APPENDICES

Stocking rate and organic waste type affect development of three *Chrysomya* species and *Lucilia sericata* (Diptera: Calliphoridae): implications for bioconversion

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Appendix A

Biochemical assays used to determine body composition of pre-pupal larvae

Body composition of individual pre-pupal larvae fed on the different waste products were determined using gravimetric methods for water content and by using the methods described by Foray *et al.* (2012) The latter, based on van Handel's method (van Handel, 1985a,b), permits an estimate of the total dissolvable protein, lipid and carbohydrate content in the same individual organism.

The method explained by Foray *et al.* (2012) and used in this study is a relatively novel approach for insect bioconversion studies that quantifies protein, carbohydrate and lipid content present in an individual insect. In comparison with other methods that quantify body composition, this method can determine body composition of an individual insect, is relatively cheap, and carbohydrates are measured directly. However, despite the high sensitivity of this method for detecting proteins, carbohydrates and lipids, there are limitations. The lipids detected are primarily stored lipids and therefore complete lipid content may not be detected. Detected proteins are limited to those that can be solubilised by the buffer that is used, with some proteins that are not solubilized remaining in the pellet (Foray *et al*., 2012). However, no protein assay is uniformly sensitive to all protein types. The majority of methods to determine protein content of larvae involve determining the nitrogen content of a sample and then multiplying it by 6.25 to obtain an approximation of the absolute protein content. However, because there are differences in the ratio of nitrogen to protein present, this method can also introduce inaccuracies that should be accounted for. The most important of these is the nitrogen that is present in chitin, which is non-digestible and therefore does not contribute to the total protein present in the insect, leading to an overestimation of protein content (Mariotti *et al.*, 2008; Pieterse & Pretorius, 2013; Jonas-Levi *et al,*, 2017).

Each larva was weighed to the nearest 0.1 mg using an analytical balance (CPA2P, Sartorius AG, Germany), freeze-dried, then weighed again to determine moisture content and dry mass. Each freeze-dried larva was then placed individually into a 2 mL tube with a zirconium bead and 180 µL of aqueous phosphate-lysis buffer solution. The solution was prepared using 100 mM monopotassium phosphate (KH_2PO_4,), 1 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA), at a pH of 7.4. The larva was then homogenised by shaking the tube for 90 seconds at 25 Hz in a tissue homogeniser (Beadbug, Prism R, Labnet International Inc, Edison, NJ, USA). Each homogenate was centrifuged at 200 RCF and 4˚C for

15 minutes. After this, three replicate samples of 1.5 µL of each of the resulting supernatant was transferred into a plastic disposable microplate, and 250 µL of Bradford micro-assay reagent (B6916, 500 mL, Sigma) was added. A dilution series of bovine serum albumin (0, 0.5, 1, 2, 4 $mg.mL^{-1}$; SH30574.01, HyClone) suspended in the same buffer was used as a standard. The microplate with the samples, standards and Bradford micro-assay reagent was incubated at ambient room temperature for 20 minutes. Protein concentration was determined using a microplate reader (Eon™, BioTek, Instruments Inc, Winooski, Vermont, USA) set at 595 nm. The microplate was gently shaken for three seconds by the microplate reader to disrupt protein-dye aggregates that may affect the readings. Readings were taken by the microplate reader at room temperature.

Carbohydrates were dissolved by adding 20 µL of 20% sodium sulphate (Na_2SO_4) solution to the left over homogenate, along with 4.5 µL of the extraction buffer to replace the removal of 4.5 µL homogenate. This solution was then mixed with 1500 µL of a chloroform-methanol solution (1:2 v/v). This solubilised the total lipids and the water-soluble carbohydrates. Each sample was centrifuged for 15 minutes at 200 RCF and 4˚C. The supernatant was removed for subsequent analysis. Total carbohydrate content was determined using a classical colourimetric method using anthrone reagent. Anthrone reagent was prepared in low volumes and used within two days as it decreases in stability over time. Anthrone reagent was prepared by mixing anthrone powder (319899-25G, Sigma) with 70% sulphuric acid (H_2SO_4) to a concentration of 1.42 $g.L^{-1}$ and stored in a dark glass bottle, covered in aluminium foil and kept out of direct sunlight. A borosilicate microplate was used for all subsequent assays.

