Sludge reduction by aquatic worms in wastewater treatment

with emphasis on
the potential application of *Lumbriculus variegatus*

Promotor
Prof. dr. ir. W. H. Rulkens
Hoogleraar Milieutechnologie

Copromotor
Dr. ir. A. Klapwijk
Voormalig universitair hoofddocent, sectie Milieutechnologie

Samenstelling promotiecommissie

Prof. dr. H. J. P. Eijsackers	Wageningen Universiteit
Prof. ir. J. H. J. M. van der Graaf	Technische Universiteit Delft
Prof. dr. ir. A. J. M. Stams	Wageningen Universiteit
Dr. ir. P. F. M. Verdonschot	Alterra, Wageningen Universiteit en Researchcentrum

Sludge reduction by aquatic worms in wastewater treatment

with emphasis on
the potential application of *Lumbriculus variegatus*

Hellen J. H. Elissen

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M. J. Kropff,
in het openbaar te verdedigen
op dinsdag 18 december 2007
des ochtends te elf uur in de Aula

Elissen, H. J. H., 2007. Sludge reduction by aquatic worms in wastewater treatment with emphasis on the potential application of *Lumbriculus variegatus*.
PhD thesis Wageningen University, Wageningen, the Netherlands - with references – with summaries in English and Dutch, 192 pages. ISBN 978-90-8504-777-3.

╡Abstract╞

In wastewater treatment plants (WWTPs), large amounts of biological waste sludge are produced. In the Netherlands, the application of this sludge in agriculture or disposal in landfills is no longer allowed, mainly because of its high heavy metal content. The sludge therefore generally is incinerated. Sludge processing costs are estimated to be half of the total wastewater treatment costs. This thesis focuses on the application of aquatic worms to reduce the amount and volume of the excess sludge. Several worm species, belonging to the Aeolosomatidae, Tubificidae (including Naidinae) or Lumbriculidae have specific characteristics that could make them suitable for such an application. A 2.5-year survey of free-swimming Aeolosomatidae and Naidinae in WWTPs showed that their growth was hard to control and their effect on the treatment process unclear.

The sessile species *Lumbriculus variegatus* was selected for further research, because of its stable and quantifiable growth and sludge reduction. In addition, batch experiments indicated that *L. variegatus* has more potential for sludge reduction than sessile Tubificidae. Different municipal waste sludges could be digested by *L. variegatus*. This process typically was twice as fast as endogenous sludge digestion and on average 0.09 (±0.04) mg sludge/ mg worm/ day (dry matter based) was digested. However, the final reduction percentage was the same for both processes (around 50 % dry matter based) and 17 (±6) % could be attributed to digestion by the worms. The resulting worm faeces had a distinct compact shape and a highly improved initial settling rate and settleability (low sludge volume index of around 60 mL/ g), which could be beneficial to further processing. Around 7 % of the sludge (dry matter based) was converted into new worms after asexual reproduction by division and biomass growth. This protein-rich biomass can easily be separated from the sludge and re-used, e.g. as aquarium fish food, because the concentrations of most heavy metals in the worms were lower than in the waste sludge. A reactor set-up for sludge reduction with *L. variegatus* was developed, based on immobilization of the worms in a carrier material and a complete separation of waste sludge and worm faeces. It showed promising results in a sequencing batch experiment, but should be further optimized as sludge reduction percentages as well as other performance parameters varied considerably. The process has high potential for full-scale applications, but the feasibility depends on the unknown maximum effective worm population density that can be maintained, and the production, re-use possibilities and value of the produced worm biomass.

Keywords: Waste sludge reduction, wastewater treatment, aquatic worms, *Lumbriculus variegatus*

Opgedrage aan mien auwers, miene broor en mien groetauwers

╡Contents╞

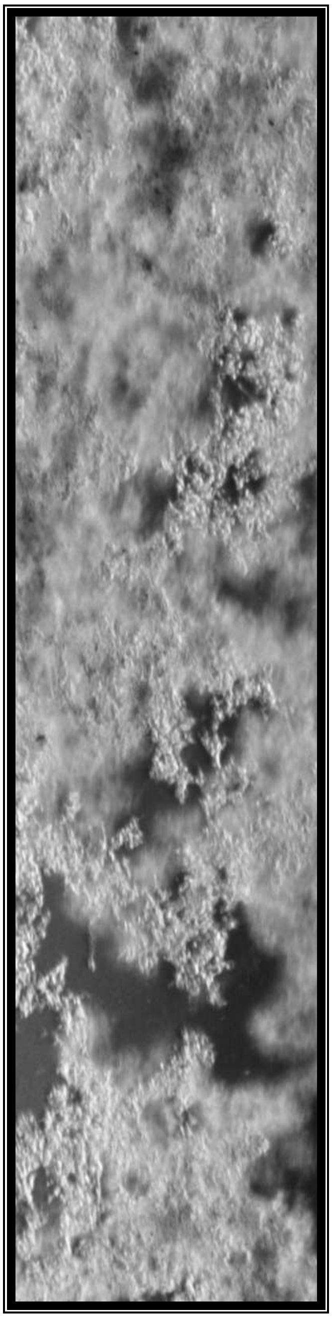
⫞ Frequently used terms and abbreviations ⫟

Annelida	Animal phylum consisting of segmented terrestrial, freshwater and marine worms
Aphanoneura	Class of Annelida consisting of one (mainly) aquatic family, the Aeolosomatidae
Ash	Inorganic fraction of waste sludge. Calculated by subtracting the VSS from the TSS
AT	Aeration tank in wastewater treatment plant
Consumption	Ingestion/uptake of sludge by worms
Digestion by worms	Sludge (TSS) breakdown after consumption during worm gut passage due to metabolic processes (e.g. maintenance, growth)
D	Linear sludge digestion rate by worms (dry matter based; in d^{-1})
Dry weight	Dead worm weight, determined after drying overnight at 105 °C in porcelain crucible
Endogenous digestion	Sludge (TSS) breakdown due to endogenous respiration —i.e. oxidation of bacterial tissue— in sludge without external substrate (adapted from van Loosdrecht & Henze, 1999)
Faeces percentage	Visual estimation of the worm faeces as fraction of the total amount of waste sludge plus worm faeces. A faeces percentage of 100 % indicates that worms have consumed all the sludge flocs
Free-swimming	Common term in wastewater treatment to describe (small) species of aquatic worms (like Naidinae (Tubificidae) and Aeolosomatidae) that are abundant in the mixed liquor (in or on the sludge flocs) of wastewater treatment plants
G	Linear worm biomass growth rate (dry matter based; in d^{-1})
Gn	Linear worm number growth rate (in d^{-1}). Measure of reproduction
n	Number of worms
N	Number of samples (unless indicated otherwise, e.g. Chapter 3)

Oligochaeta	Class of Annelida consisting of aquatic families like Tubificidae (including the Naidinae) and Lumbriculidae, but also terrestrial families like earthworms
Reduction or digestion	General term used to describe (TSS or VSS) breakdown of sludge by (combinations of) biological, chemical, mechanical or physical methods
Sessile	Common term in wastewater treatment to describe species of (larger) aquatic worms (like Tubificidae (except for the Naidinae) and Lumbriculidae) that are mainly found on surfaces in wastewater treatment plants, e.g. the reactor walls or carrier materials
Sludge age	Or SRT (solids retention time). The average period of time the sludge has remained in the system. Also indicated with 'θ'
SVI	Sludge volume index of sludge (in mL/ g). Volume of 1 g of sludge TSS after settling of the sludge flocs. SVI_{30} is the SVI after 30 minutes. SVI values decrease with improved settleability
TSS	Total suspended solids. Portion of the total solids retained on a filter with a specified pore size, measured after being dried at 105 °C
VSS	Volatile suspended solids or organic fraction of the waste sludge. Those solids that can be volatilized and burned off when the TSS are ignited at 600 °C
W/S ratio	Worm to sludge ratio (dry matter based; dimensionless)
Waste sludge	Surplus (activated) sludge produced in WWTPs
Wet weight	Live worm weight, determined after removing adhering water on a perforated piece of aluminium foil on tissue paper
Worm faeces	Fraction of sludge excreted as faecal pellets by worms after consumption and digestion
WWTP	Wastewater treatment plant
Y	Yield. Worm biomass produced from sludge digested by worms only (dry matter based; dimensionless or percentage)

╡Chapter 1╞

Introduction to sludge reduction research with aquatic worms



1.1 The problem of waste sludge production in wastewater treatment

In aerobic WWTPs (wastewater treatment plants), large amounts of biological waste sludge are produced with an average sludge production of 0.4 kg VSS (volatile suspended solids) per kg COD (chemical oxygen demand) of incoming wastewater removed (Tchobanoglous *et al.*, 2003). A worldwide average of 20-40 kg dry matter waste sludge is produced per population equivalent per year (Kroiss, 2004). Waste sludge is a complex mixture of water (up to more than 95 %), bacteria, dead organic and inorganic materials, containing phosphorous and nitrogen compounds and various pollutants (e.g. heavy metals, organic pollutants and pathogens) (Rulkens, 2004). In Europe, wastewater is treated in more than 40,000 WWTPs, which produce around 7 million tonnes of dry waste sludge yearly (Roman *et al.*, 2006; Spinosa, 2007). In the Netherlands, almost 400 municipal WWTPs currently produce a total amount of 350,000 tonnes dry sludge mass per year (Statistics Netherlands (CBS), 2007). Even though production in the Netherlands is expected to stabilize the coming years within a range of 5 % (Loeffen & Geraats, 2005), waste sludge production worldwide is only expected to increase (Neyens *et al.*, 2004). The cost of treatment and disposal of waste sludge —from municipal as well as non-municipal (industrial) sources— is estimated to be half of the total costs of wastewater treatment (Wei *et al.*, 2003b; Kroiss, 2004). In the Netherlands for example, these costs (and environmental 'costs' in the form of for example CO_2-emissions) mainly result from incineration, but also from the fact that most of the produced waste sludge has to be transported to central sludge processing plants, while this waste sludge in general still consists of 70-75 % water (de Jong, 2007). Therefore, minimizing sludge production and the costs for further processing of waste sludge has a high priority in wastewater treatment (Boehler & Siegrist, 2006).

In Europe, most settled sludges are stabilised, thickened by anaerobic digestion and then disposed (Roman *et al.*, 2006). Traditional methods for sludge disposal are application as fertilizer in agriculture, disposal in landfills or the sea, or incineration (Spinosa, 2004). However, due to stricter regulations —and these regulations will become even stricter due to the upcoming version of the European Urban Waste Water Treatment Directive (UWWTD)— there is a strong need to develop technologies for decreasing waste sludge production and alternatives for disposal, such as recycling of valuable components in the waste sludge (e.g. Low & Chase, 1999; Spinosa, 2001; Wei *et al.*, 2003b). Several authors wrote reviews on current technologies for waste sludge minimization, involving chemical, physical, mechanical and biological technologies and combinations thereof (e.g. Wei *et al.*, 2003b; Ødegaard, 2004; Ramakrishna & Viraraghavan, 2005; Andreottola & Foladori, 2006; Pérez-Elvira *et al.*, 2006). These technologies for example influence/promote lysis-cryptic growth, uncoupling metabolism, anaerobic digestion, maintenance metabolism and predation on bacteria in the sludge matrix. In addition, several authors wrote reviews about recovery options for materials and energy from waste sludge (e.g. Ødegaard *et al.*, 2002; Spinosa, 2004; Kroiss, 2004; Rulkens, 2004; Rulkens & Bien, 2004; Hospido *et al.*, 2005; Pérez-Elvira *et al.*, 2006). These options include nutrients, organic materials, biogas, carbon, fuel and building materials. For example, 104 WWTPs in the Netherlands have anaerobic sludge digesters for sludge reduction and biogas generation (Coenen *et al.*, 2005).

In conclusion, key factors for improving the processing of waste sludge are reducing the amount of dry solids, reducing the volume and recovering valuable components.

1.2 A biological option for reducing waste sludge production and recovering valuable components

In the Netherlands, the majority of WWTPs, especially the smaller plants, do not have facilities for anaerobic sludge digestion. In addition, some of the sludge reduction technologies above have a high potential, but require the addition of chemicals or the input of a substantial amount of extra energy (e.g. Wei *et al.*, 2003b; van Rens *et al.*, 2005; Nowak, 2006). A biological technology for processing waste sludge, that addresses the three mentioned key factors and in theory makes use of the natural food chain only, is waste sludge reduction by worms. This technology applies worms that feed and grow on biological waste sludge and it can be divided into sludge reduction with earthworms (vermicomposting) or with aquatic worms. While feeding on the sludge, its dry mass is reduced by metabolic processes in the worms, its volume is reduced due to compacting of the sludge flocs and at the same time protein-rich worm biomass is produced, with several re-use options and thus added value.

Sludge reduction with earthworms is a promising and relatively common technology, especially in developing countries in small-scale settings (e.g. Hornor & Mitchell, 1981; Hartenstein *et al.*, 1984; Ndegwa & Thompson, 2001; Cardosa & Ramirez, 2002; Bajsa *et al.*, 2003; Bukuru & Jian, 2005). An important consideration for the applicability of this technology is the moisture content of waste sludge, which is optimal at around 80 % (e.g. Lotzof, 1999; Singh *et al.*, 2004; Ratsak & Verkuijlen, 2006).

The second option is sludge reduction with aquatic worms, the subject of this thesis. This technology applies worms that naturally occur in WWTPs and there is no need for sludge thickening as with earthworms. The occurrence of aquatic worms in WWTPs and their growth on waste sludge has been described by several authors since more than 60 years ago (e.g. Reynoldson, 1939b; Curds & Hawkes, 1975; Learner, 1979; Poole & Fry, 1980; Aston & Milner, 1981; Densem, 1982; Learner & Chawner, 1998). Papers on the actual application (mostly in laboratory set-ups) however have mainly appeared in more recent years. Currently, no full-scale systems for sludge reduction with aquatic worms are operational (or the results have not been published). Most research on sludge reduction by aquatic worms has been conducted in China (e.g. Wei *et al.*, 2003a; Wei & Liu, 2005; Liang *et al.*, 2006a; Liang *et al.*, 2006b; Wei & Liu, 2006; Guo *et al.*, 2007; Huang *et al.*, 2007), Japan (e.g. Zhang, 1997; Luxmy *et al.*, 2001) and the Netherlands (e.g. Ratsak, 1994; Rensink & Rulkens, 1997; Janssen *et al.*, 1998;

Ratsak, 2001; Janssen *et al.*, 2002). Table 1.1 shows an overview of the most important results from these researches.

Table 1.1 Overview of the main results from researches on sludge reduction by aquatic worms. Abbreviations used: A.h. = *Aeolosoma hemprichi*, C_{sludge} = carbon content of sludge, N.e. = *Nais elinguis*, P.a. = *Pristina aequiseta*, SVI = sludge volume index, TOC = total organic carbon, TP = total phosphate, TSS = total suspended solids, T.t. = *Tubifex tubifex*, Tub = sessile Tubificidae, VSS = volatile suspended solids, W/S ratio = calculated approximate **maximum** worm to sludge ratio (dry matter based), Y = sludge yield in kg TSS per kg $COD_{removed}$, ↓ = decrease, ↑ = increase.

Author	Main worm species (+W/S ratio)	Main results	Controls
China			
Wei *et al.* (2003a)	A.h., P.a., N.e. (~0.9)	Y = 0.14 SVI↓	Control Y = 0.22
Wei & Liu (2005)	Tub	TSS↓ 59 % SVI↓	Control TSS↓ 14 %
Liang *et al.* (2006a)	A.h. (~0.07)	TSS↓ 39-65 % VSS↓ 0.5-6.3 mg/ mg worm/ d Y = 0.10-0.27 SVI↓	Control deducted in calculations Control Y = 0.25-0.49
Liang *et al.* (2006b)	A.h., T.t.	VSS↓ 0.5-0.8 mg C_{sludge}/ mg worm/ d	Control deducted in calculations
Wei & Liu (2006)	Tub (~0.6)	TSS↓ 48 (±45) %	No control
Guo *et al.* (2007)	Tub	TSS↓ 46 %	No control
Huang *et al.* (2007)	T.t. (~0.1)	VSS↓ 0.2-0.8 mg/ mg worm/ d SVI↑ Effluent TP↑	Control deducted in calculations
Japan			
Zhang (1997)	A.h. (~0.07)	TSS↓ 40 %	Control
Luxmy *et al.* (2001)	Unknown worms	No reduction	Control
the Netherlands			
Rensink & Rulkens (1997)	Tub	COD↓ 18-67 % Y = 0.15 SVI↓ Nitrate, phosphate↑	Control COD↓ 20 % Control Y = 0.4
Janssen *et al.* (1998)	Tub, A.h., N.e. (~0.3)	TSS↓ 10-50 % Y = 0.17 SVI↓ Nitrate, phosphate↑	Control TSS↓ 10-15 % Control Y = 0.22
Ratsak (1994 & 2001)	N.e. (~0.4)	Sludge disposal or TSS↓ 25-50 % SVI↓	Control
Janssen *et al.* (2002)	Tub, A.h.	TSS↓ 30 %	Control TSS↓ 10 %

In summary, most researchers mention a substantial TSS reduction and a lower SVI (i.e. improved sludge settleability) as a result of waste sludge digestion by worms. Ratsak & Verkuijlen (2006) gave an extensive overview of the applicability of this technology in full-scale wastewater treatment and concluded that the main challenges lie within the control of the process. Some points of consideration are unstable worm growth (and sludge digestion) in relation to unknown optimal conditions, surface area of a reactor, extra energy input (e.g. for aeration) and the fate of heavy metals that are present in waste sludge. As they pointed out, available data from these researches are highly variable and sometimes incomplete, contradictory or difficult to interpret. Therefore, a critical view on sludge reduction research with aquatic worms is essential, because several factors are often overlooked, but cause serious overestimations of the ability of aquatic worms to digest sludge. These factors include the worm to sludge ratio, increased endogenous sludge digestion as a result of increased sludge ages, oxygen concentrations or temperatures, sludge accumulation in reactors, carrier materials (in full-scale experiments) and worms (in case of pilot-scale experiments). It may well be that worm presence often coincides with or results from changes in certain process characteristics or process performance, but not causes them. In addition, even though the maximum biodegradability of waste sludge by various technologies lies around 80 % (Ramakrishna & Viraraghavan, 2005; Pérez-Elvira *et al.*, 2006), this does not mean that aquatic worms alone are capable of reaching this percentage, as the variable sludge reduction percentages of 10-93 % in Table 1.1 illustrate.

1.3 Past research on waste sludge reduction with aquatic worms in the Netherlands

Research on waste sludge reduction by aquatic worms started more than 30 years ago at the Sub-department of Environmental Technology at Wageningen University. In 1973, during a student research aimed at reducing the occurrence of bulking sludge, large populations of the aquatic worm *Aeolosoma hemprichi* were observed in pilot-scale oxidation ditches fed with synthetic dairy wastewater. Their high population densities caused the sludge to colour red and waste sludge production and effluent COD concentrations seemed to decrease. Even though the worms were initially regarded as a negative factor for the treatment system, their application for waste sludge reduction was considered for the first time. In 1995, this research field became much more relevant, due to the Dutch 'BOOM' regulations. These regulations prohibit the application of waste sludge as fertilizer in agriculture, when certain heavy metal concentrations are exceeded (Appendix II, Table A3). As a result, waste sludge was no longer applied in agriculture in the Netherlands.

