



RESEARCH ARTICLE

Safety of black soldier fly (*Hermetia illucens*) larvae reared on waste streams of animal and vegetal origin and manure

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Abstract

In Europe, commercial and scientific interest in black soldier fly larvae (BSFL, *Hermetia illucens*) as a new feed source has grown substantially, primarily because this species can be reared on waste-streams which are otherwise unsuitable. However, before BSFL may legally be reared on such materials, and subsequently fed to livestock animals, safety must be guaranteed. Many hazards could be relevant, depending on the origin of the waste stream. Small- and large-scale experiments were performed in which BSFL were reared on the organic wet fraction of municipal household waste (OWF), kitchen/fast food waste (FFW), mushroom feet stems (MF), pig manure liquid slurry mixed with roadside silage grass (PMLSG), pig manure solids (PMS), and secondary sludge from slaughter waste (SW). Larval yields were highest on the control (chicken feed + water) and the FFW. Substrates and larvae were analysed to determine the presence of heavy metals, acrylamide, pesticides, veterinary drugs, and pathogenic bacteria. Cadmium (Cd) bioaccumulated in larvae reared on all substrates, in line with previous research. Some pesticides and veterinary drugs were found in the substrates: concentrations in the larvae were low, but potential formation of metabolites could be studied further. Acrylamide was present in the FFW, but not in the larvae reared on it: more research is needed to determine the (metabolic) fate. *Bacillus cereus* and traces of *Salmonella* spp. were found on some larval samples, but appropriate processing is anticipated to minimize potential risks. Based on these results, we conclude that most tested substrates are suitable for rearing BSFL, and do not appear to present major safety concerns, aside from the need for monitoring Cd concentrations in the substrates, and control measures for pathogenic bacteria. Further verification to account for variance in contamination of substrates is needed for definitive conclusions on the safety.

Keywords

insects – residual streams – circularity – contaminants – pathogens

1 Introduction

Black soldier fly larvae (BSFL, *Hermetia illucens* (L.); Diptera: Stratiomyidae) have shown the capacity to convert low quality organic waste into protein-rich ingredients for feed use (Bosch *et al.*, 2019; Nyakeri *et al.*, 2017; Spranghers *et al.*, 2017). Underutilized biowaste streams could thus be valorised when used as a source of valuable nutrients for these insects. However, such streams may also contain chemical residues which could bioaccumulate in the insects (Meyer *et al.*, 2021), or pathogenic bacteria which could be transferred (Vandeweyer *et al.*, 2021). These feed safety hazards are primarily dependent on the insects' substrate: for example, residues of antibiotics and antiparasitic drugs are regularly found in pig or chicken manure and can be present in slaughterhouse waste (Berendsen *et al.*, 2015; Hoek van den Hil *et al.*, 2022; Massé *et al.*, 2014). Pesticides residues can be found in organic waste of, for instance, vegetable origin (Kuchheuser and Birringer, 2022; Taube *et al.*, 2002). Current European Union (EU) legislation restricts the use of biowastes containing animal products, originating from catering or commercial kitchens formulations, as feed source (Regulation (EC) No 1069/2009), and prohibits the use of manure for all livestock rearing (Regulation (EC) No 767/2009). Therefore, necessary feed safety data must be gathered to pave the way for possible legalization of several of these underutilized streams.

Previous research has shown a tendency of BSFL to bioaccumulate heavy metals from their substrate: high bioaccumulation factors were observed for cadmium (Cd) in particular (Biancarosa *et al.*, 2018; Diener *et al.*, 2015; Van der Fels-Klerx *et al.*, 2016; Van der Fels-Klerx *et al.*, 2020). Acrylamide is a processing contaminant which is formed when products rich in starch and free amino acids such as proteins (e.g. French fries) are fried at high temperatures (Baskar *et al.*, 2018; Taubert *et al.*, 2004). Acrylamide is classified as potentially carcinogenic (EFSA, 2015). To date, research on the bioaccumulation and/or effects of acrylamide on commercially reared BSFL has not been performed. Subsequently, pesticides may be present in animal feed commodities such as maize and wheat (Kumar *et al.*, 2019), as well as in organic vegetal waste (Kuchheuser and Birringer, 2022; Taube *et al.*, 2002); and veterinary drugs may be present in manure and slaughterhouse waste (Hoek van den Hil *et al.*, 2022). Finally, all animal feed products are, in principle, subject to microbiological contamination – which may include a variety of human pathogens such as *Salmonella* spp., *Listeria monocytogenes*, and *Bacil-*

lus cereus (Vandeweyer *et al.*, 2021). The objective of this study was to investigate the suitability (in terms of conversion efficiency and safety) of several types of waste streams with the potential to be used as feed for commercially reared BSFL, as a feed ingredient for food producing animals. To this end, an experiment was conducted on small- and large-scale to gain more insight into the potential of BSFL to be safely reared on a variety of organic biowaste streams.

2 Materials and methods

The experiment was conducted on small- and large-scale (abbreviated as SS and LS, respectively). The same diet was used for both experiments. Seven-days-old BSFL were reared on six biowaste sources for seven days: the organic wet fraction of municipal household waste (OWF), kitchen/ fast food waste (FFW), mushroom feet stems (MF), pig manure liquid slurry mixed with roadside silage grass (PMLSG), pig manure solids (PMS), secondary sludge from slaughter waste (SW), and a control feed consisting of commercial chicken feed. All substrates are brought to 35% dry matter (DM) by adding tap water and/or cellulose/wood shavings to decrease or increase the DM in the respective substrates. For the small-scale experiment, larvae were manually counted to obtain $n = 100$ larvae, which were reared in insect breeding dishes (cylindrical dishes with a diameter of 100 mm \times 40 mm (height) with a ventilation cap of 40 mm, Novolab NV, the Netherlands) at 28 °C, 70% humidity, and a 12:12 hr light regime. Per treatment, three biological replicates/dishes were used. After seven days, insects were manually counted, washed, gently dried with a paper tissue and weighted. Substrates were weighted per dish at the start and at the end of the experiment. All samples for microbiological analyses were collected in glycerol (40%), which was used as a cryoprotectant to stabilise the frozen bacteria and to keep them alive by preventing damage to the cell membranes (Almeida *et al.*, 2017; Urbanek *et al.*, 2020). This step allowed for an extended period of storage prior to analysis, although it must be noted that this may have affected the accuracy of the reported total microbial counts. All samples per replicate, including the harvested insects, substrate (pre-experiment) and frass (post-experiment) were stored at -20 °C for further analysis. For the large-scale experiment, 1850 larvae were reared for seven days in crates (75 \times 47 \times 15 cm) with 10 kg substrate, as described by Naser El Deen *et al.* (2023).

