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The effects of saline toxicity and food-based AD digestate on the earthworm *Allolobophora chlorotica*

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Abstract

Anaerobic digestion (AD) is used to produce biogas and can offer a solution in waste management. Digestate, the AD by-product, can be applied to soil to improve fertility. However, the response of soil biological communities is not fully understood. There are mixed reports on its impact on earthworm survival. This study aimed to investigate digestate effects on earthworm mortality, and to elucidate potential mechanisms underlying it, if observed, after digestate application to soil. Juvenile and adult *Allolobophora chlorotica* were used as model organisms and added to microcosms prepared in a glasshouse trial. Five replicated treatments were: liquid Digestate; Osmotic-Stress (i.e. same salt concentration as digestate); Labile-C (i.e. same Biological Oxygen demand as Digestate); Synthetic-Digestate a mixture of Osmotic-Stress and Labile-C (i.e. same salt concentration and BOD as digestate); and Water as the control. Treatments were applied at two different standardised rates equivalent to the digestate's N content (i.e. 150 kg N ha^{-1} eq. or 300 kg N ha^{-1} eq.). The two development stages of *A. chlorotica* had different responses to treatments. Adult

biomass was significantly greater in the Water control R150 treatment than in Digestate. Significantly lower juvenile biomass was observed in the Digestate R300 treatment than in the Labile-C and Water control treatments. The biomass of adults in the Labile-C R300 treatment was significantly greater than in the Digestate, Osmotic-Stress, Synthetic-Digestate and Water control treatments. Both life-stages exhibited a decline in biomass across all treatments, but the adults had higher mortality rates. The biomass of adults and juveniles declined, respectively, by 90 % and 62 % for Digestate applied at the lower rate, and by 96 % and 90 % at the higher rate. Whereas the abundance of adults and juveniles suffered 80 % and 24 % drop at the lower rate, and a 90 % and 84 % drop at the higher rate. This study demonstrates that digestate can have negative impact on earthworm morbidity and mortality when applied to soil at 60% water filed pore space, with most of the total weight loss per pot due to reduced earthworm abundance. A likely hypothesis could be the osmotic stress induced by salts present in the digestate. However, there are other factors that interact with this effect, including possibly anaerobic impacts caused by high water content soils, as well as other mechanisms that have not been fully elucidated through this experimental design. Nevertheless, this work provides the basis for further ecotoxicology studies on the impact of digestate applied to soil. Further, while this works has shown that digestate can negatively impact *A. chlorotica* survival, whether the same is true for other earthworm species, ecotypes and life-cycle stages warrants further investigation. Considering the important role that worms play in soil health, field scale studies are also required to monitor the impacts of repeated digestate application on earthworm communities.

Key words: Lumbricidae; ecological category; toxicology; soil health; soil amendments; bioindicators.

1. Introduction

Earthworms are ecosystem engineers with functional roles within the soil profile that affect ecological processes and properties. They modify soil properties through bioturbation processes, including their burrowing and feeding action (Huber *et al.*, 2008; Ritz *et al.*, 2009; Blouin *et al.*, 2013). As such, they have been widely used as bioindicators in soil monitoring networks and environmental assessments (Huber *et al.*, 2008).

Allolobophora chlorotica Savigny (Lumbricidae), is an intermediate earthworm (in the epi-endo-anecic ecological category), that develops as a pale or green morph (Satchell, 1967; Bottinelli *et al*. 2020). They are the most commonly found earthworm in England, especially in neutral to base-rich grasslands and arable soils, where they are usually found in the rhizosphere (Jones and Eggleton, 2014). Their tolerance to various degrees of soil moisture is morph dependent, and both morphs can be found in different soil types of pH ranging from 4.5-8.2 (Satchell, 1967; Sims and Gerard, 1999). They create horizontal burrows and excrete casts, usually within the soil rather than on the surface. Earthworm casts are rich in plant available nutrients (Lee, 1985; Vos *et al.*, 2014) and their feeding action can facilitate the formation of soil aggregates (Kavdir and Ilay, 2011).

Soil organisms known to be beneficial ecosystem engineers like earthworms are often used in toxicity studies. These studies generally rely on commercially available epigeic and anecic earthworms. However, these are not best suited to soil toxicity studies or for determining the impact of nutrient mobility and availability (Sizmur and Hodson, 2009). For example, epigeics can be directly exposed to soil

amendments but, like anecics, feed mostly on plant matter. Conversely, epi-endo-anecic, such as *A. chlorotica*, are exposed to and feed on accumulated residues within the soil profile and so may be better models for soil toxicity and nutrient mobility studies (Van-camp *et al.*, 2004; Sizmur *et al.*, 2017).

It has been estimated that 180 million tonnes of digestate, a by-product of anaerobic digestion (AD), are produced in the EU28 every year. A variety of feedstocks can be used to produce energy through AD processes, such as manures, crop residues, energy crops and food waste (Corden *et al.*, 2019). Digestate is mostly used as a soil amendment in agricultural systems. Its composition is variable, mainly dependent on the feedstock digested. Its application to land has potential environmental risks, such as ammonia emissions, heavy metal contamination and/or a high salt content, and its impact on soil biological communities is not well understood (Taylor *et al.* 2011; EA and WRAP, 2014; Moller, 2015; Corden *et al.*, 2019). The impact on earthworm survival following the application of food-based digestate to land has been shown to be site dependent WRAP (2015); abundance reduced in some sites, whereas in others no significant difference was observed. Another study found that the spreading of digestate could reduce the abundance of endogeics, and that *A. chlorotica* was missing from such treatment at a particular site (Koblenz *et al*. 2015). Sizmur *et al.* (2017) observed an increase in the biomass of anecic earthworms following the application of digestate incorporated with straw.

The main factors affecting digestate impacts on earthworm populations remain unknown but are likely to include osmotic stress due to salts present, increased anaerobicity due to digestates' biochemical oxygen demand (BOD), chemical oxygen demand and pH impacts due to the presence of volatile fatty acids (WRAP, 2015).

This study aimed to elucidate mechanisms associated with the effect of BSI PAS 110 (British Standards Institution Publicly-Available Specification) food-based digestate on *A. chlorotica* survival following application to soil, and whether there is a different response between the juvenile and adult stages. This was done to test the following hypotheses:

1. Digestate application to soil increases *A. chlorotica* mortality
2. Increased *A. chlorotica* mortality is caused by salt stress
3. Increased *A. chlorotica* mortality is caused by anaerobicity resulting from the digestate application

2. Materials and Methods

2.1 Experimental design

The experiment used independent measures in a randomised block design. Microcosms were constructed using 10.3 L white food-safe polypropylene boxes (28.6 x 19.8 x 27.3 cm) with six 1.5 mm drainage holes. The hook side of self-adhesive hook & loop tape, 2.5 cm wide, was attached around the internal rim of each box to prevent earthworms escaping (Lubbers and van Groenigen, 2013).

The boxes were filled with loamy sand topsoil (80.6 % sand, 14.2 % silt, 5.2 % clay, 3.0 % SOM, pH 6.6 H_2O) collected from Crabtree Leasow field, Harper Adams University (HAU), UK (Latitude: 52.772627, Longitude: -2.424008) to 20 cm depth. Soil was homogenised by removing plant material, gravel, and rocks (> 5 mm), sieving (4 mm), passing it through a shredder (Royer Pneulec, 240v soil/compost shredder) and repeated mixing using a spade. Pots were packed to a dry bulk density of 1.3 g cm^{-3} (10.4 kg dry soil per pot) and maintained

gravimetrically at 60 % water filed pore space (WFPS) for the duration of the experiment by watering every two days with tap-water.

