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Effect of *Miscanthus* × *giganteus* ash on survival, biomass, reproduction and avoidance behaviour of the endogeic earthworm *Aporrectodea caliginosa*

Claire Brami[1,2,3] · Guénola Pérès[1] · Safya Menasseri-Aubry[1] · Jane Darcy Byers-Woods[2] · Thierry Jacquet[3] · Christopher Nathan Lowe[2]

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Abstract

To achieve the EU's targets for reducing energy production from fossil fuels, the use of energy crops, such as *Miscanthus* × *giganteus*, is increasing resulting in a corresponding increase in waste ash from incineration. The chemical properties of Miscanthus ash (e.g. phosphorus and potassium content) may allow this waste material (currently landfilled) to be used as a fertiliser, but no information exists on the effect of the ash on the biological properties of soil. The main aim of this study was to determine the potential impact of Miscanthus ash on earthworms by assessing the effect on survival, change in biomass, reproduction and avoidance behaviour of the geophagous, soil dwelling earthworm, *Aporrectodea caliginosa*. Tests utilised a range of Miscanthus ash doses from 0 to 50 t ha^{-1} (0, 1, 2.5, 5, 10, 25, 50). Results showed that Miscanthus ash had no significant impact on *A. caliginosa* survival, biomass and reproduction, but negative trends were observed for biomass from 2.5 t ha^{-1} and for reproduction from 10 t ha^{-1}. In contrast, a significant avoidance response was observed in the 25 and 50 t ha^{-1} treatment and according to ISO guideline 17512 there is a negative impact of the Miscanthus ash on soil habitat function at 25 t ha^{-1} and above as more than 80% of earthworms were in the control soil. It is suggested that this negative effect on soil habitat function could be attributed to a range of factors including the presence of heavy metals in the ash and a change in substrate pH, texture and/or osmotic stress. Further laboratory-based studies conducted over extended time periods with a more refined range of ash doses and associated field-based studies are required to validate the results and determine a more precise assessment of the threshold ash value inducing a loss of soil habitat function.

Keywords *Aporrectodea caliginosa* · *Miscanthus* × *giganteus* · Biomass ash · Ecotoxicology · Avoidance behaviour

Introduction

In the last decade the use of biomass to produce energy has increased across Europe. The potential of *Miscanthus* × *giganteus* (here after called Miscanthus) as an energy crop is widely recognised due to low maintenance costs (Lewandowski et al. 2000), high yields of 10–25 t ha^{-1} yr^{-1} (Lewandowski et al. 2000) and low moisture content of harvested biomass, reducing energy losses during the combustion process (Morandi et al. 2016). In addtion, incineration of Miscanthus leads to ash production of between 2 and 3.5% (European Biomass Association 2017), substantially below the maximum permitted value of 6% stated in the solid biofuel standard (ISO, 17225-6 2014). In 2016 there were 20,000 ha of Miscanthus in Europe utilised mainly for heat and power generation (Lewandowski et al. 2016), with a potential annual ash production of between 4000 and 17,500 tonnes (authors own calculation).

Currently the main outlet for biomass ash is landfill (RECORD 2016). However, it has been suggested that, due to high levels of key nutrients such as phosphorus and potassium, the ash could be used as fertiliser (Cruz-Paredes et al. 2017). Nevertheless, the benefits of ash as a fertiliser depend on source, application rate and the target plant/crop. For example, Ots et al. (2017) recorded an increase in birch productivity following application of a 25 t ha^{-1} mixture of