TABLE 1 Overview of analyses performed for samples of each treatment, prioritized according to anticipated presence of each hazard category / waste stream

Treatment	Heavy metals	Acrylamide	Pesticides	Veterinary drugs	Pathogens
Control (chicken feed)	×	×	×	×	×
Organic wet fraction of municipal household waste (OWF)	×		×		×
Kitchen/ fast food waste (FFW)	×	×			×
Mushroom feet stems (MF)	×				×
Pig manure liquid slurry mixed with roadside silage grass (PMLSG)	×			×	×
Pig manure solids (PMS)	×			×	×
Secondary sludge from slaughter waste (SW)	×			×	×

The results of the large-scale study – in terms of the exact diet nutrient composition and larval performance, larval yield and composition – were discussed by Naser El Deen *et al.* (2023). The focus of this article is on food and feed safety, therefore all the results of the safety analyses of both experiments will be discussed in this paper. For the small-scale study, measures of larval performance in terms of yield and survival will be presented in this paper. Survival was defined as the number of larvae at the end of the experiment for every replicate, as a percentage of the $n = 100$ larvae added at the start. Larval yield was defined as the mean individual biomass weight of larvae per replicate after cleaning and drying, so as to remove the adhering frass.

Samples were analysed to determine the presence and concentrations of heavy metals, acrylamide, pesticides, veterinary drugs, and pathogenic bacteria. Not all analysed hazards are relevant for all types of tested substrate. The analyses were therefore prioritized for the relevant diets according to anticipated presence of each hazard in the waste streams as explained in the introduction (see Table 1). All analyses were performed with $n = 3$ samples for each of the three matrices (substrate; SS larvae; LS larvae).

Heavy metals

Substrate and larval samples of all treatments in the SS and LS experiment were analysed for heavy metals. Samples were analysed for the heavy metals Cd, arsenic (As), lead (Pb) and mercury (Hg). All chemicals used for elemental analysis were of analytical grade. Concentrated nitric acid (HNO_3 , 67-70% RS Superpure for trace analysis, Carlo Erba, Val de Reuil, France) was used for the digestion of the samples. External calibration curves for Hg were constructed using a Hg standard

solution (Merck, Darmstadt, Germany) and for As, Cd and Pb using a mixed standard solution (Quality control standard 21, Perkin Elmer, Waltham, MA, USA). Palladium (II) nitrate ($\text{Pd}(\text{NO}_3)_2$) and magnesium nitrate ($\text{Mg}(\text{NO}_3)_2$) solutions (Merck) were used for production of the matrix modifier for atomic absorption spectrometry (AAS) measurements. Tin (II) chloride dihydrate (SnCl_2), used as a reducing agent during cold-vapor atomic fluorescence spectrometry (CV-AFS) measurements, was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Samples were weighed with 0.8 g for substrate and 0.5 g for larvae into TFM microwave vessels (CEM Corporation, Matthews, NC, USA). After addition of 10 ml of concentrated HNO_3 , samples were digested using a microwave oven (MARS express, CEM Corporation) at a maximum temperature of 210 °C. The digests were quantitatively transferred to 50 ml polypropylene centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) and diluted with de-ionized water to a final volume of 50 ml. The determination of Cd, Pb and total As concentration was performed using an electrothermal atomic absorption spectrophotometer (ETAAS, AAnalyst 800, Perkin Elmer, Waltham, MA, USA), equipped with a graphite furnace and Zeeman background correction system. Cd, Pb and As were measured at wavelengths of 228.8; 283.3 and 193.7 nm, respectively. To improve the analytical measurements a 0.1% Pd and 0.12% $\text{Mg}(\text{NO}_3)_2$ matrix modifier was used. Hg concentrations were determined using cold vapor atomic fluorescence spectroscopy (CV-AFS, Mercur, Analytik Jena, Jena, Germany). Ionic Hg is reduced to gaseous elemental Hg using SnCl_2 . Hg atoms are excited using a Hg lamp at a wavelength of 253.7 nm and subsequent fluorescence is detected at the same wavelength. ERM-CE278k

muscle tissue, BCR 482 lichen and BCR 032 Moroccan phosphate rock (all obtained from Institute for Reference Materials and Measurements, IRMM, Geel, Belgium) were used as certified reference materials for quality control. The limit of quantification (LOQ) was 0.0093 mg/kg for Cd, 0.038 mg/kg for Pb, 0.1 mg/kg for As and 0.0038 mg/kg for Hg.

For heavy metals, the bioaccumulation factor was calculated as described by Van der Fels-Klerx *et al.* (2016), as the concentration in the larvae divided by the concentration in the substrate, with both concentrations normalized for an equivalent dry matter content of 12%.

Acrylamide

Substrate, frass, and larval samples of the control and FFW treatments in the LS and SS experiment were analysed for acrylamide. Acrylamide ultra-pure [CAS No. 79-06-1] was obtained from VWR (Amsterdam, the Netherlands). The labelled Acrylamide-d3 was purchased from Sigma-Aldrich Netherlands. The dispersive solid phase extraction (d-SPE) columns containing 900 mg magnesium sulphate (MgSO₄) + 150 mg Primary Secondary Amine (PSA) were from Waters. The UPLC water used was purchased at Actua-chemicals (the Netherlands). The acetonitrile (ACN) came from Biosolve (Valkenswaard, the Netherlands). Ammonium acetate (NH₄CH₃CO₂) and the sodium chloride (NaCl) from Merck (Søborg, Denmark). The acetic acid (CH₃COOH) was from Merck (Darmstadt, Germany) and the MgSO₄ was from VWR chemicals (Leuven, Belgium). All stock solutions of acrylamide and acrylamide-d3 were prepared in closed brown bottles (1 mg/kg; 10 µg/kg; 1 µg/kg; 100 ng/kg) in 50% or 90% ACN with 1% CH₃COOH and stored at 4 °C. The calibration standards (2.5-100 ng/ml) were prepared fresh before analyses.

