

The carbon to nitrogen ratio in isoenergetic wheat based diets controls the growth rate of the aquatic worm *Lumbriculus variegatus*

B. Laarhoven^{1,2*}, H.J.H. Elissen³, C.J.N. Buisman^{1,2} and H. Temmink^{1,2}

¹Sub-department of Environmental Technology, Wageningen University and Research Centre, P.O. Box 8129, 6700 EV Wageningen, the Netherlands; ²Wetsus, European Centre of Excellence for Sustainable Water Technology, P.O. Box 1113, 8900 CC Leeuwarden, the Netherlands; ³ACRRES, Wageningen University and Research Centre, P.O. Box 430, 8200 AK Lelystad, the Netherlands; b.laarhoven@hotmail.com

Received: 3 July 2015 / Accepted: 11 June 2016

© 2016 Wageningen Academic Publishers

OPEN ACCESS



RESEARCH ARTICLE

Abstract

The aquatic worm *Lumbriculus variegatus* (Lv) contains high levels of proteins and can provide an excellent fish food. Large-scale production of Lv on low value organic substrates, such as by-products produced during wheat processing, therefore can be a promising and sustainable concept for the aquaculture industry. Growth and reproduction of Lv on different combinations of wheat based derivatives was studied at fixed isoenergetic levels (expressed by the chemical oxygen demand of the food), but at different carbon to nitrogen (C:N) mass ratios under controlled conditions in specifically designed test-beaker tests. Growth and reproduction rates were compared to those on Tetramin[®], a substrate known to give excellent growth of Lv. Although Lv did exhibit a growth response on single as well as on mixed wheat fractions, growth was mainly controlled by the C:N ratio of the diets. Lower C:N ratios of typically 6-7 gave a much better performance than high C:N ratios of approximately 20. It was discussed this is probably caused by Lv relying on the presence of proteins for their carbon and energy source. Although growth and reproduction rates were not as high as on the control diet, the results are promising for the development of a worm biomass production system operating on by-products from the wheat processing industry.

Keywords: *Lumbriculus variegatus*, aquatic worm, wheat, gluten, aquaculture, food

1. Introduction

Because of its fatty acid and amino acid composition the freshwater worm *Lumbriculus variegatus* (Lv) (Oligochaeta, Lumbriculidae) is an excellent fish food (Elissen *et al.*, 2015; Mount *et al.*, 2006). It contains high levels of protein and like most other sediment-feeding invertebrates is capable of growing on complex mixtures of decomposing organic matter, algae, and microorganisms (Moore, 1978). For this reason production of Lv biomass on safe, low value organic substrates could add a lot of value to the aquaculture sector. Suitable substrates can be found among by-products and waste streams originating from the food industry where food quality and content are carefully monitored and controlled.

According to the FAO, European food and agricultural production of terrestrial plant based resources is dominated by wheat, sugar beet, potatoes, maize and barley with wheat the most important one with an annual production of 195 million tons (<http://faostat3.fao.org>). If by-products generated during wheat processing could be efficiently assimilated by Lv, large-scale, safe and sustainable worm biomass production comes within reach. Direct inclusions of wheat based by-products in aquaculture feeds are feasible to some extent but comes with nutritional and technical challenges (Apper-Bossard *et al.*, 2013; Draganovic *et al.*, 2013); complete fishmeal replacement is not feasible for carnivorous fish species like salmon and trout. The conversion of wheat by-products by Lv biomass could perhaps improve the indirect use of those plant based resources in aquaculture feeds.

How growth (biomass yield and production rate) of Lv is affected by the quantity and elemental composition of its food has not yet been studied. More insight in the effect of food composition on growth can improve selection of organic (waste) substrates that are suitable for Lv production. Aquatic invertebrates such as Lv may react in many ways to foods of different composition (C:N:P ratio), source (bacterial, plant or animal) and quantity by changing their feeding rate, production efficiency and growth and reproduction rate. The possibility to grow Lv on different crude wheat sources like gluten, wheat germ oil and starch and their effect on worm growth and reproduction rate was investigated. To be able to compare the results between different wheat sources experiments were performed under fixed, high isoenergetic food levels to minimise food limitation. The effect of the foods' macro and elemental composition, in particular the C:N ratio, on worm growth will be discussed because this is very important for a selection of suitable by-products and waste streams enabling large-scale aquatic worm production.

2. Materials and methods

Experimental design

Experiment 1 (without additional phosphorus) and 2 (with additional phosphorus) were performed with different combinations of wheat based derivatives and different types of saccharides (Table 1).