✉ Claire Brami
c.brami@phytorestore.com

[1] UMR SAS, Institut Agro, INRAE, 35000 Rennes, France

[2] University of Central Lancashire, Preston, UK

[3] Phytorestore, 53 avenue Philippe Auguste, 75011 Paris, France

wood and oil shale ash while Füzesi et al. (2015) recommended to limit annual application rates of wood ash to between 1 and 5 t ha^{-1} in order not to exceed optimum nutrient values for white mustard and ryegrass crops. Ash application has also been recommended for use in phytostabilization projects as it can increase soil pH, and so reduce bioavailability of heavy metals such as Cd, Pb and Zn (Bidar et al. 2016; Lopareva-Pohu et al. 2011; Mortensen et al. 2018). In contrast to these positive effects, the presence of heavy metals in ash can also have negative effects, especially associated with uptake by soil fauna and bioaccumulation in food chains (Mortensen et al. 2018). Assessment of the effect of biomass ash on soil organisms has received increasing interest because it is now recognised that these organisms provide key ecosystem services in agricultural systems and are indicators of soil quality (Hooper et al. 2005; Kibblewhite et al. 2008; Pulleman et al. 2012). Recent work demonstrated that the effect of wood ash on microbial communities is often minimal but depending on the site, it can increase phylogenetic diversity of bacterial communities (Noyce et al. 2016). Furthermore, Qin et al. (2017) established that the lowest observed effect concentration (LOEC) of wood ash on survival and reproduction of Collembola (*Onychiurus yodai*) was 17.4 t ha^{-1}.

A limited number of studies (McTavish et al. 2020; Pukalchik et al. 2018; Singh et al. 2017) have evaluated the effect of biomass ash on earthworms. These studies showed that wood ash has a negative effect on earthworm biomass (McTavish et al. 2020; Singh et al. 2017). However, this effect decreased following the input of organic matter (as a food source) and varied according to the species studied, with the epigeic species *Eisenia fetida* less affected than the anecic species *Lumbricus terrestris* (McTavish et al. 2020; Pukalchik et al. 2018; Singh et al. 2017).

The composition of biomass ash (including P, K and metal content) and corresponding effect varies widely depending on the plant type and the soil on which it was grown (Cruz et al. 2019; Nordin 1994; Vassilev et al. 2013; 2014). Although studies have focussed on the composition of Miscanthus ash (Baxter et al. 2012; Lanzerstorfer 2017; Michel et al. 2012), to our knowledge there have been no studies assessing the impact of Miscanthus ash on soil quality. Given ongoing developments in the commercial-scale growth of Miscanthus and associated increase in ash production, this topic should be considered timely and of real interest.

In this study, we have focused our attention on the effect of Miscanthus ash on earthworms because they represent the largest edaphic zoomass (Gobat et al. 2004), play an important role in soil function (Blouin et al. 2013) and are recognised as key bioindicators in ecotoxicology (Bart et al. 2018; Fründ et al. 2011; Spurgeon et al. 2003). Our work concentrated on *Aporrectodea caliginosa* as this geophagous soil-dwelling species often dominates earthworm communities of temperate agroecosystems (Sims and Gerard 1999) and has been proposed as a model species in toxicity tests (Bart et al. 2018).

The aim of this study was to assess the effect of different concentrations of Miscanthus ash (from a crop grown on uncontaminated soil) on survival, change in biomass, reproduction and avoidance behaviour of *Aporrectodea caliginosa*, and utilise the results to contribute to an assessment of the potential for utilising the ash as a soil conditioner.

Materials and methods

To assess the effects of Miscanthus ash on *A. caliginosa*, three different endpoints were assessed in laboratory experiments: (i) survival and change in biomass, (ii) reproduction, (iii) avoidance. Earthworms were exposed to ash doses ranging from 0 to 50 t ha^{-1} (0, 1, 2.5, 5, 10, 25, 50 t ha^{-1}). The doses where selected based on a range of identified biomass application rates. In the UK the annual limit for wood ash amendment to agricultural land is 1 t ha^{-1} (HMSO, 2014). In Hungary, Füzesi et al. (2015) recommended between 1 and 5 t ha^{-1} for routine agronomic application of wood ash while 5 t ha^{-1} is the recommended maximum application dose in Nordic countries (Huotari et al. 2015) and a French case study (ADEME, DVNAC 2001) recommended wood ash application from 2 to 15 t ha^{-1} every 3 years depending on associated fertiliser applications and heavy metal content of the ash. In addition, ash doses of 25 and 50 t ha^{-1} were selected to establish earthworm responses in soil at levels above current recommended thresholds.

