fraction of available and biodegradable organic matter. The cow manure and liquid dairy manure had lower chemical contamination and similar characteristics with lower biodegradable fractions of their organic matter. No chronic phytotoxicity was evidenced: on the contrary, particularly with sewage sludge, the germination rate and aerial and root biomass of the two plants increased. Considering earthworms, their biomass increased considerably during the reproduction assays in soil amended with sewage sludge, which contained the more bioavailable organic matter. Nonetheless, the earthworms presented an inhibition close to 78% of the production of juveniles when exposed to sewage sludge exceeding 20 g·kg<sup>-1</sup> DW (that means 2 times the agronomic dose). This reprotoxic effect was also observed in the presence of liquid dairy manure, but not with cow manure. At the end of the assays, the glycogen and protein reserves in earthworms exposed to sewage sludge were inferior to that of control earthworms, respectively around 50% and 30%. For the earthworms exposed to liquid dairy manure, protein and lipid reserves increased. In the case of liquid dairy manure, this reprotoxic effect did not appear to be linked to the presence of micropollutants. In conclusion, our results confirm the need to use several ecotoxicity assays at different biological levels and with different biological models to assess the ecotoxic impacts of soil amendments. Indeed, although certain organic wastes present a strong nutritional potential for both plants and earthworms, a not inconsiderable risk was apparent for the reproduction of the latter. An integrated ecotoxicity criterion that takes into account a weighted sum of the different results would guide the utilization of organic amendments while ensuring the good health of agricultural ecosystems.

### 1. Introduction

In the context of developing the circular economy in every sector of the economy, the exploitation of organic wastes and agricultural residues as organic amendments is a major challenge with respect to substituting chemical amendments, improving soil quality, and carbon

sequestration (Cucina et al., 2021, 2019; Scotti et al., 2015). Organic amendments generally comprise livestock effluents and organic wastes resulting from the treatment of household organic waste, and urban and industrial sewage sludge (Collivignarelli et al., 2019; Loyon, 2017).

These organic amendments are often characterized by their high percentage of organic matter and nutritive elements (Goss et al., 2013).

\* Corresponding author.

E-mail address: [JeanPhilippe.BEDEL@entpe.fr](mailto:JeanPhilippe.BEDEL@entpe.fr) (J.-P. Bedell).

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Consequently, spreading them represents a major advantage for fertilizing soils and increasing crop yields (Zuo et al., 2019). Indeed, organic waste products are sources of nutrition for decomposing organisms such as earthworms and microorganisms, whereas inorganic nutritive elements such as nitrogen, phosphorous and potassium are assimilated by plants. Moreover, these organic amendments improve soil quality by reorganizing and stabilizing soil (Khaliq and Abbasi, 2015). Indeed, high organic matter content improves the soil's water holding capacity (WHC), its porosity and hydraulic conductivity (Głab et al., 2018). All these improvements lead to promoting plant growth and soil biodiversity by stimulating microbial activity and decomposing organisms. Thus, analyses of the chemical and physical composition of these organic amendments lead to better understanding of their fertilizing effect on soils. Methods combining biochemical extraction and characterization of organic matter also provides important information on their bioavailability, biodegradability, and their mineralization rate in soil. Availability is defined as the specific volume of a chemical product that is or can become available within a given period of time and conditions (Reichenberg and Mayer, 2006). This fraction therefore becomes mobile and available for processes such as biodegradation and digestive absorption (Loehr and Webster, 1996; Mayer et al., 1996).

However, pollutants, whether sequestered or not in organic matter, are contained in these amendments and are liable to contaminate the fertilized soil. Indeed, according to the origin of the wastes introduced in the processes that produce these organic amendments, persistent and non-persistent metallic trace elements (MTEs) and organic pollutants were evidenced at quantifiable contents (Fijalkowski et al., 2017; Goss et al., 2013). To limit soil contamination, several European directives aim at controlling the spreading of certain pollutants in agricultural soils by imposing a maximum threshold for the main MTEs (7), polycyclic aromatic hydrocarbons (3) and polychlorobiphenyls (7), as well as the maximum spreading doses for sewage sludge (EC, 1986). What is more, organic amendments can also generate soil acidification and salinization, linked to readily biodegradable organic matter, with impacts on the biological activity of the soils fertilized (Bonanomi et al., 2014).

In view to assessing the risks of spreading these organic amendments, the use of laboratory bioassays has been generalized and standardized with, in particular, acute ecotoxicity assays such as the earthworm lethality test ISO 11268-1:2015 (ISO, 2015a) and the plant germination root growth test ISO 18763:2020 (ISO, 2020). However, the application of these tests at agronomic doses of organic amendments in soils does not allow highlighting ecotoxic effects linked to spreading (Roques et al., 2023). In addition, the contribution of organic matter by organic amendments modifies the characteristics of soil, generating short-term positive effects on terrestrial organisms (Roques et al., 2023). Setting up chronic ecotoxicity assays appears pertinent for observing the ecotoxicity of organic amendments. The scientific literature mentions a large number of potential assays used to assess the chronic ecotoxicity of these organic amendments like the higher plant emergence and growth test of (Žaltauskaitė et al., 2016), the earthworm reproduction assay based on counting the number of cocoons and juveniles produced (Urionabarrenetxea et al., 2022; Parente et al., 2021; Huguier et al., 2015) and the springtail reproduction assay (Kończak and Oleszczuk, 2018).

The organic matter present and the type of soil will play a major role in the chronic ecotoxicity of these organic amendments on terrestrial organisms. Indeed, modifying the properties of soil leads to responses that vary as a function of the biological model exposed. In our study, the chronic ecotoxicity of organic amendments was assessed by exposing two types of organisms belonging to two different trophic levels: earthworms and plants. The plant response mainly depends on the mineral composition of the fertilized soil. On the contrary, earthworm response depends on the available fraction and the biodegradation of organic matter in the soil. Due to its major role in the degradation and recycling of organic matter, the earthworm is a pertinent bioindicator for assessing the risk of spreading these organic amendments. These two

organisms appear to be complementary for assessing the ecotoxicity of organic amendments. According to the literature, a reduction of earthworm reproduction has already been observed during exposure to poultry manure and sewage sludge (Žaltauskaitė et al., 2016; Parente et al., 2011). Some studies have also shown reduced germination and growth in plants (Žaltauskaitė et al., 2016).

The study of biomarkers during chronic toxicity assays can also provide information on the impact of pollutants on organisms of different levels of biological complexity (Lagadic et al., 1997). Most of the biomarkers studied concern analyses relating to the biochemical responses linked to stress caused by the external environment. These biochemical responses make it possible to show metabolic impacts and are potential indicators of stress. The main biomarkers of the biological stress of earthworms concern cell integrity, enzymatic activity and energy reserves (Urionabarrenetxea et al., 2022; Parente et al., 2021; Parelho et al., 2018; Pelosi et al., 2016). The energy of organisms is fixed and the implementation of detoxification mechanisms linked to environmental stress leads to a reduction of energy reserves and the reduction of the quantity of energy allocated to other mechanisms like growth and reproduction (Calow, 1991).

The biological activity of the soil can therefore be impacted in the long-term by the spreading of organic amendments on agricultural land. Observing the acute toxic effects of organic amendments on organisms is difficult and often not discriminant. Thus, it requires carrying out chronic ecotoxicity assays to observe measurable effects on soil organisms. In addition, organic amendments can have positive and negative effects. The aim of this study is to develop an analytical approach by using chronic ecotoxicity assays to evidence the effect of spreading organic amendments on agricultural land. To do this, two ecotoxicity bioassays on earthworms and plants were used to assess the chronic ecotoxicity of three organic amendments: the plant seedling emergence and growth test (ISO, 2013) and the earthworm reproduction test (ISO, 2015). This approach was also complemented by the characterization of the organic matter of the three organic amendments studied: sewage sludge, dairy cow manure, and liquid dairy manure as well as an energy reserve assessment aspect for earthworm component. Moreover, an organic matter balance was implemented during the earthworm reproduction test to avoid masking ecotoxic effects caused by the presence of a larger quantity of organic matter in the fertilized soils.