Two adult and five juveniles of *A. chlorotica* (pale morph only) were added to each box and allowed to adapt for five days before treatments were applied. The total earthworm biomass did not significantly differ between replicates or across treatments.

Ten treatments were replicated five times each to give a total of 50 experimental units. Treatments were prepared to replicate the application of anaerobic digestion (AD) digestate, raw liquid phase, at rates equivalent to 150 kg N ha^{-1} and 300 kg N ha^{-1}, R150 (16.6 tFW ha^{-1}) and R300 (33.2 tFW ha^{-1}) respectively.

2.2 Treatments

The AD digestate (Table 1) was collected from a plant located in Shropshire, managed to British Standards Institution Publicly-Available Specification, BSI PAS 110 (industry specification verifiable for consistent quality and fit for purpose). The conditions were: Mesophilic (44°C); Hydraulic Retention Time (HRT) of 40 days. Feedstock: 50% food waste from processed food factories; 40% non-animal by-products (e.g. dairy derived, products of animal feed production); 7 - 7.5% poultry litter; 2.5 - 3% compost leachate (i.e. liquid that seeps from decomposing organic material).

The results obtained from characterisation measurements on the digestate (i.e. salinity, electrical conductivity, biological oxygen demand and pH) were used to develop synthetic treatments mimicking labile-carbon (C) content, salt content and a mix of both salt and labile C in the digestate. Three solutions were produced and standardised: hereafter Labile-C, Osmotic-Stress or Synthetic-Digestate, respectively.

159 Table 1: Characteristics of whole digestate, units as appropriate relative to fresh
160 matter (FM) or dry matter (DM).

Parameter	Units	Result
pH	pH units	8.4 (FM)
Oven dry matter (TS)	% m/m	5.13 (DM)
Volatile solids (VS)	% m/m	3.13 (DM)
Total Nitrogen (N)	% m/m	0.904 (DM)
Ammoniacal Nitrogen (NH_4-N)	mg kg^{-1}	6668 (FM)
Total Potassium (K)	mg kg^{-1}	4017 (DM)
Total Sodium (Na)	mg kg^{-1}	2869 (DM)
Total Calcium (Ca)	mg kg^{-1}	1686 (DM)
Total Phosphorus (P)	mg kg^{-1}	1359 (DM)
Total Magnesium (Mg)	mg kg^{-1}	157 (DM)
Biological Oxygen Demand (BOD)	mg L^{-1}	8720 (FM)
Electrical Conductivity (EC) at 21°C	mS	51.6 (FM)

161

162 The Labile-C treatment consisted of a solution of glucose, L-glutamic acid and
163 starch prepared to match biological oxygen demand (BOD level of 8718 mg L^{-1}, pH
164 8.2, EC 19 ±2 mS, Temp 21 ±1°C) obtained from the AD digestate. Biological
165 oxygen demand values provide an indication on the concentration of labile-C
166 (Taylor *et al.,* 2011).

167 The Osmotic-Stress treatment was formulated using a saline solution comprised of
168 sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$) and
169 magnesium chloride ($MgCl_2$) in accordance with the main ions found in the AD
170 digestate. The concentration ratios were replicated (i.e. NaCl : KCl : $CaCl_2$: $MgCl_2$,
171 19:16:9:1 g L^{-1}) to match digestate's electrical conductivity (EC) and pH adjusted
172 with hydrochloric acid (EC = 51 ±2 mS, pH = 8.4, Temp = 21 ±1°C). Electrical

conductivity provides a proxy measure for quantifying salts in soils and can be used for assessing saline toxicity (Owojori and Reinecke, 2014).

The Synthetic-Digestate treatment was produced using the solution as of Labile-C and mixing in the same concentration of salts as in the Osmotic-Stress solution (EC = 47 ±2 mS, pH = 8.5, Temp = 21 ±1°C). Deionized water was used as the Water control treatment.

All treatments were applied to the soil surface, R150 received 100 ml from each treatment per respective replicate, and R300 received 200 ml, increasing WFPS to *c.*61 %. Earthworms were not provided with any food during the experiment beyond the organic matter content of the treatment applied. Continuous observation was done for 1 h post application of treatments, and subsequent observations were made on the hour for six hours on the day. The worms that surfaced after treatments were applied and died were collected, washed and pat-dried and their mass recorded.

2.3 Earthworm sampling and analysis

Allolobophora chlorotica were collected from a grassland site south of Shrewsbury, UK (Latitude: 52.614207; Longitude: -2.695704) by digging to 20 cm depth and hand sorting earthworm species *in situ*. Both colour morphs and development stages were collected but only the pale morph was used in the experiment. Juveniles were differentiated from other species through their characteristic curling behaviour and excretion of coelomic fluid when handled (Sims and Gerard, 1999). They were kept in 50 L plant pots filled with a composite of oven dried at 105°C soil and fresh soil from the sampled pits at a ratio of 75:25. Soil was free from roots, living plants and macrofauna. Diced carrots (~ 180 g), apples (~ 200 g) and

199 green beans (~ 100 g) were mixed into the soil on a weekly basis. The culture was
200 kept at 18°C, in the dark, for 3 months prior to the start of the trial (Butt and Lowe,
201 2010). This period allowed standardisation of feeding, while also ensuring that
202 earthworms were randomly applied to each treatment with no pit collection effects.

203 During experimental setup, earthworms were removed from culture, rinsed with
204 deionised water, and kept in a plastic container with moist paper towel for 24 h to
205 void their guts. Their mass was then recorded as each worm was placed in the
206 experiment.

207 The experiment was concluded after 29 days, at which point the soil in the boxes
208 was removed by hand, earthworms and cocoons hand sorted, and developmental
209 stages counted. Following this, earthworms were placed in moist plastic boxes for
210 24 h at 18°C ± 2°C. Excess moisture was then removed with paper towels and the
211 worms individually weighed.

212

213 *2.4 Soil analysis*
214

215 Soil texture was determined using the pipette method (MAFF/ADAS, 1986) and
216 experimental boxes were packed with it.

217 Soil was collected at the end of trial from different depths (increments of ~ 5 cm)
218 during hand sorting to obtain a representative sample. Each sample was
219 homogenised by passing it through a 4 mm sieve before air drying at 30°C. The
220 exchangeable fraction of soil cations was analysed because it gives an indication
221 of their bioavailability. An aliquot of soil from the composite sample was collected
222 and analysed for Na^+, K^+, Mg^{2+} and Ca^{2+} ions by extracting with 1 M ammonium
223 nitrate (NH_4NO_3), 1:5 ratio of soil to NH_4NO_3 horizontally shaken for 30 minutes
224 and filtered through Whatman No. 2 filter paper (MAFF/ADAS, 1986). Their

exchangeable fraction was analysed by inductively coupled plasma mass spectrometry ICP-MS (Perkin Elmer NexION 2000). Total nitrogen (tN) content in soil was analysed by combustion (950°C) using Leco FP528. Soil bioavailable phosphorus was extracted using the Olsen-P method (MAFF/ADAS, 1986) using 0.5 M sodium bicarbonate solution adjusted to pH 8.5 at 20°C. Absorbance of the final blue complex concentration was read in a spectrophotometer (Jenway 6305) at 880 nm.

Soil organic matter was determined by loss-on-ignition (LoI) (MAFF/ADAS, 1986) by first oven drying soil (10 g) at 105° C and then measuring the mass loss after further heating at 450° C for 4 h in a furnace (Carbolite AAF1100).

2.5 Statistical analysis

Statistics were computed using R-programming (R Core Team, 2019) and additional packages: 'MASS' (Venables and Ripley BD, 2002), 'car' (Fox and Weisberg, 2019), 'dplyr' (Wickman *et al*., 2020), 'lme4' (Bates *et al*., 2015), 'vegan' (Oksanen *et al*. 2019), 'rcompanion' (Mangiafico, 2016).