Experimental design

Miscanthus ash was obtained from a crop grown on an unpolluted site: the Phytorestore Bioferme at La Brosse-Montceaux, France (GPS Coordinate 48° 21'8.08'N; 3° 1'24.98 E). The harvested Miscanthus was processed in a biomass plant (Power Corn, Guntamatic) in the RAGT Energie laboratory (Albi, France) and the bottom ash collected. Chemical properties of the ash were determined at the RAGT Energie laboratory (Table 1). All experiments employed Kettering Loam (obtained from Boughton Loam Ltd) as a soil substrate (see Table 1 for physical and chemical properties). The loam is widely used in earthworm research and recommended for culture of temperate soil dwelling species (Brami et al. 2017; Lowe and Butt 2005).

To calculate the weights of Miscanthus ash (MA) required to obtain the different ash doses, i.e. 0, 1, 2.5, 5, 10, 25 and 50 t ha^{-1} (MA0, MA1, MA2.5, MA5, MA10,

Table 1 Physico-chemical characteristics of Miscanthus ash, Kettering Loam and compost used in the experiments

		Methodological standard	Miscanthus ash	Kettering Loam	Compost	Limit values in MTE of the standard NF U44-095
Clay	%		–	24	–	
Silt	%		–	18	–	
Sand	%		–	58	–	
pH	–	NF EN 12176 (1998)	11.8	6.8	4.5	
Organic matter	g kg^{-1}	NF EN 12879 (2000)	42.0	67.2	161.4	
C_{org}	g kg^{-1}	ISO, 10694 (1995)	21.0	–	138.1	
N_{tot}	g kg^{-1}	RAGT intern method	0.2	–	11.8	
Ratio C:N	–		105	–	11.7	
CaO	mg kg^{-1}	NF EN ISO, 11885 (2009)	65,600	–	59,600	
P_2O_5	mg kg^{-1}	NF EN ISO, 11885 (2009)	29,700	–	19,590	
K_2O	mg kg^{-1}	NF EN ISO, 11885 (2009)	58,900	–	5330	
Cd	mg kg^{-1}	NF EN ISO, 11885 (2009)	0.1	–	2.4	3
Cr	mg kg^{-1}	NF EN ISO, 11885 (2009)	3.59	–	89.6	120
Cu	mg kg^{-1}	NF EN ISO, 11885 (2009)	24	–	135.3	300
Ni	mg kg^{-1}	NF EN ISO, 11885 (2009)	1.9	–	55	60
Pb	mg kg^{-1}	NF EN ISO, 11885 (2009)	0.82	–	173.7	180
Zn	mg kg^{-1}	NF EN ISO, 11885 (2009)	73.9	–	542.1	600
Co	mg kg^{-1}	NF EN ISO, 11885 (2009)	0.52	–	–	
B	mg kg^{-1}	NF EN ISO, 11885 (2009)	32.9	–	–	
Fe	mg kg^{-1}	NF EN ISO, 11885 (2009)	1400	–	–	
Hg	mg kg^{-1}	NF ISO, 16772 (2004)	<0.03	–	0.63	2
Mn	mg kg^{-1}	NF EN ISO, 11885 (2009)	978	–	–	
Mo	mg kg^{-1}	NF EN ISO, 11885 (2009)	2	–	–	

n. a. not analysed, *MTE* metal trace elements

MA25 and MA50, respectively), the following equation was used:

$$D_l = \frac{V_l \times D_f}{V_f}$$

Where D_l is the amount of ash applied in the container in the laboratory (g), D_f is the amount of ash applied in the field (g), V_l is the volume of soil in the container in the laboratory (L) and V_f is the volume of soil in the field in an area of 1 ha and at a depth of 10 cm (L) which corresponds to the depth at which ash is ploughed into the soil before crop sowing.

Dry components of the substrate (soil and Miscanthus ash) were homogeneously mixed and water added to obtain a moisture content of approximately 25%, calculated by mass of dry soil equivalent (Lowe and Butt 2005). Substrate pH was assessed in each treatment according to ISO, 10390 (2005) and 5 replicates of each treatment established.

Earthworms were collected from un-polluted pasture at two farms in Preston, UK (53.746°N, 2.682°W and 53.707°N, 2.676°W) and maintained in laboratory cultures as described by Lowe and Butt (2005) prior to experimental use.