## 2. Materials and Methods

### 2.1. Origins and preparation of matrices

The reference soil used in this study was sampled between 0 and 80 cm depth in November 2019 in a meadow having received no spreading and located in the Arve area, France (46°07'16.0"N 6°18'37.3"E, Scientrier, 74930). After having removed all the plant elements, this soil was homogenized, air dried and sieved at 2 mm, then conserved at ambient temperature with a water content of  $4.63 \pm 0.24\%$ . It was composed of sand (51.1%), silt (45.6%) and clay (3.2%). An artificial ISO soil was prepared according to standard ISO 17512-1 (ISO, 2020) with a mixture of 69% fine sand, 20% kaolin clay, 10% peat moss and 1% calcium carbonate. The characterization techniques are detailed in the article by Roques et al. (2023), and the information obtained is mentioned in [supplementary material 1](#).

The three organic amendments were selected for their representativeness of spreading practices in the study area located in the Arve catchment area in France. The selection comprised a sewage sludge, a cow manure and liquid dairy manure. The sewage sludge was collected at the wastewater treatment plant of Bellecombe, located in Scientrier (Haute-Savoie department, France). It was a secondary type sludge resulting from urban and hospital effluents, collected after dehydration in a glasshouse. The two organic amendments from cattle livestock comprised a liquid manure and manure from dairy cows from two separate farms located few kilometers from Annemasse (Haute-Savoie

department, France). Pre-treatments before conservation were performed on two different matrixes. The sewage sludge was sieved at 2 mm by hand grinding and fraction > 2 mm, was discarded and excluded from the experiment. The cow manure was crushed by a cutter mill to obtain smaller straws with a maximum length of 5 cm. The liquid manure was dried in a ventilated thermostat-controlled oven at 25 °C to reduce its water content, initially  $90.56 \pm 1.64\%$ . The three organic amendments were homogenized by manual mixing of all the stock and kept at  $-20\text{ °C}$  with water contents (mass percentage) of  $53.97 \pm 0.44\%$  for the sewage sludge,  $82.39 \pm 0.50\%$  for the manure and  $78.07 \pm 3.14\%$  for the liquid manure.

The characteristics of the three organic amendments in terms of fertilizing values and pollutant contents are described previously in the article by Roques et al. (2023) and detailed in [supplementary material 2](#).

## 2.2. Characterization of organic matter by coupling chemical fractionation and analysis of organic extracts by fluorescence spectroscopy

The organic amendments were freeze-dried and sieved (1 mm). Sequential chemical extraction was carried out according to the method described by Jimenez et al. (2015), to characterize organic matter and provide information on its bioavailability (for details see [supplementary material 3](#)). Each fraction was characterized by measuring the chemical oxygen demand (COD) according to protocol ISO 15705: 2002 (ISO, 2002). In this experimental protocol, the fractionation of organic matter (ISBAMO\*: Index of Stability and Bioavailability of Organic Matter) carried out on a dry sample of 0.5 g is automated using an extraction system accelerated by solvents: Thermofisher™ ASE™ 350. Only the fourth step was manual as it required the use of sulfuric acid at 72%, a solvent incompatible with the device.

In brief, 5 fractions were obtained by sequential extraction:

- Soluble Extractable Organic Matter (SPOM). The SPOM fraction was extracted using a solution of  $\text{CaCl}_2$  at 10 mM (to avoid the possible rupture of the cell walls). This soluble fraction in aqueous phase mainly contained sugars and proteins.
- Readily Extractable Organic Matter (REOM). The REOM fraction was extracted using a saline sodium - hydroxide solution ( $\text{NaCl} = 10\text{ mM}$  /  $\text{NaOH} = 10\text{ mM}$ ,  $\text{pH} = 11$ ). This fraction mainly contained proteins, nucleic acids, sugars and lipids.
- Slowly Extractable Organic Matter (SEOM). The SEOM fraction was extracted using a sodium hydroxide solution ( $\text{NaOH} = 100\text{ mM}$ ,  $\text{pH} = 12$ ). This fraction mainly contained proteins, humic substances and lipids.
- Poorly Extractable Organic Matter or lignocellulosic fraction (PEOM). This PEOM fraction, based on Van Soest fractionation, was extracted using a concentrated commercial sulfuric acid (72%). This fraction contained cellulose and hemicellulose.
- Non-extractable Organic Matter (NEOM). This fraction was obtained by subtracting the sum of the fractions from the total mass of organic matter determined for the intact sample. This fraction was mainly composed of lignocellulosic type organic matter.

The organic matter extracted in the different fractions was characterized by 3D fluorescence spectroscopy (type of compounds of each compartment). The fluorescence spectra of each of the fractions was acquired with a Perkin Elmer LS55spectrophotometer. The excitation wavelength varied from 200 to 600 nm with increments of 10 nm. With the method described in Jimenez et al. (2015), the spectra obtained were decomposed into seven zones according to the fluorescence of major biochemical types (proteins, humic acids, fulvic acids). Then, an index representing the ratio of complexity was calculated as per Muller et al. (2014) for each fraction. This index is defined as the ratio between the sum of the most complex fluorescence zones (zones no. 4–7) over the sum of the protein type fluorescence zones (zones no. 1–3).

## 2.3. Choice of application doses of organic amendment

Whether for reproduction tests on earthworms or for plant growth tests, the choice of spreading dose is based on the doses imposed by European regulations. For sewage sludge, the maximum spreading dose over 10 years on agricultural soil in France (EC, 1986),  $30\text{ t ha}^{-1}$  dry equivalent, was divided by 3, i.e.  $10\text{ t ha}^{-1}$  dry equivalent, corresponding to an application dose of sewage sludge of about  $10\text{ g kg}^{-1}$  of dry soil (1 ha of soil = 1250 t of dry soil on 10 cm depth and for a density estimated at  $1.25\text{ g cm}^{-3}$ ). Thus, the doses tested were 1 x, 2 x or 5 x the average spreading dose. For the two agricultural amendments tested during this study, the calculation of the average spreading dose was based on the agricultural practices of the area studied, i.e. a spreading dose of  $35\text{ t ha}^{-1}$  of raw material, that is to say a concentration of agricultural organic amendment of  $10\text{ g kg}^{-1}$  of dry soil for a dry matter content of 30%. The doses tested were therefore 0.5 x, 1 x, 2 x or 5 x the average spreading dose of the area studied. These ranges of concentrations tested were determined following results obtained during earthworm lethality bioassays performed by Roques et al. (2023). Thus, the doses tested were 1–2x the average spreading dose for plant trials and 0.5–5x for earthworm trials.

## 2.4. Bioassays

### 2.4.1. Plant germination and growth

The chronic higher plant emergence and growth bioassay was performed by adapting the standardized procedure ISO 11269–2: 2013 (ISO, 2013). Two species were selected: the alfalfa *Medicago sativa* and the mustard *Sinapis alba*. These two species are representative of the crops planted in the area and are recommended in standard ISO 11269–2. The test was performed in a thermostat controlled climatic chamber at  $20 \pm 2\text{ °C}$  and a photoperiod of 16 h day/8 h night. The soil-amendment mixtures were prepared 5 days before starting the bioassay to permit the recolonization of the mixture by the microorganisms and the stabilization of water content fixed at 70% of WHC for all the mixtures tested.

The bioassays were performed with reference soils fertilized with sewage sludge, cow manure and liquid dairy manure at the following concentrations: 0, 10, 20  $\text{g kg}^{-1}$  dry weight (DW) of dry soil. Terracotta pots with a hole in their base (diameter 14 cm, height 13.5 cm) were covered with aluminum foil to prevent exchanges of water and pollutants between the fertilized soil and the terracotta pots. The pots were then filled with the reference soil or the fertilized reference soil up to 2 cm below the edge of the pots. Then, 75 seeds were placed homogeneously on the surface of the soil and then covered with a 1 cm layer of the reference soil or the fertilized reference soil. Each treatment was performed in triplicate. To maintain an appropriate water content in the soil during the bioassays, the pots were watered 3 times a week and placed on a dish filled with demineralized water to ensure humidification by capillarity. The water content was checked by weighing every week.

Seed germination and growth were monitored for 28 days for *M. sativa* and 21 days for *S. alba*. At the end of the bioassay, the aerial parts of the plants were cut at the separation with the root, counted and measured by numerical treatment with ImageJ© software. The aerial and root parts were cleaned by successive ultrasound baths. Lastly, the aerial and root parts were weighed before and after drying for 96 h in an oven at  $50\text{ °C}$ .