Raw data was visualised using boxplots and outliers identified from chemical analysis. The means of the identified chemical parameters were recalculated for each treatment with outliers (i.e. data points more than three standard deviations away from the mean) excluded. These were one data point from each of the treatments applied, specifically from the Digestate (R300), Water control (R300, R150), Osmotic-Stress (R300) and Labile-C (R150).

Data was tested for homogeneity of variance using Lavene's test and for normality with Shapiro-Wilk test. Tukey's ladder transformation of data was applied if results did not satisfy the necessary assumptions of linear regression. The transformed

variables were: Na R150, K R300 and P R300 (transformation applied = $-1 * x^{\lambda}$); Biomass of adults R300, and juveniles and adults R150, Na R300, Mg R300 and EC R300 (transformation applied = x^{λ}).

Multiple regression models were applied to continuous data, i.e. earthworm biomass (juveniles or adults from the R150 or R300 treatments as the response variables) and soil chemical analysis (i.e. Na, K, Mg, Ca, tN, P, pH, EC, SOM) as explanatory variables with treatments as the explanatory categorical data. Variables were included in the model to test which mechanism, if any, explained the response to treatments of either the juvenile or adult stages.

Simplified models were compared to initial model using ANOVA to test the significance of factors set as the explanatory variables. The Akaike information criterion (AIC) was used to determine whether model simplification led to the loss of information.

One-way ANOVA was then computed with the biomass as the response variable with treatments as the explanatory variable. Tukey Honest Significant Difference post-hoc tests were computed on significant models (< 0.05) for all individual comparisons.

A Generalised Linear Model (GLM) using a quasi-Poisson error structure test was used for count data (i.e. earthworm abundance of adults or juveniles as the response variables).

3. Results

3.1 Earthworm Biomass Response to Treatments

The combined initial biomass of both juvenile and adult stages before they were added to boxes did not differ across treatments applied at R150 lower rate ($p = 1.0$, ANOVA) or R300 higher rate ($p = 0.9$, ANOVA).

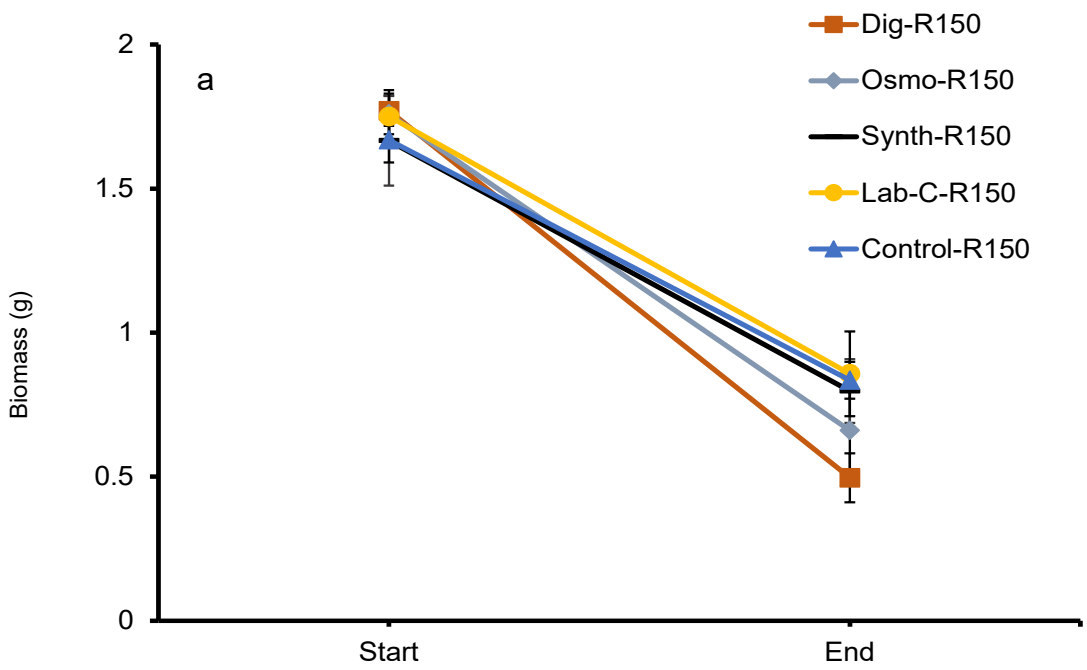
Earthworm biomass at the end of the experiment significantly declined compared to initial biomass for both stages at both application rates, R150 (juveniles $p < 0.001$, adults $p < 0.001$) or R300 (juveniles $p < 0.001$, adults $p < 0.001$), across all treatments, including the Water control (Fig. 1). The biomass of adults and juveniles declined, respectively, by 90 % and 62 % for Digestate applied at the lower rate, and by 96 % and 90 % at the higher rate.

The concentration of salts (as the covariate in the model) did not explain biomass decline of juveniles (as response variable) when compared to the Water control treatments (as explanatory variables) that were either added at R150 rate (Na $p = 0.7$, K $p = 0.6$, Mg $p = 0.7$, Ca $p = 0.8$) or R300 (Na $p = 0.7$, K $p = 0.6$, Mg $p = 0.7$, Ca $p = 0.8$). Additionally, salt concentration did not explain biomass loss of adults as response variable in the R150 treatments (Na $p = 0.9$, K $p = 0.5$, Mg $p = 0.3$, Ca $p = 0.2$) or R300 treatments (Na $p = 0.7$, K $p = 0.1$, Mg $p = 1.8$, Ca $p = 0.4$).

Treatments' electrical conductivity (EC) (explanatory variables and respective covariates) was also not able to explain biomass (as the response variable) loss in comparison with Water's EC, regardless whether it was from the lower rate R150 treatments (Digestate: juveniles $p = 0.9$ or adults $p = 0.9$; Labile-C: juveniles $p = 1.0$ or adults $p = 0.6$; Osmotic-Stress: juveniles $p = 0.9$ or adults $p = 0.8$; Synthetic-Digestate: juveniles $p = 0.9$ or adults $p = 0.9$) or the higher R300 rate (Digestate: juveniles $p = 0.4$ or adults $p = 0.4$; Labile-C: juveniles $p = 0.6$, adults $p= 0.5$; Osmotic-Stress: juveniles $p = 0.3$, adults $p = 0.9$; Synthetic-Digestate: juveniles $p = 0.9$, adults $p = 0.5$).

Biomass was not significantly affected when multiple regression was conducted to compare R300 treatments with the Water control, using SOM (juveniles $p = 0.1$ or adults $p = 0.5$), tN (juveniles $p = 0.2$ or adults $p = 0.8$) and P (juveniles $p = 0.6$ or adults $p = 0.7$) as explanatory variables. Similar analyses with the R150 rate indicated that SOM (juveniles $p = 0.8$ or adults $p = 0.3$), tN (juveniles $p = 0.9$ or adults $p = 0.5$) and P (juveniles $p = 0.9$ or adults $p = 0.2$) had no effect on biomass.