From each sample, 1 g was taken in a centrifuge tube and 25 ml of 10 µg/ml acrylamide-d3 as internal standard and 5 ml of UPLC water was added and vortexed shortly. The samples were left to rest for 10 min before adding 10 ml of ACN and vortexing again. The samples were horizontally shaken for 15 min at 200 bpm. Then 1 g of NaCl and 5 g of MgSO₄ were added to each sample and were centrifuged for 5 min at 4,000 rpm and 10 °C (Rotica from Hettich Zentrifugen, Dusseldorf, Germany). The top layer of approximately 10 µl of the samples was transferred to a clean glass tube and evaporated under a nitrogen flow until 1.5 ml. Then 0.5 ml of acidified ACN was added. The final step of the extraction was a clean-up. This clean-up was performed on a disperse SPE Colom, which contained 900 mg MgSO₄ and

150 mg PSA. The 2 ml liquid samples in glass tube were transferred to the d-SPE column, shaken firmly and centrifuged for 5 min at 3,000 rpm at 10 °C. In a LC-MS vial with insert, a final amount 0.3 ml clear sample was transferred and ready for analysis.

For the separation of acrylamide, an Acquity UPLC BEH Hilic column, 1.7 µm 2.1 × 150 mm (Waters, Etten-Leur, the Netherlands) was used on a Shimadzu UPLC system (Shimadzu Benelux, 's-Hertogenbosch, the Netherlands). The injection volume of the samples was 2 µl which was eluted with a gradient which was initially set at 3% of 0.1% CH₃COOH, 1 gr NH₄CH₃CO₂ in water and 97% ACN and was brought to 50% of each within 2.5 min and remained at 50% until 5 min runtime. Finally, the gradient was brought back to its initial settings in the last 3 min. A valve was used to set a selected time window for MS measurement of acrylamide, to prevent contamination of the MS system from matrix components.

The MS-MS detection was realized on a SCIEX 6500 with Analyst software (Sciex, Nieuwerkerk aan den IJssel, the Netherlands). The electrospray was set in the positive ion mode with the capillary voltage of 3.5 kV, the collision energy was set at 30 to 16 V and the source temperature at 700 °C. There was 20 psi curtain gas used and the declustering potential was set at 60 V. The multiple reaction monitoring (MRM) mode of the fingerprint patterns *m/z* 72 → 55 *m/z* 72 → 27 (acrylamide) and *m/z* 75 → 58 (acrylamide-d3) were used for quantification. The results were generated with the MultiQuant software incorporated with the Sciex system.

Pesticides

Control and OWF substrate samples were screened for the presence of active substances of pesticides by means of an accredited GC-MS/MS and LC-MS/MS based procedure. The larval samples were thereafter analysed for the active substances positive in the substrate samples by an LC-MS/MS based procedure. Lists with the 268 active substances and metabolites thereof in scope for both methods are provided in Supplementary Tables S1 and S2 for the GC (88 substances) and LC-MS/MS (180 substances), respectively.

The QuEChERS based substrate extraction procedure and analysis by LC-MS/MS and GC-MS/MS were described by Meijer *et al.* (submitted) with a slight modification concerning the applied LC-MS/MS equipment. LC-MS/MS identification and quantification was performed by using a Waters Acquity LC system (Etten-Leur, the Netherlands), and a Sciex (Framingham, MA, USA) 6500 triple quad MS using multiple reaction mon-

itoring (MRM) in both the positive and negative mode. Separation was obtained using a Waters (Etten-Leur, the Netherlands) HSS-T3, 1.8 μm , 2.1 \times 100 mm UPLC column and a water/95% methanol (MeOH) gradient (both with 5 ml 1M ammonium formate (NH_4HCO_2) and 1 ml formic acid (FA, HCOOH)) with a cycle time of 14 minutes, a flow of 0.3 ml/min and a column temperature of 35 \times . Five μl of sample extract, reference solution and quality control solution was injected. The MS/MS was operated with collision gas: -2, curtain gas: 20 psi, ion source gas 1: 55 psi, ion source gas 2: 55 psi, an ion spray of 5,500 V (-4,500 V in negative mode) and a source temperature of 500 $^\circ\text{C}$.

Veterinary drugs

Substrate, frass, and larval samples of the control, PMLSG, PMS, and SW treatments in the LS and SS experiment were analysed for the presence of residues of the veterinary medicines, belonging to the classes of antibiotics and antiparasitic compounds (see Supplementary Table S3 for all analysed compounds). Extraction and analysis was done using an LC-MS/MS method, as described in Hoek van den Hil *et al.* (2022). The antibiotic analysis was performed with an Acquity UPLC system, connected to a Waters TQXS (Waters, Milford, MA, USA). Chromatographic separation was carried out with an ACQUITY UPLC HSS T3 2.1 \times 100 mm, 1.8 μm analytical column (Waters, Milford, MA, USA), this was placed in a column oven at 40 $^\circ\text{C}$. The mobile phases applied were 2 mm NH_4HCO_2 and 0.016% FA in water (Solvent A) and 2 mm NH_4HCO_2 and 0.016% FA in MeOH (Solvent B). The flow rate was 0.4 ml/min, the gradient was 0-1 min, 0% B, 1-2.5 min, followed by a linear increase to 25% B, 2.5-5.4 min, then a linear increase to 70% B, 5.4-5.5 min, and a linear increase to 100% B and a final hold of 1 min and 1 min equilibration. The injection volume was 5 μl . Transitions of compounds were as described previously in Berendsen *et al.* (2015), Janssen *et al.* (2019) and Hoek van den Hil *et al.* (2022), in addition to details in Supplementary Table S4 for Oxytetracycline. Data processing was done using Masslynx 4.2 software (Waters, Milford, MA, USA). The response of the compounds was corrected using the corresponding isotopically labelled internal standards. A matrix calibration curve consisting of aliquots (2 g) of blank material (insects, substrate or frass) were spiked at relevant levels. For insects, the range was 0-0.1 mg/kg, for frass this was 0-0.1 mg/kg. The limit of quantification for oxytetracycline was 1 $\mu\text{g}/\text{kg}$ for insects and 10 $\mu\text{g}/\text{kg}$ for substrate and frass matrix. Antiparasitic analyses were identical to Hoek van den Hil *et al.* (2022).