In order to determine the carbohydrate content, two replicate samples of 150 µL of the supernatant of each homogenate was transferred to a microplate and allowed to evaporate at ambient room temperature for approximately 40 minutes until a volume of 10 µL was reached. A dilution series of D-glucose (0, 0.5, 1.0, 2.5, 5 $\mu g.\mu L^{-1}$; SAAR2676020EM, Merck) was used as a standard and two replicates of 10 µL of each standard was added to the microplate. 240 µL of anthrone reagent was added to each well and incubated for 15 minutes at room temperature. The microplate was then carefully covered and heated in a water bath for 15 minutes at 90˚C. Carbohydrate concentration of the final solution was determined using a microplate reader set at 625 nm.

The total amount of lipids within each sample was determined using the vanillin assay. Vanillin reagent is light sensitive and was stored in a glass bottle wrapped in aluminium foil and used

within two days. A dilution series of glycerol trioleate (92860-5mL, Sigma) was used as a standard (0.1, 0.2, 0.5, 1.0 µg.µL^{-1}). A total of 100 µL of supernatant was transferred into a borosilicate microplate and heated at 90˚C in a water bath until the solvent had evaporated completely. Subsequently, 10 µL of 98% sulphuric acid was then added to each microplate and further heated at 90˚C in a water bath for two minutes, covered in foil. After this, the microplate was cooled on ice and 190 µL of vanillin reagent was added to each microplate well. The samples were incubated at room temperature for 15 minutes and the absorbance was measured using a microplate reader at 525 nm.

An electronic multipipette (Multipipette Stream, Eppendorf AG, Hamburg, Germany) and low-retention tips were used to minimise experimental errors while working with very small volumes.

References

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Appendix B

Table B.1. Average estimat (a) ·ginal mean lipid content (mg) of different species of pre-pupal larvae fed on different waste types at different initial stocking rates in 100 g of waste, with standard error

Species	Stocking rate	Waste		
		Kitchen waste	Abattoir waste	Swine manure
C. chloropyga	20	1.763 ± 0.153	1.423 ± 0.168	0.846 ± 0.171
	50	2.173 ± 0.135	1.224 ± 0.183	0.710 ± 0.176
	100	1.609 ± 0.152	1.524 ± 0.183	0.875 ± 0.171
C. megacephala	20	1.113 ± 0.136	1.186 ± 0.161	1.173 ± 0.155
	50	1.747 ± 0.123	1.188 ± 0.157	1.128 ± 0.161
	100	1.571 ± 0.110	1.057 ± 0.148	0.884 ± 0.146
C. putoria	20	1.626 ± 0.178	1.148 ± 0.129	1.062 ± 0.160
	50	1.465 ± 0.178	0.970 ± 0.139	1.030 ± 0.175
	100	2.251 ± 0.178	1.420 ± 0.144	0.913 ± 0.168
L. sericata	20	1.441 ± 0.133	1.092 ± 0.138	0.875 ± 0.177
	50	1.841 ± 0.182	1.458 ± 0.155	0.759 ± 0.165
	100	1.428 ± 0.185	1.627 ± 0.168	0.932 ± 0.185

Table B.2. Average estimated marginal mean body water content (mg) of different species of pre-pupal larvae fed on different waste types at different initial stocking rates in 100 g of waste, with standard error

Species	Stocking rate	Waste		
		Kitchen waste	Abattoir waste	Swine manure
C. chloropyga	20	30.672 ± 0.790	38.952 ± 0.996	36.000 ± 0.903
	50	32.881 ± 0.708	33.330 ± 1.019	34.376 ± 0.980
	100	33.098 ± 0.793	34.696 ± 1.014	37.066 ± 0.936
C. megacephala	20	32.416 ± 0.734	33.141 ± 0.878	35.613 ± 0.804
	50	34.462 ± 0.624	32.986 ± 0.833	37.497 ± 0.851
	100	33.894 ± 0.588	32.158 ± 0.802	37.673 ± 0.753
C. putoria	20	34.453 ± 0.956	31.475 ± 0.684	36.135 ± 0.848
	50	35.010 ± 0.952	32.614 ± 0.722	36.761 ± 0.854
	100	31.260 ± 0.954	32.889 ± 0.736	36.726 ± 0.853
L. sericata	20	33.390 ± 0.715	33.244 ± 0.750	35.547 ± 0.982
	50	29.160 ± 0.951	33.828 ± 0.814	36.192± 0.919
	100	32.937 ± 0.952	34.090 ± 0.864	36.781 ± 1.018