### 2.4.2. Reproduction for *Eisenia fetida*

Earthworms of the species *Eisenia fetida* were provided by a breeding facility in Germany. After reception, these earthworms were kept in a medium composed of horse dung from an organic breeding facility and coco peat soil (50/50) with a water content close to 70% of WHC and a temperature of  $20 \pm 2\text{ °C}$ . Only adult earthworms between 350 mg and 600 mg in fresh weight with a visible and well-developed clitellum were



selected for the chronic ecotoxicity bioassays. Before starting the assays, the earthworms were cleaned with demineralized water by rinsing, weighed and fasted for 24 h.

The reproduction test for the *Eisenia fetida* earthworms was performed for 56 days by adapting procedure ISO 11268-2: 2015 (ISO, 2015). It was performed in a room climatized at  $20 \pm 2$  °C and a photoperiod of 16 h day/8 h night. Each mixture was prepared 5 days before the starting the assay to permit the recolonization of the medium by microorganisms and the stabilization of the water content. The water content was adjusted to 70% of the WHC of the mixture. These bioassays were performed in glass jars with a volume of 1 L (Le Parfait®), 11 cm in diameter, 16.5 cm high and closed by plastic covers uniformly perforated to prevent the earthworms from escaping and ensure the oxygenation of the medium. Thus, 450 g of dry mass of the reference soil or the fertilized reference soil was introduced in each jar covered at the base by a strip of aluminum foil 6 cm high to maintain darkness in the soil. Then, 10 earthworms comprising 3 replicates per treatment were exposed for 28 days at the following concentrations: 10, 20 and 50 g kg<sup>-1</sup> DW (1 x, 2 x and 5 x the average spreading dose) for the sewage sludge and the cow manure; 5, 10, 20 g kg<sup>-1</sup> DW (0.5 x, 1 x and 2 x the average spreading dose) for the liquid dairy manure. A fourth replicate was performed for exposure to the liquid dairy manure in order to anticipate the variability linked to this matrix texture in regards of acute results obtained previously (Roques et al., 2023). A negative control was performed for each of the tests in an artificial ISO soil defined by standard ISO (ISO, 2015). The water content was checked each week by weighing and corrected if necessary with demineralized water.

To avoid masking the possible ecotoxic effects of the organic amendments tested by the presence of a higher content of organic matter, a reverse organic carbon gradient was applied between the different conditions tested by adding organic horse dung. The latter was used as basic food in the earthworm breeding facility. According to the results detailed in [supplementary material 2](#), this amendment is free of any organic contaminant and contains low MTE contents. A quantity of about 20 g of dry organic horse dung, i.e. 8.36 g of carbon, was reached for the control assays. This quantity was sufficient to ensure the effective growth of the earthworms for a month according to the recommendations of the ISO reproduction test. No nutritive addition was made during the rest of the test.

After 14 days of exposure, the adult earthworms were counted to check their mortality, then reintroduced in the assay medium mixed manually to ventilate it. After 28 days of exposure, the adult earthworms were extracted, counted, and weighed after 24 h fasting to assess mortality (Eq. 1) and the gain and loss of the earthworm biomass (Eq. 2). The earthworms were considered alive if able to respond to mechanical stimulus. The earthworms were then frozen at  $-20$  °C to analyze their energy reserves.

$$z = \left(\frac{l}{T}\right) \times 100 \quad (1)$$

Where  $z$  = percentage of dead earthworms,  $l$  = number of dead earthworms and  $T$  = initial number of earthworms.

$$g = \left(\frac{mf - mi}{mi}\right) \times 100 \quad (2)$$

Where  $mf$  = mass of live earthworms at the end of the experiment and  $mi$  = mass of earthworms alive at the start of the experiment.

The bioassay continued for 56 days, the juveniles and unhatched cocoons were withdrawn from the soil using a water filtration system on which a sieve was superposed (1 mm, 630 µm and 200 µm) and then counted. The validity of the test was checked against the control assays by a mortality lower than 10%, a loss of fresh biomass not exceeding 20% and the production of juveniles higher than 30 with a variability lower than 30%. The pH and electric conductivity were measured at 0, 28 and 56 days of the reproduction assay by following ISO standard

protocols (ISO, 2005, 1995). These two parameters allowed monitoring the degradation of organic matter through the release of protons and ions in solution.

## 2.5. Analyses of energy reserves of exposed earthworms

The analysis of the energy reserves allowed detecting the response of the earthworms following the presence of contaminants or a mixture of contaminants in the medium. The expression of stress in the organism can be identified by a decrease or increase of these energy reserves (Beaumelle et al., 2014). All the biochemical measurements were performed on earthworms individually and selected at random in each of the treatments. For each treatment, the biochemical analyses of the energy reserves were performed on 7 earthworms according to the protocol described by Pelosi et al. (2016) and details were given in the methods below (e.g. 'see sections 2.51–2.53'). The frozen earthworms were homogenized individually using a homogenizer (Ultra-Turrax IKAT10, 15 000 rpm) in 1 mL of iced phosphate buffer at 100 mM (pH 7.2 and 1 mM EDTA) for 100 mg of earthworm.

### 2.5.1. Proteins

The protein content was determined by using the BCA (BiCinchoninic acid Assay) method described by Smith et al. (1985). The earthworm homogenate was introduced in a microplate where 200 µL of bicinchoninic acid reagent was added (22 mL of bicinchoninic acid and 0.44 mL of copper sulfate at 4%). The microplate was then incubated for 30 min at 37 °C. The chelation between the copper ions (Cu<sup>2+</sup>) and the bicinchoninic acid formed a complex measurable at 562 nm. The protein content was calculated by using a calibration curve established with bovine albumin serum (BAS).

### 2.5.2. Lipids

A sample of 500 µL of earthworm homogenate was extracted using an organic solvent composed of 1.25 mL of methanol and 0.65 mL of chloroform, by vortex shaking (3 min) then by centrifugation for 5 min at 1800 g at 10 °C. The lipids were then separated from the previous extract by adding 2.37 mL of chloroform and 2.73 mL of water by shaking (vortex 1 min). Following another centrifugation at 1800 g for 5 min at 10 °C, the extract in the chloroform was introduced into Pyrex tubes and the chloroform evaporated under a nitrogen flux. The hydrolysis of the lipids contained in the Pyrex tubes was then performed by incubating the sample with 5 mL of sulfuric acid (18 mM) in a water bath at 100 °C for 10 min before cooling in ice for 5 min. Lastly, 200 µL of the mixture was sampled and mixed with 3 mL of phosphoric vanillin acid reagent prepared according to Knight et al. (1972). After incubation for 15 min in a water bath at 37 °C to form a lipid-phospho-vanillin complex, the absorbance at 525 nm was measured and the lipid content of the samples was calculated using a calibration curve established with olive oil.

### 2.5.3. Glycogen

The glycogen was quantified in the earthworm homogenates using the Glucose Assay Reagent HK kit (Sigma). A volume of 250 µL homogenate of tissue was incubated in 1 M of NaOH at 80 °C for 3 h, then 100 µL of the extraction mixture was incubated again for 2 h at 37 °C with 50 µL of α-amyl glucosidase (at 20 mg mL<sup>-1</sup>) in 850 µL of acetate buffer (0.25 M, pH 4.4). After incubation, the mixture was centrifuged for 10 min at 10 000 g at 40 °C. The glucose formed by the enzymatic hydrolysis was quantified using the reagent from the kit, leading to the formation of NADH measurable by absorbance at 340 nm. The glycogen contents were calculated using a calibration curve determined with glucose (1 mg mL<sup>-1</sup>, 1 M NaOH).

## 2.6. Statistical analyses

The aerial parts of the plants, plant biomass and earthworms, the

number of juveniles and the quantity of energy substrates of the earthworms are expressed by means and standard deviations. The normality and homoscedasticity of these data were checked by Shapiro-Wilk and Bartlett tests respectively. For the parameters checking the normality of the data, an analysis of parametric variances ANOVA) was performed. The significant differences between the different treatments were extracted by a post-hoc Student's test. When the conditions were not verified, a non-parametric analysis of variances was performed with the Kruskal-Wallis test (and the Scheirer-Ray-Hare test for a 2-way ANOVA) followed by Dunn's multi-comparison test. The statistical tests were analyzed with the RStudio© software with a significance threshold equal to  $p < 0.05$ .