Pathogenic bacteria

Substrate and larval samples of all treatments in the LS experiment were analysed for pathogenic bacteria. After sampling, material was suspended in glycerol as described in section 2 above and did not undergo further processing prior to analysis. Samples were analysed for *Salmonella* spp., *Listeria monocytogenes*, and *Bacillus cereus*, using dedicated accredited methods for food and/or feed matrices. Methods for *Salmonella* spp. and *Listeria monocytogenes* were equivalent to ISO 6579-1 and ISO 11290-2, respectively. The first component of the analysis of *Bacillus cereus* consisted of a horizontal method for the enumeration of presumptive *Bacillus cereus*, in accordance with ISO 7932. Secondly, bacteriological isolates were confirmed using a MALDI Biotyper system (Bruker, Leiderdorp, the Netherlands).

Statistical analysis

GraphPad Prism 5 (version 5.02, Graphpad Software, Inc., San Diego, CA, USA) was used for statistical analysis. One-way analysis for variance (ANOVA) followed by a Bonferonni's Multiple Comparison Test was used to compare the larval weight of all treatments. Two-way ANOVA followed by a Bonferonni's post-test was used to compare the concentrations found in the larvae or frass with the concentrations found in the respective substrates.

3 Results

Larval yield

All results of larval performance in the SS experiment are shown in Supplementary Table S5. For comparison, the mean individual larval fresh weight of the LS experiment as reported on by Naser El Deen *et al.* (2023) is also included in that table. Results for the SS experiment, in terms of larval yield, are shown in Figure 1. The mean larval weight for the control was significantly the highest with a value of 128 ± 33 mg, followed by the FFW with a value of 81 ± 39 mg. The mean larval weight for the remaining five substrates (OFW, MF, PMLSG, PMS, SW) was between 24 and 39 mg, of which PMLSG and SW were significantly lower than the other three. Survival was at or near 100% for all treatments (98 ± 3.5). Some differences were observed in mean individual larval weight between the SS and LS experiment: the FFW seemed to perform better than the control in the LS experiment.

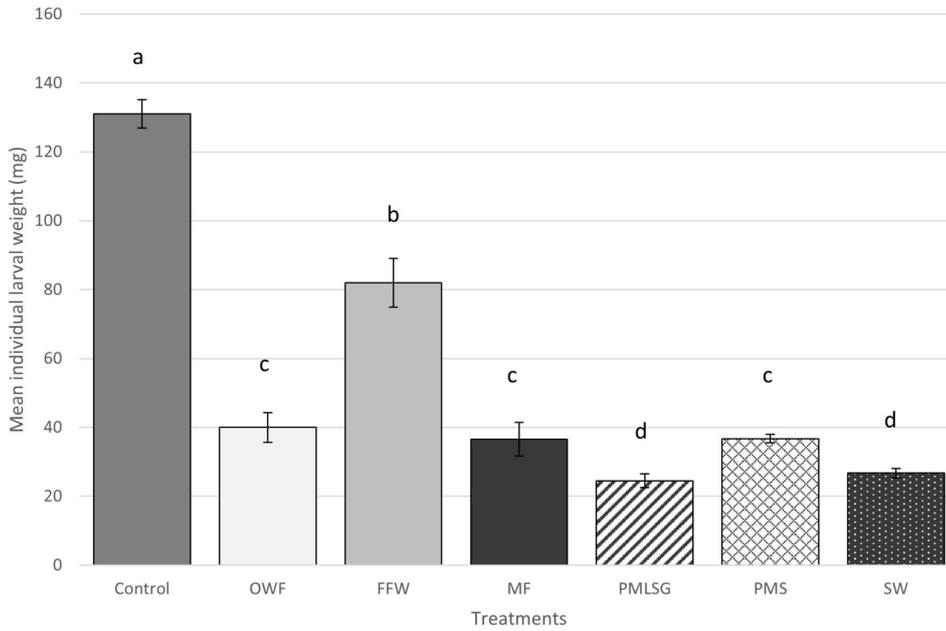


FIGURE 1 Mean individual larval fresh weight (mg) of black soldier fly larvae (BSFL, *Hermetia illucens* (L.) Diptera: Stratiomyidae), after rearing on selected substrates. Treatments: organic wet fraction of municipal household waste (OWF), kitchen rests / fast food waste (FFW), mushroom feet stems (MF), pig manure liquid slurry mixed with roadside silage grass (PMLSG), pig manure solids (PMS), and secondary sludge from slaughter waste (SW). Data were presented as mean \pm SD (standard deviation) ($n = 3$), different letters indicate statistical differences between the treatments ($P < 0.05$).