ANOVA was run with the final biomass of juveniles ($p = 0.001$) or adults ($p < 0.001$) (end of experiment) as the responsive variables and R300 treatments as the explanatory variables. Post-hoc test revealed that the biomass of juveniles was significantly greater in the Labile-C ($p = 0.002$) and Water control ($p = 0.001$) than in the Digestate R300 treatment. Whereas the biomass of adults was significantly greater in the Labile-C R300 treatment than Digestate ($p < 0.001$), Water control ($p < 0.001$), Osmotic-Stress ($p = 0.01$) and Synthetic-Digestate ($p < 0.001$) R300 treatments. Moreover, significantly lower biomass was observed in the Synthetic-Digestate treatment in comparison with Osmotic-Stress ($p = 0.04$). No significant difference in biomass of juveniles between the treatments applied at the lower rate R150 ($p = 0.68$). In contrast, post-hoc test from the R150 treatments found that adult biomass was significantly greater in the Water control than in Digestate ($p = 0.03$).


a
Biomass (g)
2
1.5
1
0.5
0
Start
End
Dig-R150
Osmo-R150
Synth-R150
Lab-C-R150
Control-R150

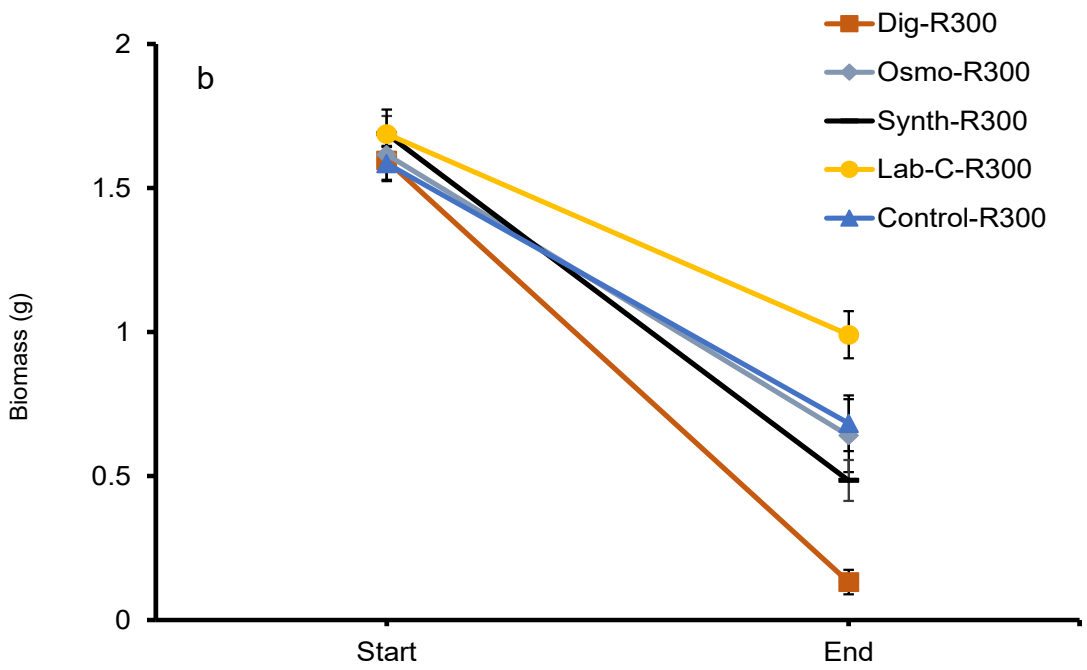

b
Biomass (g)
2
1.5
1
0.5
0
Start
End
Dig-R300
Osmo-R300
Synth-R300
Lab-C-R300
Control-R300

Figure 1: *Allolobophora chlorotica* total mean biomass of combined juvenile and adult stages at the start of the experiment before treatments were applied and at the end. Biomass is grouped by treatments applied at either the lower rate (R150, panel a, $p < 0.001$) or higher rate (R300, panel b, $p < 0.001$). Treatments are: Dig-Rx = Digestate; Osmo-Rx = Osmotic-Stress-Rx; Synth-Rx = Synthetic-Digestate-Rx; Lab-C-Rx = Labile-C-Rx; Control-Rx = Water-Rx. ± Standard error of the mean (SEM)s, n = 5.

3.2 Earthworm Abundance Response to Treatments

The Digestate, Osmotic-Stress and Synthetic-Digestate salt containing treatments resulted in some earthworms surfacing and dying shortly after these were applied at both R150 ($p = 0.1$) or R300 rates ($p < 0.001$) (Table 2). Only one cocoon per each R150 treatment was found at the end of the experiment, except in the Labile-C treatment where none were found. In the R300 treatments, only one or two cocoons were found in the Synthetic-Digestate or Labile-C treatments, respectively (Table 2). No significant differences between the number of dead earthworms or cocoons were found between the Digestate treatment applied at either R150 or R300 rate and Osmotic-Stress (R150, dead $p = 0.7$, cocoon $p = 1.0$; R300, dead $p = 1.0$, cocoon $p = 1.0$), Synthetic-Digestate (R150, dead $p = 0.3$, cocoon $p = 1.0$; R300, dead $p = 0.1$, cocoon $p = 0.9$), Labile-C (R150 dead $p = 1.0$, cocoon $p = 0.9$; R300 dead $p = 1.0$, cocoon $p = 0.9$) or Water control (R150 dead $p = 1.0$, cocoon $p = 1.0$; R300 dead $p = 1.0$, cocoon $p = 1.0$).

The overall abundance of earthworms declined at the end of the experiment regardless of life stage, juveniles or adults, (R150 juveniles $p < 0.001$, adults $p < 0.001$; R300 juveniles $p < 0.001$, adults $p < 0.001$; Fig. 2) as determined by the generalised linear models. The abundance of adults and juveniles suffered 80 % and 24 % drop at the lower rate, and a 90 % and 84 % drop at the higher rate.

The concentration of salts in the R150 treatments could not explain the decline in abundance of juvenile (Na $p = 0.07$, K $p = 0.07$, Mg $p = 0.34$ or Ca $p = 0.07$) or adult (Na $p = 0.64$, K $p = 0.54$, Mg $p = 0.52$ or Ca $p = 0.57$). Similar results were observed with the R300 treatments, both juveniles (Na $p = 0.48$, K $p = 0.42$, Mg $p = 0.84$ or Ca $p = 0.98$) and adult numbers (Na $p = 0.10$, K $p = 0.90$ or Ca $p = 0.21$).

There was an exception with Mg in the R300 rate that showed a significant greater number of adults ($p = 0.02$), but this could not be explained as a treatment effect (R300 Digestate $p = 1.0$, Labile-C $p = 1.0$, Osmotic-Stress $p = 1.0$, Synthetic-Digestate $p = 1.0$).

The decline in juvenile and adult numbers in the Digestate R150 and R300 treatments could be explained by the simpler model with treatments only as the explanatory variable, respectively. The number of juveniles ($p < 0.001$) and adults ($p = 0.03$) were significantly lower in the Digestate treatment, applied at the rate of R300 and R150 respectively, than the Water control (Table 3). Greater final number of adults were observed in the Labile-C ($p = 0.01$) than in the Water control R300 treatments (Table 3).

Electrical conductivity results of each treatment applied at either rate, R150 or R300, could not explain total mean number of juveniles (R150 Digestate $p = 0.6$, Labile-C $p = 0.5$, Osmotic-Stress $p = 0.6$, Synthetic-Digestate $p = 0.6$; R300 Digestate $p = 0.3$, Labile-C $p = 0.8$, Osmotic-Stress $p = 0.4$, Synthetic-Digestate $p = 0.7$) or adult stages at the end of the experiment (R150 Digestate $p = 0.7$, Labile-C $p = 0.5$, Osmotic-Stress $p = 0.8$, Synthetic-Digestate $p = 0.7$; R300 Digestate $p = 1.0$, Labile-C $p = 1.0$, Osmotic-Stress $p = 1.0$, Synthetic-Digestate $p = 1.0$).