### 3. Results and discussion

#### 3.1. Physicochemical characterization of organic amendments

The organic carbon contents of the three organic amendments ranged between 371 and 412 g.kg<sup>-1</sup> DW (supplementary material 2). The fertilizing values N, P, K for the two agricultural effluents were in the same range with total nitrogen values of  $27.48 \pm 1.02$  g.kg<sup>-1</sup> DW and  $30.09 \pm 4.95$  g.kg<sup>-1</sup> DW, phosphorous of  $4.66 \pm 0.17$  g.kg<sup>-1</sup> DW and  $6.48 \pm 0.17$  g.kg<sup>-1</sup> DW and potassium of  $33.84 \pm 3.64$  g.kg<sup>-1</sup> DW and  $46.21 \pm 3.74$  g.kg<sup>-1</sup> DW for the manure and liquid manure respectively. However, the sewage sludge stood out due to its fertilizing properties with clearly higher total nitrogen and phosphorous values (respectively  $74.39 \pm 1.14$  g.kg<sup>-1</sup> DW and  $25.73 \pm 2.02$  g.kg<sup>-1</sup> DW) but a lower value for potassium ( $7.24 \pm 0.14$  g.kg<sup>-1</sup> DW). A high value for ammonia nitrogen or organic nitrogen characterizes a considerable fertilizing value for organic amendments (Houot et al., 2014). Indeed, sewage sludge are known to present a high and readily mineralized content in organic nitrogen (Cucina et al., 2021). On the contrary, manures are known to have a lower fertilizing value. What is more, the organic C/N ratio is an interesting indicator for predicting the short-term availability of nitrogen (Gutser et al., 2005). In our study, the organic C/N ratio of the sewage sludge (5.40) was much lower than that of the agricultural wastes (13.53 for the manure and 13.90 for the liquid dairy manure). However, a lower ratio indicates a strong release of nitrogen in soil and thus better soil fertilization (Gutser et al., 2005).

Chemical analyses showed a considerable diversity of pollutants in the organic amendments (supplementary material 2). The MTEs were sought and quantified in the organic amendments using classical methods. In study of Goss et al. (2013), copper and zinc were the MTEs whose concentrations were the highest in the organic amendments. In our study, the sewage sludge presented a value of  $483.43 \pm 9.48$  mg.kg<sup>-1</sup> DW in zinc and  $208.41 \pm 1.78$  mg.kg<sup>-1</sup> DW in copper, i. e. relatively low concentrations for the agricultural effluents. Furthermore, few organic pollutants were found in the two agricultural amendments, contrary to the sewage sludge selected in our study. Indeed, the analyses of the sewage sludge showed the presence of fluoroquinolone antibiotics at high concentrations such as ofloxacin ( $957.7 \pm 239.4$  µg.kg<sup>-1</sup> DW) and ciprofloxacin ( $129.0 \pm 32.0$  µg.kg<sup>-1</sup> DW). Biocides were also present in high concentrations such as dimethyl-didecyl ammonium ( $36.9 \pm 9.2$  mg.kg<sup>-1</sup> DW), chlorhexidine ( $2066.7 \pm 517.0$  µg.kg<sup>-1</sup> DW), triclosan ( $21.4 \pm 5.3$  µg.kg<sup>-1</sup> DW) and a metabolite of isoxazole ( $55.9 \pm 14$  µg.kg<sup>-1</sup> DW). Other organic pollutants such as certain polycyclic aromatic hydrocarbons, polychlorobiphenyls and brominated flame retarders were also present with contents at considerable levels in the sewage sludge with phenanthrene ( $74.09 \pm 3.90$  µg.kg<sup>-1</sup> DW), pyrene ( $44 \pm 0.84$  µg.kg<sup>-1</sup> DW), some brominated flame retarders ( $28.87$  µg.kg<sup>-1</sup> DW for  $\Sigma$  3 HBCDDs) and non-dioxin like polychlorobiphenyls ( $19,72$  µg.kg<sup>-1</sup> DW) (see supplementary material 2). Nonetheless these values are consistent with those found in the literature for this type of amendment of urban origin.

With respect to the literature, some of these pollutants detected in the organic amendments present chronic risks for organisms.

Fluoroquinolone antibiotics were present in large doses ( $> 0.05$  g.kg<sup>-1</sup> DW) in an artificial ISO soil leading to the inhibition of earthworm reproduction (Li et al., 2015). In addition, triclosan is known to have effects at low doses in an artificial ISO soil ( $35.0 \pm 2.5$  µg.kg<sup>-1</sup> DW) on earthworms (Chevillat et al., 2018). The presence of these pollutants in mixture in these organic amendments confirms the importance of assessing chronic ecotoxicity on terrestrial organisms. Some of them are known endocrine disruptors, such as HAPs and triclosan.

#### 3.2. Characterization of organic matter

The characterization of organic matter in the different organic amendments showed different fractionation profiles (Fig. 1). Thus, we could compare the distribution of available compartments to non-extractable fractions for the three amendments. The two agricultural organic amendments presented non-extractable fractions of 18.2–26.1% respectively for cow manure and slurry. Moreover, a large proportion of COD (around 40%) was found in the lignocellulosic fraction (PEOM) relating to the presence of straws in these two matrices. The sewage sludge presented the largest non-extractable fraction with 35.7% and a considerable proportion of COD was also found in the PEOM fraction linked to the presence of fibers. The study by Jimenez et al. (2014) also observed that the largest fractions composing the organic matter of sewage sludge are the non-extractable fraction (NEOM) and the lignocellulosic fraction (PEOM).

The cow manure presented a sum of SPOM+REOM+SEOM, corresponding to the three most available fractions of 43.2% of the total COD of the manure. Thus, the manure was mostly composed of simple and complex proteins, lipids and humic and fulvic acids, related to the presence of transformed fecal matter. Regarding the sludge and the liquid manure, the sum of the 3 most available fractions was 29.7% and 30.8%, respectively. Although the size of these three fractions was similar for these two matrices, the fluorescence signature was very different, with complexity indexes always higher for the liquid manure. Regarding the latter, this emphasizes the presence in these available fractions of complex proteins and humic and fulvic acids. On the contrary, the sewage sludge presented available fractions at lower complexity indexes, thus containing more sugars and simple proteins. It was also the amendment with the highest SPOM fraction (10.6%), as this fraction was the most available (extractable in water = SPOM).

We therefore had three amendments that presented different fractionation profiles and which is consistent with what has been observed before in similar samples (Jimenez et al., 2015, 2017). Thus, there was a

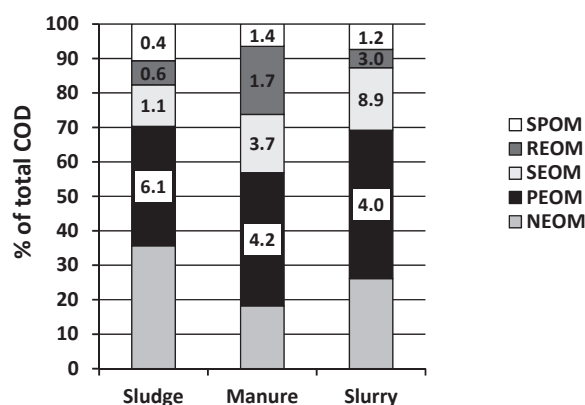


Fig. 1. Fractionation profile of samples of the different matrices studied (in % of total COD). The numbers in each of the different fractions represent the ratio of complexity calculated from the fluorescence spectra (supplementary material 3). Soluble extractable organic matter (SPOM). Readily extractable organic matter (REOM). Slowly extractable particulate organic matter (SEOM). Poorly extractable organic matter (PEOM). Non-extractable organic matter (NEOM). Extractions were done only one time for each matrices.

sewage sludge with a small proportion of available but quite simple fractions, undoubtedly suggesting faster biodegradability for these available fractions. Conversely, the cow manure presented a rather large proportion of available but more complex fractions, suggesting much slower biodegradability. The liquid dairy manure could be situated between the two other amendments. Indeed, these three matrixes presented a very large proportion of poorly or non-extractable organic matter, placing them among relatively stable amendments. According to the predictive regression model developed by Jimenez et al. (2017), the mineralizable carbon reached a higher value of 41.3% in sewage sludge and 25.8% and 27.8% respectively for the cow manure and the liquid dairy manure. The organic matter composing the sewage sludge was therefore more available and more biodegradable than that of the agricultural organic amendments with similar biodegradability according to the predictive model.