Worm growth and reproduction were monitored on single fractions or mixtures of these fractions. Each diet (Table 2) was tested in duplicate for 56 days and diets were replaced three times a week to avoid the effect of food depletion caused by worm consumption and bacterial degradation. Wheat derivatives combination (Table 2) were mixed on the basis of their chemical oxygen demand (COD):N ratios and not on the basis of their carbon ratios as carbon analysis

was only performed on the final composed diets used in experiment 1 and 2. In experiment 1, combined diets except for diet 5 were constituted targeting an overall COD:N of 50. In experiment 2, each combination of wheat based derivatives was mixed to constitute, a high and a low C:N ratio version by targeting a COD:N ratio of either 50 or 17. Sodium dihydrogen phosphate (SKU: S9638; Sigma-Aldrich, Darmstadt, Germany) was added to each diet in experiment 2 to maintain similar high phosphorus levels in all diets matching the levels seen in the control diet. For the addition of dihydrogen phosphate the low phosphorus levels of the single fraction were neglected, only the COD:P ratio of the control diet (109) and the COD values of each test diet were used for this matter.

Food was dosed isoenergetically using COD as an indication for energy content. Isoenergetic food dosing makes it possible to compare the growth only on the basis of the diets C:N and C:P ratios. Using COD in feed studies is uncommon but there is a direct relationship between energy in terms of Joules or kWh and COD. The conversion factor for that is: 1.47×10^7 joules energy production per kg COD oxidised or 3.86 kWh/kg COD (McCarty *et al.*, 2011). TetraMin[®] fish food (Tetra, Germany) was used as a control diet as it was proven to be a suitable diet to support growth of Lv and other aquatic worms (Ducrot *et al.*, 2007; Elissen *et al.*, 2015).

Preliminary tests with the control diet showed that no further increase in growth rate occurred above 100 mg COD per test beaker and that growth rate slowed down above 140 mg COD per beaker (Laarhoven *et al.*, 2016). Therefore, 127 mg COD per test beaker for all diets was used as a maximum food dose representing food saturation. To prevent mineral and vitamin limitation, 19 mg yeast extract (BBL 211929, Bacto™ Yeast Extract; Technical, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was added for each gram of food.

Table 1. Composition of resources used in diet experiment 1 and 2. Chemical oxygen demand (COD), total nitrogen (N) and total phosphorus (P) all are expressed in g/kg dry matter (\pm RSD).

Food source	Experiment	COD	N	COD:N	P
TetraMin [®] (control)	1, 2	1,330 (47)	75.0 (1.35)	18 (0.7)	13.5 (0.24)
Crude wheat starch	1	1,120 (39)	1.9 (0.03)	602 (23.7)	n.a
Crude wheat gluten	1	1,210 (42)	40.7 (0.73)	30 (1.2)	n.a
Wheat germ oil	1	3,091 (108)	0.3 (0.01)	9,721 (383)	n.a
Wheat native starch	2	1,210 (42)	1.9 (0.03)	651 (25.6)	1.1 (0.02)
Maltodextrin	2	1,010 (35)	1.9 (0.03)	546 (21.5)	1.0 (0.02)
Glucose	2	896 (31)	<0.05 (0.00)	>17,920 (705)	0.0 (0.00)
Vital wheat gluten	2	1,320 (46)	129.0 (2.32)	10 (0.4)	1.4 (0.03)
Hydrolysed wheat gluten	2	1,360 (48)	129.0 (2.32)	11 (0.4)	1.6 (0.03)
Yeast (micro-nutrient inclusion)	1, 2	1,020 (36)	114.0 (2.05)	9 (0.4)	12.7 (0.23)

Table 2. Diet composition and growth performance overview. Weight percentage (dry matter) for each gluten (G), sugar (S) and lipids (L) in each combination, chemical oxygen demand (COD), carbon (C), organic nitrogen (N), phosphorus (P) and iron (Fe). RSD for COD<3.5% for C, N, P <1.8% and Fe<3.5%. In experiment 1., crude wheat gluten was used in combination with crude starch and/or germ oil. Vital wheat gluten was used in diets of experiment 2, except for diet 8 in which hydrolysed wheat gluten (h) was used. Extra addition of NaH₂PO₄ in diets of experiment 2, starch (w), maltodextrin (md) and glucose (gl). Growth rates \pm standard deviation.¹