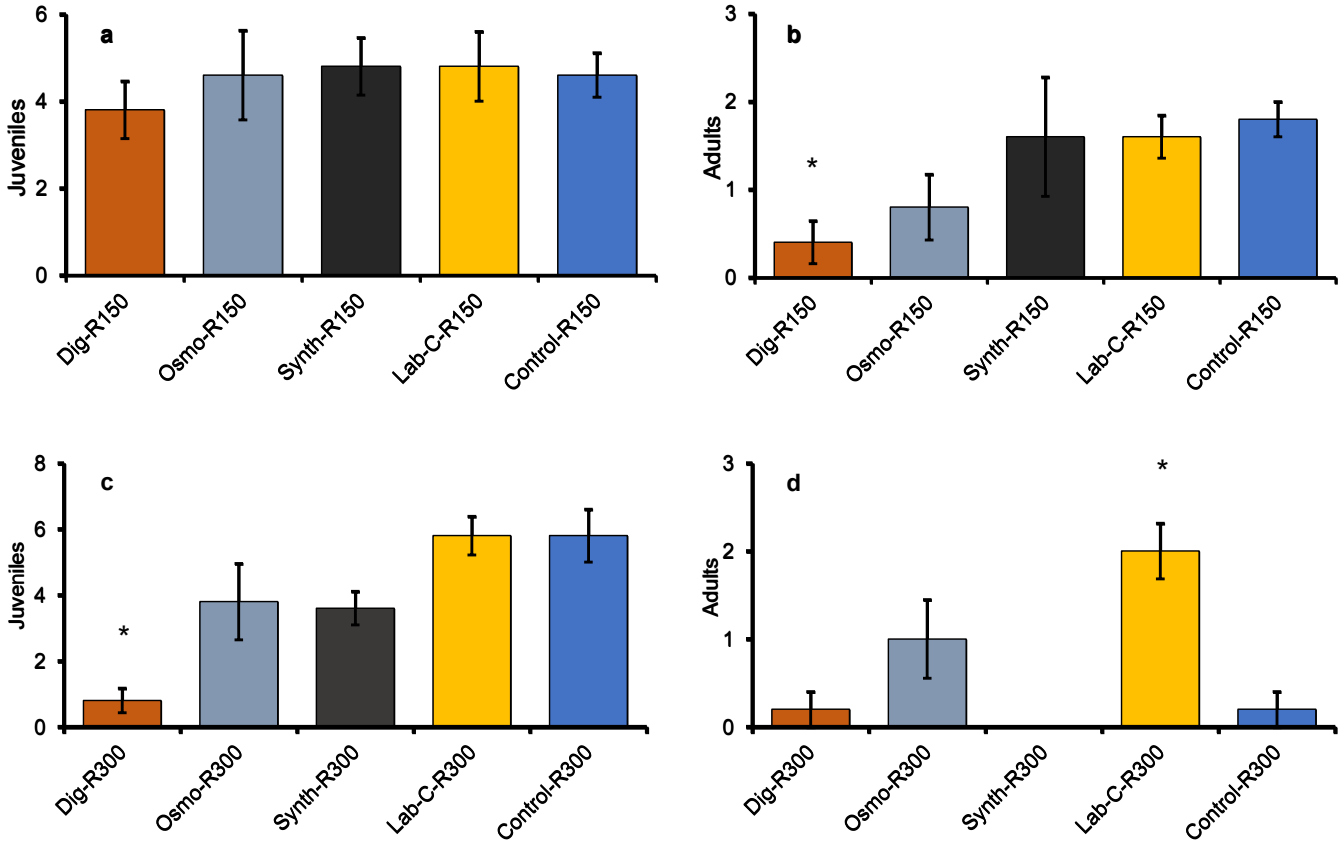


Figure 2: Mean abundance of juveniles and adults at the end of the experiment of all treatments applied at the lower R150 (a & b) and higher R300 (c & d) rate. Dig-Rx = Digestate; Osmo-Rx = Osmotic-Stress; Synth-Rx = Synthetic-Digestate; Lab-C-Rx = Labile-C; Rx = R150 or R300. Error bars equal Standard Error of the Mean (± SEM). Juveniles n = 5, adults n = 2. Asterisks (*) symbolise significantly different ($p < 0.05$) results in comparison with the Water control treatment.

412 Table 2: Total overall number of dead earthworms found at soil surface soon after
413 treatments were applied, and total cocoons at the end of trial for both lower (R150)
414 and higher (R300) rate. All treatments started with a total of seven juvenile and
415 adult earthworms per replicate, a grand total of 35 earthworm per treatment.
416

Treatment	Rate 150		Rate 300	
	Dead	Cocoons	Dead	Cocoons
Digestate	2	1	2	0
Labile-C	0	0	0	2
Osmotic-Stress	3	1	7	0
Synthetic-digestate	5	1	7	1
Water control	0	1	0	0

417
418

Table 3: Outputs of generalised linear models computed with the abundance of either juvenile or adult stages as the response variable against treatments applied at either rate as the explanatory variable. Significance of regression models was set at ≤ 0.05. Models' explained deviance (pseudo R^2): R150 model, juveniles = 5.8 %, D.F. = 20, adults = 30 %, D.F. = 20; R300 model, juveniles = 57 %, D.F. = 20, adults = 61 %, D.F. = 20.

	Juvenile Counts R150		Adults Counts R150		Juvenile Counts R300		Adults Counts R300	
	Parameter value	P	Parameter value	P	Parameter value	P	Parameter value	P
Intercept	1.53	0.000	0.59	0.044	1.76	< 0.001	-1.61	0.060
Digestate	-0.19	0.538	**-1.50**	**0.029**	**-1.98**	**0.000**	0.00	1.000
Labile-C	0.04	0.884	-0.12	0.771	0.00	1.000	**2.30**	**0.013**
Osmotic-Stress	0	1.000	-0.81	0.115	-0.42	0.118	1.61	0.083
Synthetic-Digestate	0.04	0.884	-0.18	0.771	-0.48	0.085	-17.69	0.996

3.3 Soil Chemical Analysis – R150

Regression analysis showed significant difference in bioavailable Na for the four treatments in relation to the Water control treatment. Concentrations of Na were greater in the Digestate ($p < 0.001$), Labile-C ($p = 0.004$), Osmotic-Stress ($p < 0.001$) and Synthetic-Digestate ($p < 0.001$) (Fig. 3).

Concentrations of K in soil were significantly greater in the Digestate ($p = 0.004$), Osmotic-Stress ($p < 0.001$) and Synthetic-Digestate ($p = 0.02$) than in the Water control water treatment (Fig. 3).

In comparison to the Water control treatment, Mg concentrations were lower in the Labile-C ($p = 0.045$), Osmotic-Stress ($p = 0.04$) and Synthetic-Digestate ($p = 0.04$) (Fig. 3).

Concentrations of bioavailable Ca (Digestate $p = 1.0$, Labile-C $p = 0.4$, Osmotic-Stress $p = 0.5$, Synthetic-Digestate $p = 0.6$) and Olsen-P (Digestate $p = 0.8$, Labile-C $p = 0.1$, Osmotic-Stress $p = 0.7$, Synthetic-Digestate $p = 0.4$), and SOM fraction (Digestate $p = 0.06$, Labile-C $p = 0.6$, Osmotic-Stress $p = 0.6$, Synthetic-Digestate $p = 0.2$) did not significantly differ across all treatments. The fraction of total-N was greater in the Labile-C ($p = 0.04$) than in the Water control treatment.

The results of the EC were significantly higher in the Digestate ($p = 0.003$), Osmotic-Stress ($p = 0.005$) and Synthetic-Digestate ($p = 0.020$) than in the Water control treatment.