### 3.3. Bioassays

#### 3.3.1. Plant seed germination and growth

The germination parameters and the size of the aerial parts of the plants were monitored during the exposure of two types of plant (supplementary material 4). The rate of mustard and alfalfa seed germination in control was higher than  $73.78 \pm 7.34\%$  (supplementary material 4). According to the species exposed and the organic amendment studied, significant positive effects on germination were observed. Indeed, the rates of mustard seed germination were higher in a soil fertilized with sewage sludge and cow manure. Germination rates reached  $93.33 \pm 5.81\%$  and  $92.44 \pm 2.04\%$  at application doses of  $20 \text{ g.kg}^{-1}$  DW for the sewage sludge and cow manure respectively versus  $77.33 \pm 14.85\%$  for controls. A slight non-significant stimulation of mustard seed germination had already been observed for these three organic amendments during exposure for 5 days (Roques et al., 2023). In the same study, a hormesis effect was also observed on the root growth of mustard seeds exposed to application doses of  $10\text{--}50 \text{ g.kg}^{-1}$  DW of sewage sludge. For the alfalfa seeds, the fertilization of soil with liquid dairy manure and sewage sludge significantly improved their rate of germination. It reached  $95.56 \pm 7.58\%$  for liquid dairy manure of  $10 \text{ g.kg}^{-1}$  DW and  $84.00 \pm 9.24\%$  for a dose of liquid dairy manure of  $20 \text{ g.kg}^{-1}$  DW whereas controls presented a germination rate of  $73.78 \pm 7.34\%$ . Only the application dose of  $10 \text{ g.kg}^{-1}$  DW of sewage sludge led to a significantly higher germination rate, reaching  $82.67 \pm 4.00\%$ . Regarding the size of the aerial parts, the size of the aerial parts of the control alfalfa plants was  $87.98 \pm 40.74 \text{ mm}$  after 28 days exposure and the mustard  $53.90 \pm 22.95 \text{ mm}$  after 21 days exposure respectively. Only the alfalfa seeds exposed to a dose of  $10 \text{ g.kg}^{-1}$  DW of sewage sludge and mustard

seeds exposed to a dose of  $10$  and  $20 \text{ g.kg}^{-1}$  DW of cow manure showed respectively a significant decrease than an increase in the size of the aerial parts (supplementary material 4). The size of the aerial parts reached  $102.09 \pm 44.66 \text{ mm}$  and  $60.17 \pm 25.56 \text{ mm}$  respectively.

Biomass gain is the parameter that most efficiently expresses crop yields. During our works, monitoring the fresh biomass of aerial parts showed a significant increase for the alfalfa exposed to the three amendments (supplementary material 4). This considerable increase in fresh biomass had already been observed during the exposure of three types of plants to sewage: cress, sorghum, and mustard (Kominko et al., 2022). However, the analysis of fresh biomass can be influenced by greater water retention and/or the stage of plant development. During our study, the dry biomass of the aerial and root parts of the plants was monitored and represented a more robust and pertinent indicator or real gain in plant biomass (Fig. 2). The soils amended with sewage sludge presented a dry biomass of the aerial parts of alfalfa and mustard significantly higher than controls. Indeed, the dry biomass of the aerial parts of alfalfa reached  $17.39 \pm 7.27 \text{ mg}$  and  $25.05 \pm 1.80 \text{ mg}$  for the soils fertilized with sewage sludge at  $10$  and  $20 \text{ g.kg}^{-1}$  DW versus  $7.62 \pm 1.32 \text{ mg}$  for controls. With the strongest application dose of sewage sludge, the dry biomass of the mustard stems reached  $14.16 \pm 2.74 \text{ mg}$  whereas controls presented a dry stem biomass of  $6.72 \pm 0.43 \text{ mg}$ . The aerial part of alfalfa also presented a slightly greater dry biomass in soils fertilized at  $20 \text{ g.kg}^{-1}$  DW of liquid dairy manure ( $12.48 \pm 1.47 \text{ mg}$ ). For the root parts, the dry biomass of alfalfa showed the highest increase for sewage sludge. The dry biomass of the alfalfa root parts reached  $10.91 \pm 0.24 \text{ mg}$  versus  $2.17 \pm 0.24 \text{ mg}$  for controls. Regarding the dry biomass of mustard roots, it was not measured due to experimental problems when extracting the roots from the soil.

Globally, the organic amendments at the doses of application tested in the soils showed positive effects on the two species of plants studied. Due to its higher fertilizing value (total nitrogen at least twice more and phosphorous around four time more) than the other organic amendments studied, exposure to sewage sludge significantly increased the biomass of the aerial and root parts of the plants studied. This increase of biomass of the plants exposed to low concentrations of sewage sludge is often observed in the literature (Žaltauskaitė et al., 2016). The works of Orman et al. (2014) also showed an increase in the fresh and dry biomass of the stems and roots of alfalfa at low concentrations in soils fertilized with sewage sludge. The increase of the plant biomass of alfalfa was also highlighted in the study by Antolín et al. (2010) after adding sewage sludge to a soil in comparison to a non-fertilized soil. These positive effects are consistent with the aim of improving fertilization by spreading organic amendments.

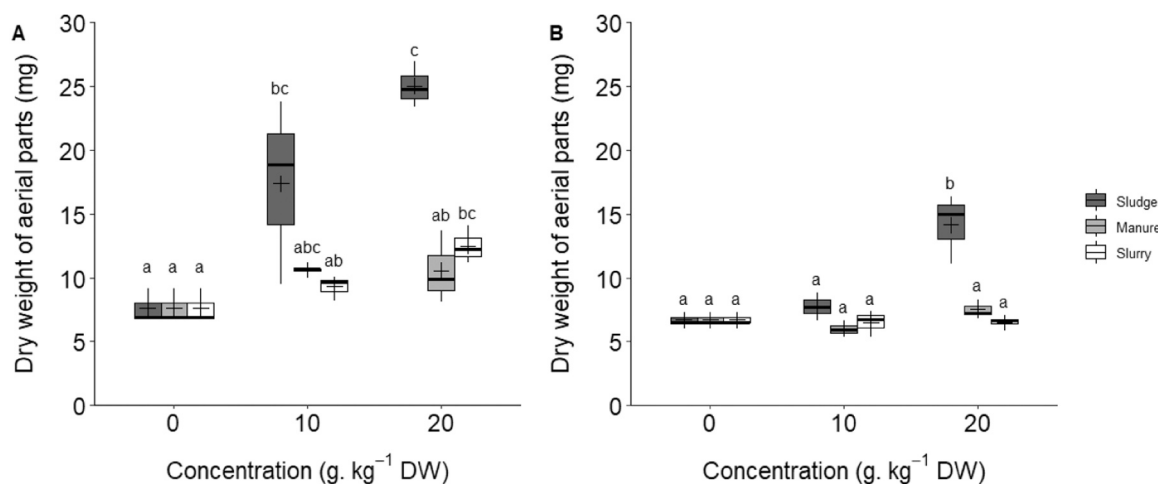


Fig. 2. Dry biomass of aerial parts of alfalfa (*Medicago sativa*) (A) and mustard (*Sinapis alba*) (B) exposed to different concentrations of organic amendments. Mean values ( $n = 3$ ). The same letters indicate the same statistical group (at 95%).

### 3.3.2. Earthworm reproduction

**3.3.2.1. Evolution of fertilized soil – earthworms.** During the earthworm reproduction bioassay, the pH and electric conductivity of the soil were measured at the beginning and at the end of the exposure of adult earthworms to different concentrations of agricultural amendments (Table 1). At the beginning of the experiment, the application of agricultural amendments of origin in the reference soil increased the pH, which changed from  $6.41 \pm 0.06$  without amendment to  $7.75 \pm 0.02$  for a manure amendment of  $50 \text{ g.kg}^{-1}$  DW and to  $7.63 \pm 0.02$  for liquid dairy manure at  $20 \text{ g.kg}^{-1}$  DW. Since the sewage sludge had the same pH as the reference soil, fertilizing the soil with sludge did not modify the pH which was initially 6.7. However, after 28 days exposure of the earthworms to different agricultural amendments, the pH of the soil fertilized with sludge fell considerably, reaching  $5.08 \pm 0.05$  for an application of sewage sludge of  $50 \text{ g.kg}^{-1}$  DW. On the contrary, the pH remained stable for the soils fertilized with agricultural amendments after the exposure of the earthworms. Most of the earthworms tended to acidify their medium through their activity (Springett, 1983). This acidification of soils fertilized by sludge may be attributed to the activity of earthworms in the mineralization of nitrogen and phosphorous, which were found in high concentrations in sewage sludge, in nitrate and orthophosphate forms, or with the bioconversion of organic matter into intermediate species like organic acids (Ndegwa et al., 2000).