Research in the period between 1973 and 2001 focused initially on the free-swimming family Aeolosomatidae (mostly *A. hemprichi*) and later also on sessile

Tubificidae mixtures[1]. From 2001 on, the research focused mainly on the sessile species *Lumbriculus variegatus* (family Lumbriculidae), because this species displayed much more stable sludge reduction and worm growth than the before-mentioned families (this thesis). Further information on the characteristics of these worm families can be found in Chapter 2.

The batch or continuous experiments with Aeolosomatidae and sessile Tubificidae were done with sludges produced from municipal and non-municipal (dairy and beer) wastewaters. A variety of systems was studied, ranging from laboratory glassware to pilot-scale trickling filters and activated sludge systems, to full-scale WWTPs. The Aeolosomatidae, which do not need a carrier material, displayed population peaks. After a fast growth period of 3-4 weeks with enormous population densities of up to 600 specimens per mL, their individual size became smaller and the populations usually disappeared after some more weeks. There were no clear indications as to what caused these peaks and their disappearance. Growth of sessile Tubificidae, usually in carrier materials that they needed for attachment, was more stable, but populations regularly disappeared also. Beneficial conditions for aquatic worm populations seemed to be > 2 mg O_2/ L, pH 5-9, low ammonia concentrations, temperatures around 15-25 °C, low turbulence and sludge retention times lower than the doubling times of the worms. However, applying these conditions did not guarantee the presence of high worm densities and growth under different conditions was also observed. The most important effects of aquatic worms were decreases in sludge production (up to twice that of a system without worms), SVI and effluent COD and increases in nitrate, phosphate and turbidity of the effluent, but results were variable and sometimes contradictory. In addition, verifying these conclusions now is not easy from the available data and is often hampered by changes in process conditions during experiments (e.g. sludge loading rate, pH, temperature and sludge age), missing analytical details and missing control experiments.

Even though the results obtained so far were promising, there were still too many problems associated with maintaining stable worm populations and with constructing an experimental set-up that exactly quantified the sludge reduction capacity of aquatic worms and their influence on other process characteristics.

1.4 Objective and outline of this thesis

Based on the past results, it was too early for scaling up this technology for practical applications in wastewater treatment. Further research at Wageningen University on this technology therefore focused on three main subjects. The natural

[1] Free-swimming species are abundant in the mixed liquor (in or on the sludge particles) of WWTPs. Sessile species are mainly present on surfaces in the plants, e.g. the reactor walls or carrier materials. Tubificidae can be subdivided into sessile species (i.e. sessile Tubificidae) and free-swimming species (i.e. Naidinae) (Chapter 2).

occurrences and population dynamics of aquatic worms in relation to process characteristics and process performance were studied in WWTPs and pilot-scale continuous reactors. In batch experiments, the effects of different worm species on sludge reduction and sludge characteristics were studied. The research was carried out in close cooperation with Bas Buys (Sub-department of Environmental Technology, Wageningen University, the Netherlands). This thesis describes part of the research and specifically aims to identify factors that are important for the application of this technology (especially with the sessile aquatic worm *Lumbriculus variegatus*) in wastewater treatment.

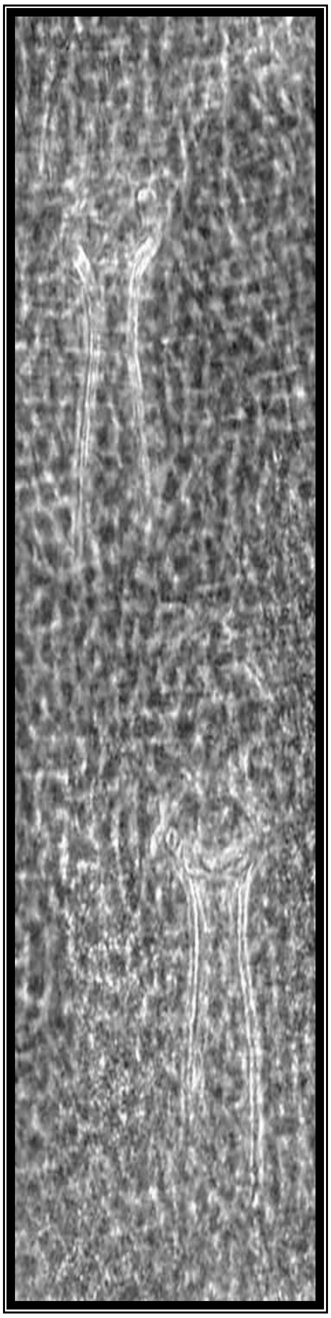
Chapter 2 gives an overview of the aquatic worms (Aeolosomatidae, Tubificidae (including Naidinae) and *L. variegatus*) that are described in this thesis, with short descriptions of their appearance, habitat, food, reproduction and characteristics that make them suitable for sludge reduction processes. **Chapter 3** describes a 2.5-year survey, in which populations of free-swimming worms in the aeration tanks of four Dutch WWTPs were counted regularly. The relation of these populations with process characteristics and process performance of the sampled WWTPs was analysed by multivariate analyses to evaluate their applicability in wastewater treatment.

The sessile aquatic worm *L. variegatus*, which was found in mixtures of sessile Tubificidae, was selected for further investigation. This was based on initial observations of sludge reduction and growth by this species (Buys, 2005; own data), the variability in literature data for other sessile and free-swimming aquatic worm species and the unstable growth of these free-swimming species in the survey described in Chapter 3. **Chapter 4** (a joint chapter with Bas Buys as first author) describes batch experiments that investigated the basic mechanisms of sludge reduction by *L. variegatus*. **Chapter 5** describes batch experiments that evaluated factors, which possibly influence sludge digestion by *L. variegatus* and worm growth. It focuses on sludge characteristics (sludges from different WWTPs and sludges pre-treated by digestion, sterilization or sieving into different particle size classes), worm characteristics (high population densities, high worm to sludge ratios and worm size) and process conditions (ferric iron addition and light/dark conditions). **Chapter 6** describes batch experiments that investigated the influence of sludge digestion by *L. variegatus* on sludge characteristics. These characteristics include settleability, dewaterability and degradability of sludge, turbidity of the sludge water phase and concentrations of proteins, carbohydrates and heavy metals. **Chapter 7** describes batch experiments that compared the sludge reduction capacity and worm growth of *L. variegatus* with that of sessile Tubificidae and a mixed culture of both worm types. In addition, heavy metal bioaccumulation by both worm types was compared. **Chapter 8** describes initial batch experiments with a pilot-scale reactor for the application of *L. variegatus* in sludge reduction processes. In this set-up, the worms are immobilized in a carrier material and waste sludge and worm faeces are separated. Finally, **Chapter 9** discusses the consequences of the data found in this research for the applicability of *L.*

variegatus for sludge reduction in full-scale wastewater treatment. The overall feasibility of this concept and suggestions for further research are discussed as well.

╡Chapter 2╞

Descriptions of aquatic worms in sludge reduction research



Abstract

This thesis refers to various species of aquatic worms. An overview of the three involved families Aeolosomatidae, Tubificidae (including the subfamily Naidinae) and Lumbriculidae is presented in this chapter. It describes their appearance, natural habitat, food, reproduction and use in research on sludge reduction in wastewater treatment. Of the Lumbriculidae, only *Lumbriculus variegatus* is described. This overview shows that each of the described (sub)families has characteristics that are advantageous or disadvantageous for application in sludge reduction processes. Species with the most optimal combination of these characteristics under the conditions in WWTPs should be selected. Alternatively, specific conditions in a separate worm reactor can be optimized for the selected species.

2.1 Introduction

This thesis refers to various species of aquatic worms (phylum Annelida), belonging to the three families Aeolosomatidae, Tubificidae (including the subfamily Naidinae) and Lumbriculidae. Representatives of these families are commonly found in surveys of natural waters. This chapter gives a short overview of their appearance, natural habitat, food, reproduction and use in research on sludge reduction in wastewater treatment. The information in this chapter focuses mainly on the species of each family that are important in the current research.

The species under study can be classified according to their mode of movement or attachment to substrates into the categories 'free-swimming' or 'sessile'. Free-swimming species are abundant in the mixed liquor (in or on the sludge particles) of WWTPs (wastewater treatment plants), while sessile species are mainly found on surfaces in the plants, e.g. the reactor walls or carrier materials. In natural aquatic environments, the sessile species typically forage in a head-down position in the sediment. The free-swimming species include representatives of the family of Aeolosomatidae and subfamily Naidinae (family Tubificidae). The sessile species include representatives of other Tubificidae than the Naidinae and of the family Lumbriculidae. Even though Naidinae were long regarded as a separate family —the Naididae—, and still are classified as such in literature on sludge reduction research (e.g. Wei & Liu, 2006), they recently have been re-classified as subfamily Naidinae within the family Tubificidae based on 18S rDNA sequences (Erseus *et al.*, 2002). Due to this recent re-classification, most literature on Tubificidae does not refer to the Naidinae. In this thesis, 'sessile Tubificidae' therefore refers to the sessile species, but not to the free-swimming Naidinae. The information on the family Lumbriculidae focuses on the species *Lumbriculus variegatus,* because no other members of this family are mentioned in the remainder of this thesis.

2.2 Identification and appearance

The following identification keys for aquatic worms were used: Sperber (1950), Brinkhurst (1971), Brinkhurst & Jamieson (1971) and Timm (1999). Exact identification is often only possible after examination of the chaetae ('bristles') or reproductive organs in mature specimens. Therefore, the specimens are mounted in polyvinyl lactophenol or Canada balsam (e.g. Sperber, 1948; McElhone, 1982) and studied under a microscope. Table 2.1 shows an overview of the four (sub)families with their general characteristics. Table 2.1 includes length, reproduction, motion and aspects of appearance that facilitate identification.

Table 2.1 Some general characteristics of aquatic worm (sub)families described in this thesis.

(Sub)families	Length (mm)	Reproduction	Appearance
Free-swimming			
Aeolosomatidae	1-2	Asexual	Transparent, gliding, oil droplets
Naidinae	5-10	Asexual	Transparent, swimming, sometimes eyes or proboscis (snout) present
Sessile			
Sessile Tubificidae	20-60	Sexual (eggs)	Light red, coiling, waving tail
L. variegatus	40-60	Asexual	Dark red, escape reflex, steady tail

Worm sizes (and weights) can vary greatly during life history and also between different food sources and species of the same genus. To get an indication of this range, an overview of wet and dry weights from literature, Buys (2005) and the author's observation is given in Table 2.2. Worm dry weight was determined after drying overnight at 105 °C. The dry to wet weight percentage of Aeolosomatidae and Naidinae is around 5-10 %, and of sessile Tubificidae and *L. variegatus* around 17 and 13 % respectively.

Table 2.2 Some individual wet and dry weights of Aeolosomatidae, Naidinae, sessile Tubificidae and *L. variegatus* from literature and own observations.

	Wet weight	Dry weight	Food source	References
Aeolosomatidae	(μg)	(μg)		
Aeolosoma spp.	8-14	0.2-3	Sewage sludge	Buys (2005), Liang *et al.* (2006a), own data
	130	0.1-11	Bacteria & yeast	MacMichael *et al.* (1988)
Naidinae	(μg)	(μg)		
Nais spp.	140	11-16	Sewage sludge	Buys (2005), own data
	110-130		Agar based	Lochhead & Learner (1983)
	220		Detritus	Schönborn (1985)
		30	Sediments	Petersen *et al.* (1998)
Pristina sp.	90		Agar based	Lochhead & Learner (1983)
C. diastrophus	10-40		Protozoa	Schönborn (1984)

Table 2.2 Continued.

Species	Wet weight	Dry weight	Food source	References
Sessile Tubificidae	(mg)	(mg)		
T. tubifex	3-8		Sediments	Appleby & Brinkhurst (1970)
	2-23	0.4-3	Sewage sludge	Finogenova & Lobasheva (1987)
Limnodrilus spp.	0.1-20		Sediments	Finogenova & Lobasheva (1987), Wiederholm *et al.* (1987)
		0.6	Sediments	Reible *et al.* (1996)
L. variegatus	(mg)	(mg)		
	5-18		Fish food	Conrad *et al.* (2002), Leppänen & Kukkonen (1998a)
	10-50	1-7	Sewage sludge	Buys (2005), own data

2.3 Descriptions of free-swimming species

2.3.1 Aeolosomatidae (Class Aphanoneura)

In the past, Aeolosomatidae were considered to belong to the class of Oligochaeta, but based on their distinct and primitive characteristics they were assigned to a separate class, the Aphanoneura (e.g. Hessling & Purschke, 2000). In sludge reduction research however, they still are usually classified as Oligochaeta (e.g. Wei & Liu, 2006). The aeolosomatid species referred to in this thesis are *Aeolosoma hemprichi*, *A. variegatum* and *A. tenebrarum*.

Appearance Aeolosomatidae are transparent, a few mm long and most of them possess coloured 'oily' droplets in their body (Stephenson, 1930; Singer, 1978). Their movement can be described as 'gliding'. They possess a typical enlarged ciliated prostomium (head section) (Kamemoto & Goodnight, 1956). Figure 2.1 shows a general view of *A. hemprichi*.

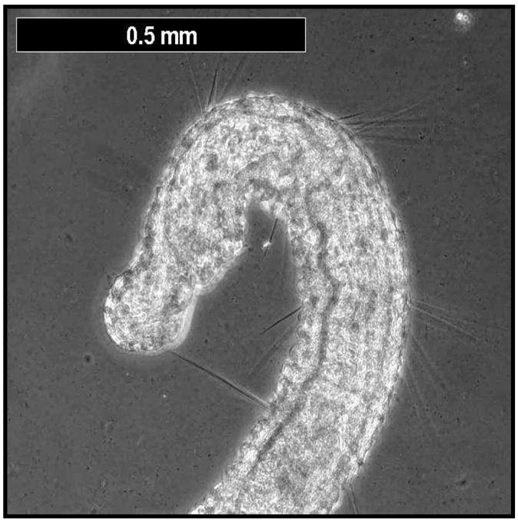


Figure 2.1 General view of *Aeolosoma hemprichi.*

Habitat Aeolosomatidae have a worldwide distribution (Stephenson, 1930) and mainly are fresh-water inhabitants, but can also be found in shallow brackish waters (Jørgensen & Jensen, 1978). They are collected from ponds, lakes and rivers from decaying plant materials (Singer, 1978; Niederlehner *et al.*, 1984). The optimal temperature for growth and reproduction of *A. hemprichi* and *A. variegatum* is between 20 and 30 °C and at 10 °C reproduction stops (Kamemoto & Goodnight, 1956). Various authors recorded mass presences of Aeolosomatidae, especially *A. hemprichi*, in WWTPs (Curds & Hawkes, 1975; Jørgensen & Jensen, 1978). More recently, several authors described the use of *A. hemprichi* for sludge reduction (Inamori *et al.*, 1983; Inamori *et al.*, 1987; Inamori *et al.,* 1990; Kuniyasu *et al.*, 1997; Zhang, 1997; Wei *et al.*, 2003a; Wei & Liu, 2005; Liang *et al.*, 2006a; Liang *et al.*, 2006b).
Food Aeolosomatidae feed on plant tissue, detritus, Protozoa, bacteria and algae (Kamemoto & Goodnight, 1956; Singer, 1978).
Reproduction Aeolosomatidae reproduce through asexual transversal fission (paratomy) and sexually mature specimens are rare (Christensen, 1984). Paratomy involves regeneration of worms before fragmentation and leads to a chain of connected specimens (zooids). The doubling time of Aeolosomatidae is short and usually lies between 1 and 4 days (Kuwahara & Yamamoto, 1981; MacMichael *et al.*, 1988; Inamori *et al.*, 1990).

2.3.2 Naidinae (Class Oligochaeta, family Tubificidae)

The naidine species referred to in this thesis are *Nais elinguis*, *N. communis*, *N. variabilis*, *Pristina aequiseta* and *Chaetogaster diastrophus*. However, the representatives of the genus *Nais* were often not identified to species level. Learner *et al.* (1978) have written an extensive review on the ecology of Naidinae.
Appearance Most Naidinae are colourless and transparent (Stephenson, 1930) and usually smaller than 1 cm (Learner *et al.*, 1978). Members of the genus *Nais* often possess pigmented eyespots, while those of the genus *Pristina* have an elongated proboscis (Brinkhurst, 1971). Naidinae move by swimming or by crawling (Sperber, 1948). Figure 2.2 shows a general view of *Nais* sp.

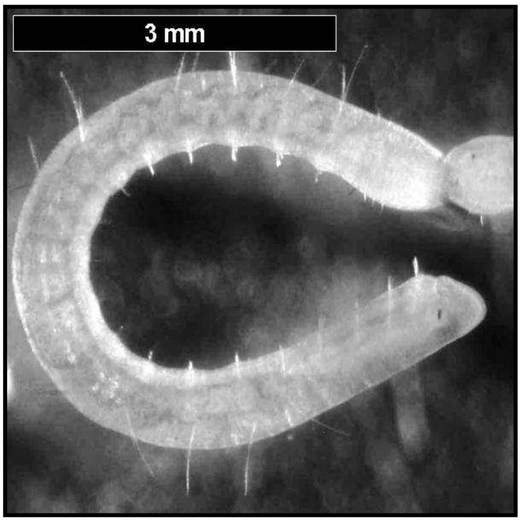


Figure 2.2 General view of *Nais* sp.

Habitat Naidinae are mostly freshwater inhabitants found in rivers, lakes and estuaries, but can also be found in brackish waters (Brinkhurst & Jamieson, 1971; Harper *et al.*, 1981a; Little, 1984). They have a worldwide distribution (Stephenson, 1930). Some species, like *N. elinguis*, can be abundant at organically enriched sites (Learner *et al.*, 1978; Petersen *et al.*, 1998), but in general, they are less tolerant to oxygen depletion and pollution than sessile Tubificidae (Learner *et al.*, 1978; Marshall & Winterbourn, 1979). The occurrence of species that are also investigated in this thesis in WWTPs and their possible application in sludge reduction processes was mentioned by various authors (e.g. Curds & Hawkes, 1975; Learner, 1979; Lochhead & Learner, 1983; Inamori *et al.*, 1983; Ratsak, 1994; Kuniyasu *et al.*, 1997; Ratsak, 2001; Wei *et al.*, 2003a; Wei & Liu, 2005). Optimal temperatures for feeding and growth lie around 20 °C (Petersen *et al.*, 1998). At 5 °C, growth is almost absent (Lochhead & Learner, 1983). Examples of typical maximum densities are 70,000 specimens per m^2 in freshwater habitats and 200,000 specimens per m^2 in polluted habitats (Wachs, 1967; Szczesny, 1974).

Food Naidinae can feed on bacteria, detritus and algae, but they can also use dissolved free amino acids (McElhone, 1978; Harper *et al.*, 1981a; Harper *et al.*, 1981b; Bowker *et al.*, 1985; Schönborn, 1985; Petersen *et al.*, 1998). Some species, like *C. diastrophus*, are mainly predatory (e.g. feeding on Protozoa) (Schönborn, 1984).

Reproduction Naidinae reproduce asexually by transverse fission (paratomy) all year round, but in certain seasons they also reproduce sexually and produce eggs in cocoons (Christensen, 1980). Their densities display large seasonal fluctuations (Smith, 1985). It is largely unknown how the percentage of worms reproducing by paratomy or sexual reproduction is related to environmental physico-chemical parameters (Smith, 1985; Smith, 1986), but asexual reproduction seems to be related to favourable environmental conditions (temperature and food supply), in contrast to sexual reproduction (Loden, 1981; Juget *et al.*, 1989). For the species mentioned in this thesis densities are related to water temperature, oxygen concentrations, alkalinity, pH, lack of predation, abundant food supply and lack of competition (e.g. McElhone, 1978; Lochhead & Learner, 1983;

Smith, 1985; Smith, 1986). Doubling times lie between 2 and 23 days (Lochhead & Learner, 1983; Schönborn, 1985; Smith, 1986).