Experiment	Diet	Combinations (%)			COD (g/kg DM)	Elemental analyses (g/kg DM)				Ratios				Growth rates		
		G	S	L		C	N	P	Fe	COD:N	C:N	C:P	N:P	SGR (%/day)	SRR (%/day)	
1	1	Control ²	0	0	0	1,304	481	77.6	12.0	0.28	17	6.2	40.3	6.5	1.53 \pm 0.09 ^a	2.42 \pm 0.06 ^a
1	2	G	100	0	0	1,198	462	44.2	2.0	0.05	27	10.5	235.2	22.5	0.74 \pm 0.08 ^b	1.80 \pm 0.14 ^b
1	3	G+L	75	0	25	1,468	547	33.1	1.8	0.04	44	16.5	300.1	18.2	0.48 \pm 0.03 ^b	1.12 \pm 0.18 ^b
1	4	G+S	50	50	0	1,129	437	22.7	1.4	0.05	50	19.3	302.5	15.7	0.65 \pm 0.10 ^b	1.79 \pm 0.12 ^b
1	5	S+L	0	87	13	1,276	489	3.7	1.1	0.02	344	132	464.5	3.5	0.16 \pm 0.08 ^c	0.60 \pm 0.21 ^c
1	6	G+S+L	60	30	10	1,271	479	26.3	1.5	0.03	48	18.2	313.3	17.2	0.66 \pm 0.08 ^b	1.56 \pm 0.02 ^b
2	1	Control ²	0	0	0	1,304	481	77.6	12.0	0.28	17	6.2	40.3	6.5	1.10 \pm 0.14 ^a	2.52 \pm 0.06 ^a
2	2	G+S(w)	17	83	0	1,073	429	24.4	13.4	0.03	44	17.6	31.9	1.8	0.05 \pm 0.03 ^b	1.58 \pm 0.29 ^b
2	3	G+S(w)	55	45	0	1,146	455	67.4	13.1	0.05	17	6.7	34.6	5.1	0.01 ³	0.02 ³
2	4	G+S(md)	15	85	0	1,046	423	20.2	12.3	0.02	52	20.9	34.4	1.6	0.00 \pm 0.00 ^b	1.70 \pm 0.00 ^b
2	5	G+S(md)	51	49	0	1,154	458	63.2	11.7	0.04	18	7.2	39.1	5.4	0.46 \pm 0.03 ^c	2.18 \pm 0.12 ^c
2	6	G+S(gl)	13	87	0	994	406	19.8	12.8	0.03	50	20.5	31.6	1.5	0.05 \pm 0.01 ^b	1.38 \pm 0.22 ^b
2	7	G+S(gl)	48	52	0	1,111	432	60.3	11.5	0.04	18	7.2	37.6	5.2	0.44 \pm 0.10 ^c	2.12 \pm 0.11 ^c
2	8	G(h)+S(md)	15	85	0	1,085	430	21.1	10.7	0.04	51	20.4	40.1	2.0	0.00 \pm 0.00 ^b	1.51 \pm 0.10 ^b

¹ a-c refer to significant groups based on Posthoc testing.
² Commercial mixture.
³ Single test therefore standard deviation not calculated.

Pretreatment of wheat fractions and diet formulation

Different gluten and starch fractions were provided by the industrial wheat milling industry (Cargill, Bergen op Zoom, the Netherlands) and wheat germ oil (Jacob Hooy & Co BV, Limmen, the Netherlands) was purchased from a local retail shop. Maltodextrin and glucose were purchased from VWR® (Radnor, PA, USA). Gluten and starches were frozen at -20 °C for temporary storage and freeze dried (Modulyo freeze dryer; Edwards High Vacuum International, Crawley, UK) to remove moist. Prior to diet formulation and chemical analysis, samples were finely ground to create food particles suitable for unhindered worm ingestion and blended as stated in Table 2 to obtain a wide variation of C:N ratio in the diets.

Test organisms

Lv from a recirculating culture system were retained in plastic flow-through tanks. The cultures were fed three times per week with the Tetramin® control diet. Water temperature was maintained at 19 °C and circulated over

a nitrifying filter to prevent ammonia and nitrite levels above 0.4 mg/l. Prior to entering the tanks, the water was aerated to ensure oxygen levels above 6 mg/l. Fully developed worms from cultures experiencing growth with no indications of recent fragmentation or abnormalities were selected and purged overnight in clean tap water to remove their gut content. In experiment 1 and 2, 75 and 50 worms were used, respectively.