The addition of agricultural matter into soils led to an increase in electric conductivity at the beginning of the experiment (Table 1). Indeed, electric conductivity increased by 336.7% in the soil following the application of  $50 \text{ g.kg}^{-1}$  DW of manure. The application of sewage sludge and liquid dairy manure showed a similar increase of electric conductivity of 100.1% and 134.2% respectively at the strongest concentrations tested in the soils. The addition of charged compounds (anions/cations) through the application of organic amendments in the soils was responsible for modifying these physicochemical characteristics. However, after 28 days' exposure, the electric conductivity

**Table 1**  
Measure of pH and electric conductivity (EC) during exposure to different agricultural amendments during the earthworm reproduction test.

Agricultural amendments	Agricultural amendment concentration in the reference soil ( $\text{g kg}^{-1}$ DW)	pH		EC ( $\mu\text{S cm}^{-2}$ )	
		0 day	28 days	0 day	28 days
Sewage sludge	0	6.56 $\pm 0.05$	7.03 $\pm 0.06$	168.87 $\pm 8.99$	81.43 $\pm 4.55$
	10	6.75 $\pm 0.03$	6.68 $\pm 0.09$	189.67 $\pm 17.25$	230.17 $\pm 16.80$
	20	6.74 $\pm 0.01$	5.64 $\pm 0.10$	224.87 $\pm 18.60$	931.00 $\pm 28.84$
	50	6.67 $\pm 0.02$	5.08 $\pm 0.05$	326.50 $\pm 18.90$	1468.80 $\pm 66.44$
	Cow manure	0	6.41 $\pm 0.06$	6.99 $\pm 0.01$	163.13 $\pm 10.95$
Cow slurry	10	7.09 $\pm 0.02$	7.33 $\pm 0.03$	260.47 $\pm 20.90$	127.60 $\pm 6.55$
	20	7.44 $\pm 0.19$	7.65 $\pm 0.03$	363.73 $\pm 24.32$	187.70 $\pm 3.87$
	50	7.75 $\pm 0.02$	7.80 $\pm 0.02$	712.43 $\pm 25.11$	593.80 $\pm 25.07$
	0	6.74 $\pm 0.05$	7.03 $\pm 0.01$	113.20 $\pm 11.63$	89.73 $\pm 6.55$
	5	7.20 $\pm 0.02$	7.20 $\pm 0.02$	159.63 $\pm 7.31$	137.63 $\pm 1.86$
10	7.36 $\pm 0.01$	7.41 $\pm 0.02$	239.47 $\pm 4.96$	184.33 $\pm 3.59$	
20	7.63 $\pm 0.02$	7.75 $\pm 0.02$	382.03 $\pm 3.42$	307.17 $\pm 5.59$	

Mean values ( $n = 3$ ) and  $\pm$  standard deviation.

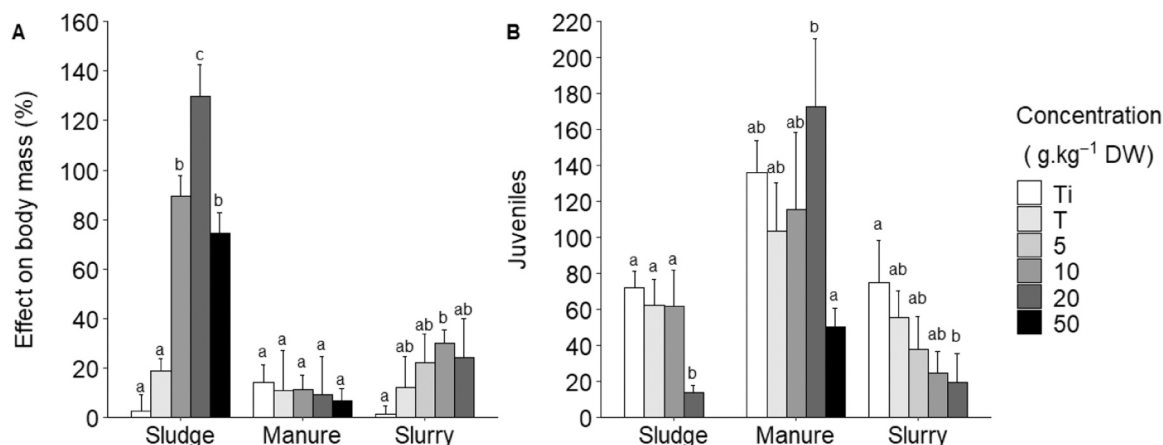
decreased by about 20% in the soils receiving an agricultural organic amendment at the strongest concentrations. The works of Garg et al. (2006) also showed in the context of vermicomposting a decrease of electric conductivity in soils fertilized with different agricultural organic amendments. Nonetheless, the sludge amendment stood out through a considerable increase of electric conductivity after 28 days' exposure of earthworms. The electric conductivity of fertilized soil increases up to  $931 \pm 28.84 \mu\text{S cm}^{-2}$  at  $20 \text{ g.kg}^{-1}$  DW and  $1468.80 \pm 66.44 \mu\text{S cm}^{-2}$  at  $50 \text{ g.kg}^{-1}$  DW sludge. The increase of electric conductivity of soil is probably due to the metabolization of the organic matter resulting from the sewage sludge, leading to the release of salts/ions in the fertilized soil.

After withdrawing the earthworms from the soil after 28 days, the electric conductivity and pH of the different treatments stopped changing until the end of the experiment (after 56 days). The measures of pH and electric conductivity in the soil therefore showed the degradation of the organic matter of the agricultural amendments by the microbial biomass of the soil. In our case, the mineralization of C, N and P was greater in the soils fertilized by sewage sludge than by the organic amendments. However, the modification of the pH and electric conductivity generated by the application of agricultural amendment and the degradation of its organic matter has already been mentioned as one of the factors limiting the survival and growth of earthworms (Pivato et al., 2014; Gunadi and Edwards, 2003). The laboratory bioassays revealed considerable biases with reality in the field since soil leaching increases the physicochemical stability of the medium.

**3.3.2.2. Measures of effects: effect on biomass and reproduction.** At the end of the 28 days' exposure, the mortality rate of the earthworms in each of the treatments was low ( $\leq 10\%$ ). Only the exposure of the earthworms to the highest concentration of sewage sludge presented a high mortality rate ( $36.67 \pm 28.87\%$ ). The variation of earthworm biomass and reproduction were monitored during the reproduction test for 56 days (Fig. 3). A gain of biomass from 10% to 20% was observed for the reference soil controls (T) and from 0% to 20% for the ISO artificial soil controls (Ti) (Fig. 3A). The slight differences of biomass between the controls resulting from the two types of soils stemmed from their distinct compositions. Indeed, the artificial ISO soil is a medium less rich in essential nutrients and organic matter in comparison with the reference soil. The growth of earthworms greatly depends on the biochemical quality of the organic amendments, their bioavailability and the ease with they are metabolized into nutrients (Vodounnou et al., 2016; Edwards, 1988). The biomass of the earthworms after 28 days exposure the sewage sludge increased considerably with the increase in concentration of the sludge in the soil. This gain of biomass reached  $89.41 \pm 8.45\%$  to  $10 \text{ g.kg}^{-1}$  DW of sludge,  $129.85 \pm 12.75\%$  at  $20 \text{ g.kg}^{-1}$  DW of sludge and  $74.18 \pm 8.69\%$  at  $50 \text{ g.kg}^{-1}$  DW of sludge. As sludge is rich in easily decomposable organic compounds (sugars and proteins), spreading it facilitates the growth of earthworms through this nutritional contribution. Effectively, the fractionation of the sludge sample with the three most available fractions (SPOM+REOM+SEOM) representing 29.7% of total COD may explained this biomass gain. Moreover, these three fractions showed the lowest ratio of complexity (respectively 0.4, 0.6, 1.1%; see Fig. 1) of the three amendments which reflected their highest degradability. Furthermore, the stimulation of the microflora of the soil fertilized by sewage sludge indirectly contributed to a larger share on the food of the earthworms (Pramanik and Chung, 2011; Flack and Hartenstein, 1984). In the case of the bioassays with the spreading of cow manure and liquid dairy manure, the gains of earthworm biomass were the same and did not vary significantly in comparison to the controls.