For each experiment, earthworms were placed on the soil surface and when all individuals had burrowed into the substrate, the vessel was covered with a plastic lid, pierced with a mounted needle to allow gaseous exchange, and kept in 24 h darkness in a temperature-controlled incubator (LMS Ltd). The survival and biomass experiment was conducted at 10 °C because this temperature is close to the mean annual field temperature in the UK. Reproduction and avoidance experiments were conducted at 15 °C which corresponds to the optimal temperature for *A. caliginosa* activity and cocoon production (Lowe and Butt 2005). At each sampling point earthworm survival was recorded, individuals washed, carefully blotted dry with a paper towel and individually weighed.

Survival and change in biomass

Five Miscanthus ash treatments (MA0, MA1, MA2.5, MA5 and MA10) were established in plastic containers (11 × 11 × 6.2 cm). Sub-adult and adult (clitellate) earthworms with masses ranging from 100 to 1000 mg were used (mean weight: 450 ± 200 mg). Two individuals were randomly selected, weighed and added to the surface of each replicate to ensure that there was no significant difference in the initial mean biomasses between treatments ($p = 0.9618$, One-way ANOVA). To mimic field conditions and to provide organic matter, compost obtained from the Phytorestore Bioferme (La Brosse-Montceaux, see Table 1), was added at a rate of 10 t ha^{-1}. This dose is equivalent to field application rates and provides potassium and phosphorus without exceeding maximum annual input levels stated in the NF U44-095 (2002) standard. Sieved horse manure (1% of dry mass) was incorporated in the substrate as an additional food source. Earthworm survival and individual biomass were recorded after 28, 56 and 84 days.

Reproduction

Six MA treatments (MA0, MA1, MA2.5, MA5, MA10, MA25) were established, following an adapted version of ISO, 11268-2 (2015) utilising the same vessel type as the survival / biomass experiment with the addition of dried and sieved horse manure (2.5%, which is equivalent to 10 g $adult^{-1}$ $month^{-1}$ as recommended by Lowe and Butt (2005)). Adult (clitellate) earthworms (mean weight of 900 ± 300 mg) were used with two individuals of similar mass added to the surface of each replicate (a density more suitable for *A. caliginosa* reproduction than 10 individuals as suggested in the standard (Bart et al. 2018)). The experiment was sampled after 28 and 56 days, at which point cocoons were collected by wet-sieving the substrate following ISO, 11268-2 (2015) and counted. At initial sampling (28 days), adult earthworms were placed into new substrate prepared as described previously.

Avoidance

A two-choice chamber avoidance test, based on ISO, 17512-1 (2008), was performed to evaluate the behavioural (avoidance) response of *A. caliginosa* to 6 MA treatments (MA0, MA2.5, MA5, MA10, MA25, MA50). In this experiment, plastic containers with a volume of 2600 mL (18.5 × 14 × 11 cm) were used. In each vessel, one half of the container was filled with 700 g (to a depth of 9 cm) of treatment soil (spiked with MA) and the other half filled with the same volume of control soil (without ash). During construction, the two sections were separated with a central plastic divider which was removed prior to earthworm addition. Adult (clitellate) *A. caliginosa* (mean weight: 500 ± 200 mg) were used and five individuals (a density considered optimal for the soil volume (Bart et al. 2018)), weighed and placed on the soil surface in the middle of each container. After 48 h the divider was re-inserted and the number of individuals in each side of the container recorded. Avoidance rate A (%) was calculated according to the following equation:

$$A = \left(\frac{n_c - n_t}{N}\right) \times 100$$

Where n_c is the number of worms in the control soil, n_t is the number of worms in the test soil and N is the total number of worms per replicate. Individuals cut by introduction of the divider were attributed as 0.5 per section independent of the length of the two parts. Additionally, each earthworm was washed, carefully blotted dry with a paper towel and individually weighed.

Statistical analysis

Data were analysed with XLSTAT (2014) software. To assess differences between treatments in all experiments, assumptions of normality (Shapiro–Wilk test) and homoscedasticity (Bartlett test) were determined. When validated, a one-way ANOVA followed by Dunnett's post-hoc test was performed. A Kruskal–Wallis test was used when normality and homoscedasticity conditions were not respected, followed by a Dunn test with a Bonferroni correction when differences were significant. Spearman's rank was used to evaluate the relationship between treatments and avoidance response. In addition, results of the two-choice chamber avoidance test were analysed with a binomial test, with R software (R Core Team 3.6.1, 2019), to assess if the observed number of individuals in control and treatment sections differ from a theorical distribution of 0.5.