2.4 Descriptions of sessile species

2.4.1 Sessile Tubificidae (Class Oligochaeta)

The tubificid species referred to in this thesis are *Tubifex tubifex*, *Limnodrilus hoffmeisteri*, *L. udekemianus* and *L. claparedianus*. However, the sessile Tubificidae were often not identified to genus or species level.

Appearance Sessile Tubificidae are usually reddish in colour, several cm in length (up to 20 cm) and less than 2 mm in diameter (Stephenson, 1930; Whitten & Goodnight, 1966a). They move by crawling and typically forage in a head-down position in sediments, with their tails protruding upwards and waving for the uptake of oxygen. When disturbed, they often coil their body (Alsterberg, 1924). Figure 2.3 shows a general view of sessile Tubificidae.

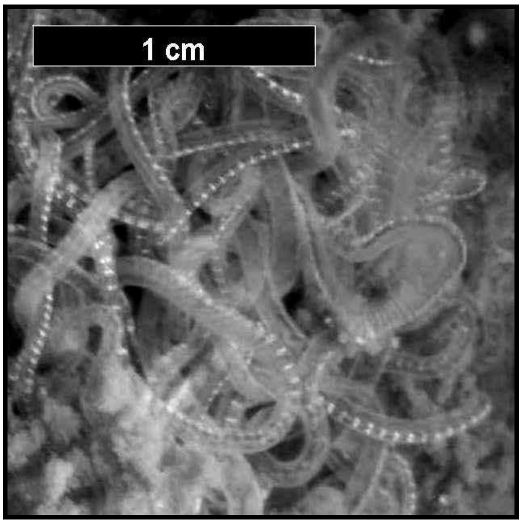


Figure 2.3 General view of sessile Tubificidae (mainly tails of *L. udekemianus*).

Habitat Sessile Tubificidae include both freshwater (e.g. lakes and often slowly flowing waters) and marine species and are mostly found in muddy and sandy sediments (Appleby & Brinkhurst, 1970; Kosiorek, 1974). The far most studied species is *T. tubifex* (e.g. Kosiorek, 1974; Finogenova & Lobasheva, 1987). The species of sessile Tubificidae referred to in this thesis are known for their tolerance to high eutrophic conditions (especially *T. tubifex* and *L. hoffmeisteri*) and are regarded as indicators of organic pollution (Brinkhurst & Kennedy, 1965; Whitley, 1968; Aston, 1973; Chapman & Brinkhurst, 1984). Various authors reported their occurrence in WWTPs and their application (usually *T. tubifex*) for sludge reduction in wastewater treatment (Whitten & Goodnight, 1966a; Rensink & Rulkens, 1997; Luxmy *et al.*, 2001; Wei & Liu, 2005; Liang *et al.*, 2006b; Wei & Liu, 2006; Huang *et al.*, 2007; Guo *et al.*, 2007). Sessile Tubificidae populations can reach very high densities of 400,000 specimens per m²

(Whitten & Goodnight, 1966a) or even more. In addition to their pollution tolerance they are able to deal with anoxic conditions for long periods (up to 25 days) by switching to an anaerobic metabolism, as was described for *T. tubifex* (Alsterberg, 1922; Degn & Kristensen, 1981) and sessile Tubificidae in general (Whitten & Goodnight, 1966a). The optimal temperature of *T. tubifex* is 20-25 °C (Kosiorek, 1974). At 4 °C, feeding and growth rates of *L. hoffmeisteri* and *T. tubifex* are very low (Appleby & Brinkhurst, 1970). The role of sessile Tubificidae as bioturbators, i.e. by mixing the sediment surface and thereby altering conditions, is often described (e.g. Reible *et al.*, 1996; Wegener *et al.*, 2002; Delmotte *et al.*, 2007). In addition, they (especially *T. tubifex* and sometimes *L. hoffmeisteri*) are used as test organism in bioassays and water quality assessment studies (e.g. Milbrink, 1987a; Wiederholm *et al.*, 1987; Reynoldson *et al.*, 1991; Millward *et al.*, 2001; Mosleh *et al.*, 2005). Mixtures of sessile Tubificidae (mainly consisting of *Limnodrilus* species and some *T. tubifex*, but mistakenly called 'Tubifex') are sold in pet shops as fish food.

Food Like most aquatic worms, sessile Tubificidae feed on detritus, and researches by Brinkhurst & Chua (1969) and Wavre & Brinkhurst (1971) indicated that they feed mainly on the bacteria in sediments, which they extract during the continuous ingestion of sediment particles.

Reproduction Sessile Tubificidae usually reproduce sexually by producing eggs in cocoons, with up to 300 eggs per worm in 100 days (Finogenova & Lobasheva, 1987). Asexual reproduction by fragmentation is rarely observed, but asexual reproduction by parthenogenesis (involving egg production without fertilization) is also common (Christensen, 1980). The duration of a typical life cycle for *T. tubifex* is 20-62 days (Kosiorek, 1974; Finogenova & Lobasheva, 1987).

2.4.2 *Lumbriculus variegatus* (Class Oligochaeta, family Lumbriculidae)

Extensive information on this species can be found on the website of the late Charles Drewes (Drewes, 2005).

Appearance *L. variegatus* has a red colour, but is usually somewhat darker than sessile Tubificidae. Specimens are on average 4-6 cm long (up to 17 cm) and up to 1.5 mm in diameter (Drewes, 2005). Figure 2.4 shows a general view of *L. variegatus* specimens.

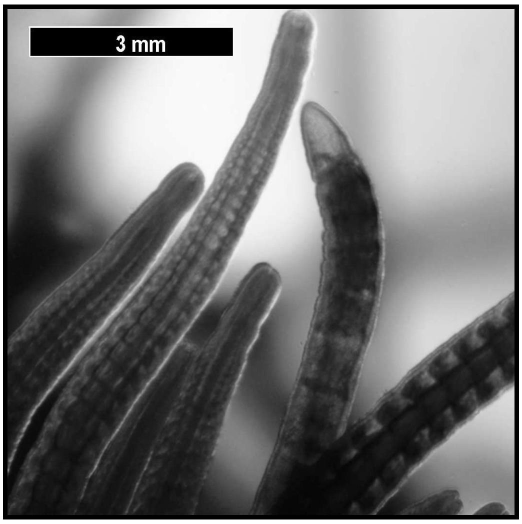


Figure 2.4 General view of *Lumbriculus variegatus* specimens.

Its wet weight (mg) to length (in mm) percentage when grown on sludge is around 55 % (Figure 2.5). Weight and length are dependent on the food source (as illustrated by Table 2.2 for several worm species), because worms grown on waste sludge are in general larger than those grown on fish food and other substrates are (e.g. 5-10 mg as found by Conrad *et al.* (2002)). In addition, Gnaiger & Staudigl (1987a) found a different wet weight to length percentage of only 24 % on plants and bacteria as food source.

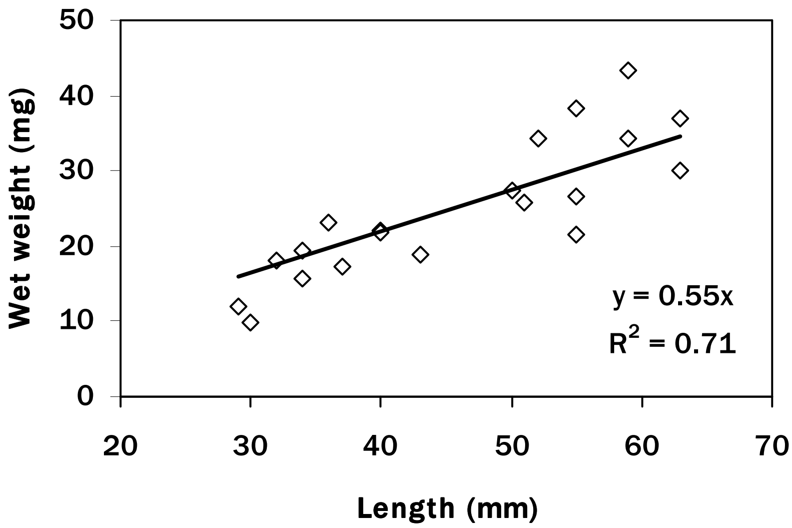


Figure 2.5 Wet weight (mg) of *Lumbriculus variegatus* as a function of length (in mm) grown on waste sludge.

Like sessile Tubificidae, *L. variegatus* feeds in a head-down position in sediment, with its tail protruding upwards for the uptake of oxygen. However, it does not wave its tail as sessile Tubificidae, but keeps a fixed position (Drewes, 2005). *L. variegatus* moves by crawling and swimming for short distances (Drewes, 1999). When disturbed, it displays a highly characteristically quick escape reflex (Drewes & Fourtner, 1989).

Habitat *L. variegatus* is a cosmopolitan species, that is mainly found in Europe and North-America in freshwater and benthic environments, but was also introduced into Asia, Africa, Australia and New Zealand (Brinkhurst & Jamieson, 1971; Pickavance, 1971). In these environments, it is a very common species as was described for example for Ireland by Trodd *et al.* (2005). It can especially be found in leaf litter along the shallow margins of marshes and ponds (Drewes, 2005). The abundance of this species is usually negatively correlated with the pollution level and nutrient enrichment of the habitat, in contrast to members of the sessile Tubificidae (Marshall & Winterbourn, 1979). Probably as a result of this, *L. variegatus* is not commonly found in WWTPs, but occasional records of mass occurrences (for example in trickling filters) exist (Solowiew, 1924; Sperry, 1943; Curds & Hawkes, 1975; Learner & Chawner, 1998). There is also scarce information that *L. variegatus* can sometimes be found at nutrient enriched sites (Marshall & Winterbourn, 1979; Learner & Chawner, 1998) and can survive for 12 hours up to 7 days without oxygen by switching to anaerobic metabolism as was also described for sessile Tubificidae (Putzer *et al.*, 1990; Reh, 1991; Penttinen, 1997). Natural population densities never exceed 12,000 specimens per m^2 (Cook, 1969; Williams, 2005). Chapman *et al.* (1999) state that the optimal temperature range for *L. variegatus* is 20-25 °C, which is the same as for *T. tubifex*. Quinn *et al.* (1994) found LT50 values (i.e. the lethal temperature for 50 % of the worms) of 27-30 °C. These test animals were acclimatized to 15 °C prior to testing, and it was suspected that raising this temperature increased their tolerance to higher temperatures. Nevertheless, *L. variegatus* is considered a thermal sensitive species. Leppänen & Kukkonen (1998a) described that *L. variegatus* stops reproducing at 6 °C. *L. variegatus* can sometimes be found in the sessile Tubificidae mixtures from pet shops. Stephenson (1930) also described mixed populations. *L. variegatus* is commercially available from specialized laboratory suppliers, because it is one of the most frequently used standard benthic test organisms for bioaccumulation and toxicity assays (e.g. Leppänen, 1999; Ingersoll *et al.*, 2000; Williams, 2005).

Food *L. variegatus* feeds on algae and most likely on a mixture of food particles that accumulate in benthic environments, like decaying plant material, bacteria and fungi (Moore, 1978; Williams, 2005).

Reproduction *L. variegatus* reproduces almost exclusively by fragmentation (architomy) and subsequent regeneration (Christensen, 1980). Architomy differs from paratomy (as in Aeolosomatidae and Naidinae) and is more primitive. Doubling times of *L. variegatus* growing in sediments lie between 14 and 40 days (Williams, 2005). Sexually mature specimens of *L. variegatus* are extremely rare but a few authors mentioned sexual reproduction in laboratory cultures (Drewes, 2004) or marshes (Lesiuk & Drewes, 1999). It is not clear if sexual reproduction is linked to a particular season and there is no consensus between different authors (Pickavance, 1971). The same is true for asexual reproduction and the exact factors controlling the fragmentation of *L. variegatus*. Leppänen & Kukkonen (1998b) found that the minimum wet weight required for asexual reproduction is 9 mg (which corresponds to a length of 2 cm in Figure 2.5) and that reproduction is followed by a 6-7 day non-feeding period. They also found that the wet weight and frequency at which worms divide increases with favourable culture conditions and this may explain why Lesiuk & Drewes (1999) mentioned a length of 4 cm, before asexual reproduction took place.

2.5 Conclusions

Each of the (sub)families described above has specific characteristics that are advantageous for application in sludge reduction processes. These characteristics are for example the fast growth of free-swimming Aeolosomatidae and Naidinae, the pollution tolerance of sessile Tubificidae and the continuous asexual reproduction of *L. variegatus*. At the same time, each (sub)family also has characteristics that may be disadvantageous for application in sludge reduction processes: The growth of Aeolosomatidae and Naidinae seems hard to control, sessile Tubificidae need a suitable environment for egg deposition and *L. variegatus* is not often found at organically enriched sites.

Each characteristic will have consequences for sludge reduction and worm growth in an experimental set-up or practical application. Research on application of these families for sludge reduction therefore has to focus on finding the species with the most optimal combination of characteristics under the specific process conditions in WWTPs. Alternatively, when a separate reactor for sludge reduction with worms is constructed, the process conditions can be optimized for the selected worm species.

Acknowledgments

We thank Piet Verdonschot (Alterra, Wageningen University and Research Centre) for his valuable comments on this chapter and an anonymous reviewer of Chapter 3 for pointing out the recent re-classification of Naidinae.

╡Chapter 3╞

Population dynamics of free-swimming Annelida in four Dutch wastewater treatment plants in relation to process characteristics

Based on submitted paper for Hydrobiologia (Elissen, Peeters, Buys, Klapwijk & Rulkens)

Abstract

Free-swimming Annelida, belonging to the Aeolosomatidae and Naidinae (Tubificidae), occasionally occur in very high densities in WWTPs (wastewater treatment plants) and are nowadays applied for waste sludge reduction, but their population growth is uncontrollable. To get more insight in the population dynamics of these free-swimming Annelida, and relate their presence to process characteristics, nine ATs (aeration tanks) of four Dutch WWTPs were regularly sampled over a 2.5-year period. For each species or genus, peak periods in worm population growth were defined and population doubling times and half-lives calculated, and compared to those in natural systems. Data of the process characteristics were provided by the plant operators. By means of multivariate analysis, these process characteristics, as well as sampled WWTP, sampling year and sampling month were related for the first time to the worm populations in full-scale WWTPs.

The species composition in the WWTPs was limited and the most abundant free-swimming Annelida were *Aeolosoma hemprichi, A. tenebrarum, A. variegatum, Nais* spp., *Pristina aequiseta* and *Chaetogaster diastrophus*. This latter species had never been found before in WWTPs. Worm absence was sometimes related to the presence of anoxic zones. Worms were present all year round, even in winter, but no yearly recurrences of population peaks were observed, probably as a result of stable food supply and temperature, and the lack of predation in the WWTPs. Peak periods were similar between the ATs of each WWTP. The duration of the peak periods was on average 2-3 months for all species and the population doubling times in the peak periods were low (on average 2-6 days), which also corresponds to a stable favourable environment. The disappearance of worm populations from the WWTPs was presumably caused by declining asexual reproduction and subsequent removal with the waste sludge.

Multivariate analysis indicated that 36 % of the variability in worm populations was due to variations in sampled WWTP, sampling year and month only. In addition, no more than 4 % of the variability in worm populations was related to variations in process characteristics only, of which sludge settleability was the most important characteristic. The presence of most worm species was associated with better sludge settleability. In conclusion, our data from full-scale WWTPs suggest that population growth of free-swimming Annelida still seems uncontrollable and that their effects on treatment performance are unclear, which makes stable application in wastewater treatment for sludge reduction difficult.

3.1 Introduction

Several species of annelid worms, mainly belonging to the family Tubificidae (including the subfamily Naidinae) are associated with polluted environments characterized by organic enrichment and oxygen depletion (e.g. Aston, 1973; Schönborn, 1985). Their species distribution is regarded as an indicator of environmental quality (Chapman *et al.*, 1982a). Specific examples of these extreme environments are aerobic WWTPs (wastewater treatment plants). The main processes in these plants are aerobic conversion of organic components from municipal or non-municipal (industrial) wastewater into biomass (activated sludge), water and CO_2, and nutrient removal.

Protozoa are the most common and well-described bacterivorous grazers in WWTPs (Curds & Hawkes, 1975; Ratsak, 1994) and several authors described their role as indicators of plant performance and their influence on process characteristics of these WWTPs (e.g. Madoni *et al.*, 1993; Martín-Cereceda *et al.*, 1996; Lee *et al.*, 2004). However, the presence and role of metazoan organisms (Annelida, but also Nematoda and Rotifera) in several types of WWTPs were only occasionally studied in the past. It is not exactly known how Annelida end up in WWTPs, but it is likely that they originate from surrounding water bodies or are transported by birds into the ATs (aeration tanks) (Milbrink & Timm, 2001).

Annelida in WWTPs can be classified as 'free-swimming' (in the sludge) or 'sessile' (on surfaces in the plants, e.g. the reactor walls or carrier materials). They often belong to the class Aphanoneura (Aeolosomatidae) or to the class Oligochaeta (Tubificidae, Enchytraeidae, Lumbriculidae and Lumbricidae) (Curds & Hawkes, 1975). Within these classes, the family Aeolosomatidae and subfamily Naidinae (Tubificidae) are mostly free-swimming species, while the other families and remaining subfamilies of the Tubificidae are more sessile. Annelida feed on organic material in the activated sludge, but some species like *Chaetogaster diastrophus* feed mainly on Protozoa (Schönborn, 1984). Research on Annelida in full-scale WWTPs has mainly focused on biofilter systems or activated sludge systems.

For biofilter systems (e.g. filter beds), the occurrence of Enchytraeidae was studied in many field and laboratory studies. Reynoldson (1939a & 1948) extensively described the life cycles of *Lumbricillus lineatus* and *Enchytraeus albidus*. Constant humidity, high temperatures, good food supply and lack of predators supported large year-round populations. The worms prevented the filter beds from clogging with suspended solids, thus maintaining their treatment efficiency. He also mentioned the presence of *Aeolosoma* sp. and *Pristina* sp. in the beds. Williams *et al.* (1968) described the ecology and seasonal patterns of two Enchytraeidae species (*Lumbricillus rivalis* and *Enchytraeus coronatus*) in a biofilter system. According to Hawkes & Shephard (1972), the worm populations in these filters were only influenced by seasonal fluctuations. Learner & Chawner (1998) described a study in which the macro-invertebrate fauna of 67 biofilters in 48 WWTPs in Britain was sampled once or twice and related to the physico-chemical characteristics (such as F/M (food to micro-organism) ratio, temperature, sampled location and pH) of the biofilter environment. *Nais* sp. and/or *Pristina* sp. were found in more than 40 % of the surveyed biofilters and their occurrence was positively correlated to certain geographical locations and low F/M ratios. *Aeolosoma* sp., *Chaetogaster* sp. and sessile Tubificidae were only found occasionally.

For activated sludge systems, only a few studies have been done on the occurrence of Annelida. Poole & Fry (1980) found stable populations of Annelida (Aeolosomatidae and sessile Tubificidae) in three ATs during 2 months. They concluded that high TSS (total suspended solids) concentrations were beneficial to the Aeolosomatidae. Because high TSS concentrations usually coincide with higher sludge ages (i.e. the average residence times of the sludge in the ATs), their results suggest a positive effect of high sludge ages on worm densities. Inamori *et al.* (1983) mentioned the presence of large quantities of *Aeolosoma* sp., *Pristina* sp., *Nais* sp., *Dero* sp. and *Chaetogaster* sp. in the ATs of WWTPs.