Reproduction was assessed by counting the number of juveniles after 56 days' exposure (Fig. 3B). Statistical analysis did not show significant interaction between the type of amendment and the application dose on the number of juveniles (Scheirer-Ray-Hare test:  $p\text{-value} = 0.47$ ). The





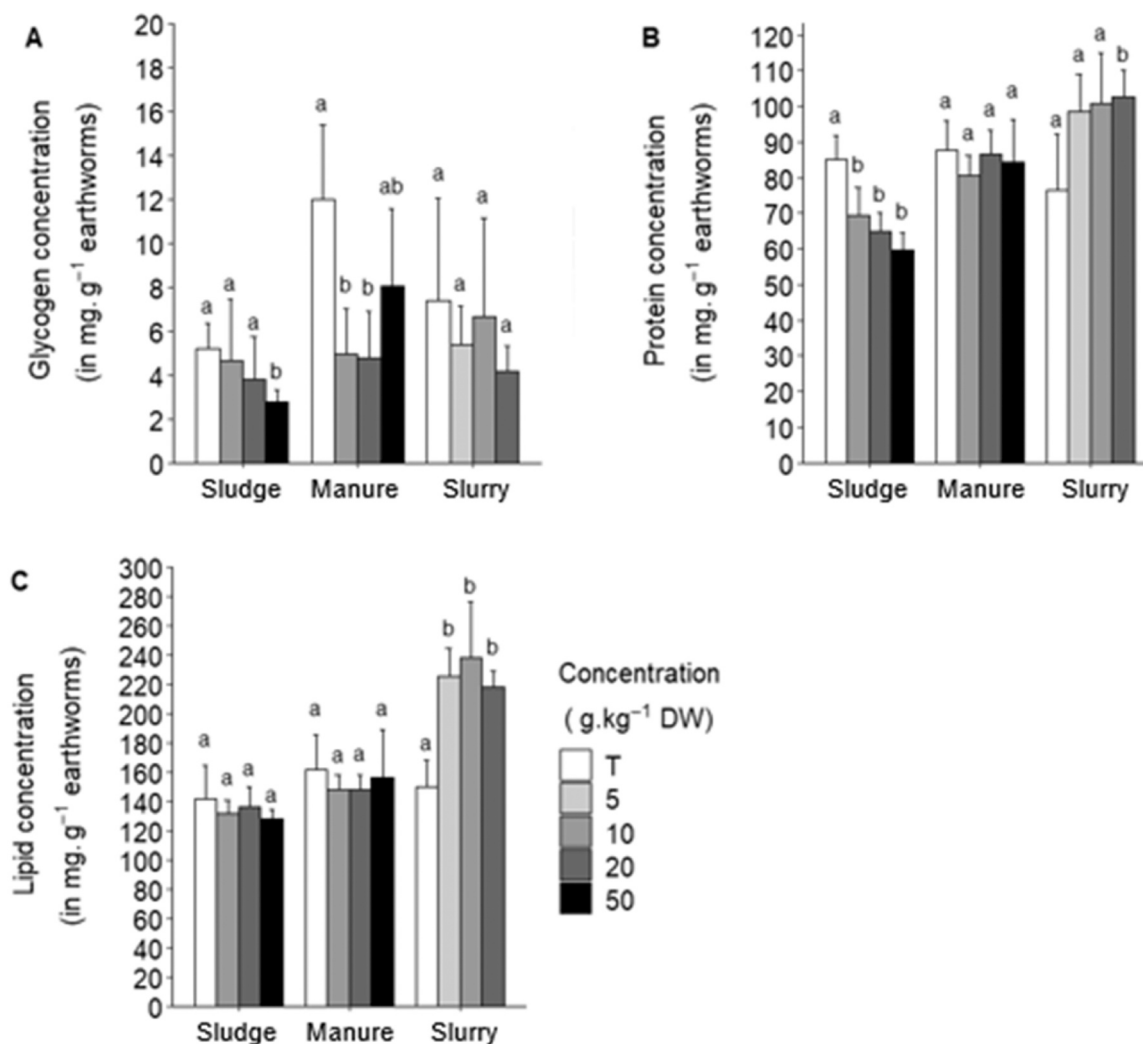
**Fig. 3.** Effect on the biomass (A) and reproduction (measured by number of juveniles) (B) of earthworms *Eisenia fetida* exposed to a reference soil fertilized by three types of different organic amendment at different concentrations for 28 days. Ti = artificial non-fertilized ISO soil, T = non-fertilized reference soil. Mean values ( $n = 3$  or 4) and standard deviation. The same letters indicate the same statistical group (at 95%) performed independently between each organic amendment.

reproduction data were therefore analyzed independently between the different organic amendments. The exposure of the earthworms to the sewage sludge showed a significant decrease in the number of juveniles above a sludge content of  $20 \text{ g.kg}^{-1} \text{ DW}$ . Indeed, the number of juveniles was  $61.33 \pm 20.55$  for the controls versus a number of juveniles produced of  $13.67 \pm 4.04$  for the exposure tests at  $20 \text{ g.kg}^{-1} \text{ DW}$ . In  $50 \text{ g.kg}^{-1} \text{ DW}$  concentration of sludge in the soil, the absence of juveniles was attributed to non-negligible earthworm mortality observed. The inhibition of earthworm reproduction in a soil in the presence of sewage sludge has already been observed (Žaltauskaitė et al., 2016). Despite the gain in nutritive elements associated with the addition of sewage sludge, the earthworms produced fewer juveniles. The works of Pivato et al. (2014) highlighted a clear fall in reproduction despite the addition of biomass by spreading composted sludge, considered more stable and less decomposable than a sludge that had simply been dried. The hypothesis put forward by these authors is that the high bioavailability of organic matter and the presence of pollutants are responsible for this fall in fertility. Modifications of physicochemical conditions such the increase of electric conductivity and the reduction of pH could also be one of the causes of the mortality and lower reproduction of earthworms (Pivato et al., 2014; Owojori et al., 2009; Gunadi and Edwards, 2003). Indeed, the electric conductivity values measured at the end of the exposure of the earthworms in our study have already shown an effect on earthworm reproduction (Owojori et al., 2009). The decrease of pH observed in the soils fertilized with sludge do not appear to be sufficient to be a factor responsible for decreasing reproduction (Wu et al., 2020). Nonetheless, a decrease of pH can lead to an increase in the availability of metallic pollutants for earthworms (Leal et al., 2013). The decrease of the number of juveniles could also be due to delayed hatching. The hatching of cocoons is also a reproduction parameter liable to be disturbed by the presence of pollutants (Yasmin and D'Souza, 2010). In our study, the number of unhatched cocoons reached  $8.67 \pm 2.08$  for a sludge content of  $20 \text{ g.kg}^{-1} \text{ DW}$  and  $14.0 \pm 3.61$  for a content of  $50 \text{ g.kg}^{-1} \text{ DW}$  that must be linked to the poor number of cocoons in control of with  $1.0 \pm 1.0$  (data not presented). The largest number of juveniles was produced by the earthworms exposed to a soil fertilized with a manure concentration of  $20 \text{ g.kg}^{-1} \text{ DW}$ . The number of juveniles reached  $172.67 \pm 38.08$  but the ANOVA test did not allow confirming a significant difference in comparison to the control assays. Regarding the liquid dairy manure, a downward trend for the number of juveniles was observed. The production of juveniles reached a mean of  $19.00 \pm 16.51$  in the soil fertilized at  $20 \text{ g.kg}^{-1} \text{ DW}$  with liquid dairy manure, i.e. an inhibition of 77.81%, if we compare this result with the control artificial ISO soil ( $74.50 \pm 23.63$  juveniles produced) which is not treated and which can be qualified as preferential controlled conditions.