Test beaker and system configuration

A controlled temperature and water flow system with plastic flow-through beakers (working volume of 770 \pm 8 ml and a bottom surface area of 57 cm²) was used (Figure 1). Each test beaker contained 0.6% agar-gel (20 ml), 10 g fine sand (0.1-0.35 mm grain size), 40 g coarse sand (0.71-1.25 mm grain size) and 127 mg COD of each diet supplied as a finely ground dry powder. The diets were mixed with sand and agar gel to provide suitable conditions for food uptake while reducing (microbial) food hydrolysis at the same time. Water was continuously discharged with a drain pipe positioned approximately 2 cm above the artificial

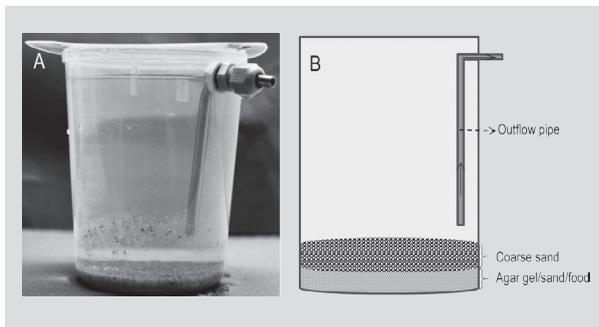


Figure 1. (A) Photograph and (B) schematic drawing of the test beaker set-up.

sediment. The beakers were submerged in a temperature controlled bath at 19 ± 1 °C and retained in dark conditions until sediment replacement and/or sampling. Water was supplemented at a flow rate of 4.34 ml/min, resulting in refreshment rates of 8 per day. The water (pH 7.2, hardness 77 mg CaCO₃) was a mixture of 70% tap water (Leeuwarden, the Netherlands) and 30% softened water. The water was disinfected with UV-C light to prevent inoculation of the beakers with microorganisms and was aerated to keep oxygen at saturated levels. The pH was controlled by dosing HCL (37%) with the use of a pH controlled dose pump (StepDos O8S, KNF Neuberger Inc., Trenton, NJ, USA). Water was supplied on the surface of each beaker by a needle dispenser, creating a constant flow of small droplets.

Replacement of artificial sediment and worm handling

The test beakers were withdrawn from the temperature controlled bath and approximately 70% (v/v) of the overlying water was removed. The beakers were gently shaken to

fragment the sediment layer which incited most worms to perform their typical escape reflex (Drewes, 1999) into the overlying water. This impulse made it possible to collect the worms by pouring off the overlying water. Repeating this three times resulted in complete removal of the worms from the sediment. The collected worms were cleaned by flushing them with clean water, and all of the sediments were thoroughly inspected for remaining worms. The worms were placed directly into clean test beakers with new sediments and 200 ml of water. Once per week, the total wet worm weight was measured after 3 to 4 hours of gut purging by using a pre weighted fine mesh. Worms were collected on top of the mesh, and paper towels were gently pressed against the back of the mesh for 10 seconds to absorb adherent water. The total number of worms was determined by counting the worms using a glass pipette.

Estimation and comparisons of worm specific growth rates based on weight and number

Weekly measurements of total weight and number of worms collected during substrate replacement were used to estimate the specific growth rate (SGR) based on weight and specific reproduction rate (SRR) based on number, both expressed in %/day. Figure 2 shows an example of the growth and reproduction response of the worms. This response, consisting of three distinct phases, was observed with all the diets. During phase 1 the individual worm weight increased until a certain (average) individual worm weight was reached. During phase 2 asexual reproduction by fragmentation took place. Phase 3 gave a stable reproduction rate (>20% of the population) and from this phase the SGR and SRR were estimated. All the datapoints in phase 3 were used for this purpose and fitted