In the Netherlands, most (366 out of 375) of WWTPs are nowadays activated sludge systems (Statistics Netherlands (CBS), 2007). Up to date, there has been only one long-term (1.5 years) monitoring of an annelid species (*Nais elinguis*) in one of these WWTPs (Ratsak, 2001). She concluded that high densities of this worm resulted in decreases of the SVI (sludge volume index), the energy consumption for oxygen supply and, depending on the temperature, the waste sludge production. She also found that the density of worms varied both per season (high densities were both found in warm and cold periods) and per AT for unknown reasons. Population densities displayed peaks (also called 'worm blooms'), which were invariably followed by a sudden disappearance of the population, a well-known phenomenon in WWTPs.

In 2001, a telephone survey of 23 WWTPs in the Netherlands (Janssen *et al.*, 2002) showed that several worm species were often found in the consulted WWTPs. Sessile Tubificidae were found all year round, but *Aeolosoma* spp. and *Nais* spp. were usually found in summer or at high temperatures. Species of the latter genera were sometimes present in such high densities, that the sludge in the ATs had a reddish colour. In half of the plants surveyed, worm presence was assumed to coincide with decreases in waste sludge production and/or better sludge settling characteristics. In recent years, increasingly more researchers focused on applying Annelida (mostly *Aeolosoma hemprichi, Nais* sp. and sessile Tubificidae) for this purpose in laboratory set-ups (e.g. Wei *et al.*, 2003a; Wei & Liu, 2005; Liang *et al.*, 2006a). The largest problems in this research field were and are (next to highly variable results) the before-mentioned uncontrollable population dynamics of especially free-swimming worms (Wei *et al.*, 2003b; Ratsak & Verkuijlen, 2006).

A long-term study of worm population dynamics and possible interactions in and between full-scale WWTPs between process characteristics and worm species that are used for sludge reduction research could provide useful insight for maintaining stable worm populations and sludge reduction rates. Therefore, free-swimming Annelida in the ATs of four Dutch WWTPs (one including a biofilter) were regularly sampled over a 2.5-year period. To explain the population dynamics in these artificial highly enriched ecosystems, peak periods, population doubling times and half-lives were compared to those found in natural water bodies. In addition, the plant operators provided data of the process characteristics during that period. Process characteristics that were expected to have an influence on (e.g. sludge loading rate, sludge age) or to be influenced by the occurrence of worms (e.g. waste sludge production, TSS concentration and nutrient concentrations) were selected. For the first time, a multivariate analysis was performed to quantify possible relationships between long-term worm population growth and process characteristics of full-scale WWTPs.

3.2 Materials and methods

3.2.1 WWTPs

The WWTPs were chosen based on their worm diversity found in the telephone survey (Janssen *et al.*, 2002). WWTPs Drachten (one AT), Nijmegen (three ATs), Renkum (three ATs) and Zwolle (two of four ATs) in the eastern and northern part of the Netherlands were sampled frequently (in total about 75 times per AT, which equals about once every 2 weeks). Plants were sampled from July 2000 through December 2002, but the sampling of WWTP Renkum was terminated in June 2002, due to plant alterations. The plants were all activated sludge systems with a low F/M ratio (< 0.2 kg BOD (biological oxygen demand)/ kg TSS/ d), with parallel ATs acting as separate systems with return waste sludge going to the AT, from which it originated. The wastewater was routed through a series of channels constructed in the AT. Wastewater was first treated in a pre-settler to remove particles by sedimentation, but in WWTP Drachten wastewater was first treated in a trickling filter (biofilter) where soluble and particulate BOD was removed and partly converted into bacteria. Furthermore, in WWTP Nijmegen, the pre-settled influent was heated with cooling water from the nearby waste incinerator from November 2001 on and the average temperature in these ATs was therefore 22 °C instead of 16 °C in the other plants. Table 3.1 shows differences in phosphorus (P) and nitrogen (N) removal steps between the plants.

Table 3.1 P- and N-removal in the sampled WWTPs.

WWTP	P-removal (chemical)	N-removal
Drachten	From January 2002 on	Only nitrification
Nijmegen	Yes	Anoxic pre-denitrification 1)
Renkum	No	Anoxic pre-denitrification 2)
Zwolle	Yes	Anoxic pre-denitrification 3)

1) All ATs contained anoxic zones, 2) Only AT1 in WWTP Renkum contained an anoxic zone for denitrification (non-aerated; nitrate> 0 mg/ L), 3) Anoxic zones were established in all ATs of WWTP Zwolle in the following periods: August-October 2001 and April-October 2002

3.2.2 Worm sampling

At the sampling dates, a sludge sample from each AT was taken in the mixed liquor phase of the aerated zone with a plastic sample container tied to a long pole. The containers were sent by regular mail (which took 2 (±2) days) to our lab, except for the samples from WWTP Renkum, which were analysed the same day. The containers from WWTPs Drachten, Nijmegen and Zwolle were about half-filled with 100-250 mL sample ensuring the presence of oxygen. This was regularly checked after arrival of the samples with a WTW Oximeter 330 and DO (dissolved oxygen) concentrations were on average 2.3 (±1.6) mg O_2/ L.

Two or three samples of 1 mL each were taken by Pasteur pipette with a cut off tip from the well-mixed sludge in the containers, divided into droplets and diluted with tap water on a Petri dish. Live specimens were counted under an Olympus SZ40 stereomicroscope with a Clay Adams laboratory counter and identified to genus level (*Nais* spp.) or species level (all other frequent species) according to Brinkhurst (1971), Brinkhurst & Jamieson (1971) and Timm (1999). Identification was regularly confirmed after live mounting in polyvinyl lactophenol using an Olympus BHT microscope.

3.2.3 Process characteristics

The plant operators provided data of the process characteristics of the WWTPs during the sampling period. Characteristics from the ATs (Table 3.2a), effluent and other miscellaneous characteristics (Table 3.2b) that were expected to have an influence on or to be influenced by the occurrence of worms were selected. Because characteristics were not always analysed at the same days as the worm densities, values within 5 days before and 5 days after (when available) were averaged.

Table 3.2a Overview of selected process characteristics from the ATs (averages in bold, standard deviations in italic, minimum and maximum values, no. of samples between brackets). Abbreviations used: θ = sludge age, Ash = ash content of sludge, D = WWTP Drachten, N = WWTP Nijmegen, R = WWTP Renkum, Z = WWTP Zwolle, numbers 1-4 behind D, N, R and Z indicate ATs, t behind D, N, R and Z indicates the total WWTP.

AT	Aeration tanks							
	pH	DO (mg/ L)	T (°C)	TSS (g/ L)	Ash (%)	θ (d)	SVI (mL/ g)	F/M ratio (g BOD/ g TSS/ d)
Dt			**15** ±*3*	**4** ±*1*	**33** ±*2*		**65** ±*8*	
			10-20	2-7	30-36		42-79	
			(27)	(29)	(27)		(29)	
N1	**7** ±*0*		**21** ±*3*	**4** ±*1*			**74** ±*17*	**0.07** ±*0.02*
	6-7		15-27	2-5			46-143	0.02-0.14
	(77)		(77)	(77)			(77)	(74)
N2	**7** ±*0*		**21** ±*3*	**4** ±*1*			**69** ±*13*	**0.07** ±*0.02*
	6-7		15-27	2-6			45-102	0.02-0.12
	(73)		(72)	(73)			(73)	(70)
N3	**7** ±*0*		**20** ±*3*	**4** ±*1*			**70** ±*14*	**0.07** ±*0.02*
	6-7		15-27	2-6			42-103	0.03-0.20
	(77)		(76)	(77)			(77)	(72)
Nt								
R1	**7** ±*0*		**16** ±*4*	**4** ±*1*	**23** ±*3*	**16** ±*8*	**69** ±*15*	**0.07** ±*0.02*
	6-7		8-23	3-7	18-31	4-49	44-131	0.04-0.12
	(70)		(69)	(70)	(28)	(59)	(68)	(29)
R2	**7** ±*0*		**16** ±*4*	**4** ±*1*	**23** ±*3*	**15** ±*8*	**76** ±*16*	**0.07** ±*0.03*
	6-7		8-24	3-11	16-27	2-54	33-121	0.04-0.18
	(67)		(67)	(69)	(30)	(58)	(67)	(23)
R3	**7** ±*0*		**16** ±*4*	**4** ±*1*	**22** ±*2*	**17**	**121** ±*29*	**0.06** ±*0.02*
	6-7		8-23	2-6	17-27	±*12*	65-218	0.04-0.13
	(64)		(62)	(66)	(24)	3-68	(64)	(30)
						(55)		
Rt								
Z3		**2** ±*1*		**4** ±*1*		**12** ±*6*	**81** ±*26*	**0.07** ±*0.04*
		0-4		3-7		1-47	43-217	0.02-0.22
		(81)		(80)		(81)	(80)	(74)
Z4		**3** ±*1*		**4** ±*1*		**14** ±*7*	**74** ±*22*	**0.07** ±*0.04*
		0-8		3-7		6-38	43-133	0.02-0.23
		(81)		(80)		(81)	(80)	(72)
Zt								

Table 3.2b Overview of selected process characteristics from the effluent and other miscellaneous characteristics (averages in bold, standard deviations in italic, minimum and maximum values, no. of samples between brackets). Abbreviations used: Same as Table 3.2a, plus Iron = ferric iron dosage to the influent for phosphate removal, N (mg/ L) = sum of NH_4^+, NO_3^- and NO_2^- concentrations as indication of denitrification efficiency, NH_4^+ = indication of nitrification efficiency, Rain = daily rainfall, Waste sludge = daily waste sludge production.

	Effluent				**Miscellaneous**			
AT	**NH_4^+ (mg/L)**	**N (mg/L)**	**BOD (mg/L)**	**TSS (mg/L)**	**Rain (mm)**	**Iron (mL/m³)**	**Waste sludge 10^3 kg**	**Waste sludge 10^3 m³**
Dt	**3** ±*3* 0-9 (27)	**17** ±*6* 9-30 (27)	**4** ±*2* 2-9 (27)	**7** ±*3* 5-14 (27)	**3** ±*2* 1-7 (27)			
N1						**22** ±*7* 7-37 (73)		
N2						**23** ±*7* 3-37 (69)		
N3						**22** ±*8* 2-37 (73)		
Nt	**1** ±*2* 0-6 (77)	**23** ±*5* 9-34 (77)	**2** ±*1* 1-5 (77)	**3** ±*2* 1-11 (77)	**2** ±*3* 0-20 (77)			
R1	**3** ±*3* 0-15 (53)	**24** ±*8* 5-39 (53)					**0.7** ±*0.4* 0-2.2 (61)	**0.1** ±*0.1* 0-0.5 (76)
R2	**4** ±*6* 0-36 (52)	**25** ±*9* 7-40 (52)					**0.8** ±*0.7* 0-5.1 (59)	**0.1** ±*0.1* 0-0.5 (76)
R3	**5** ±*5* 0-21 (50)	**16** ±*6* 6-34 (50)					**0.8** ±*0.6* 0-2.7 (58)	**0.1** ±*0.1* 0-0.4 (75)
Rt			**4** ±*2* 1-10 (66)	**7** ±*2* 5-15 (26)				**0.3** ±*0.2* 0-1.2 (75)
Z3							**1.3** ±*0.8* 0.4-6.7 (81)	**0.2** ±*0.1* 0-0.8 (81)
Z4							**1.0** ±*0.5* 0-4.7 (81)	**0.1** ±*0.1* 0-0.6 (81)
Zt	**2** ±*3* 0-16 (76)	**21** ±*10* 5-55 (77)	**3** ±*2* 1-15 (78)	**6** ±*4* 5-38 (75)	**2** ±*4* 0-18 (87)	**62** ±*30* 12-177 (81)	**4.0** ±*1.5* 1.5-13.7 (81)	**0.6** ±*0.2* 0.2-2.2 (81)

3.2.4 Data analysis

Population dynamics (peak periods, population doubling times and half-lives) For each species and AT, worm densities that exceeded the average density plus two standard deviations were considered to represent peak periods. The densities outside the peak periods were averaged and this was the background density. The start of a peak period was defined as the date at which the density became higher than this background density and the end of a peak period as the date at which the density became lower. For species that never exceeded 15 specimens per mL (e.g. *C. diastrophus*), no peak periods were calculated. In addition, peak periods that lay partially outside the sampling period were excluded from calculations on duration of the peak periods. A peak period could contain several (up to three) peaks, when the worm densities did not become lower than the background densities.

Population doubling times (t_d) and half-lives (t_h) in days in the population peaks were calculated using exponential functions (Nandini & Sarma, 2004), that included population growth rates and decay rates. 'Mechanical' loss of worms by sludge wasting was also taken into account in the calculations. This loss was dependent on the sludge ages (θ) during the peak periods. For WWTPs Renkum and Zwolle, sludge age values (ranging from 9 to 48 days) were provided by the plant operators. For WWTP Nijmegen, the average sludge age was assumed the average sludge age in the other WWTPs, 18 days. Furthermore, the supply of worms to the ATs with the influent and removal of worms from the plant with the effluent were both negligible, as we observed in our pilot plants that were representative of full-scale WWTPs. The population growth rates in the downward phases of the peaks were zero, because dividing worms were rarely observed in these phases. The population decay rates were the same in the upward and downward phases of the peaks, because there was no indication for increased mortality in the downward phases. The observed population growth and decay rates (μ_{obs} and r_{obs}) are thus the result of the real population growth and decay rates (μ and r) and the inverse of the sludge age according to the following equations:

r_{obs}:	**$r + 1/\theta = \ln(X_0 / X_t)/(t-t_0)$**	(d^{-1})	**(1)**
Parameters:			
r	**Real population decay rate**	**(d^{-1})**	
1/θ	**Loss rate via waste sludge**	**(d^{-1})**	
θ	**Sludge age**	**(d)**	
X_t	**Total density of worms at time t**	**(specimens per mL)**	
X_0	**Total density of worms at time t_0**	**(specimens per mL)**	
t, t_0	**Time at end or top of peak**	**(d)**	

μ_{obs}:	μ-r-1/θ = ln(X_t / X_0)/(t-t_0)	(d^{-1})	**(2)**
Parameters:			
μ	Real population growth rate	(d^{-1})	
t, t_0	Time at top or start of peak	(d)	
Population doubling times t_d and half-lives t_h in days were calculated as ln(2)/μ and ln(2)/r, respectively.			

Multivariate analysis To find out if and how the worm species composition was related to the abiotic variables (sampled WWTP and AT, sampling year and month and process characteristics of the WWTPs) a multivariate analysis was performed. Multivariate analyses are suitable to recognize latent patterns in large datasets (Jongman *et al.*, 1987). They can graphically summarize complex datasets in low dimensions. For a proper use, it is important to choose the appropriate response model (linear or unimodal). A preliminary DEtrended CORrespondence ANAlysis (DECORANA) was performed with log transformed abundance data of the worms and invoking the option 'down-weighting of rare species'. The length of the gradients as calculated by DECORANA was larger than 3.5 and thus the unimodal response model was assumed to be appropriate for this dataset (ter Braak, 1986). To analyse the importance of the different abiotic variables on the worm species composition in the ATs, a direct ordination analysis was performed (ter Braak, 1986). The contribution of the different variables was quantified using the variance partitioning method as proposed by Borcard *et al.* (1992), which was also successfully applied to quantify the effects of contaminants in aquatic ecosystems (e.g. Peeters *et al.*, 2001). The method was applied to the dataset containing the information of all four WWTPs as well as for the separate WWTPs, excluding WWTP Drachten due to the low number of samples. All ordination analyses were performed using the software program CANOCO (CANOnical COrrespondence analysis) developed by ter Braak & Smilauer (1998). CANOCO extracted four axes and calculated scores for samples, species and abiotic variables. The sequence of the extracted axes was determined by the amount of information they contained. Ordination diagrams were created by using the calculated scores to visualize the main structure in the multivariate dataset in two dimensions (the first and second axes). The statistical significance of the effect of each set of explanatory variables was tested by a Monte Carlo Permutation test (ter Braak, 1990).

3.3 Results

3.3.1 General

Annelida were found in all ATs and almost all species belonged to the Aeolosomatidae (*Aeolosoma hemprichi*, *Aeolosoma variegatum* and *Aeolosoma tenebrarum*) or Naidinae (*Nais* spp., *Pristina aequiseta* and *Chaetogaster diastrophus*). All these species reproduce mainly asexually (Loden, 1981; Christensen, 1984; Bell, 1984) and worms with reproductive organs were indeed rarely found. Table 3.3 gives an overview of average densities, standard deviations, maximum densities and frequencies of occurrence in all the samples of one AT (or the ATs in one WWTP) for the six main species. Unidentified worms or worms belonging to the genus *Dero* or to the sessile Tubificidae were rarely found and these counts were not further analysed.

Table 3.3 Overview of free-swimming Annelida in nine ATs of four Dutch WWTPs (average worm densities in specimens per mL in bold with standard deviations in italic, maximum worm densities in specimens per mL, and frequencies (%) of occurrence in all the samples of the indicated AT (or ATs) between brackets). Minimum worm densities were always zero in all ATs. Abbreviations used: D = WWTP Drachten, N = WWTP Nijmegen, N* = no. of samples, R = WWTP Renkum, Z = WWTP Zwolle, numbers 1-4 behind D, N, R and Z indicate ATs.

	Naidinae			**Aeolosomatidae**			**Total**
AT	*Nais* spp.	*Pristina aequiseta*	*Chaetogaster diastrophus*	*Aeolosoma hemprichi*	*Aeolosoma variegatum*	*Aeolosoma tenebrarum*	
D	**0** ±*0*			**0** ±*35*	**1** ±*12*		**1** ±*46*
N* = 84	3 (14)			178 (15)	79 (11)		259 (27)
N1	**5** ±*8*		**0** ±*1*	**8** ±*18*	**0** ±*0*	**0** ±*1*	**14** ±*21*
N* = 72	55 (72)		3 (17)	103 (51)	3 (3)	5 (3)	115 (83)
N2	**6** ±*5*		**1** ±*2*	**11** ±*23*	**0** ±*1*	**0** ±*1*	**18** ±*24*
N* = 67	19 (88)		8 (30)	132 (56)	5 (2)	3 (11)	141 (97)
N3	**5** ±*6*	**0** ±*0*	**0** ±*2*	**10** ±*21*	**0** ±*0*	**3** ±*13*	**18** ±*32*
N* = 74	29 (78)	2 (1)	12 (23)	110 (54)	1 (4)	79 (27)	194 (89)
N1+2+3	**16** ±*16*		**1** ±*2*	**19** ±*34*	**0** ±*1*	**3** ±*13*	**48** ±*61*
N* = 65	87 (94)		9 (48)	146 (62)	6 (5)	79 (22)	250 (97)
R1	**0** ±*1*			**0** ±*0*	**0** ±*0*		**0** ±*1*
N* = 45	4 (4)			1 (4)	2 (2)		7 (7)
R2	**13** ±*19*		**0** ±*1*	**11** ±*21*	**17** ±36		**41** ±*46*
N* = 73	88 (84)		6 (19)	96 (49)	158 (56)		168 (88)
R3	**9** ±*10*		**0** ±*0*	**13** ±*29*	**55** ±*81*		**76** ±*81*
N* = 72	40 (81)		2 (8)	121 (37)	297 (66)		298 (86)
R1+2+3	**22** ±*25*		**1** ±*1*	**23** ±*33*	**67** ±*88*		**113** ±*103*
N* = 69	113 (87)		6 (23)	122 (64)	301 (70)		348 (88)
Z3	**2** ±*4*	**4** ±*5*		**2** ±*6*			**8** ±*10*
N* = 80	29 (56)	25 (75)		34 (29)			50 (83)
Z4	**2** ±*6*	**8** ±*9*	**0** ±*1*	**5** ±*14*	**0** ±*1*		**16** ±*21*
N* = 81	30 (46)	46 (79)	5 (12)	66 (31)	9 (9)		115 (81)
Z3+4	**4** ±*8*	**12** ±*12*	**0** ±*1*	**7** ±*18*	**0** ±*1*		**24** ±*28*
N* = 79	35 (66)	52 (80)	5 (13)	100 (39)	9 (8)		133 (86)

Nais spp. were the most common species (59 % of all samples) and were followed in decreasing order by *A. hemprichi* (37 %), *P. aequiseta* (19 %), *A. variegatum* (17 %), *C. diastrophus* (12 %) and *A. tenebrarum* (5 %). To our knowledge, this latter species was never found before in WWTPs. WWTP Nijmegen displayed the most diverse worm population. *A. hemprichi*, *A. variegatum* and *Nais* spp. were found in all WWTPs, but not in all ATs. *C. diastrophus* was found in all WWTPs except WWTP Drachten, *A.*

tenebrarum only in WWTP Nijmegen and *P. aequiseta* mostly in WWTP Zwolle (except for one sample of WWTP Nijmegen AT3).