Results

Survival and change in biomass

The effect of MA treatments on earthworm survival and biomass are presented in Fig. 1. At the end of the experiment, 100% survival was recorded in all treatments. There were no significant differences in mean biomass of earthworms between treatments at day 28 ($p = 0.9217$), day 56 ($p = 0.8276$) and day 84 ($p = 0.6313$). Although differences are not significant, individuals in MA0 and MA1 had the highest mean biomass (0.63 ± 0.19 g, 0.66 ± 0.25 g respectively). Percentage changes in biomass between day 0 and day 84 were higher in MA0 and MA1, compared to other treatments (MA2.5, MA5 and MA10) with 36 ± 46%, 35 ± 24%, 8 ± 18%, 27 ± 16%, 22 ± 20% respectively but no significant differences between treatments were recorded ($p = 0.468$). This trend of decreasing growth rates with increasing ash content over the three-month period may suggest that MA applications from 2.5 t ha^{-1} restrict earthworm development over a longer time period.

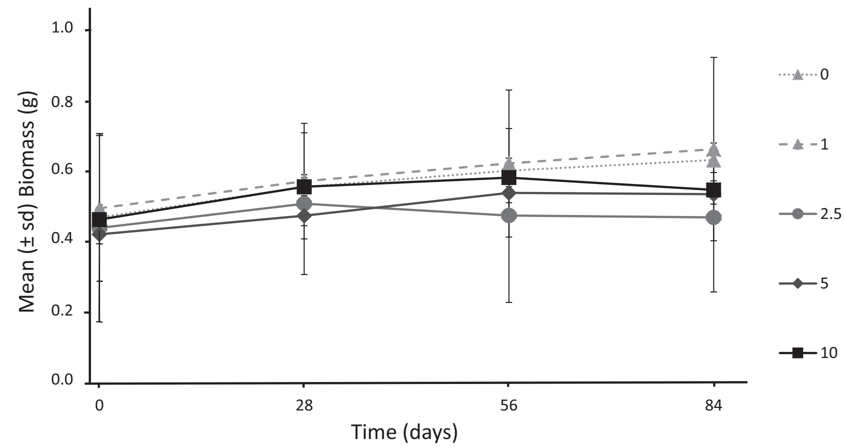

Fig. 1 Mean (± sd) biomass of *A. caliginosa* in MA (Miscanthus ash) treatments (MA0, MA1, MA2.5, MA5, MA10 t ha^{-1}) after 0, 28, 56 and 84 days

Reproduction

No significant differences ($p > 0.05$) in cocoon production were recorded between treatments at each sampling point (Table 2). The lowest survival rate (90%) was recorded in MA25 and is explained by the death of one individual. After 56 days cocoon production was greatest in MA1 and MA2.5 and lowest in MA10 and MA25 treatments.

Table 2 *A. caliginosa* cocoon production after 28 and 56 days and reproduction rate in the MA (Miscanthus ash) treatments (0, 1, 2.5, 5, 10, 25 t ha^{-1}) after 56 days (mean ± sd)

Treatment	Cocoon production		Overall reproduction rate
	Day 28 (No replicate^{-1})	Day 56 (No replicate^{-1})	(Cocoon worm^{-1} day^{-1})
MA0	9.6 ± 6	7 ± 4	0.15 ± 0.08
MA1	12.4 ± 3	7 ± 6	0.20 ± 0.08
MA2.5	10.6 ± 3	10 ± 2	0.20 ± 0.03
MA5	11 ± 4	12 ± 3	0.18 ± 0.06
MA10	8.8 ± 4	8.6 ± 4	0.12 ± 0.06
MA25	8.2 ± 3	4.6 ± 4	0.13 ± 0.05

Avoidance

After 48 h, the percentage of individuals found in the control section at MA0, MA2.5, MA5, MA10, MA25 and MA50 treatments was 54 ± 21%, 60 ± 24%, 62 ± 18%, 68 ± 11%, 84 ± 22% and 100 ± 0% respectively. The number of earthworms located in the control soil increased with increase in MA and significant differences in the number of earthworms located in control and treated sections was recorded for MA50 ($p = 2.98\text{e-}08$) and MA25 ($p = 0.0004$) and was close to being significant in MA10 ($p = 0.0538$).