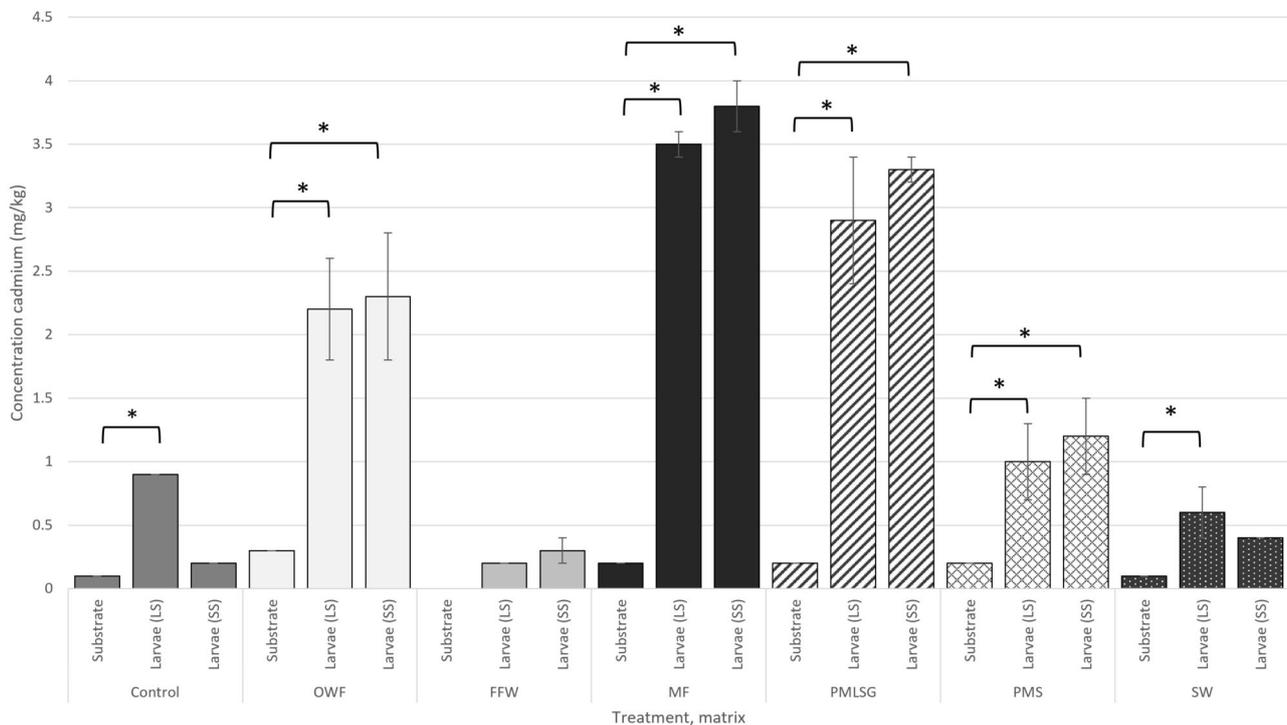


FIGURE 2 Concentrations (mg/kg corrected for 12% moisture content) of Cd in different analysed matrices (substrate, and black soldier fly larvae of the large-scale (LS) or small-scale (SS) experiment), for all treatments: organic wet fraction of municipal household waste (OWF), kitchen rests / fast food waste (FFW), mushroom feet stems (MF), pig manure liquid slurry mixed with roadside silage grass (PMLSG), pig manure solids (PMS), and secondary sludge from slaughter waste (SW). Data were presented as mean \pm SD ($n = 3$). Asterisks indicate statistical different concentrations found in the larvae compared to the respective substrates ($P < 0.05$).

Heavy metals

Figure 2 shows the Cd concentrations corrected for the dry matter content in the substrate and larvae reared

on all tested substrates, from both experiments. The dry matter contents used to correct for concentrations,

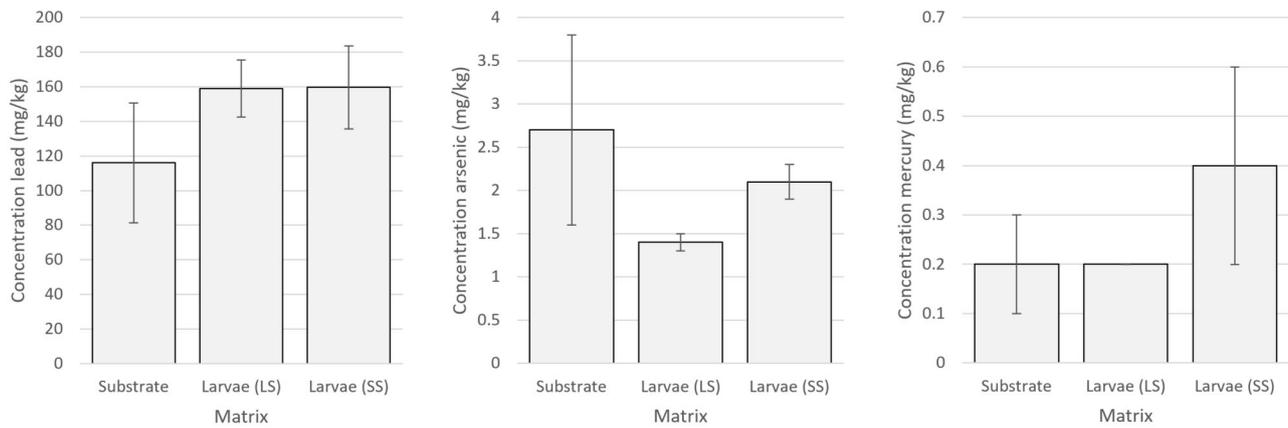


FIGURE 3 Concentrations (mg/kg corrected for 12% moisture content) of Pb, As, and Hg in different analysed matrices (substrate, and larvae of the large-scale (LS) and small-scale (SS) experiment), for the treatment organic wet fraction of municipal household waste (OWF). Data were presented as mean \pm SD ($n = 3$). No statistical differences were found comparing concentrations found in the larvae with the respective substrates ($P < 0.05$).

are shown in Supplementary Table S6. Concentrations found in the larvae were significantly higher than the concentrations in the substrates, implying bioaccumulation of Cd in the larvae, except for the treatments FFW, small scale SW, and small scale control. Comparatively high mean bioaccumulation factors (BAFs) were found in both experiments: especially so ($BAF > 10$) for the MF (LS: 16.6; SS: 17.8) and PMLSG (LS: 13.5; 15.1) treatments. For the control treatment, the mean Cd BAF in the LS experiment was relatively high at 14.8, compared to 4.2 in the SS experiment.

For all substrates except the OWF, As, Hg and Pb could not be detected ($<LOQ$) or concentrations were relatively low. The same was found for concentrations of these heavy metals in the corresponding larvae. As such, only the Pb, As, and Hg concentrations in the OWF treatment are shown in Figure 3. No statistical differences were found of the concentrations in the larvae compared to the respective substrates. The BAF was around 1 for As, Pb and Hg.