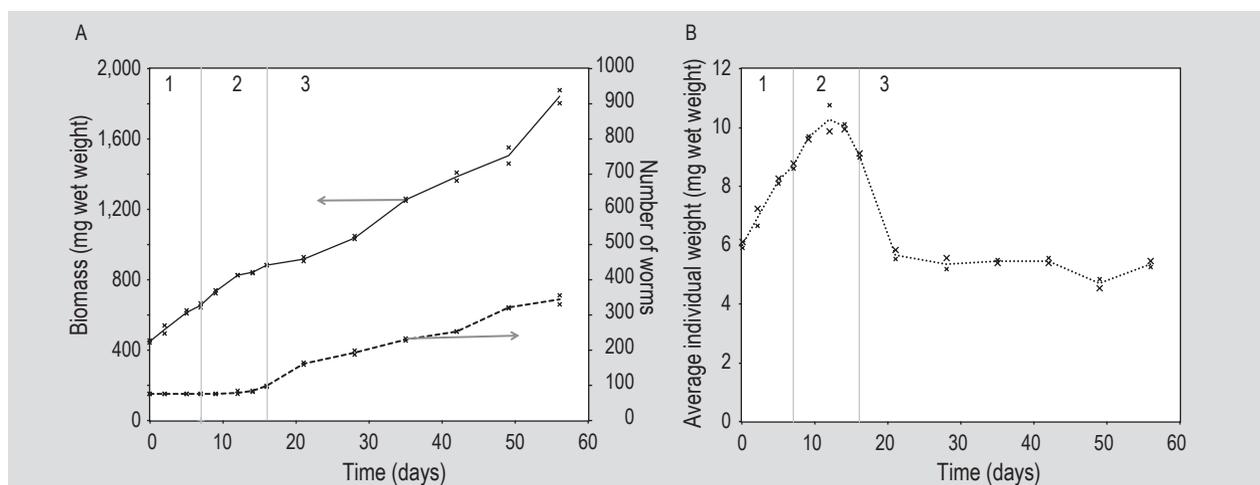


Figure 2. (A) Growth, reproduction and (B) the resulting average individual weight for 75 worms starting with similar individual start weights on a Tetramin® diet. Phase 1, growth without reproduction. Phase 2, growth with less than 20% of the population reproducing. Phase 3, growth with stable reproduction rate (>20% of the population). Specific growth rates for phases 1 and 3 were 5.4% ($R^2=0.97$) and 1.8%/day ($R^2=0.98$) per day. Specific reproduction rates for phases 3 was 2.8% ($R^2=0.90$), no reproduction took place during phase 1.

assuming exponential growth and by using a least-squares method to obtain estimates for SGR and SRR. To identify differences between diets the SGR and SRR were compared with ANOVA and Posthoc testing using Tukey HSD.

Chemical analyses

The resources, diets and previously cultured worm biomass fed with Tetramin® were analysed according to standard national methods for COD (NEN 6633:2006; NEN, 2006), total nitrogen (NEN-EN 16169:2012; NEN-EN, 2012), total ammonium-N (NEN-ISO 7150-1:2002; NEN-ISO, 2002), total P (CEN/TS 16188:2012; NEN, 2012) combined with ICP-OES, total organic C (NEN-EN 15936:2012; NEN-EN, 2012), and dry matter (DM) (NEN 7432:1998; NEN, 1998). All analyses were performed by the Department of Environmental Technology at Wageningen University (Wageningen, the Netherlands).

3. Results

General observations

The different diets that were tested (Table 2) contained a COD of $1,274 \pm 114$ and $1,092 \pm 54$ g/kg DM in experiment 1 and 2, respectively. The N content varied between 3.7 and 77.6 g N/kg DM. In the diets of experiment 1, the P content varied between 1.1 and 13.4 g P/kg DM, whereas in experiment 2 it was kept at a constant level of 12.4 ± 0.9 g/kg DM.

Growth and reproduction of Lv with single and mixed crude wheat fractions (experiment 1)

Lv could grow and reproduce on single as well as on mixed crude wheat fractions. Coefficients of determination for SGR and SRR estimates were all above 0.81. Only with the sugar and lipids (S+L) diet very poor estimates were obtained because growth did not follow the expected exponential growth relationship. Observed differences between the diets were significant for both SGR (ANOVA, $F_{5,6}=63.9$, $P<0.001$) and SRR (ANOVA, $F_{5,6}=40.4$, $P<0.001$).

In table 2 the SGR and SRR are given and can be compared with C:N and C:P ratio of each diet. Because reproduction of Lv is size related SRR and SGR exhibit similar patterns. Within the experimental diets only a significant reduction for SGR and SRR was seen for diet 5 containing only sugar and lipids but without crude wheat gluten (S+L diet). Overall, a positive trend of faster growth and reproduction was observed for diets with a lower C:N or C:P ratio. However, the SRR and SGR in the experimental diets still were 31-48% lower than those obtained with the control diet.

In the experimental diets of experiment 1, N and P were mainly associated with the crude gluten and a low C:N

ratio was always accompanied by a low C:P ratio. Whether growth was limited by N or P thus remains unclear. The range of the N:P ratio in the experimental diets also was rather narrow: between 15.7 and 22.5 (Table 2, S+L diet excluded). Therefore, a second experiment was carried out with more concentrated wheat gluten fractions enriched with phosphorus to reach nitrogen and phosphorus levels similar to those in the control diet. In the same experiment different saccharides were added to investigate if the type of sugar has an effect on growth and reproduction of Lv.