3.3.2 Population dynamics

For each species, peak periods were calculated and an overview of these peak periods for each worm species according to season, year and AT is shown in Table 3.4.

Table 3.4 Overview of peak periods for each worm species according to season (in which the highest worm density was reached), year and AT. Abbreviations used: Ah = *A. hemprichi*, At = *A. tenebrarum*, Aut = autumn, Av = *A. variegatum*, D = WWTP Drachten, N = WWTP Nijmegen, Na = *Nais* spp., Pa = *P. aequiseta*, R = WWTP Renkum, Spr = spring, Sum = summer, Win = winter, Z = WWTP Zwolle, numbers 1-4 behind D, N, R and Z indicate ATs.

	2000		2001				2002	
	Sum	Aut	Win	Spr	Sum	Aut	Win	Spr
D	Ah, Av							
N1	Na							Ah
N2	Na						Ah	
N3	Na, Ah		Na					Ah, At
R1								
R2	Av			Ah, Na				
R3				Av		Ah, Na		
Z3	Pa			Ah, Na, Pa				Ah
Z4			Ah	Ah, Na, Pa				Ah

Most importantly, no yearly patterns in the peak periods were observed. The presence of a worm species did not always lead to peak periods. In WWTP Drachten, peak periods were found in the first month of the sampling period but thereafter no worm densities higher than 1 specimen per mL were found. The three ATs of WWTP Nijmegen had similar peak periods of *A. hemprichi* and *Nais* spp. In addition, only in AT3 a peak period of *A. tenebrarum* was found. In AT1 of WWTP Renkum, no peak periods were observed because of very low worm densities (Table 3.4) and the ATs differed in peak periods, although AT2 and AT3 showed similarities in species and densities. AT3 and AT4 of WWTP Zwolle showed similar peak periods. The average durations of the peak periods for *A. hemprichi*, *A. tenebrarum*, *A. variegatum*, *Nais* spp. and *P. aequiseta* were 91 (±50), 84, 64, 96 (±37), and 58 (±21) days respectively.

Table 3.5 shows an overview of calculated average (plus standard deviations), minimum and maximum population doubling times and minimum and maximum population half-lives in days for each species in the exponential phases of the peaks.

Table 3.5 Average (plus standard deviations), minimum and maximum population doubling times (t_d) and minimum and maximum population half-lives (t_h) in days for each species in the exponential phases of the peaks.

Species	t_d	t_h
Nais spp.	5 ±3 1-12	2-∞
P. aequiseta	6 ±1 5-6	∞
A. hemprichi	4 ±1 2-6	1-∞
A. variegatum	3 ±2 1-6	1-∞
A. tenebrarum	2 ±1 1-3	2-8

The population doubling times for the Naidinae (on average 5-6 days) were somewhat longer than for the Aeolosomatidae (on average 2-4 days). Infinitely long population half-lives are biologically impossible, but were calculated when the observed population decay rates were only slightly higher than the inverse of the sludge age. This resulted in very low real population decay rates (equation **(1)**) and subsequently, in infinitely long population half-lives. These values thus signify a large influence of sludge age (and not worm death) on the disappearance of the worm populations and this confirms that worm population growth simply stops after a certain time.

3.3.3 Multivariate analysis

Figure 3.1 shows ordination diagrams with the results of an analysis in which the worm species composition was directly related to the abiotic variables (sampled WWTP and AT, sampling year and month, and process characteristics of the WWTPs).

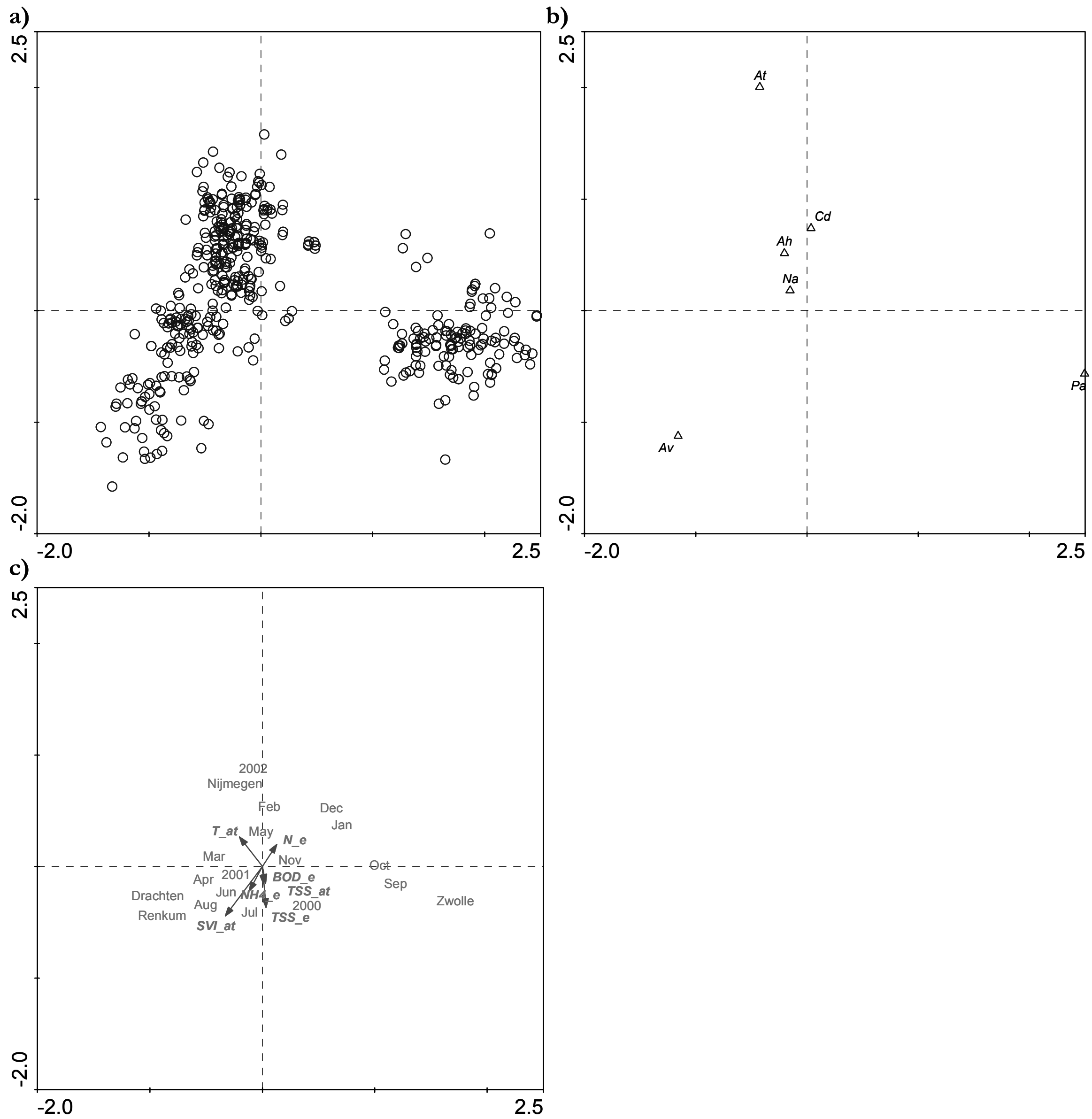


Figure 3.1 Ordination diagrams of the direct ordination of the worm species composition in the ATs of four Dutch WWTPs related to the abiotic variables (sampled WWTP and AT, sampling year and month, and process characteristics of the WWTPs). The distribution for the first two axes is given for **a)** samples, **b)** species, and **c)** abiotic variables. In **a)**, the worm samples are positioned as circles, with samples similar in species composition and worm density at the smallest distance and samples different in species composition and worm density at the largest distance. In **b)**, the species are positioned as triangles, with species similar in frequency and worm density in the samples at the smallest distance, and species different in frequency and worm density in the samples at the largest distance. In **c)**, the abiotic variables that are related to the worm species composition are shown. The positions of WWTPs, years and months (nominal variables) are shown in regular font, while process characteristics (continuous variables) are represented by arrows and their abbreviation in italic font. The length of each arrow is a measure of the importance of the process characteristic, while the arrowhead points in the direction of increasing influence. <u>Abbreviations used:</u> **b)** *Ah* = *A. hemprichi*, *Av* = *A. variegatum*, *At* = *A. tenebrarum*, *Cd* = *C. diastrophus*, *Na* = *Nais* spp., *Pa* = *P. aequiseta* **c)** _at indicates characteristics of the ATs; _e indicates characteristics of the effluent.

Approximately 57 % of the variation in the species composition was explained by the variables included in the analysis. Sampled WWTP, sampling year and month, as well as process characteristics of the WWTPs all affected the worm species composition. The samples from WWTP Zwolle were positioned in the right part of the diagram and clearly apart from the samples of the other WWTPs (Figure 3.1a & 3.1c). This was mainly related to the much higher abundances of *P. aequiseta* (Figure 3.1b). Figure 3.1 also shows that the samples of WWTP Nijmegen were positioned in the upper part of the diagram corresponding with higher abundances of *A. tenebrarum*. In addition, the samples of WWTP Renkum were mostly situated in the left lower corner of the diagram coinciding with higher abundances of *A. variegatum*. The partitioning of the variance indicated that the sampled WWTP explained most of the variance in worm species composition (Table 3.6) followed by sampling year and month. The gross percentages were calculated with only the listed variables as explanatory, while the pure percentages were calculated with the listed variables as explanatory and all others as covariables (i.e. their effects were removed).

Table 3.6 Overview of the percentages variance in worm species composition that were explained by the abiotic variables (sampled WWTP, sampling year and month, and process characteristics), calculated by partitioning of the variance obtained from the partial canonical correspondence analyses. All analyses were significant ($p<0.010$) according to the Monte Carlo Permutation Test.

Variables	Variance explained (%)	
	Gross [1)]	Pure [2)]
WWTP	40.3	24.4
Sampling date	19.5	11.3
Year	12.7	4.8
Month	9.0	6.1
Process characteristics	16.0	4.3
Effluent (NH_4^+,N,TSS, BOD)	8.4	1.1
AT (TSS, SVI, T)	12.5	2.1
Shared by WWTP, sampling date and process characteristics		16.8
Total	56.8	56.8

[1)] calculated through a direct analysis with only the listed variable(s) as explanatory, [2)] calculated through a direct analysis with the listed variable(s) as explanatory and all others as covariables.

Table 3.6 shows that the process characteristics by themselves explained approximately 4 % of the variation in the species data and this contribution is significant.

Subsequently, an analysis of the worm species composition with the process characteristics as explanatory and sampled WWTP, sampling year and month as covariables was performed (Figure 3.2).

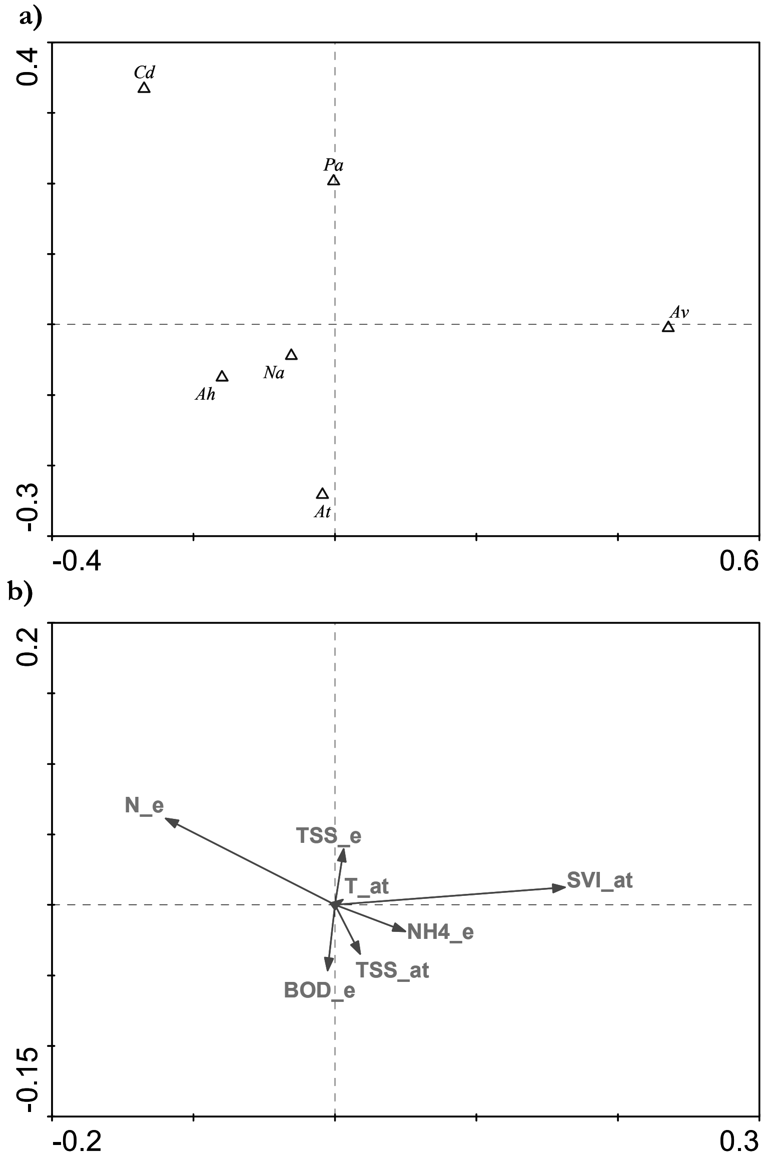


Figure 3.2 Ordination diagrams of a partial correspondence analysis in which the worm species composition was related to the process characteristics (the explanatory variables) after removing the effects of sampled WWTP, sampling year and month (the covariables). WWTP Drachten was not included in this analysis. The distribution for the first two axes is given for **a)** species and **b)** process characteristics. In **a)**, the species are positioned as triangles, with species similar in frequency and worm density in the samples at the smallest distance, and species different in frequency and worm density in the samples at the largest distance. In **b)**, the process characteristics (continuous variables) that are related to the worm species composition are shown. They are represented by arrows and their abbreviations. The length of each arrow is a measure of the importance of the process characteristic, while the arrowhead points in the direction of increasing influence. Abbreviations used: Same as Figure 3.1.

Figure 3.2b shows that the SVI was most important, since this variable has the longest arrow. Figure 3.2a shows that especially the species *A. variegatum* was associated with higher SVI values. *C. diastrophus* seemed to be associated with lower denitrification efficiencies (higher values of N in the effluent) whereas *A. tenebrarum* was associated with higher BOD concentrations in the effluent. The partitioning of the variance indicated that the SVI was associated with worm species composition in all three WWTPs (Table 3.7).

Table 3.7 Overview of the percentages of the variance in the worm species composition that were explained by the process characteristics of WWTPs Nijmegen, Renkum and Zwolle, calculated by variance partitioning. WWTP Drachten was not included in this analysis.

WWTP	AT	Effluent	Variance explained (%)
Nijmegen	SVI, T	NH_4^+	5
Renkum	SVI	N	7
Zwolle	SVI, TSS		6

3.4 Discussion

3.4.1 General

In the sampled WWTPs, almost exclusively Naidinae and Aeolosomatidae were found, as was also observed by other authors (e.g. Curds & Hawkes, 1975). Relatively few species were found, possibly due to the extreme conditions in the plants: high organic pollution levels and turbulence in the ATs. In WWTPs Drachten and Zwolle and AT1 of WWTP Renkum worms were (temporarily) completely absent during the sampling period. The worm absence from WWTP Drachten during most of the sampling period cannot be explained, but the absence from the latter two WWTPs may have been due to the presence of anoxic zones, through which the sludge and worms are routed (Table 3.1). In contrast, WWTP Nijmegen also contained anoxic zones but this did not decrease worm population growth. The reasons for this are unknown, but we hypothesize that the positive effect of the higher influent temperatures in this WWTP on worm population growth enabled them to cope with losses under temporary anoxic conditions. Several authors found that population growth rates on sterilized activated sludge were positively correlated with temperature with optima of 25-35 °C for the species that were encountered in our research (Inamori *et al.*, 1983; Kuniyasu *et al.*, 1997). Furthermore, *A. tenebrarum*, *C. diastrophus* and *P. aequiseta* were not present in all WWTPs and worm dispersal in the WWTPs may also be related to their natural occurrence in the direct surroundings of the WWTP.

The maximum worm densities for the three main species/genera in our research (*Nais* spp., *A. hemprichi* and *P. aequiseta*) were 88, 178 and 46 specimens per mL respectively. Maximum worm densities found by other researchers in different wastewater treatment systems were variable. In full-scale plants, the maximum densities varied from 0.3 Naidinae (Learner & Chawner, 1998) and 30 Aeolosomatidae

(Poole & Fry, 1980) up to 160 *N. elinguis* per mL (Ratsak, 1994). In contrast, the maximum densities in pilot-scale systems were much higher. Inamori *et al.* (1983) found maximum densities of 1,000 *Aeolosoma* sp., 200 *Nais* sp. and 200 *Pristina* sp. per mL and Wei *et al.* (2003a) found maximum densities of around 700 and 125 specimens per mL for the former two species.

3.4.2 Population dynamics

The occurrence of peak periods did not follow a yearly pattern, but usually showed high similarities within each WWTP. Ratsak (1994) also found that the density of *N. elinguis* in a WWTP varied both per season and even per AT for unknown reasons. We cannot rule out longer-term patterns even though in natural populations of aquatic Annelida annual population growth patterns are common. E.g., during a seven-year period, Loden (1981) always found peaks in spring in field populations of Naidinae. Only *P. aequiseta* showed peaks in autumn but we did not observe this in the WWTPs either. Schönborn (1985) also found that the densities of several naidine species in a polluted river were highest in spring and he concluded that this was a food issue. Therefore, the stable food supply and temperatures could explain the absence of annual population growth patterns in the WWTPs. This was also supported by asexual reproduction of the species in our research, which usually indicates favourable conditions like food availability, higher temperatures and low NaCl concentrations (Learner *et al.*, 1978; Loden, 1981). In addition, top-down predation as observed in field situations by for example fishes (Wallace & Webster, 1996) is virtually absent from WWTPs, which is another explanation for the seemingly random population dynamics. The absence of seasonal patterns for no apparent reason was also sometimes observed for other invertebrates like Nematoda (Michiels & Traunspurger, 2004).