A positive correlation between increasing ash content and avoidance ($p = 0.0001$, $r_s = 0.66$) is shown in Fig. 2. However, a significant difference in avoidance response (%) was only observed in the MA50 treatment ($p = 0.0013$).

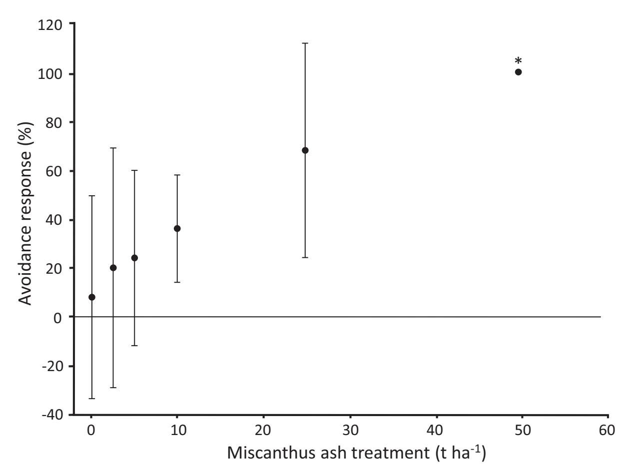

Fig. 2 Mean avoidance rate (±sd) of *A. caliginosa* after 48 h in MA (Miscanthus ash) treatments (0, 2.5, 5, 10, 25, 50 t ha^{-1}), (*statistically significant, $p < 0.05$)

Discussion

Only a few published studies have assessed the impact of ash on earthworms and these have tended to focus on ash from wood (McTavish et al. 2020; Pukalchik et al. 2018; Singh et al. 2017) or coal (Demuynck et al. 2014; Grumiaux et al. 2010; 2007; Muir et al. 2007; Yunusa et al. 2009). In addition, these studies have utilised different forms of ash e.g. fly ash (pulverised fuel ash) (McTavish et al. 2020; Pukalchik et al. 2018) or do not indicate whether it is bottom ash or fly ash (Singh et al. 2017), while our work focuses on bottom ash that are recovered from the boiler ash pan (ESCo 2014). As a result, it is important to recognise that the paucity of previous related research limits the scientific relevance of direct comparison with the current study but also highlights the novelty of the information provided.

Survival and change in biomass

In the survival and biomass change experiment, 100% survival in all treatments was recorded at the end of the experiment suggesting that, under the experimental conditions, Miscanthus ash does not have a lethal effect on *A. caliginosa* even at application rates up to 10 t ha^{-1}.

Biomass increases remained relatively small in all treatments, including the control (from 36 ± 46 to 22 ± 20% for MA0 and MA10 respectively) when compared to the results obtained by other authors for the same species (70 and 50% for Booth et al. (2000) and Owojori et al. (2009) respectively after one month). Different factors may explain these results, such as temperature (Eriksen-Hamel and Whalen 2006), which was 20 °C in Booth et al. (2000) and Owojori et al. (2009), or quality and quantity of the food provided (Bart et al. 2019b), which was grass-meal in Booth et al. (2000) and oat-meal in Owojori et al. (2009). In addition, Booth et al. (2000) used juvenile individuals whose relative growth rates are greater than when earthworms are reproductively active (Mathieu 2018).

In our experiment, biomass tended to increase more rapidly in treatments with the lowest ash content (MA0 and MA1), suggesting a negative effect of ash on *A. caliginosa* development in treatments greater than 1 t ha^{-1}. This negative effect was reported previously in the literature and was explained by the low palatability of ash with Singh et al. (2017) observing that biomass and cast production of *E. fetida* (epigiec species) were lower in soil spiked with wood ash compared to soil spiked with cow dung or rice husks. The negative effects could also be related to an increase in soil electrical conductivity as reported by Owojori et al. (2009) and changes in soil texture due to ash application (Demeyer et al. 2001). *A. caliginosa* is a geophagous species and a change in soil texture may influence feeding behaviour leading to a change in biomass (Baker et al. 1998; Lapied et al. 2009; Singh 2018). In the current study these proposed negative effects did not have a significant impact on biomass and it is suggested that their influence may have been ameliorated/explained by (i) earthworms utilising horse manure as a source of organic matter (food) balancing the low palatability of ash, and (ii) the low amount of ash applied.