Growth and reproduction of Lv at low and high C:N ratios at fixed C:P ratios using concentrated wheat gluten and different saccharides (experiment 2)

Significant differences were found for the SGR and SRR between the control diet and all other test diets (ANOVA, $F_{7,8}=20.3$, $P<0.001$). In Table 2 the SGR and SRR are given and can be compared with C:N and C:P ratio of each diet. Wheat gluten based diets with C:N ratios of around 7 gave a significantly higher SGR and SRR than the other diets with a C:N ratio close to 20 (ANOVA, $F_{7,8}=10.76$, $P<0.002$), but were 29-41% lower than growth rates with the control diet. Because growth was almost absent in diets with a C:N ratio of 20, rate estimates for this ratio could not be established in a reliable way. For the gluten and starch diet (C:N~7) an outlier was observed with a very low SGR estimate of 0.0012 per day. This could in retrospective be explained by a lack of overlying water refreshment during this test. This value was therefore not included for further analysis or discussion.

Although the additional phosphorus gave much lower C:P ratios than in experiment 1 (Table 2), this did not stimulate the growth rates to achieve a level similar to those in the control diet. Apparently the growth rates were mainly controlled by the C:N ratio. Table 2 also shows that the type of saccharide (starch, maltodextrin or glucose) and the gluten fraction did not exhibit an effect on the growth rates. Finally, also hydrolysed wheat gluten did not result in a higher growth rate compared to a diet containing vital wheat gluten. This suggests that digestibility of the wheat protein matter was not an important factor for growth and reproduction at high C:N ratios.

4. Discussion

A surplus of chemical energy (COD) and micro-nutrients was supplied to all diets, and therefore only the bioavailability (digestibility) and nutrient composition expressed in terms of C:N and C:P ratios of the food were possible factors limiting growth and reproduction. P was equally dosed in all test diets of experiment 2 and therefore P limitation in comparison with the control diet can be neglected. Applying hydrolysed wheat gluten did not result in higher growth or reproduction rates compared to native

wheat gluten. This implies that the digestibility of this particular wheat fraction also was not a limiting factor, and Lv or the microbial population it harbours in its gut system has a high digestible power for this proteinaceous matter.

Although worm biomass composition was not determined in this experiment, a culture of Lv fed with the same control diet (Tetramin®) for several months was gut purged and dried overnight at 105 °C for elemental analysis. This gave a C:N mass ratio of 4.5, which is a reflection of the relatively high protein content of Lv of 62-66% (Elissen *et al.*, 2010). The C:P mass ratio for Lv was 32.8, which is much lower in comparison with other aquatic detritivores which have an average C:P mass ratio of approximately 95 (Frost *et al.*, 2006).

Generally, higher nitrogen fractions in the diets (typically at a C:N ratio around 7) gave much higher growth and reproduction rates than lower nitrogen fractions (typically at a C:N ratio of approximately 20) Table 2. The ideal C:N ratio for Lv apparently is much lower than for terrestrial worms like *Eisenia foetida*, which has an ideal C:N mass ratio of 15-35 (Neuhauser *et al.*, 1980). Because Lv and *E. foetida* have similar protein levels (Stafford and Tacon, 1985), it is suggested this difference in ideal food C:N ratios is related with the natural differences between terrestrial and freshwater food webs. Terrestrial food webs are often extremely nutrient-poor and autotrophic, with C:N ratios that are much higher than in lake particulate matter (Elser *et al.*, 2000). *E. foetida* can grow on relatively high C:N ratios as a result of the high enzymatic activity related with the digestion of non-proteinaceous matter. In contrast, Lv may depend more on proteins as their source of energy and biomass. This is also supported by the observation that addition of sugars to the diets of experiment 2 did not stimulate growth and reproduction of Lv. Mass balances for COD and N performed on a Lv reactor for biological waste sludge reduction also revealed that Lv more selectively digested the protein fraction of this sludge than other fractions (Hendrickx *et al.*, 2009, 2010). Approximately 40% of the nitrogen and 12% of the phosphorus in the waste sludge that was digested by the worms was used for the formation of new worm biomass.