The average durations of the peak periods were quite similar, all around 2-3 months, but variability within one species was high for unknown reasons. Once worm population growth was triggered, the population doubling times were low (on average 2-6 days), which also indicated favourable conditions. They were slightly higher for the Naidinae than for the Aeolosomatidae. In experiments with sterilized activated sludge (Inamori *et al.*, 1983; Kuniyasu *et al.*, 1997) doubling times for *A. hemprichi*, *Pristina* sp. and *Nais* sp. were also 1-6 days depending on TSS concentration. Similar to our results, the highest population doubling times were found for *Nais* sp. Under more natural conditions (laboratory set-ups with polluted river water and detritus as food source), the population doubling times of several Naidinae were higher (3-16 days) (Lochhead & Learner, 1983; Schönborn, 1985).

The sudden disappearance of the population peaks could be due to the observed lack of dividing worms observed in the downward phases of the peaks, which was possibly caused by senescence phenomena (Martinez & Levinton, 1992). This was illustrated by the high values for the population half-lives, which indicated a low population decay rate but a big influence of removal with waste sludge.

3.4.3 Multivariate analysis

The results from the multivariate analysis suggested that the variability in worm populations was mostly due to differences in sampled WWTP, sampling year and month. This was possibly caused by the before-mentioned absence of certain worm species from some WWTPs (e.g., *A. tenebrarum* was only present in WWTP Nijmegen). Process characteristics of the WWTPs could be related to only 4 % of the variability in worm populations. There were indications that worms were related to the SVI, which was also found by other authors (e.g. Ratsak, 1994; Wei *et al.*, 2003a). The present study indicated that *A. variegatum* was associated with higher SVI values, whereas the other species were associated with lower SVI values. The latter can be explained by compacting of the sludge flocs by the worms, which increased the settleability. The association of *C. diastrophus* with higher denitrification efficiencies could be due to removal of denitrifying bacteria populations, but this is not likely, since *C. diastrophus* mainly feeds on Protozoa. The association of *A. tenebrarum* with higher BOD concentrations in the effluent could be due to the release of suspended solids in the water phase because of sludge consumption. It is unknown why other species did not show these correlations.

In contrast, other authors suggested many more though variable correlations between the presence of worms and several process characteristics. Ratsak (1994) concluded that high worm densities not only resulted in a low SVI but also lower energy consumption for oxygen supply and, depending on the temperature, less sludge production. The worms had no influence on the effluent quality in terms of BOD and nutrients. Wei *et al.* (2003a) concluded the same for especially *Nais* sp. and to a lesser extent for *A. hemprichi*, but he reported a decrease in effluent quality in terms of TSS and BOD. In addition, population growth of *A. hemprichi* was slightly positively correlated to T and negatively to pH, while population growth of *N. elinguis* was slightly positively correlated to TSS, T and DO and negatively to F/M ratio. Inamori *et al.* (1987) and Zhang (1997) reported similar phenomena.

However, these researches did not consider interactions between variable process characteristics, like the sludge age, which is inversely correlated to sludge production (van Loosdrecht & Henze, 1999). Wei & Liu (2006) for example concluded from their research that not all results from their study could be attributed to the presence of worms due to the lack of a control system. The available process characteristics of the studied Dutch WWTPs could hardly be related to the worm composition and densities in the samples from those WWTPs. These data suggest that the population peaks of free-swimming Annelida are phenomena that are hard to explain and control, and confirms that their supposed effects on WWTP process performance, like waste sludge production, should be interpreted with much care.

3.5 Conclusions

In this chapter, a long-term survey of free-swimming Annelida in the aeration tanks of four Dutch wastewater treatment plants was described. The survey showed that:

- Worm absence sometimes seemed to be related to the presence of anoxic zones.
- No yearly recurrences of population peaks were seen in the WWTPs, probably because of stable food supply and temperature, and the lack of predation.
- Peak periods were similar between the ATs of each WWTP. The duration of the peak periods was on average 2-3 months for all species and the doubling times were low (on average 2-6 days). The disappearance of worm populations from the WWTPs was presumably caused by declining asexual reproduction and subsequent removal with the waste sludge.

Multivariate analysis of the worm species composition and the abiotic variables indicated that:

- The variability in worm populations was largely due to differences in sampled WWTP, sampling year and month.
- Process characteristics of the WWTPs had a small but significant contribution with SVI as the most important one. Most worms were associated with lower SVI values, except for *A. variegatum* that was associated with high SVI values.

This survey suggested that population growth of free-swimming Annelida could not be explained by the investigated process characteristics. In addition, they seemed to have little influence on process performance. This would seriously hamper the stable application of these species in wastewater treatment for sludge reduction.

Acknowledgments

We thank Dennis Piron (WWTP Nijmegen), Annelies van der Ham, Janny van Vliet en Hans Huijsman (WWTP Zwolle), Jan Talsma (WWTP Drachten) and the other employees of the plants for sending the samples and providing additional information. In addition, we thank Christa Ratsak for her help with sample and data processing and finally, we thank two anonymous reviewers assigned by Hydrobiologia for their valuable comments.

╡Chapter 4╞

Development of a test to assess the sludge reduction potential of *Lumbriculus variegatus* in waste sludge

Based on submitted paper for Bioresource Technology (Buys, Elissen, Klapwijk & Rulkens)

Abstract

A quantitative sludge reduction test was developed using the sludge-consuming aquatic worm *Lumbriculus variegatus* (Oligochaeta, Lumbriculidae). Essential in the test were sufficient oxygen supply and the presence of a non-stirred layer of sludge for burrowing of the organisms. The test eliminated the unwanted effects of the macroscopic movements of the organisms, so-called bioturbation, on oxygen transport and (therefore) on sludge reduction. Non-treated waste sludge grown on municipal wastewater was used, in order to stay as close to the daily practice of sludge treatment as possible. By separating sludge and worms after the test, sludge reduction and worm growth were quantified independently and accurately. Sludge digestion by *L. variegatus* was approximately twice as fast as the endogenous digestion rate of waste sludge, but did not affect the endpoint of sludge reduction. In addition to endogenous digestion around 19 % sludge VSS (~ 16 % sludge TSS) was digested by *L. variegatus*. A minimum initial W/S ratio (ratio of worm to sludge dry matter) of about 0.4 was required. Under the test conditions, 20 to 40 % of the digested sludge was converted into worm biomass (organic matter based). *L. variegatus* seemed to release more ammonium during sludge consumption than was expected based on the TSS digestion percentage.

The sludge reduction test is simple, reproducible and accurate and can be done with equipment generally available in any laboratory, yielding results within a few days. The test can also be used to evaluate the application of mixtures of different aquatic organisms or cascaded sludge consumption on sludge reduction.

4.1 Introduction

The disposal of waste sludge of WWTPs (wastewater treatment plants) is still one of the major challenges of sustainable wastewater engineering. In activated sludge WWTPs, each ingoing kilogram of organic pollution results in the production of 250-400 grams of waste sludge as dry solids. This sludge contains micro-organisms, slowly biodegradable and non-biodegradable organic and inorganic materials. The costs for waste sludge treatment in activated sludge WWTPs may amount to 50-60 % of the operational costs and this has stimulated research into alternative sludge treatment technologies and scenarios aimed at minimizing sludge production.

A biological option for waste sludge reduction is the consumption and digestion of this sludge by higher organisms. Theoretically, energy is lost at every transition in a natural food chain whereby part of the organic material is converted into CO_2 and water (Dajoz, 1977). The food chain in biological wastewater treatment starts with the conversion of pollutants into bacterial biomass and may be extended by introducing higher organisms that feed on the bacterial biomass. This will result in less sludge production and thus in fewer costs for sludge treatment. Moreover, after separation of worms and the remaining sludge (including worm faeces) after treatment, the worm biomass becomes available for re-use.

Different aquatic worms that are frequently found in WWTPs (Oligochaeta and Aphanoneura) were suggested or investigated for sludge reduction: sessile Tubificidae

(Densem, 1982; Rensink & Rulkens, 1997) and free-swimming Naidinae (a subfamily of the Tubificidae) (Ratsak, 1994; Wei *et al.*, 2003a) and Aeolosomatidae (Zhang, 1997; Wei *et al.*, 2003a). However, in spite of a substantial research effort, up to date no systems for sludge reduction with any of these organisms have been put into practice (or the results have not been published). This may be caused by the lack of an adequate testing method that closely approximates the practice of wastewater treatment.

Aquatic worms are usually assumed to use live bacteria as a food source (Brinkhurst & Chua, 1969; Wavre & Brinkhurst, 1971; Densem, 1982; Ratsak *et al.*, 1993). Using sterilized sludge for a sludge reduction test, as suggested by Liang *et al.* (2006b), may therefore change the test result, since sterilization results in the lysis of live bacteria. We developed a test method that uses non-treated waste sludge, so that test results may be translated to real-life WWTPs without any assumptions. The sessile aquatic oligochaete *Lumbriculus variegatus* was selected as model consumer. *L. variegatus* is several cm long when grown on waste sludge, so that manual separation of worms and sludge is feasible. A testing method should allow for separate quantification of sludge reduction and worm growth, in order to prove that the two are clearly linked.

The main problem of a test using crawling or otherwise moving organisms in a non-stirred (stagnant) layer of sludge is the effect of the movements of these organisms on the oxygen balance of the sludge layer and the supernatant water layer. This process is in fact a type of 'bio-stirring' and is referred to in literature as 'bioturbation' (Wang & Matisoff, 1997; Mermillod-Blondin *et al.*, 2003). Bioturbation is the process of mixing and transfer of solutes and particulate material through the mechanical working of sediments by (in this case) Oligochaeta. For example, increased sediment oxygen uptake rate, denitrification and ammonia mobilization were reported in the presence of sessile Tubificidae (Pelegrí & Blackburn, 1995). More specifically the reduction of activated sludge is slowed down by a lack of oxygen (Kim & Hao, 1990), so that movements of the worms may increase sludge reduction. This is an unwanted effect in the study of the effect of consumption by higher organisms on sludge reduction. Waste sludge reduction in the presence of crawling sludge-consuming organisms is thus caused by a combination of three simultaneous processes: 1) digestion of sludge solids in the digestive track of worms, which converts sludge into CO_2, worm biomass and worm faeces, 2) endogenous sludge digestion by oxygen input through diffusion, and 3) endogenous sludge digestion by additional oxygen input through bioturbation. The batch experiments described in this chapter were aimed at distinguishing between these processes and the separate quantification of the effect of sludge consumption by worms on sludge reduction.

4.2 Materials and methods

4.2.1 Sludge cultivation

Activated sludge for the batch experiments was grown in a pilot scale system treating pre-settled wastewater of mainly municipal origin from a real-life WWTP in the Netherlands. The system consisted of a selector of 50 L, an AT (aeration tank) of 530 L and a secondary clarifier of 190 L (Figure 4.1). It was operated for COD (chemical oxygen demand) removal and nitrification. Excess activated sludge was daily wasted directly from the AT and the sludge age in the system varied between 8 to 19 days at a SLR (sludge loading rate) of 0.2-0.6 g COD / g TSS (total suspended solids)/ d.

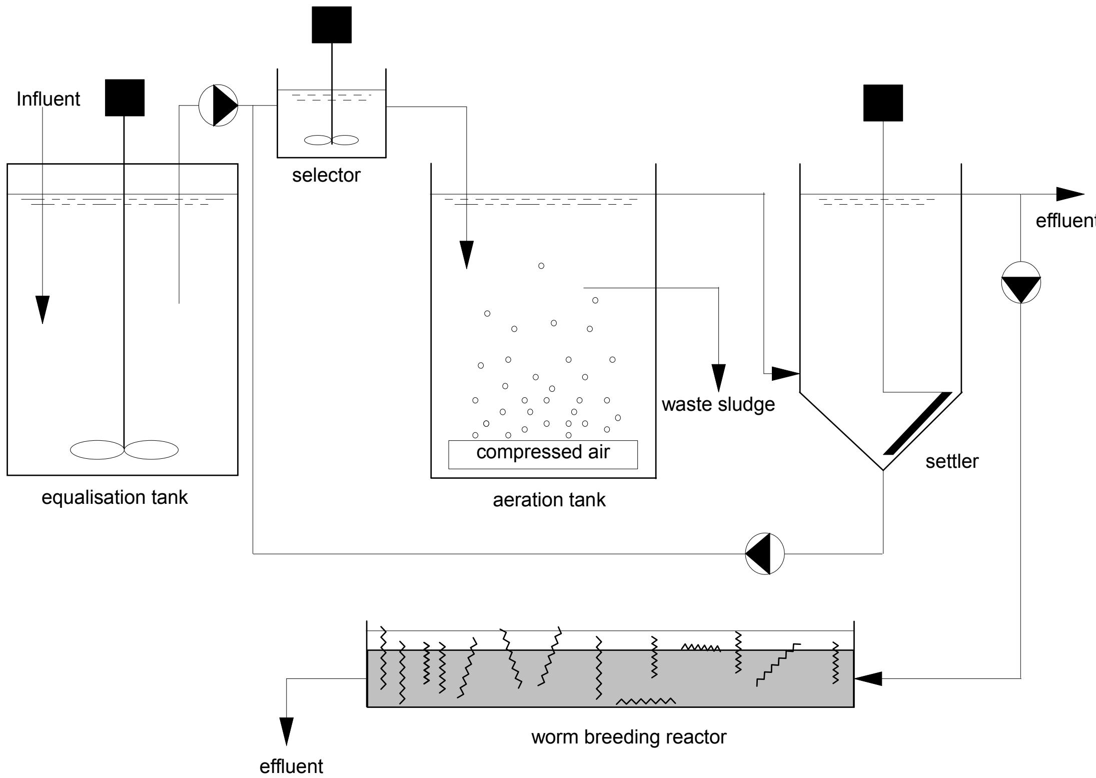


Figure 4.1 Layout of the pilot-scale system used for growing activated sludge and breeding worms on the sludge flocs washed out with the effluent.

4.2.2 Cultivation of *L. variegatus*

L. variegatus was grown in a Plexiglas ditch (400x28 cm^2), which was fed continuously with 550 L/ d of effluent containing flushed out sludge flocs of the AT (Figure 4.1). On the bottom of this ditch Oligochaeta were growing in and on top of a 2 cm settled sludge layer. The Oligochaeta were originally bought in a pet shop as a mixture of sessile Tubificidae (mainly *Limnodrilus hoffmeisteri*, *Limnodrilus udekemianus* and *Tubifex tubifex*) and *L. variegatus*, originating from polluted rivers in Eastern Europe. After several months, the population in the ditch had evolved into a near monoculture of *L. variegatus*, which inhabited the sludge layer for a period of several years. *L. variegatus* was identified after mounting in polyvinyl lactophenol, using an Olympus BHT microscope (Brinkhurst, 1971; Timm, 1999).

4.2.3 Batch experiments on sludge reduction with *L. variegatus*

We compared waste sludge reduction with and without (controls) *L. variegatus* in two types of batch experiments. In the first type, we monitored progressive sludge reduction in time. In the second type, sludge reduction was measured only at the end, when in some batches of a series with worms the faeces percentage was 100 %. These faeces have a pellet like shape that can easily be discerned from waste sludge flocs that have not been consumed by worms (own observations). Therefore, the degree of consumption can be estimated from the structure change of sludge flocs into worm faeces.

Batch experiments were chosen as most appropriate, because the amount of sludge can be quantified accurately. Batch experiments were carried out in non-aerated Petri dishes (Ø 18.5 cm, glass) or Erlenmeyer flasks (250-300 mL, Duran borosilicate glass) with forced aeration through electrical air pumps and plastic tubes (PVC, Ø 4 mm). As *L. variegatus* is a sediment dwelling worm, the experimental set-up needed to simulate sediment conditions, i.e. a more or less stagnant layer of sludge at the bottom and a layer of supernatant liquid. This implies that sludge with worms could not be completely mixed or stirred. Therefore, in the Erlenmeyer flasks with worms, the aeration was adjusted so that the sludge was not completely mixed, but just slightly mixed with most of the sludge and the worms on the bottom. Control flasks were aerated more intensely to prevent oxygen limitation and to ensure complete mixing.

L. variegatus specimens were counted and kept in tap water 16-24 hours to allow for gut purging (Densem, 1982; Mount *et al.*, 1999). Before the start of the experiment, the worms were washed several times with tap water and then weighed in lots of about 100 specimens after gut purging. At the start of the experiment, sludge was taken from the AT of the pilot-scale system and diluted with effluent of this system to a concentration of 2-3 g TSS/ kg sludge in a 10 L bucket. Sludge was taken from this well-stirred bucket and transferred in portions of 200-300 g to the Petri dish or Erlenmeyer flask. Next, *L. variegatus* was added to some of the batches. At the end of each experiment, the worms were separated from the sludge manually and washed several times with tap water. This wash water, containing some sludge flocs, was added to the rest of the sludge of the batch. Great care was taken to include all of the sludge in the solids determinations. The worms were kept 16-24 h in tap water for gut purging and then washed repeatedly. The amount of faeces in this wash water was determined in a separate suspended solids measurement and this was at most 1.2 % of the total solids at the end of the batch experiment.

4.2.5 Analytical methods

Substantial attention was paid to the determination of TSS and VSS (volatile suspended solids) because they determine the validity of our sludge reduction research. They were determined according to Standard Methods (APHA, 1998), with the following additions or modifications:

1. At the start of an experiment, sludge samples for determination of solids concentration were taken from a well-stirred beaker of 2-3 L, directly poured into 65 mL centrifuge tubes and weighed. The relative errors in the TSS and VSS determinations, which reflect weighing and sampling errors, were 1.5 % and 3 % respectively.
2. At the end of a batch experiment, the sludge from each batch was concentrated by centrifuging for 10 min. at 2000 g, pouring the supernatant through the filter and adding the pellets to the same centrifuge tube, until all the solids were concentrated in one tube. The relative errors in the TSS and VSS determinations, which reflect weighing and transfer errors, were 1 % and 2 % respectively.
3. Both Whatman GF/C glass fibre filters (retention 1.2 µm, Ø 55 mm) and Schleicher & Schuell 589^1 black ribbon ash-free filters (retention >12-25 µm, Ø 55 mm) were used for solids determinations. No difference was found between these two types.
4. Standard Methods (APHA, 1998) recommends limiting the solids sample to no more than 200 mg dried residue, in order to avoid the formation of a water-trapping crust on the residue. We used substantially more dried residue (up to 800 mg) and found a maximum decrease of 1 % in mass after redrying the crushed residue overnight. This was equal to a non-crushed sample, so we concluded that no water-trapping crust was formed.
5. Solids were dried overnight, i.e. the drying time was 12-24 h. Drying time between 12-24 h did not significantly affect the TSS value.

The wet weight of *L. variegatus* was determined by squeezing them gently on a perforated piece of aluminium foil placed on dry paper tissue to remove adhering water. Dry weight was determined by drying overnight at 105 °C (Densem, 1982) and ash content by overnight ignition at 600 °C. The relative errors in the wet weight and the dry to wet weight ratio were 5 %.

DO (dissolved oxygen) concentrations were measured with a WTW Oxi-330 meter in the mixed suspension (in a system with a stagnant layer this gives a rough indication of the oxygenation conditions). pH was measured with a WTW 323 or 325 pH meter equipped with a Sentix electrode. Dissolved ammonium, nitrate and nitrite were determined in paper-filtered samples (Schleicher & Schuell, $595^{1/2}$ folded filters, retention 4-7 µm), using a Skalar auto-analyser (segmented flow analysis). Analysis is based on ISO standards: for ammonium (0-50 mg N/ L) ISO 11732, for nitrate (0-20 mg N/ L) and nitrite (0-2.5 mg N/ L) ISO 13395.