Reproduction

According to ISO, 11268-2 (2015), three criteria must be met for the results of the reproduction test to be considered valid. The coefficient of variation in the control must be ≤ 30% (it was 11% in our study), survival rate in control must be ≥ 90% (it was 100%) and the number of juveniles hatched from cocoons produced by adults must be at least 30 per replicate. However, this final condition is not applicable in our study because cocoon production of *E. fetida* /*E. andrei* (species recommended for use in the ISO standard) is between 0.35 and 1.3 per day with an incubation time of 18–26 days (Dominguez 2004; Venter and Reinecke 1988) while it is between 0.1 and 0.4 cocoon per day for *A. caliginosa* with incubation periods of 62–84 days at 15 °C (Bart et al. 2019a; Lowe and Butt 2005; Spurgeon et al. 2000). In our study, results showed that individuals produced from 0.12 to 0.20 cocoon ind^{-1} day^{-1} for treatments MA10 and MA1 respectively in accordance with results of previous authors (Bart et al. 2019a; Lowe and Butt 2005; Spurgeon et al. 2000). Therefore, as the use of endogeic species such as *A. caliginosa* in ecotoxicology testing is increasing, it would be appropriate to update ISO, 11268-2 (2015) to account for species-specific variations in reproductive output.

Miscanthus ash doses between 1 and 25 t ha^{-1} had no significant effect on *A. caliginosa* cocoon production over the 2-month experimental period and did not lead to a difference in cocoon production compared to the control treatment. Related studies have suggested that the effects of ash on earthworm reproduction rates may vary depending on the composition of ash used and the composition of metal trace elements in the ash. It is suggested that, at the doses applied, the effect of ash on MTE content and electrical conductivity of soil (Demeyer et al. 2001) is insufficient to cause a negative effect on cocoon production as reported by Khalil et al. (1996) and Owojori et al. (2009) respectively.

Moreover, our results do not support the positive effect of wood ash (at application rates equivalent to 48 t ha^{-1}) on (*E. fetida*) cocoon production after 60 days reported by Pukalchik et al. (2018). This may be explained by (i) differences in behaviour between epigeic and endogeic species, (ii) the study by Pukalchik et al. (2018) was carried out in a multi-element contaminated soil and the beneficial effect of wood ash was attributed to its ability to immobilise trace metal elements, reducing stress on the earthworms.

In the current study, the application rates of ash from Miscanthus cultivated on a non-polluted site did not detrimentally impact soil conditions sufficient to influence *A. caliginosa* fecundity. It might be expected that similar results would be obtained from Miscanthus cultivated on polluted sites as pollutant transfer from soil to above ground biomass is relatively low (Nsanganwimana et al. 2014), but further investigation is necessary to confirm this assumption.

Avoidance

The avoidance test, originally developed by Yeardley et al. (1996), is known to yield a detectable response more quickly and at lower levels of contamination than traditional endpoints such as survival or reproduction (Hund-Rinke and Wiechering 2001; van Gestel 2012; Yeardley et al. 1996). Our results showed, after 48 h, a positive correlation between MA treatments and avoidance behaviour of *A. caliginosa*. Moreover, individuals avoided the ash treatment in MA25 ($p = 0.0004$) and the number of individuals found in control soil was greater than 80% in this treatment which means that this soil has a limited habitat function, indicating that the conditions are not suitable for establishment of this species (ISO, 17512-1 (2008), Dazy et al. 2009). This could be associated with an increase in soil pH from 7.8 in MA0, MA2.5, MA5, MA10 to 8.2 and 8.4 in MA25 and MA50 treatments respectively. Earthworms are highly sensitive to changes in pH (Muir et al. 2007) and Edwards and Lofty (1975) recorded that *A. caliginosa* has an optimal pH range between 5.0 and 6.0 with a decreasing abundance when pH was below or above these values. Therefore, the increase of 0.4 pH points in the MA25 treatment could be responsible for the avoidance response as Chan and Mead (2003) recorded that a pH increase of 0.5 in acidic soil doubled the abundance of *Aporrectodea trapezoides*. Other soil parameters that are modified by ash input may have also influenced the avoidance response such as electrical conductivity, which is positively correlated with the avoidance behaviour of *A. caliginosa* (Owojori and Reinecke 2009) and increases with the addition of wood ash (Demeyer et al. 2001). Moreover, according to Riehl et al. (2010) the detrimental effect of ash on earthworms could be linked to an increase in soil water retention and a change in soil structure and texture. Indeed, Demuynck et al. (2014) recorded that changes in soil texture and increased MTE content associated with the application of 233 t ha^{-1} of coal fly ash to contaminated soil resulted in *E. fetida* avoidance behaviour greater than 70%.