Acrylamide

All results of the acrylamide analyses are shown in Supplementary Table S7. Acrylamide could not be detected ($<20 \mu\text{g}/\text{kg}$) in the control substrate samples but was present at $117.4 \pm 2.8 \mu\text{g}/\text{kg}$ in the FFW substrate samples. However, acrylamide could not be detected in the larvae reared on the control and FFW substrate, implying no bio-accumulation occurred.

Pesticides

The results of the pesticide analyses in the control and OWF treatments are shown in Figure 4 and Table S7. In the substrate of the control treatment, three different

substances were detected, compared to 15 substances in the OWF – but there were no similarities in identified substances between the two types of substrates. Despite the comparatively elevated concentrations of diazinon and permethrin in the OWF substrate, neither these nor any of the analysed pesticides exceeded the LOQ in the larval samples.

Veterinary drugs

Of the antibiotics and anti-parasitic compounds analysed in the substrates (control, PMLSG, PMS, and SW treatments) and corresponding larval samples of both experiments, only oxytetracycline was detected, and this compound was only detected in the two treatments containing pig manure (PLMSG, PMS) in the SS and LS experiment. The concentrations found in the larvae were significantly lower than the concentrations found in the substrates, implying no bioaccumulation of oxytetracycline in the larvae. These results are shown in Figure 5 and Supplementary Table S7. Mass balance calculations showed that the total amount of oxytetracycline (μg) in larvae and frass (post-experiment), as a percentage of amount of oxytetracycline in substrate (ante-experiment), was 52 ± 5 and $78 \pm 15\%$ for PLMSG and PMS in the SS experiment, respectively. These findings imply that oxytetracycline could be metabolized or degraded, since less than 100% of the amount of oxytetracycline as present in the substrate was recovered in the larvae and frass combined.

Pathogenic bacteria

Results of microbiological analyses are shown in Supplementary Table S7. The pathogen *Listeria monocytogenes* was neither detected in the substrate nor on

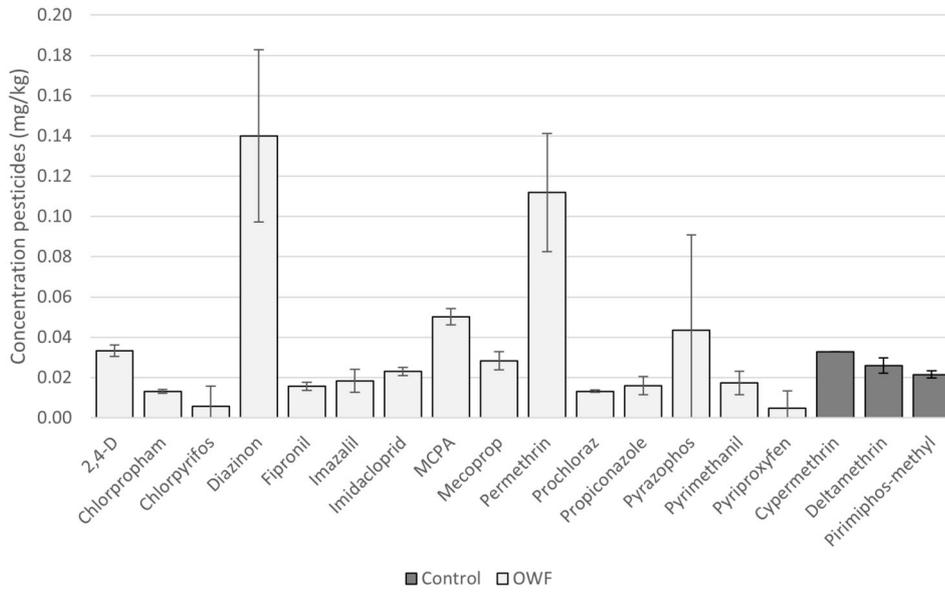


FIGURE 4 Mean concentrations (mg/kg in fresh samples) of detected pesticides in substrates of the control and organic wet fraction of municipal household waste (OWF) treatments. Data were presented as mean \pm SD ($n = 3$).