4.2.6 Calculations

TSS and VSS at the start of the experiment were calculated from sludge concentration multiplied by the volume of sludge added. At the end of the experiment all the sludge in the Petri dishes was used for the TSS and VSS determinations. A weighed sample of the sludge in the batches was used for the TSS and VSS determinations. The reduction of TSS (or VSS) was calculated relative to t_0:

TSS reduction: $(1-(TSS_t/ TSS_0))*100$		(%)	**(1)**
Parameters:			
TSS_t	TSS at t_{end}	(g)	
TSS_0	TSS at t_0	(g)	

In a batch with worms, the faeces resulting from gut purging were added to the solids in equation **(1)**. Growth of *L. variegatus* was expressed as increase in dry weight (and not in number):

Worm growth: $dw_t-(ww_0*f_{dw/ww})$		(g)	**(2)**
Parameters:			
dw_t	Dry weight of worms at t_{end}	(g)	
ww_0	Wet weight of worms at t_0	(g)	
$f_{dw/ww}$	Dry to wet weight ratio of worms	(-)	

The dry to wet weight ratio of the worms was usually determined at the start of each experiment for an extra lot of 100 specimens.

The specific growth rate of *L. variegatus* (based on dry weight) was calculated from equation **(2)** as follows:

μ_{Lv}:	**Worm growth/ $(ww_0*f_{dw/ww})/ \Delta t$**	(d^{-1})	**(3)**
Parameters:			
Δt	Experiment duration	(d)	

The growth yield of worms on waste sludge $Y_{w/s}$ was based on the VSS of the worms (their dry weight minus their ash content) and the sludge:

Y_{Lv}:	**$-\Delta VSS_{worms}/ \quad \Delta VSS_{sludge}$**	(-)	**(4)**
Parameters:			
ΔVSS	VSS_t-VSS_0	(g)	

Error propagation from measurements to calculated results was done according to standard error analysis (Rao, 2002).

4.3 Results and discussion

4.3.1 General observations

Consumption by *L. variegatus* changed the structure of the waste sludge profoundly (Figure 4.2): sludge flocs were compressed into cylinder-shaped worm faeces.

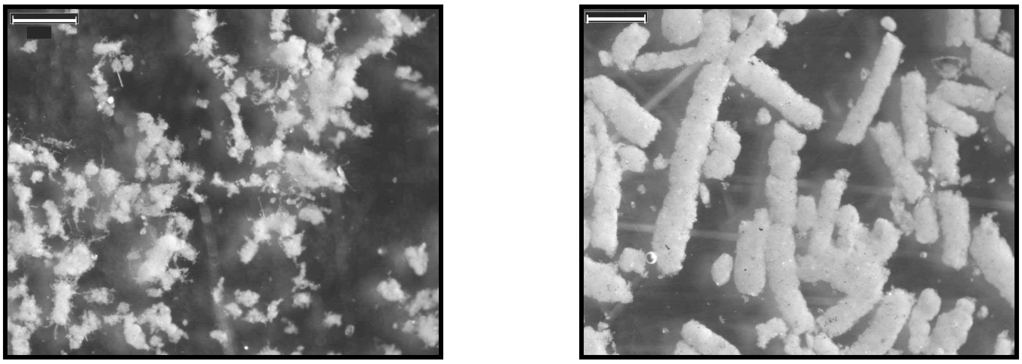


Figure 4.2 Change of waste sludge structure after consumption by *L. variegatus*. Left: before consumption. Right: after consumption. Scale bar = 0.5 mm.

The worms were either crawling around in the sludge layer, or with their heads in the sludge and their tails protruding into the supernatant water for taking up oxygen. In almost all of the experiments, we measured more sludge reduction in the worm batches compared to the controls, depending on the oxygenation conditions and the duration of the experiment. Ash content of the sludge usually increased during a batch experiment (typically from 18 to 25 %), which resulted from a larger VSS than TSS reduction percentage, because almost the entire sludge reduction concerned the organic fraction. In many cases, dissolved nitrate and nitrite completely disappeared from the water phase and pH increased, which indicates denitrification, caused by oxygen depletion in the sludge layer. This was a consequence of the experimental set-up, which needed to simulate sediment conditions: complete mixing and active aeration were therefore impossible. We present three experiments, which were performed under different conditions with sludge grown on pre-settled wastewater (Table 4.1).

Table 4.1 Conditions in the batch experiments with *Lumbriculus variegatus*. Abbreviations used: θ = sludge age, E = Erlenmeyer flask, P = Petri dish, SLR = sludge loading rate, W/S = worm to sludge ratio (dry matter based), T = temperature range, pH_{end} = pH range at end of experiment.

Exp	Par./ Fig.	Type	# worms 1) = t_0 2) = t_{end}	W/S at t_0	θ (d)	SLR (gCOD/ gTSS/d)	T (°C)	pH_{end}	Duration (d)
1	*4.3.2/* 4.3	E P	100-200 1) 98-220 2)	0.16-0.36	8	0.6	18.0-20.9	6.2-7.8	2-9.8, 52
2	*4.3.3/* 4.4	E	80-310 1) 87-306 2)	0.21-0.61	8	0.6	22.2-26.7	6.8-7.9	1.9
3	*4.3.4/* 4.5 & 4.6	E P	200 1) 201-247 2)	0.42-0.54	15	0.3	18.0-22.0	4.8-7.6	4.8

4.3.2 Sludge reduction and worm growth yield

A typical example of the progress of sludge reduction in time in Petri-dishes and the corresponding growth yield Y_{Lv} of *L. variegatus* is shown in Figure 4.3 (Experiment 1).

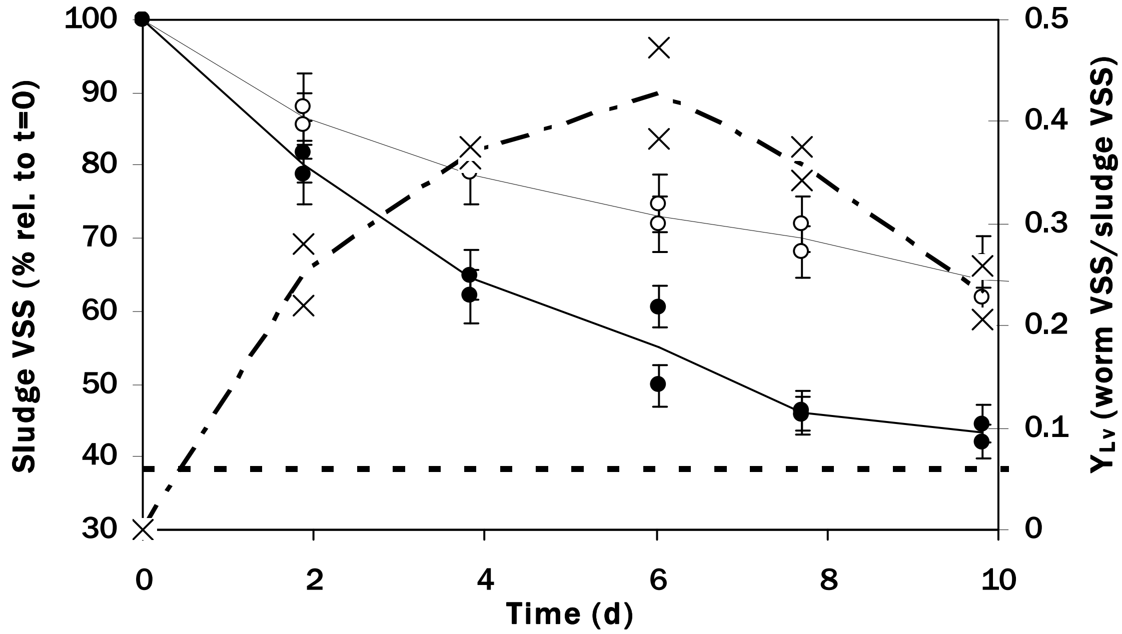


Figure 4.3 VSS reduction of waste sludge (Experiment 1) in Petri dishes during 10 d in the presence of *L. variegatus* (●) compared to controls without worms (○) and worm growth yield Y_{Lv} (×). Horizontal dotted line: sludge VSS reduction in controls after 52 days. The other lines connect the average of two duplicates. Error bars for worm growth yield were omitted for clarity reasons.

Sludge reduction proceeded at a higher rate in the presence of worms and was accompanied by growth in numbers and biomass of *L. variegatus* up to 6 days, followed by declining growth in biomass between 6-10 days. The specific growth rate μ_{Lv} of *L. variegatus* (on the time interval t = 0-6 days) was 0.05-0.11 d^{-1}. We observed that after approximately 6 days, almost all of the sludge had been converted into faeces. This coincides with the maximum in growth yield Y_{Lv}. The time to complete consumption is likely to depend on the W/S ratio: a higher ratio will result in faster consumption. Sludge can be consumed more than once, but faeces are hardly digested (Chapter 6). This may explain the decrease in growth yield after 6 days: worms started loosing weight because food uptake became limiting. The yields we found for *L. variegatus* are in the same order of magnitude as was reported for the growth of different Protozoa on waste sludge (Ratsak *et al.*, 1996), 0.16-0.54 mg Protozoa/ mg sludge.

Figure 4.3 also shows that the final sludge VSS reduction percentage reached in the control batches after 52 days was 62 % (which equals a TSS reduction of 54 %). In a similar experiment to Experiment 1, but with a duration of 63 days, we found no difference in the final sludge reduction percentage between Petri dishes with and without *L. variegatus*: 58.3 (±1.8) % and 57.1 (±1.8) % of the VSS respectively. Therefore, consumption by *L. variegatus* enhances the sludge reduction rate without affecting the final endpoint of sludge reduction. This means that the sludge components refractive to microbial biodegradation are also refractive to biodegradation by *L. variegatus*.

4.3.3 Effect of worm to sludge ratio on sludge reduction

A minimum amount of worms is needed to consume a given amount of sludge to observe a measurable effect on sludge reduction. Figure 4.4 shows the effect of the W/S ratio in Experiment 2.

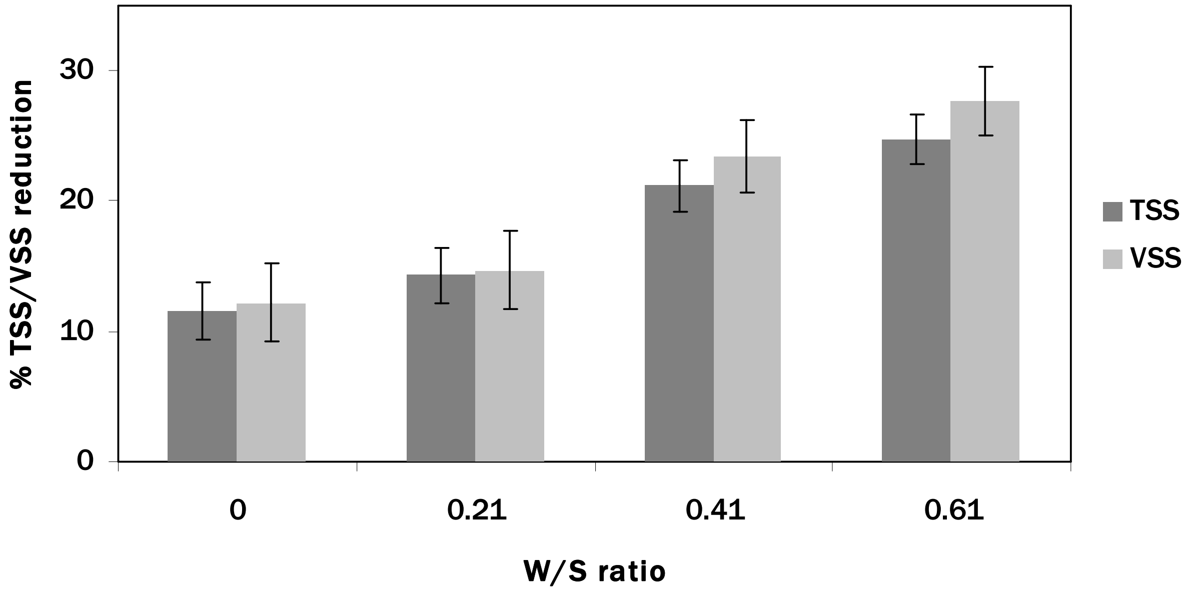


Figure 4.4 TSS and VSS reduction of waste sludge (Experiment 2) in Petri dishes after 1.9 days in the presence of 0, 80, 155 or 310 specimens of *L. variegatus*, corresponding to W/S ratios at the start of 0, 0.21, 0.41 and 0.61.

The lowest number of worms with W/S = 0.21 showed no difference with the control (W/S = 0), while W/S = 0.41 and 0.61 resulted in more sludge reduction. The difference in sludge reduction between the batches with W/S = 0.41 and 0.61 is not significant. We did not test higher W/S ratios, but expect that inhibition may occur at high worm densities when worms are competing for space, i.e. food and oxygen. Based on this experiment the minimum initial W/S ratio to observe a significant effect on sludge reduction is about 0.4. The reason why this minimum exists is endogenous sludge digestion, i.e. bacteria are feeding on (the remains of) other bacteria (van Loosdrecht & Henze, 1999). The worms therefore have to digest the sludge faster than the sludge 'digests itself' in order to make a difference in sludge reduction. This requires a minimum amount of worm biomass, which depends on the endogenous activity of the sludge: the higher the endogenous activity of the sludge, the more worms are required. Worms are thus competing with bacteria that are digesting (the remains of) other bacteria. The sludge consumer to sludge ratio is a neglected parameter in the literature on consumption of waste sludge. From the data on population peaks of Naidinae in the WWTP Deventer (Ratsak, 1994), reaching up to 160 specimens per mL sludge, we calculated a maximum W/S ratio of 0.10 (based on our own measurements on Naidinae, i.e. 28 μg dry weight per specimen). Possibly, different types of worms show an effect on sludge reduction at different (minimum) W/S ratios. The W/S ratio is one of the main design parameters for the application of the consumption concept to sludge treatment, because it determines the amount of worms required to treat a given amount of waste sludge.

4.3.4 Effect of aeration conditions on sludge reduction

Aeration has a pronounced effect on the outcome of the experiments (Figure 4.5; Experiment 3).

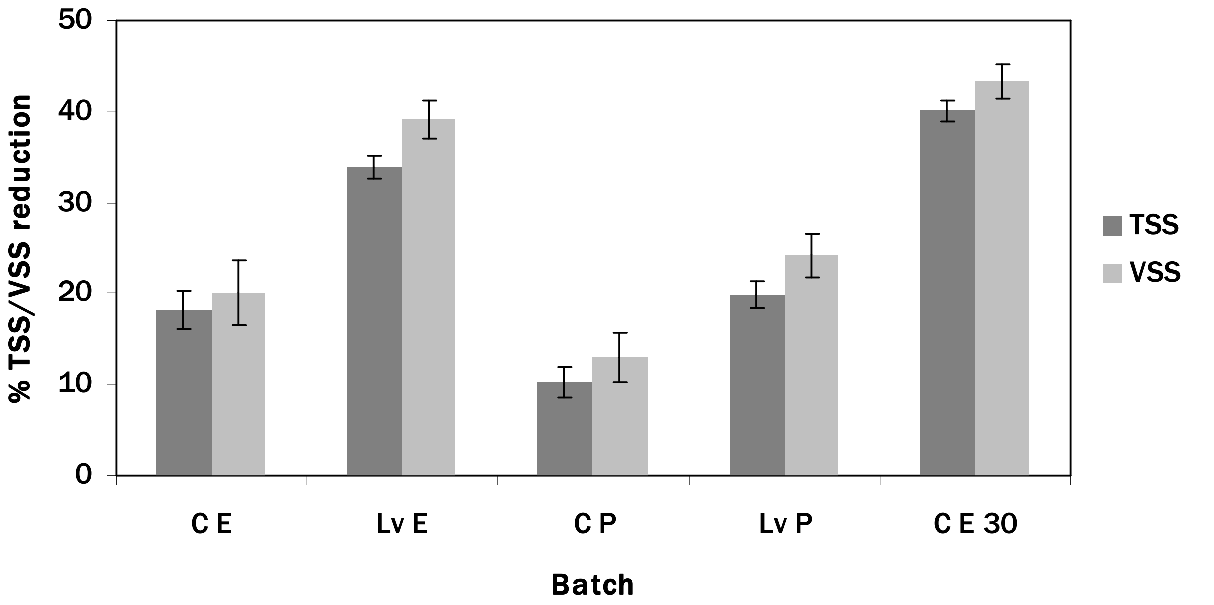


Figure 4.5 Average (N = 2, except for C E, where N = 1) TSS and VSS reduction of waste sludge (Experiment 3) under different aeration conditions after 4.8 (C E, Lv E, C P and Lv P) and 30 days (C E 30). Abbreviations used: C = control without worms, Lv = batch with *L. variegatus*, P = non-aerated Petri dish, E = aerated Erlenmeyer flask.

The sludge reduction in the control Petri dishes (C P) was lower than in the control Erlenmeyer flasks (C E), which means that this experiment was done under oxygen limited conditions. Worm growth was negligible in the dishes, probably because the dissolved oxygen concentration was low and not all of the sludge had been consumed. The highest sludge reduction was found in the flasks with worms (Lv E), where worm growth was significant. Both in the dishes and in the flasks, the sludge reduction was higher in the presence of worms than in the corresponding controls. This result is however not easy to interpret because oxygen transfer limited the sludge reduction. The worms influence the oxygen transfer process because of their respiration and their movements, but to an unknown extent. Therefore, part of the difference between control and worm reduction can be attributed to bioturbation facilitated oxygen transfer, both in the dishes and the flasks. The maximum effect of bioturbation can be estimated by comparing the control flasks: forced aeration may about double the

VSS reduction (9 versus 20 %). A lower VSS reduction rate under anoxic conditions compared to aerobic conditions is a common phenomenon (Kim & Hao, 1990). In other similar experiments (results not shown), we also found approximately a factor 2 difference in sludge reduction between sludge in closed bottles without headspace and continuously aerated sludge. Considering worm movements as a type of forced aeration, worm movement without consumption in a stagnant layer of waste sludge, may at most double the sludge reduction. However, because the contribution of bioturbation cannot be quantified exactly (only the upper limit is known), the effect of consumption by worms on sludge reduction cannot be quantified exactly when the experiments are done under conditions of oxygen limitation. An experimental method to compare the effect of consumption by different organisms needs to eliminate effects of bioturbation because different organisms will differ in their movements. Given this experiment, the best comparison is between the intensely aerated control and a lesser-aerated flask with worms. The resulting difference may be ascribed fully to the consumption effect. In Figure 4.5 C E is to be compared with Lv E, which yields: 20.1 (±3.6) % VSS reduction compared to 39.1 (±2.0) %. This equals 18.2 (±2.1) % TSS reduction in C E compared to 33.9 (±1.2) % in Lv E. Predation thus adds about 19 % of additional VSS reduction (or about 16 % of additional TSS reduction), which is almost a factor 2 compared to the endogenous digestion under conditions of excess oxygen supply. Figure 4.6 shows the changes in dissolved nutrients during Experiment 3 as percentage of TSS digestion in controls and worm batches, to compensate for the effect of increased sludge digestion in the worm batches.