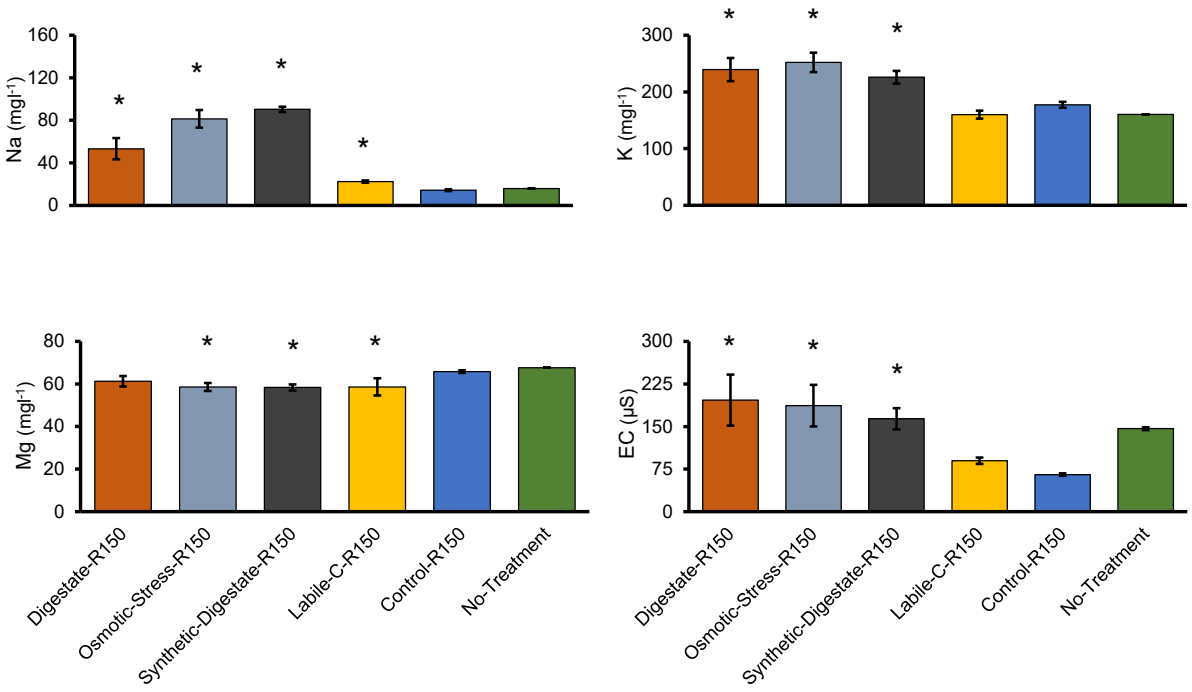


Figure 3: Concentrations of mean bioavailable Na, K and Mg in soil for each treatment applied at the lower rate (R150) and respective soil EC results. Initial soil conditions represented by green bar (No-Treatment). n = 5, ± SEM. Asterisks (*) symbolise significantly different ($p < 0.05$) results in comparison with the Water control treatment.

3.4 Soil Chemical Analysis – R300

The concentration of bioavailable Na, at the end of the experiment, could be explained by treatments containing salts. The pots that received Digestate, Osmotic-Stress and Synthetic-Digestate had significantly higher concentration of bioavailable Na in soil than the Water control ($p < 0.001$; $p < 0.001$; $p < 0.001$ respectively). Treatments with salts also influenced bioavailable K. Significantly higher soil K was observed in the Digestate ($p < 0.001$), Osmotic-Stress ($p < 0.001$), and Synthetic-Digestate ($p < 0.001$) treatments. The fraction of total-N in soil was significantly greater in the Synthetic-Digestate treatment ($p = 0.04$) than in the Water control boxes.

Bioavailable Mg (Digestate $p = 0.11$, Labile-C $p = 0.31$, Osmotic-Stress $p = 0.28$, Synthetic-Digestate $p = 0.20$), Ca (Digestate $p = 0.15$, Labile-C $p = 0.18$, Osmotic-Stress $p = 0.26$, Synthetic-Digestate $p = 0.44$), Olsen-P (Digestate $p = 0.98$, Labile-C $p = 0.59$, Osmotic-Stress $p = 0.06$, Synthetic-Digestate $p = 0.42$) and SOM (Digestate $p = 0.42$, Labile-C $p = 0.49$, Osmotic-Stress $p = 0.41$, Synthetic-Digestate $p = 0.69$) did not differ across treatments. Olsen-P was lower in the Osmotic-Stress treatment than in any other treatment but not significantly to the Water control treatment ($p = 0.06$).

EC values were higher in pots that received the treatments of Digestate ($p < 0.001$), Osmotic-Stress ($p < 0.001$) and Synthetic-Digestate ($p < 0.001$).

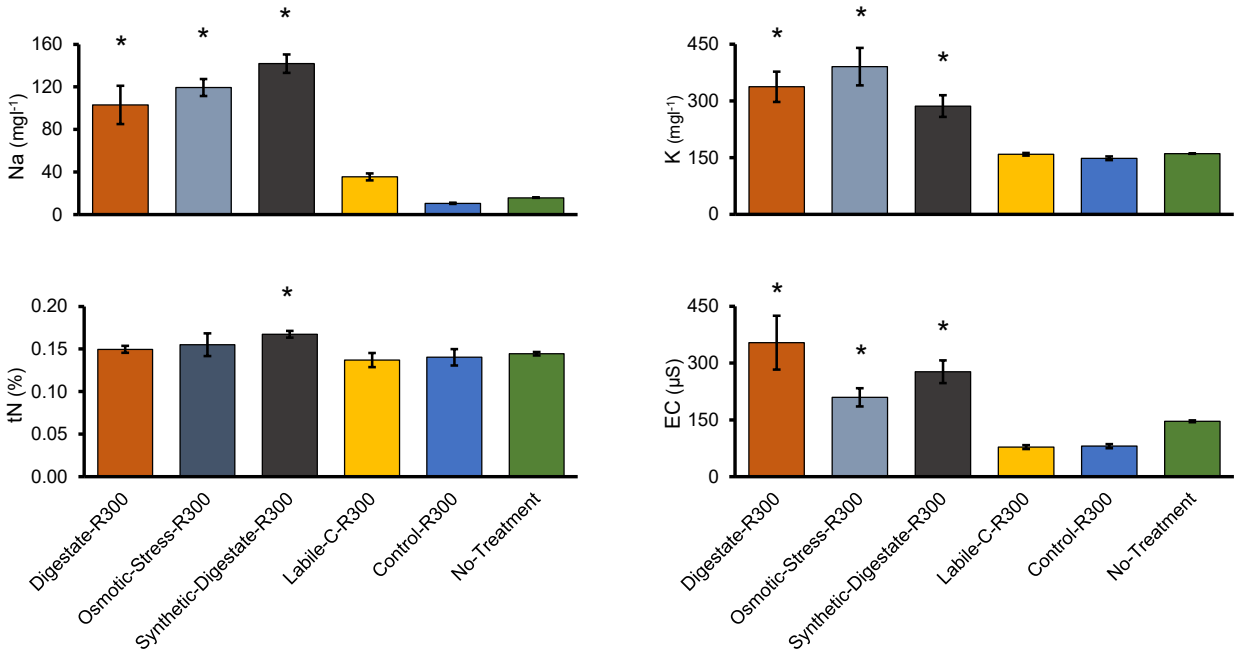


Figure 4: Concentrations of bioavailable Na and K, and Total-N in soil for each treatment applied at the higher rate (R300) and respective soil EC results. Initial soil conditions represented by the green bar (No-Treatment). n = 5, ± SE. Asterisks (*) symbolise significantly different ($p < 0.05$) results in comparison with the Water control treatment.