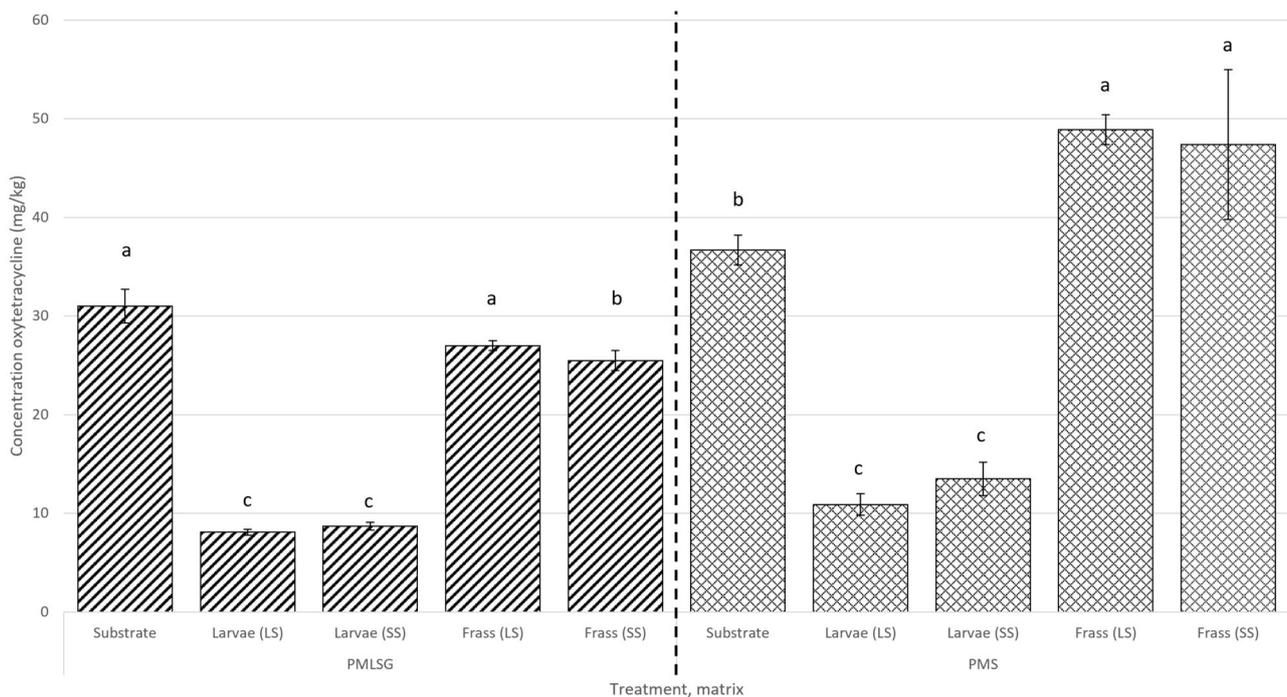


FIGURE 5 Concentrations ($\mu\text{g}/\text{kg}$ in fresh samples) of oxytetracycline in the substrate, larvae, and frass, for the treatments pig manure liquid slurry mixed with roadside silage grass (PMLSG) and pig manure solids (PMS). Larval concentrations of the large-scale (LS) and small-scale (SS) experiment. Data were presented as mean \pm SD ($n = 3$). Letters indicate statistical different concentrations found in the larvae or frass compared to the respective substrates ($p < 0.05$).

the larval samples. *Salmonella* spp. was found and confirmed in the PMS substrate, but only in one corresponding larval sample. Although *Salmonella* spp. was not found in the SW substrate, its presence was confirmed on one larval sample as well. *Bacillus cereus* was found in OWF and MF ($n = 1$) substrates as well as on

the larvae of those treatments, but also on the larvae of the control ($n = 2$) and FFW ($n = 1$) – despite its absence in the substrate of those treatments.

4 Discussion

BSF larvae were able to grow on all different biowaste streams tested in this study, although for all tested treatments in the small-scale experiment, the larvae performed sub-optimally compared to the control treatment, with only the FFW treatment nearing a similar larval yield. However, this difference was less pronounced in the large-scale experiment as reported by Naser El Deen *et al.* (2023), in which the mean individual larval weight of the control was approximately half that of the small-scale experiment, while the larvae in the FFW treatment performed better than the control treatment. The same batch was used for all substrates and for the larvae used in both large-scale and small-scale experiment, and other rearing conditions were also kept identical. Yakti *et al.* (2022) reported that differences in scale affected the speed of larval growth: this could also explain the differences observed in the two experiments reported on in this article.

Interestingly, data of the safety analyses were largely similar between the two experiments, which suggests that safety results can be confidently applied to larger scale. Nonetheless, any differences highlight some need for caution in extrapolating results of small-scale experiments to commercial conditions. In addition, the scope of this study is limited to hazards which were inherently present in the tested substrates: due to variability in the origins of the substrate material, the presence and concentrations of (other) contaminants in these substrates are unknown and depend on the specific source.

Concentrations of heavy metals As, Hg and Pb were not detected or very low for most of the tested substrates and corresponding reared larvae – with the exception of concentrations in the OWF. Maximum levels (MLs), relative to a feed with a moisture content of 12%, have been laid down in Directive 2002/32/EC on undesirable substances in animal feed. For the general product type ‘feed materials’, these MLs are 2, 0.1, and 10 mg/kg for As, Hg, and Pb, respectively – but exemptions (both higher and lower MLs) have been established for a variety of (compound) feeds. For instance, higher MLs have been established for fish intended to be used as feed for other food producing animals (Hg: 0.5 mg/kg). That ML would not have been exceeded by the BSFL samples either. Since BSFL are generally envisioned to be used in animal feed, the adoption of higher specific MLs for insect larvae to be used as animal feed in Directive 2002/32/EC may also need to be considered for BSFL, and possibly other insect species.

For all types of tested substrate, Cd bioaccumulation was observed. This finding is in line with previous studies (Biancarosa *et al.*, 2018; Diener *et al.*, 2015; Van der Fels-Klerx *et al.*, 2016; Van der Fels-Klerx *et al.*, 2020) who also found Cd to bio-accumulate in BSFL to a substantial degree. Large differences in the specific Cd bioaccumulation factor appear to be strongly related to the composition of the substrate. Ardestani *et al.* (2014) concluded that the uptake and elimination kinetics of metals in soil invertebrates were highly species-dependent, but also related to ‘exposure, temperature, and other abiotic factors’. More research is needed to determine which exact factors influence this bioaccumulation, so as to limit uptake or upregulate elimination rates of Cd in the BSFL. The Cd ML for ‘feed materials of animal origin’ in Directive 2002/32/EC is 2 mg/kg, which was exceeded by almost all larval samples (LS and SS experiment) in case of the OWF, MF, and PMLSG treatments. Other MLs in that directive are, for instance, 1 mg/kg for ‘feed materials of vegetable origin’ and ‘complete feed for cattle (except calves), sheep (except lambs), goats (except kids) and fish’. Therefore, feed materials intended to be used as BSFL substrate should be monitored to ensure low concentrations of Cd, to prevent bioaccumulation resulting in higher concentrations in the final BSFL product.

We believe this is the first study to have investigated the effects of dietary acrylamide on BSFL. Concentrations found in the FFW were below the benchmark level for ready-to-eat French fries (500 µg/kg), established in Regulation (EU) 2017/2158. Acrylamide was present in the kitchen waste substrate samples, but was absent in the larval samples. It is possible that this substance was metabolized by the larvae during the rearing process, as has been observed previously for aflatoxin B₁ (Niermans *et al.*, 2021). More research is needed on the specific metabolites being formed, particularly on the conjugate glycidamide, and secondary metabolites that are considered (in humans) as urinary biomarkers (Koszucka *et al.*, 2020).

For pesticides, of the 268 active substances and metabolites thereof that were analysed, 15 substances were found in the organic wet fraction of household waste, including insecticides and herbicides. In principle, the MRLs laid down in Regulation (EC) No 396/2005 apply to composite feeds such as this, but calculation of the exact limits should take into account changes in levels caused by processing and/or mixing (processing factors, Article 20) – which is outside the scope of this study. In the control substrate, only three insecticides (cypermethrin, deltamethrin,

and pirimiphos-methyl) were found, but all concentrations were well below the MRL for wheat (2, 1, and 5 mg/kg, respectively). None of the tested pesticides were detected in the analysed larval samples, which suggests that the feed safety risk of the final product is minimal. However, the presence of insecticide residues in the diet may also affect survival and yield of the exposed larvae, as was found for cypermethrin (0.2 mg/kg) by Meijer *et al.* (2021). Due to the complexity caused by the large number of cofactors in this study, it is uncertain to what extent the analysed insecticide concentrations may have played a role in larval survival and yields.