As a consequence of the above, (particulate) organic matter from food industries can be directly used by Lv, as long as it has a sufficiently low C:N mass ratio (typically around 6 to 7). For organic matter with much higher C:N ratios it probably is better to first convert it into microbial biomass to reduce the C:N ratio, allowing subsequent efficient worm growth on this biomass. Another option is to mix by-products from different sources such that the desirable C:N ratio is obtained.

The growth performance of Lv in this study may not reflect the growth performance in full-scale biomass

production reactors. Food hydrolysis was strongly reduced by frequent food renewals and feeding conditions which slowed down simultaneous bacterial breakdown. Most likely the contribution of microbial hydrolysis will become more important in full-scale reactors due to longer food residence times and bacterial ingrowth. This would result in lower C:N and C:P ratios and a shift to a more mixed food including bacterial biomass. This would imply that in practice also a C:N ratio higher than 7 can be used without a substantial reduction of the growth rate as was observed in this study.

The control and low C:N diets of experiment 2 unexpectedly showed lower growth rates compared to experiment 1. Variance between the control diets of each experiment suggest an influence of an unknown external factor like overall condition of the worm which was not controlled within the experiment. Although this was not measured, a possible explanation for the lower growth rates for the diets in experiment 2 could be a lower lipid content in the diets of experiment 2 as a result of using more purified gluten and saccharides without residual germs. As lipids are essential for cell membrane functions, a limited availability in experiment 2 may have reduced growth in experiment 2 in comparison to the control within that same experiment. How the fatty acids composition and lipid level in the worm are related with the fatty acid composition and lipid level of the food and how this affects growth performance is valuable information for Lv biomass production and its further use as fish feed. In amphipods it has been observed that the lipid content in the animal increases at a higher food availability (Perrone *et al.*, 2003). However, whether this also is valid for Lv needs further study.

Information about mineral use and limitation on Lv growth is scarce. Particularly iron plays an important role because its function in the oxygen-binding blood protein of Lv and other Annelida (Frossard, 1982). This also explains why addition of iron improved the growth of aquatic worms in wastewater treatments plants (Janssen *et al.*, 2002). It cannot be excluded that the iron content of the control diet was higher than of the other diets, and perhaps this explains the higher growth and reproduction rates with the control diet.

5. Conclusions

The results showed that the aquatic worm Lv can grow and reproduce on different wheat sources. Growth and reproduction were mainly controlled by the C:N ratio of the feed, with higher rates at low C:N ratios of around 7. Although growth and reproduction rates were not as high as on a control diet containing a commercial fish feed, these results are promising with respect to the development of a worm production system, fed with by-products from the wheat processing industry.

Acknowledgements

The authors thank Tiago Miguel Azevedo Marques and Klaudia Stanisława Straško for their valuable contributions to the experimental work. This study was performed in the cooperation framework of Wetsus, European Centre of Excellence for Sustainable Water Technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân, and the Northern Netherlands Provinces. The authors would like to thank the participants of the research theme 'Aquatic worms' for the fruitful discussions and their financial support.