4. Discussion

This study aimed to elucidate mechanisms associated with the effect of PAS 110 food-based digestate on *A. chlorotica* survival following application to soil, and whether there is a different response between the adult and juvenile stages. Digestate has properties that can contribute towards improving soil nutrition (Wallace *et al.*, 2011; WRAP, 2015). However, its impact on soil biological communities is not fully understood. Based on the findings of this study, earthworm biomass and survival may be affected by the application of digestate to soil, with greater negative impacts being observed on the adult stage. Specifically, a significant decline in comparison to the Water control was observed with R150 digestate. A similar decline was observed for both Digestate and Water R300 treatments, whereas a significant impact of Digestate was only observed in juveniles when digestate was applied at the highest rate (R300, i.e.300 kg N ha^{-1}eq.). While this volume exceeds common practice under agronomic decisions, the surfacing of earthworms and survival have also been reported from a field experiment receiving the rate equivalent to 70 kg N ha^{-1} (personal communication with Amy Watkins, Sustainability Project Manager, Agrii agronomy services, UK, 2020), with mixed effects reported in other studies (Koblenz *et al*., 2015; WRAP, 2015; Sizmur *et al*., 2017). For example, *A. chlorotica* was not found in one site that received 160 kg N ha^{-1} eq. of digestate, and at another site it accounted for only 6.1% out of the five species found when digestate was applied at 130 kg N ha^{-1} eq. (Koblenz *et al*., 2015). In another study, *Lumbricus terrestris* biomass only increased when digestate was applied with cereal straw but declined by 23% when digestate was applied alone (Sizmur *et al*., 2017). In contrast, the impact on the abundance of endogeics was site dependent (WRAP, 2015).

519 The variability of negative responses to salts by both adults and juveniles in this
520 study may be explained by horizontal stratification of earthworm communities
521 within the soil. Earthworms closer to the soil surface would be exposed to greater
522 concentrations of salts that likely increased mortality. Earthworms deeper in the
523 soil were exposed to less salts due to the dilution gradients that formed as the
524 salts leached through the soil profile. Toxicity response by the two stages could be
525 elucidated in further studies looking at the effect of horizontal stratification and
526 whether juveniles and adults burrowing and/or emergence behaviour is different
527 when exposed to adverse conditions.

528 A small number of earthworms emerged to the soil surface soon after applying
529 Digestate, Osmotic-Stress and Synthetic-Digestate treatments (Table 2). The few
530 that emerged died soon after surfacing. These treatments had significantly higher
531 concentrations of Na and K salt ions and EC (Fig. 3 and 4), this observation may
532 have been biased by unequal distribution of earthworms through the soil profiles of
533 the different treatments.

534 Reaction time to external stimuli. i.e. salts dissociated in soil pore water, is
535 dependent on the internal osmotic pressure of the earthworm (Parker and Metcalf,
536 1906; Laverack, 1960). In this experiment, treatment stimuli only resulted in a few
537 earthworms surfacing (Table 2). Reaction times to being exposed to NaCl or KCl
538 solutions can be species dependent. For example, *Allolobophora foetida* (Savigny,
539 1826) (accepted as *Eisenia fetida* (Savigny in Cuvier, 1826)) can react 200 times
540 faster to stimuli than *Helodrilus sp.*, and response to KCl being 7.5 times faster
541 than to NaCl. However, the authors observed biomass loss across both ecotypes
542 (Parker and Metcalf, 1906). The earthworms' body membrane is selective and
543 prevents salt molecules (i.e. $CaCl_2$, NaCl or KCl) from crossing freely (Laverack,
544 1963; Edwards and Lofty, 2013). Chloride was not measured in this study, but it is

545 unlikely that significant concentrations would be detected at the end of the trial. Its
546 anionic properties limits persistence in pore water due to soil's relatively low anion
547 exchange capacity and so concentration would decrease rapidly post application
548 (Parfitt, 1979).

549 The treatments Digestate, Osmotic-Stress and Synthetic-Digestate each had a
550 negative effect on the survival and biomass of *A. chlorotica* when applied at the
551 higher rate, R300. However, the Water control also had a similar effect on the
552 adults. These results suggest that the mechanisms behind treatment toxicity could
553 not be fully elucidated even though a significant treatment effect was observed. In
554 the conditions used in this study, the application of water alone was sufficient to
555 increase adult mortality. This suggests that the water filled pore space increased
556 to such an extent that anaerobic conditions occurred in the soil. Repeating the
557 experiment with a different soil moisture regime, a different soil texture, or with
558 different proportion of *A. chlorotica* morphs or earthworm species may have led to
559 different results. For example, the *A. chlorotica* green morph is more tolerant of
560 high moisture content soils than the pale morph (Satchell, 1967). Nevertheless,
561 these results highlight the issue of applying liquid soil amendments to soil with a
562 high moisture content (e.g. 60 % WFPS). This could be detrimental to the soil
563 biota, even if the non-water fraction of the amendment itself is harmless.

564 Biomass loss was observed for all treatments at both rates, R150 and R300, and
565 including the Water control, which suggests that osmotic stress, due to the
566 concentrations of salts, was not the only cause of death. The earthworm's
567 semipermeable membrane allows the passage of water from a hypotonic solution
568 to a hypertonic one. If internal body fluids have lower concentration of salts (i.e. is
569 hypotonic) compared to the external (hypertonic) environment when initially
570 exposed to the Digestate, Osmotic-Stress and Synthetic-Digestate treatments,

571 then internal water would pass from the earthworm to the soil causing weight loss.
572 This is because earthworm's biomass is generally about 80 % water (Laverack,
573 1963). However, for the Labile-C and Water control treatments other factors were
574 at play.

575 Exchangeable concentrations of ions were analysed in this study because the use
576 of total concentrations of metals in soil are not suited to ecotoxicology studies;
577 totals do not reflect ion bioavailability (Rowell, 1994). Higher concentrations of a
578 cation in pore water, such as Ca^{2+}, can reduce bioaccumulation of another cation
579 in Lumbricidae (Lee and Kim, 2008). However, the buffering effect of Ca^{2+} in this
580 study was unlikely because no significant differences in bioavailable Ca were
581 found in soil across all treatments applied at either rate (R150 or R300).

582 The bioavailability of toxicants can also be reduced by phosphate (PO_4^{3-}) because
583 it competes for cellular transport carriers (Lee and Kim, 2008). In our study, the
584 Osmotic-Stress R300 treatment had lower concentration of bioavailable P in
585 comparison with soil conditions before and after other treatments were applied,
586 which could be due to carrier competition between bioavailable P and ions applied
587 with the treatment. Phosphate may play a significant role in preventing toxicity if
588 the earthworms' osmotic balance remains uncompromised.

589 Earthworms' behaviour was not visually inspected throughout experiment to
590 assess whether good health had been maintained. However, earthworms were
591 checked for healthy cues such as turgidity, body shape and uncompromised
592 epidermal membrane, and mobility before being added to experimental boxes
593 (Frund *et al*., 2010). The biomass decline of *A. chlorotica* across all treatments
594 implies that feeding slowed or ceased during the experiment (Fig. 1), which could
595 have led to starvation. Therefore, an internal buffering effect through ingesting
596 Digestate or Synthetic-Digestate (which are rich in organo-compounds) is unlikely.