Of the analysed 76 different veterinary drugs, only oxytetracycline was detected in the substrate, larval and frass samples of the two treatments containing pig manure (PLMSG and PMS). Oxytetracycline is a broad-spectrum antibiotic that is commonly found in pig manure (Arikan *et al.*, 2006; Wang and Yates, 2008). No MRLs are established for insects or manure as a substrate (since it is not permitted as a feed material), but all measured concentrations were below the lowest MRL for e.g. cattle and pig muscle (100 µg/kg) (Regulation (EC) No 37/2010). As such, this implies no health concern in case these insects are used as animal feed. Larval concentrations were lower than those in the substrate and the incomplete mass balance suggests that metabolization or degradation of oxytetracycline had taken place to some extent. This finding is in line with previous research showing that BSFL are effective at biodegrading this compound (Cai *et al.*, 2018a,b; Liu *et al.*, 2022). For example, the concentration of oxytetracycline could be lower or higher in different manure samples depending on the time of the antibiotic treatment (Berendsen *et al.*, 2015). This and previous studies showed that the presence and concentrations of antibiotics, and possibly other veterinary medicines, in manure should be determined prior to selecting manure types, if to be used as substrate for insect rearing, so as to ensure safety of insect products. It is possible that (bioactive) breakdown products are formed in the insects, which should be further investigated. Overall, the present study and previous studies showed that the possible presence of antibiotics (specifically the class of tetracyclines) in manure should be assessed before using the manure as substrate for insect rearing, to ensure the safety of the insect products.

Finally, *Salmonella* spp. and/or *Bacillus cereus* were both found on the larvae of almost all treatments, with the exception of larvae grown on PMLSG. We assumed that the collecting of samples in glycerol allowed for survival of all relevant bacterial communities, but we

acknowledge that the total microbial counts may differ partially from the counts present in samples directly post-experiment. More precisely, small declines in bacterial viability due to the freezing and thawing of the samples might lead to false-negative results in samples that only contained a low abundance of the analysed food pathogens. In the control, FFW, and MF treatments, substrate levels of *Bacillus cereus* were low or below detection limits, while it was detected in the corresponding larval samples, suggesting that growth of the *Bacillus* had taken place because of, or in spite of, the presence of the larvae. It must be noted that in commercial settings, BSFL is generally further processed (e.g. using heat treatment) before use in feed: this is a legal requirement for processed animal proteins in the EU (Regulation (EC) No 142/2011). The bacterial load is therefore expected to be reduced further. Nevertheless, it must be verified that *Salmonella* is absent from the final product (Regulation (EU) No 142/2011), and controls should be in place to prevent *Bacillus cereus* from forming spores and/or toxins (Vandeweyer *et al.*, 2021). Although the feeding of live insect larvae to poultry is permitted in the EU, the presence of *Salmonella* in these larvae could present food safety issues due to transmission to humans via consumption of the meat, as highlighted by De Smet *et al.* (2021). *Listeria monocytogenes* was not present in the substrate nor on the larvae, but in case of its presence in the substrate it is suggested that appropriate control measures are taken to ensure safety of the final insect products (Grisendi *et al.*, 2022). It is recommended that the presence of other potential foodborne pathogens in relation to waste streams, such as *Escherichia coli* (Kashiri *et al.*, 2018) and *Staphylococcus aureus* (Gorrens *et al.*, 2021), are investigated further as well.

5 Conclusion and recommendations

The performance and safety of BSFL reared on a total of six different waste streams were tested in this study, in comparison to a control feed. The tested treatments consisted of organic wet fraction of municipal household waste (OWF), kitchen/fast food waste (FFW), mushroom feet stems (MF), pig manure liquid slurry mixed with roadside silage grass (PMLSG), pig manure solids (PMS), and secondary sludge from slaughter waste (SW). Most tested treatments yielded lower larval biomass than the control; but this may be compensated by lower financial costs of these materials.

Of the tested waste streams, FFW performed best in terms of larval yield in the small-scale experiment, and even better than the control in the large-scale experiment. The presence and concentrations of heavy metals in FFW were comparatively low, including Cd, for which in contrast high bioaccumulation was observed for other substrates e.g. MF and PMLSG. Although the processing contaminant acrylamide was present in the FFW substrate, it was absent in the larvae. This is a promising sign for the use of this type of substrate to rear BSFL on, but more research is required to verify the absence of (toxic) acrylamide metabolites.

Bioaccumulation of Cd is a known safety issue for BSFL from previous studies: although the Cd concentrations found in this study do not present a direct safety concern, these findings highlight the need for monitoring of Cd in BSFL substrates and verify compliance in the final product. Despite the presence of certain substances in the substrate, no alarming levels of corresponding pesticides or veterinary drugs were found in the larvae – although the possibility of other contaminants in the other batches of comparable substrates giving different results cannot be excluded. It is anticipated that any vegetative pathogenic bacteria will be neutralized by mandatory processing of the larvae, but this must of course be validated in each facility. Spores of *Bacillus cereus* may require specific forms of control measures, because they could be present and/or germinate after processing.

As far as we are aware, this is the first experimental study to assess the safety of BSFL reared on a variety of actual waste-stream materials, rather than on regular feed with contaminants of interest artificially added. These experimental results suggest that most of tested substrates are suitable for rearing BSFL, albeit some being sub-optimal. Tested alternative substrates do not appear to present additional safety concerns, aside from the need for monitoring Cd concentrations in the substrates and implementing suitable measures in processing to control for pathogenic bacteria. However, further verification to account for variance in contamination of substrates is needed for definitive conclusions on the safety of tested substrates.

Supplementary material

Supplementary material is available online at: <https://doi.org/10.6084/m9.figshare.24356719>

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Competing interests

No conflicts of interests.

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