References

- Apper-Bossard, E., Feneuil, A., Wagner, A. and Respondek, F., 2013. Use of vital wheat gluten in aquaculture feeds. *Aquatic Biosystems* 9: 21.
- Draganovic, V., Van der Goot, A.J., Boom, R. and Jonkers, J., 2013. Assessment of the effects of fish meal, wheat gluten, soy protein concentrate and feed moisture on extruder system parameters and the technical quality of fish feed. *Animal Feed Science and Technology* 165: 238-250.
- Drewes, C.D., 1999. Helical swimming and body reversal behaviors in *Lumbriculus variegatus* (Annelida: Clitellata: Lumbriculidae). *Hydrobiologia* 406: 263-269.
- Ducrot, V., Péry, A.R.R., Quéau, H., Mons, R., Lafont, M. and Garric, J., 2007. Rearing and estimation of life-cycle parameters of the tubicifid worm *Branchiura sowerbyi*: application to ecotoxicity testing. *Science of the Total Environment* 384: 252-263.
- Elissen, H.J.H., Hendrickx, T.L.G., Temmink, H., Laarhoven, B. and Buisman, C.J.N., 2015. Worm-it: converting organic wastes into sustainable fish feed by using aquatic worms. *Journal of Insects as Food and Feed* 1: 67-74.
- Elissen, H.J.H., Mulder, W.J., Hendrickx, T.L.G., Elbersen, H.W., Beelen, B., Temmink, H. and Buisman, C.J.N., 2010. Aquatic worms grown on biosolids: biomass composition and potential applications. *Bioresource Technology* 101: 804-811.
- Elser, J.J., Fagan, W.F., Denno, R.F., Dobberfuhl, D.R., Folarin, A., Huberty, A., Interlandi, S., Kilham, S.S., McCauley, E., Schulz, K.L., Siemann, E.H. and Sterner, R.W., 2000. Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408: 578-580.
- Frossard, P., 1982. The erythrocrucorin of *Eisenia fetida*. I. Properties and subunit structure. *Biochimica et Biophysica Acta* 704: 524-534.
- Frost, P.C., Benstead, J.P., Cross, W.F., Hillebrand, H., Larson, J.H., Xenopoulos, M.A. and Yoshida, T., 2006. Threshold elemental ratios of carbon and phosphorus in aquatic consumers. *Ecology Letters* 9: 774-779.
- Hendrickx, T.L.G., Temmink, H., Elissen, H.J.H. and Buisman, C.J.N., 2010. Aquatic worms eat sludge: mass balances and processing of worm faeces. *Journal of Hazardous Materials* 177: 633-638.
- Hendrickx, T.L.G., Temmink, H., Elissen, H.J.H. and Buisman, C.J.N., 2009. Aquatic worms eating waste sludge in a continuous system. *Bioresource Technology* 100: 4642-4648.
- Janssen, P.M.J., Verkuijlen, J. and Van der Roest, H.F., 2002. Slibpredatie door inzet van oligochaete wormen. Pilotonderzoek naar slibreductie op de rwzi Bennekom. Report 2002-17. STOWA, Amersfoort, the Netherlands.
- Laarhoven, B., Elissen, H.J.H., Temmink, H. and Buisman, C.J.N., 2016. Agar sediment test for assessing the suitability of organic waste streams for recovering nutrients by the aquatic worm *Lumbriculus variegatus*. *PLoS One* 11: e0149165.
- McCarty, P.L., Bae, J. and Kim, J., 2011. Domestic wastewater treatment as a net energy producer – can this be achieved? *Environmental Science and Technology* 45: 7100-7106.
- Moore, J.W., 1978. Importance of algae in the diet of the oligochaetes *Lumbriculus variegatus* (Müller) and *Rhyacodrilus sodalis* (Eisen). *Oecologia* 35: 357-363.
- Mount, D.R., Highland, T.L., Mattson, V.R., Dawson, T.D., Lott, K.G. and Ingersoll, C.G., 2006. Use of the oligochaete, *Lumbriculus variegatus*, as a prey organism for toxicant exposure of fish through the diet. *Environmental Toxicology and Chemistry* 25: 2760-2767.
- NEN, 2012. CEN/TS 16188, slib, behandeld bioafval en bodem – bepaling van de elementen na ontsluiting in koningswater en salpeterzuur – atomaire-absorptiespectrometrie vlamtechniekmethode (FAAS). NEN, Delft, the Netherlands.
- NEN-EN, 2012. 15936, slib, behandeld biologisch afval, bodem en afval – Bepaling van de totale organische koolstof (TOC) door droge verbranding. NEN, Delft, the Netherlands.
- NEN-ISO, 2002. 7150-1, water – bepaling van ammonium: handmatige spectrometrische methode. NEN, Delft, the Netherlands.
- NEN, 1998. 7432, dierlijke mest en mestproducten – bepaling van de gehalten aan droge stof en organische stof – gravimetrische methode. NEN, Delft, the Netherlands.
- NEN, 2006. 6633, water en (zuiverings)slib – bepaling van het chemisch zuurstofverbruik (CZV). NEN, Delft, the Netherlands.
- NEN_EN, 2012. 16169, slib, behandeld bioafval en bodem – bepaling van het gehalte aan Kjeldahl-stikstof. NEN, Delft, the Netherlands.
- Neuhauser, E.F., Kaplan, D.L., Malecki, M.R. and Hartenstein, R., 1980. Materials supporting weight gain by the earthworm *Eisenia foetida* in waste conversion systems. *Agric Waste* 2: 43-60.
- Perrone, F.M., Della Croce, N. and Dell'anno, A., 2003. Biochemical composition and trophic strategies of the amphipod *Eurythene gryllus* at hadal depths (Atacama trench, South Pacific). *Chemistry and Ecology* 19: 441-449.
- Stafford, E.A. and Tacon, A.G.J., 1985. The nutritional evaluation of dried earthworm meal (*Eisenia foetida*, Savigny, 1826) included at low levels in production diets for rainbow trout, *Salmo gairdneri* Richardson. *Aquaculture Research* 16: 213-222.