Uptake of hydrophobic organo-compounds mostly occurs by feeding on soil (Ma *et al.*, 1998) and metal toxicity is reduced by such compounds because they do not readily dissociate in solution and can form organometallic compounds (Artuso *et al.*, 2011). Biomass decline across all treatments, e.g. reduced internal water due to osmotic stress and/or aestivation, was a likely caused by the disruption of homeostatic mechanisms. Earthworms can stop feeding in response to sensory stimuli that activate chemoreceptors in their body membrane. *Allolobophora spp.* are sensitive to stimuli of salts and sugars origin (sucrose and glycerol) (Laverack, 1960), which supports this hypothesised mechanism. It is possible that the earthworms were stressed throughout the experiment due to the maintenance of the pots at 60 % WFPS, which may have been too high. Similar experiments should aim to use of a range of WFPS to provide insights into this potential bias.

If adverse conditions persist, *A. chlorotica* become incapable of regulating the uptake of water and salts (Laverack, 1963). In clayey soils, it was found that *A. chlorotica* coiled up at 13.5 % gravimetric soil water content (~ 34 % WFPS) (Evans and Guild, 1948). Coiling is associated with aestivation, a period of inactivity induced by adverse conditions or seasonal adaptations (Sims and Gerard, 1999). Therefore, maintaining the soil at 60 % WFPS, for experimental purposes, could have induced aestivation and various fractions of WFPS should be considered if using *A. chlorotica* pale morph in subsequent experiments.

The decline in the number of adults for all treatments applied at the higher rate R300, except for the Labile-C treatment (Fig. 2), suggests that the excretory and osmoregulatory system was compromised. Various factors could have provoked mortality and the salts in the Osmotic-Stress and Synthetic-Digestate are the most likely explanation for those treatments. However, mortality in the other treatments could have been caused by temporary anaerobic or anoxic conditions, and by the

623 accumulation of nitrogenous waste compounds (Roots, 1956; Laverack, 1963;
624 Möller and Müller, 2012; Edwards and Lofty, 2013).

625 The Water control R300 treatment is likely to have increased the volume of water
626 in soil pore space. Water immersion may not be lethal to earthworms providing
627 that toxic or noxious products do not accumulate over extensive periods and/or
628 there is no depletion of oxygen (Laverack, 1963; Edwards and Lofty, 2013).
629 However, water saturated soil reduces available oxygen within the pore space,
630 preventing critical respiration processes to take place (Abe and Buck, 1985). The
631 observed negative effects in the water control may be explained by oxygen
632 depletion. The accumulation of noxious products, i.e. nitrogenous compounds,
633 may also explain observed negative effects for both adults and juveniles in the
634 Synthetic-Digestate R300 treatment. Total-N was significantly greater in Synthetic-
635 Digestate R300 compared to Water control R300 treatment. It was also greater in
636 the Digestate and Osmotic-Stress treatments, but not significantly different.
637 Whereas it declined in the Labile-C treatment. These findings could suggest that
638 there was an accumulation of nitrogenous compounds from dead biomass in the
639 former and volatilisation of ammonium through the breakdown of glutamic acid in
640 the Labile-C treatment in the latter.

641 The adults were the most affected by treatments applied at both rates (Fig. 2). For
642 example, there was a decline in the number of individuals for the Digestate and
643 Osmotic-Stress treatments applied at both rates (R150 and R300). A decline was
644 also detected with Synthetic-Digestate and Water R300 treatments. The
645 earthworms' tubular body shape provides it with a large surface area-to-volume
646 ratio, necessary for gas exchange. Smaller earthworms, like juveniles, have
647 greater surface:volume ratio, which permits higher diffusion rates to occur as those
648 of osmotic processes. For example, processes such as exchange of metal ions

649 (i.e. Na, K, Ca and Mg), O_2 and CO_2 gases. *A. chlorotica*'s short and stout
650 morphology allows it to thrive in the rhizosphere, with juveniles having fewer
651 segments than the adults (Piearce, 1983). The shorter length of the juveniles, but
652 greater surface:volume ratio could mean that exposure to treatments is reduced
653 because diffusion rates across their semipermeable is higher leading to the
654 balancing of in- and out-ward flow that offers protection from the toxic effects of
655 treatments. *Helodrilus oculatus* is a long and thin endogeic earthworm and it has a
656 large surface:volume ratio (Dobson and Satchell, 1956). Their morphology is
657 advantageous in low oxygenated habitats because their surface area permits
658 diffusion to continue and thrive in such environments (Dobson and Satchell, 1956).
659 This sort of morphological variations could explain the different response observed
660 between the juveniles and adults. Adults being longer and stouter means that
661 diffusion processes occur at a lower rate, potentially increasing toxicity exposure.
662 However, further studies are required to determine whether the surface:volume
663 ratio is a factor affecting toxicity response by the two development stages.

664 Our results suggest that there could have been a buffering effect from Synthetic-
665 Digestate R150 and Labile-C R300, which could have reduced adult mortality in
666 these treatments (Fig. 2). Cuticle permeability and osmotic potential are likely to
667 have caused an imbalance in diffusion rates across treatments, meaning that the
668 inward flow exceeded excretion rates. Soil amendments rich in organo-compounds
669 can reduced the capacity of a soil to conduct (i.e. EC). Conductivity is affected by
670 many soil properties including soil organic matter and salinity. Organo-
671 amendments have chelating properties due to their charged properties (i.e.
672 carboxylate salts, –COO-), leading to charged sites binding with cations and
673 leaving them in an inactive state (Chehab *et al.*, 2020). Glucose and starch,
674 components of Synthetic-Digestate and Labile-C, have high sorption (binding)

675 capability and can form chemical complexes (Polaczek *et al.*, 2000). In this study,
676 EC was found to be a poor predictor of the response of earthworms to treatments.
677 For example, EC was lower in the Water control treatment than in the Digestate,
678 Osmotic-Stress or Synthetic-Digestate applied at either rate, R150 or R300 (Fig. 3
679 and 4). However, the survival of adults was lower in all four treatments in
680 comparison with Labile-C (Fig. 2). It has been suggested that the nature of salts is
681 more important than EC in forecasting potential impacts on earthworm survival
682 (Owojori and Reinecke, 2014). This demonstrates the importance of knowing the
683 chemical composition of digestates to be able to make effective predictions as to
684 their likely impacts on earthworms and the soil biota.

685

686 5. Conclusion
687

688 This study demonstrates that digestate can cause increased mortality of *A.*
689 *chlorotica*. However, the experimental setup was not conducive to earthworm
690 survival, although it was not unrealistic for field conditions. A combination of
691 different factors may have explained the variable mortality rates amongst
692 earthworms. Those factors could have been: Horizontal stratification through the
693 soil profile; the application of high-water content amendments or just water to soils
694 that are already wet; greater surface:volume ratio in the juveniles; disruption of the
695 homeostatic mechanism that prevented organometallic compounds from forming
696 and decrease toxicity.

697 This study is important because digestate is applied in agricultural systems as a
698 soil amendment but its impact on soil biology is not fully understood. It is unlikely
699 that the volume of digestate used in this study would be applied in one rate under
700 agronomic decisions. Moreover, the application of AD digestate at a rate of 300 kg
701 N ha^{-1} is not permitted in the UK within one crop season (Defra, 2010).

Nevertheless, the intention of this work was to determine a potential mechanism behind the negative effects being reported on earthworm survival after the application of AD digestate.

Other limitations with this study are that only one earthworm species and soil type was used. This preliminary study could be used to develop further work, with some focus on manipulating soil water content, soil texture and depth effect, and earthworms' diet prior to experiment. It has established a baseline from which, as far as we know, is missing from research. Habitat conditions of *A. chlorotica* were replicated in terms of soil type, depth, and area to closely resemble field conditions and reduce experimental constraints. It provides an insight into ecotoxicology research looking at the impacts of digestate on a wild earthworm, which can dominate earthworm samples from agricultural systems, as does *A. chlorotica* in the UK.

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