The estrogenic and androgenic activities of the organic amendments may also have been responsible for the increase in the reproduction of the exposed earthworms as demonstrated by Kwak and An (2021).

**3.3.2.3. Energy reserves (proteins, lipids, glycogen).** The energy reserves (proteins, lipids and glycogen) were quantified in the adult earthworms after 28 days' exposure to different concentrations of organic amendments (Fig. 4). The energy reserves of the earthworms exposed to the cow manure were not disturbed for protein and lipid, but not for glycogen. This may be due to high level of control observed, which was unexpected compared with the others controls. A reduction of the glycogen concentration was observed only for the highest content of sludge of  $50 \text{ g.kg}^{-1} \text{ DW}$  ( $2.75 \pm 0.58 \text{ mg.g}^{-1}$ ) in comparison to earthworms exposed to a non-fertilized soil ( $5.19 \pm 1.17 \text{ mg.g}^{-1}$ ) (Fig. 4A). This high concentration of sludge also led the earthworms to present a total inhibition of reproduction. Sugars are the primary source of energy utilized by organisms subject to toxic stress (Moolman et al., 2007). The reduction of glycogen and its rapid utilization have already been observed in earthworms exposed to a pesticide, epoxiconazole, and to MTEs like lead, copper and nanoparticles of zinc (Świątek and Bednarska, 2019; Beaumelle et al., 2017; Pelosi et al., 2016; Bundy et al., 2008). In the study by Świątek and Bednarska (2019), a reduction of the quantity of glycogen was measured only in the treatment of zinc nanoparticles where the biomass of earthworms decreased. The contribution of nutrient elements by sewage sludge in the other exposure conditions seemed sufficient to offset the loss of glycogen linked to stress.

Whatever the content of the sludge in the soil fertilized, the protein reserves decreased ( $85.27 \pm 6.49 \text{ mg.g}^{-1}$ ) (Fig. 4B). The protein reserves reached the lowest level of  $59.76 \pm 4.74 \text{ mg.g}^{-1}$  at the highest content of sludge of  $50 \text{ g.kg}^{-1} \text{ DW}$ . According to the literature, moderate stress conditions generate an increase of protein reserves linked to the expression of stress response proteins such as the production of glutathione to combat the accumulation of pollutants in organisms (Sokolova et al., 2012). Many studies have shown an increase of protein content in earthworms exposed to pollutants in the environment (Świątek and Bednarska, 2019; Beaumelle et al., 2017; Schreck et al., 2008). Our study seems to contradict the results classically observed in the literature. A metabolic dysfunction caused by the presence of sewage sludge in the medium may have been the cause a reduction in the protein storage capacities in the earthworms. The reduction in protein content could also have been caused by a lack of energy linked to glycogen and to the availability of mobilizable proteins to ensure the basic metabolism of the earthworms. On the contrary, the earthworms exposed to slurry showed an increase in their protein reserves at the highest concentration of slurry tested. The concentration in proteins in the earthworms



**Fig. 4.** Concentration (A) in glycogen, (B) in proteins and (C) in lipids in earthworms *Eisenia fetida* exposed to a reference soil fertilized with three types of organic amendment at different concentrations for 28 days. Mean values ( $n = 6$ ) and standard deviations. The same letters indicate the same statistical group (at 95%) performed independently between each organic amendment.

exposed to the liquid dairy manure reached a concentration of  $102.66 \pm 7.44 \text{ mg g}^{-1}$  at  $20 \text{ g.kg}^{-1}$  DW of liquid dairy manure.

The lipid reserves of the earthworms exposed to the sewage sludge and the liquid dairy manure did not decrease significantly (Fig. 4C). The utilization of lipids was not therefore necessary or was not triggered despite the fall in reproduction of the earthworms exposed to the sludge and the liquid dairy manure. Surprisingly, the lipidic concentration increased considerably for all the spreading doses tested for the liquid dairy manure. The lipid concentration in the earthworms reached values between  $218.83 \pm 10.44$  and  $238.27 \pm 38.20 \text{ mg g}^{-1}$  in the reference soil fertilized with liquid dairy manure whereas the controls presented a lipid content of  $150.49 \pm 18.27 \text{ mg g}^{-1}$ . It appears that the lipid reserves were conserved at the expense of reproduction during stress linked to the application of maximal liquid dairy manure amendment. Lipids are also an important source of energy that can be used during stress before or at the same time as glycogen. A downwards trend was nonetheless observed for the earthworms exposed to the sewage sludge.

Regarding the results, the presence of sewage sludge in the medium generated a general trend towards the reduction of the energy reserves of earthworms excepted for lipid levels. However, the sewage sludge presented readily biodegradable compounds in comparison to the other organic amendments as showed by the lowest ratio of complexity of SPOM, REOM and SEOM observed (see Fig. 1). Furthermore, the

biomass of the earthworms exposed to sewage sludge increased. Such important differences in energy reserves, notably glycogen and proteins (compared to control) and other contradictory results lead us to believe that a "disturbance" diverts these reserves from the earthworm's reproductive or growth functions. It could therefore be assumed that this reduction of storage capacities was caused by a metabolic dysfunction in regards of protein and glycogen results.

#### 4. Conclusions

Spreading organic amendments on agricultural soils modifies the physicochemical conditions of the soil. Sewage sludge presented the highest fertilizing value but with a considerable diversity of potentially ecotoxic pollutants for soil organisms. Sewage sludge also presented the most readily available and biodegradable fraction of the studied organic amendments. On the contrary to sludge, cow manure and liquid dairy manure presented less fertilizing values, and available and biodegradable organic matter fractions.

During the chronic ecotoxicity bioassays, the sewage sludge showed a positive agronomic effect and a considerable gain of biomass for the earthworms. These positive effects were a priori linked to the fertilizing properties of sewage sludge and its high biodegradability for the readily available fractions.

Contrary to cow manure, which did not present ecotoxic effects, exposing earthworms to sewage sludge had the considerable effect of reducing the number of juveniles. These results suggest a stress possibly linked on the one hand to physicochemical modifications of the exposure medium (reduction of pH and increase of conductivity) and, on the other hand, to the presence of contaminants leading to a decrease of energy reserves allocated to reproduction. Another hypothesis would be that the abundance of food would reduce the energy allocated to the reproduction of earthworms but rather to the metabolization of organic matter. This stress was also expressed by a reduction of the energy reserves, notably of protein and glycogen concentrations, in the earthworms exposed to sewage sludge. With cow manure at a dose of 20 g.kg<sup>-1</sup> DW not considerable gain in earthworm biomass was observed and can be link to the greater stability of its organic matter and led to a fall in reproduction of earthworms with higher reserves of proteins and lipids. In the case of liquid dairy manure, the ecotoxic effects did not appear to be attributable to the presence of contaminants, contrary to the sewage sludge.

Our study highlighted the ecotoxic effects of these organic amendments, but these results differed as a function of the organism exposed and the organic amendment. The dose tested is also an important criterion since no ecotoxic effect is observed when we are at agronomic doses in our study. The reproduction assay performed on earthworms appeared pertinent for assessing the ecotoxicity of these organic amendments. This risk should be considered in view to taking chronic ecotoxicity into account in spreading regulations. In addition, our results confirmed the interest of using several ecotoxicity bioassays on several biological models with the aim of better assessing the impacts of organic amendments on agricultural soils and on agricultural ecosystems. To improve the representativeness of the results, it would also be interesting to analyze the impacts of these organic amendments by using agricultural soils with a history of spreading agricultural amendments. It would also be interesting to follow the reproduction of juveniles in the longer term or to set up experimental systems closer to in situ conditions.

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## CRediT authorship contribution statement

**Olivier Roques:** Conceptualization, Preparation, Methodology, Data curation, Validation, Investigation, Writing – original draft. **Rémy Bayard:** Conceptualization, Validation, Writing – review & editing. **Julia Le Maux:** Conceptualization, Data curation, Methodology, Investigation. **Dominique Patureau:** Methodology, Writing – review & editing. **Sylvie Nélieu:** Validation, Writing – review & editing. **Sabelle Lamy:** Validation, Writing – review & editing. **Jean-Philippe Bedell:** Project administration, Supervision, Conceptualization, Validation, Project administration, Writing – review & editing.

## 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.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2023.115504](https://doi.org/10.1016/j.ecoenv.2023.115504).

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