In contrast to the study by Demuynck et al. (2014) our work revealed 100% avoidance with an ash dose of 50 t ha^{-1}. This could be explained by an increased sensitivity of the soil-dwelling geophagous *A. caliginosa* to changes induced by MA, such as osmotic stress (Qin et al. 2017), when compared with *E. fetida*, which lives on the soil surface and feeds predominantly on organic litter.

Synthesis and outlook

As a synthesis, we have demonstrated that ash from Miscanthus cultivated on non-polluted soil had no significant effect on survival, biomass and reproduction at the studied MA doses, but had an avoidance effect from 25 t ha^{-1} leading to a loss of soil habitat function. However, it is notable that this rate of Miscanthus ash application is higher than recommended values for supplementing nutrients (phosphorous, potassium) in fertilising cereal crops. For example, wheat crops which represents 15% of European agricultural land (Fertilisers Europe 2018) has phosphorus and potassium requirements of 45 and 40 kg ha^{-1} respectively for a yield target of 80 t ha^{-1} in nutrient poor soil (COMIFER, 2009). With the MA used in our study, these nutrient levels would be met from an application rate of 3.5 t ha^{-1}. Therefore, our results suggest that the amount of MA applied to fertilise crops is likely to be lower than the values eliciting limited habitat function (25 t ha^{-1}). In addition, an input of 3.5 t ha^{-1} of Miscanthus ash does not appear to be detrimental to earthworm survival and is lower than the 10 t ha^{-1} dose from which there is a non-significant trend towards a decrease in cocoon numbers. However, care should be taken because, even if it was not significant, 3.5 t ha^{-1} is higher than the 2.5 t ha^{-1} dose at which there was a tendency for the growth of individuals to stall after three months.

Ash may also be used for purposes other than mineral fertilisation, such as increasing soil pH (Demeyer et al. 2001) or aided-phytostabilisation (Lopareva-Pohu et al. 2011) when applied at substantially larger amount of ash (up to 233 t ha^{-1} (Lopareva-Pohu et al. 2011)). The most common type of ash used in aided-phytostabilisation studies is coal ash (Demuynck et al. 2014; Grumiaux et al. 2015; 2010; Leclercq-Dransart et al. 2018; Lee et al. 2014; Lopareva-Pohu et al. 2011) but the predicted increase in the production of biomass ash and decrease in the use of coal in energy generation may result in its use in such projects. As a result, careful consideration should be given to the amounts of ash applied and the potential negative impact on earthworm communities which can play a beneficial role in soil remediation and are recognised as ecosystem engineers (Blouin et al. 2013).

Our study provides novel data on the effect of Miscanthus ash on the soil dwelling earthworm *A. calignosa*. In order to determine the threshold value of ash inducing a loss of soil habitat function it is suggested that a laboratory-based multi-section avoidance test (e.g. linear avoidance test developed by Lowe et al. (2016)) and associated field-based studies are implemented. This would allow more realistic assessment of behaviour by taking into account (i) the heterogeneity of the distribution of ash after field application and (ii) extended time periods, allowing monitoring of the effect of the “flush” of alkalinity, nutrients, and potentially stressful metals and other salts that follow ash amendment (Aronsson and Ekelund 2004). Furthermore, the cumulative effect of repeated Miscanthus ash application should be investigated to ensure that there is not a deleterious effect on soil habitat function.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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