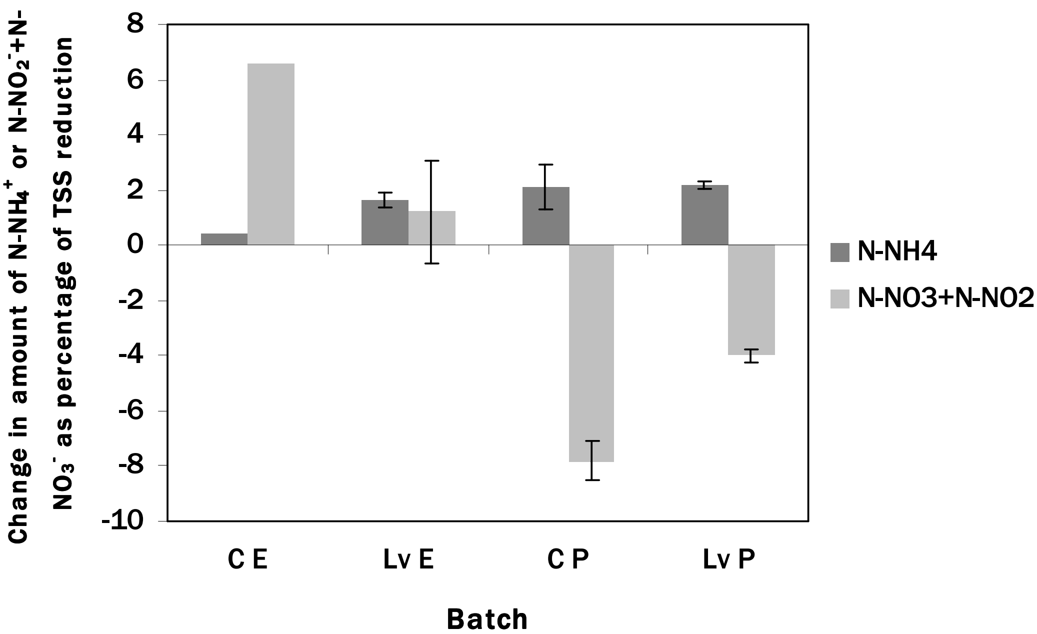


Figure 4.6 Average (N = 2, except for C E, where N = 1) changes in total amount of dissolved ammonium and nitrate+nitrite after 4.8 days in Experiment 3 (Figure 4.5) as percentage of the sludge reduction. Abbreviations used: C = control without worms, Lv = batch with *L. variegatus*, P = non-aerated Petri dish, E = aerated Erlenmeyer flask.

The dissolved nutrients shown in Figure 4.6 mainly reflect the oxygenation conditions: in the non-aerated dishes (P), denitrification was (almost) complete, also in the presence of worms. In the aerated flasks (E), nitrate usually increased during the experiment and nitrite was found in very low concentrations, but in one of the aerated flasks with worms nitrate decreased for unknown reasons. Figure 4.6 shows that ammonium accumulation occurred in all batches, but most batches with worms in both systems showed a somewhat higher relative increase. This amounts to an average extra release of 0.002-0.07 µg N/ mg dry weight/ h. Accumulation in general may have been caused by nitrification inhibition at low DO concentrations and low pH, but extra accumulation in the worm batches possibly from killing the nitrifying bacteria. The latter is an extrapolation from results on differences in survival of different types of bacteria upon passage through the gut of the aquatic Oligochaeta *T. tubifex*, *L. hoffmeisteri* and *Peloscolex multisetosus* (Wavre & Brinkhurst, 1971). In addition, sessile Tubificidae are also known to excrete ammonium at rates of 0.03-0.27 µg N/ mg dry weight/ h with full or empty guts (Postolache *et al.*, 2006). For nitrate and nitrite, the results were contradictory for both systems. In the Erlenmeyers with worms, there was a smaller increase, which is in line with a higher increase in ammonium. In the Petri dishes with worms, there was a smaller decrease, which possibly indicates that worms not only kill the nitrifying, but also the denitrifying bacteria.

4.3.5 Elimination of the effects of bioturbation

Bioturbation will only affect sludge reduction when oxygen transport is rate limiting. This is the case when the respiration rate of sludge and worms exceeds the (enhanced) diffusional oxygen influx. The oxygen influx is proportional to the surface area and the partial oxygen pressure in the gas phase. Therefore, at higher sludge respiration rates, the effect of bioturbation can be eliminated by spreading the sludge over a larger surface area or by increasing the oxygen partial pressure. In that case, experiments are best done in large trays (with a surface area of for example 1500 cm^2) with no more than about 150-200 mg sludge TSS at the start. The minimum amount of *L. variegatus* needed is then about 75-100 mg dry weight. This described methodology will always eliminate the unwanted effect of bioturbation on sludge reduction and separately quantifies sludge reduction and growth of the sludge-consuming organism.

4.5 Conclusions

In this chapter, a simple batch test using only the mass of sludge and the mass of sludge-consuming aquatic worms (*Lumbriculus variegatus*) was designed to test the suitability of biological sludge reduction. Since non-treated waste activated sludge was used, the test was as close as possible to the practice of wastewater treatment. Under the conditions applied in the test it was shown that:

- *L. variegatus* enhanced the batch wise reduction of waste sludge if the initial worm to sludge ratio was at least 0.4 (dry matter based).
- Sludge digestion by *L. variegatus* was approximately twice as fast as the endogenous digestion rate of sludge. This digestion added around 19 % VSS reduction and around 16 % TSS reduction respectively.
- Almost the entire reduction concerned the organic fraction of the sludge.
- Sludge consumption by *L. variegatus* did not affect the endpoint of sludge reduction.
- Under conditions beneficial for worm growth, 20-40 % of the sludge biomass that disappeared was converted into worm biomass (organic matter based).

In addition, the test suggested that:

- The unwanted effect of the movements of the worms on the oxygen transport into the sludge (bioturbation) in the case of high sludge respiration rates can be eliminated by either increasing the oxygen partial pressure or by spreading the sludge over a larger surface area. In that case, the true effect of sludge consumption on sludge reduction can always measured.
- *L. variegatus* seemed to release more ammonium during sludge consumption than was expected based on the TSS digestion percentage. *L. variegatus* possibly influenced (de)nitrification by killing (de)nitrifying bacteria during sludge consumption.
- The test methodology can be used to assess the suitability of other sludge-consuming organisms for sludge reduction or to optimize the composition of mixtures of different sludge-consuming organisms.

╡Chapter 5╞

Factors influencing sludge reduction by *Lumbriculus variegatus*

Abstract

For the effective application of *Lumbriculus variegatus* in wastewater treatment, it is essential to know if and how several sludge properties, worm properties and process conditions influence sludge digestion by *L. variegatus* and resulting worm growth. This was investigated in short-term batch experiments. Various municipal waste sludges from WWTPs and pilot-scale conventional and membrane bioreactor systems were digested at average rates of 0.09 (±0.04) d^{-1}. Worm biomass growth rates and worm number growth rates were on average 0.04 (±0.03) and 0.01 (±0.02) d^{-1} respectively. Of the sludge digested by worms, on average 38 (±22) % was converted into worm biomass (dry matter based). Non-municipal (Beer) sludge was also consumed, but the before mentioned rates were substantially lower. For both municipal and non-municipal sludges, the overall rates showed a high variability. However, a statistical analysis of the results of batch experiments with two of the most frequently used municipal sludges showed that this was only caused to a small extent by variations in duration (2-8 days), temperature (16-20 °C), population density (2,000-11,000 specimens per m^2), dry matter based worm to sludge (W/S) ratios (0.1-0.6), pH (4.8-7.6) and ash percentage of the sludge (13-20 %). The variability may thus be the result of unknown differences in sludge composition.

L. variegatus was able to consume all sludge floc sizes, even those smaller than 4.5 μm and larger than 300 μm. Digestion and growth rates were not affected by the different sludge floc sizes, unless the sludge concentrations of the fractions were too low. Sterilized sludge was consumed at normal digestion and biomass growth rates as long as no unknown toxic compounds were present, but reproduction (i.e. number growth rates) was negatively affected. This indicated that the worms need live bacteria in their substrate. Further batch experiments indicated that *L. variegatus* was able to increase the final reduction percentage of sludge that was pre-digested under oxic conditions. However, this effect was not clear for sludges that were pre-digested under anoxic conditions, because this often negatively affected worm growth, most likely because of the presence of toxic un-ionized ammonia.

Larger individual worm size seemed to enhance reproduction, as was expected, but the effect on sludge digestion and biomass growth was not clear. High population densities (> 39,000 specimens per m^2) and W/S ratios (> 1.4) negatively affected sludge digestion and worm growth in the batch experiments. Finally, the addition of ferric iron did not influence sludge digestion and worm growth and incubation under complete dark conditions only seemed to enhance worm number growth.

The results from these short-term batch experiments thus indicate that *Lumbriculus variegatus* can be applied in wastewater treatment for the reduction of different municipal waste sludges. In these practical applications, the major point of attention will be the avoidance of high ammonia concentrations, especially at high pH values. An interesting point for further research is the possible increase of the final reduction percentage of pre-digested sludges.

5.1 Introduction

In batch experiments, the aquatic oligochaete *Lumbriculus variegatus* consumed waste activated sludge, digested part of its organic fraction and excreted the remaining fraction as compact worm faeces (Chapter 4). Sludge digestion by worms is approximately twice as fast as the endogenous digestion rate of waste sludge. In a typical batch experiment, the combination of endogenous digestion and digestion by worms during 10 days led to a final sludge TSS (total suspended solids) reduction percentage of more than 50 %, of which around 16 % was attributable to the worms only. *L. variegatus* converted around 20-40 % of the sludge that was digested by this combined digestion into worm biomass.

For an effective and controlled application of *L. variegatus* in full-scale wastewater treatment plants (WWTPs), it is essential to know how sludge digestion and worm growth are influenced by sludge properties, worm properties and process conditions. In natural sediments, feeding rates of Oligochaeta are influenced by reproduction, body size, population density, ambient temperature, water quality parameters (e.g. dissolved oxygen, pH, ammonia) and sediment composition (particle size and organic matter content) (Williams, 2005). Similar factors will be important when applying *L. variegatus* in full-scale sludge treatment. Table 5.1 summarizes sludge properties, worm properties and process conditions that were expected to influence sludge digestion by *L. variegatus* and resulting worm growth and were investigated and/or discussed in this chapter.

Table 5.1 Variations in sludge properties, worm properties and process conditions that can affect sludge digestion and worm growth.

Sludge properties	Type of wastewater (municipal, non-municipal)
	(In)organic fraction
	Floc size
	Ammonia concentration
	(An)oxic pre-digestion
	pH
Worm properties	Individual weight
	Population density
	Worm to sludge (W/S) ratio
Process conditions	Oxygen concentration
	Temperature
	Addition of iron (Fe^{3+})
	Light/dark rhythm

5.1.1 Sludge properties

For full-scale applications of *L. variegatus,* it is essential to know how the sludge type influences digestion and growth and how tolerant they are for these types of sludges. Sludge is a complex mixture of bacteria, dead organic material and inorganic material (Gujer *et al.*, 1999). It is not known which components of sludge are ingested by *L. variegatus* and are subsequently digested or excreted after gut passage, which takes around 3 for sediment particles and around 6 hours for sand particles mixed with spinach (Gnaiger & Staudigl, 1987a; Gaskell *et al.*, 2007). Information about the food metabolism of *L. variegatus* is scarce. In terrestrial Oligochaeta like earthworms, enzymes and intestinal bacteria are involved in the digestion of biosolids (Kizilkaya & Hepsen, 2004; Frederickson & Howell, 2003). However, in aquatic Oligochaeta like Tubificidae (including Naidinae) evidence was found that the gut contained digestive enzymes, but no specific intestinal microflora (Harper *et al.*, 1981a). It may well be that the latter also applies to *L. variegatus*. Williams (2005) states that the exact composition of *L. variegatus*' diet is unknown but probably consists, like in most Oligochaeta, of a diverse mixture of small food particles that accumulate in benthic environments (e.g. algae, decaying plant material, bacteria and fungi). Not all ingested components can be digested, like certain algae species with cellulose in their cell walls (Moore, 1978). Some Oligochaeta like *Tubifex tubifex* can actively take up dissolved organic material like fatty acids through their skin, but *L. variegatus* is incapable of doing this (Sedlmeier & Hoffmann, 1989).

Next to the composition of the food source, sludge digestion and worm growth may be influenced by the pH in the medium. In nature, *L. variegatus* is known to live in a pH range of 4 to 9, and it can even survive for two days at a pH as low as 2-3 (Berezina, 2001). This was confirmed by our own observations (data not shown).

To investigate the influence of sludge composition on digestion and growth, experiments were done with different types of sludges. They were produced from the treatment of different wastewater types (municipal, non-municipal) and also differed in sludge age. Furthermore, the effect of different pre-treatment options for sludge was investigated. The size of sludge flocs may have an effect, taking into account pharynx (mouth opening) size of the worms and energy spent on gathering food particles. The influence of floc size was investigated with sieved sludge fractions. It is generally accepted that aquatic Oligochaeta mainly feed on bacteria (e.g. Wavre & Brinkhurst, 1971). The percentage of live bacteria in sludge may therefore affect digestion as well, since it is unknown whether live bacteria are an essential part of the worm diet or influence the digestion efficiency. This was investigated by feeding *L. variegatus* with sterilized sludge. Pre-digestion of sludge before feeding it to worms may also increase digestion rates and possibly the final digestion percentage, since complex components of the sludge have already been converted into smaller components. This may be important for the location of a worm reactor in a WWTP since consumption by worms may for example have more effect on sludge from a digester than on sludge from the aeration tanks. This was investigated by feeding worms with sludges that were pre-digested under oxic and anoxic conditions for varying periods.

5.1.2 Worm properties

For a full-scale application, it is essential to maintain a stable population of worms without unpredictable fluctuations in sludge digestion and growth. It is likely that there will be an equilibrium between worm population size and food (i.e. sludge) supply. A basic understanding of *L. variegatus* population dynamics in sludge is therefore essential. Three possibly important determinants of digestion and growth rates (in number and/or biomass) of *L. variegatus* are individual worm weight, population density and W/S ratio (dry matter based).

It is known that larger worms reproduce (divide) more often than smaller worms (Leppänen & Kukkonen, 1998a). They found that the worms usually reproduce when their individual wet weight is more than 9 mg, which was confirmed by Williams (2005). In contrast, it is known that in the absence of food *L. variegatus* keeps reproducing while decreasing in biomass (Buys, 2005), but this may be a survival mechanism. After reproduction, food ingestion ceases for up to 7 days (Leppänen & Kukkonen, 1998b) and this will decrease sludge digestion rates. At the same time, small worms may have a faster metabolism, since they increase mostly in biomass instead of numbers, but their smaller pharynx size may limit the uptake of larger sludge flocs. To test the influence of individual worm weight, experiments were done with large and small specimens of *L. variegatus*.

Next to individual worm weight, the W/S ratio might influence digestion. In the previous chapter, it was found that additional digestion by *L. variegatus* usually becomes significant when the W/S ratio is 0.4 or higher. This is however dependent on the endogenous activity (natural digestion rate) of the sludge used. Additional batch experiments on the influence of high W/S ratio were carried out. Next to a 'worm property', the W/S ratio can also be considered a process condition, because it can be regulated by harvesting of worms.

The third parameter population density is known to influence the excretion rate of *L. variegatus*. According to Leppänen & Kukkonen (1998a) individual excretion rates in sediment did not change up to population densities of 12,500 specimens per m^2. At higher densities, individual excretion rates decreased most likely because of competition for food. Whether population density has a similar effect on sludge digestion and possibly on worm growth was investigated in experiments with high population densities.

5.1.3 Process conditions

Next to sludge and worm properties, process conditions are likely to influence digestion and growth during sludge consumption. Applying optimal conditions can be an effective way of controlling digestion and growth. Important process conditions are oxygen concentration and temperature.

Oxygen concentration in experiments with *L. variegatus* in sludge has effects on both worms and sludge. These worms need oxygen for their metabolism. In addition, endogenous digestion is enhanced at increasing oxygen concentrations and as a result, the additional effect of *L. variegatus* on digestion decreases. When oxygen availability is not limiting *L. variegatus* in general doubles digestion rates compared to endogenous digestion (Chapter 4).

Temperature also influences sludge digestion and worm growth (Buys, 2005). Between 5 and 30 °C, both digestion by endogenous processes and worms increased but digestion by worms was always higher. At 15 and 20 °C, the digestion and growth rates were stable and in the same range, but at 30 °C the rates were sometimes higher and sometimes lower than at moderate temperatures. At 5 °C the rates were very low (Buys, 2005). Leppänen (1999) found that *L. variegatus* reproduces and feeds equally well on sediments at 15 and 20 °C and that excretion rates were a factor 3-47 higher at 20 °C than at 6 °C. Williams (2005) found that *L. variegatus* stopped feeding in sediments at 5 °C. In contrast with our results and those of Leppänen (1999), he found a significant increase in excretion rate for *L. variegatus* when temperature was raised from 15 °C to 20 °C. Chapman *et al.* (1999) state that the optimal temperature for *L. variegatus* is 20-25 °C, which explains the unstable sludge digestion and worm growth at 30 °C. Phipps *et al.* (1993) state that *L. variegatus* should not be cultured above 25 °C.

Several other process conditions may also influence the feeding behaviour of *L. variegatus*. Ferric iron (Fe^{3+}) addition to the sludge, a flocculant commonly used in wastewater treatment for phosphorus removal, and light/dark rhythm were investigated. Some plant operators have mentioned that iron addition seemed to enhance the growth of related aquatic worms in WWTPs (Janssen *et al.*, 2002). Iron is an important component of erythrocruorin, the haemoglobin-like oxygen-binding blood protein found in *L. variegatus* and many other Annelida (Frossard, 1982; Drewes, 2005). Addition of iron may therefore enhance growth. Besides, iron causes sludge flocs to compact and settle, which may facilitate food intake by the worms. The influence of iron was investigated by adding $FeCl_3$ to batches with sludge and *L. variegatus*.

L. variegatus displays negative phototaxis, i.e. avoids light and buries head-down in sediments in its natural habitat (Drewes, 2005). Complete darkness therefore may enhance sludge digestion and worm growth rates and this was further investigated in a batch experiment.

5.2 Materials and methods

5.2.1 General set-up

A detailed description of the basic set-up of the experiments was given in Chapter 4. It consisted of a series of glass Petri dishes (Ø 18.5 cm) or 250 mL Erlenmeyer flasks in which sludge was incubated with worms. As controls (without worms), Petri dishes or aerated 250 mL Erlenmeyer flasks with sludge were incubated under the same conditions. The batches in an experiment were analysed at a common endpoint and not at different intervals in time to limit the number of analyses and batches. This endpoint was reached either before the faeces percentage in the worm batches was 100 % (i.e. all the sludge was consumed) or soon after the faeces percentage in the first of a series of worm batches was 100 %. Sludge quantities, worm biomass and worm numbers were separately determined at the start and end of each experiment. In addition, temperature, pH and oxygen concentration were usually determined. Short-term experiments comprised of one run, long-term experiments of successive runs in which the same worm population was fed with fresh sludge samples.

L. variegatus specimens originated from a culture grown on effluent containing flushed out sludge flocs from a pilot-scale wastewater treatment system treating municipal wastewater from the village of Bennekom, the Netherlands. The worms were randomly selected for the batch experiments to represent all weight classes and the average individual worm wet weight was 14 (±6) mg. Population densities were between 1,100 and 11,000 specimens per m^2 and the W/S ratio was on average 0.4 (±0.2).

5.2.2 Sludge properties

To evaluate the effect of different sludge properties on digestion and growth, worms were fed in batch experiments with sludges from different WWTPs and with different pre-treatments.

Wastewater treatment plants The sludges originated from municipal and non-municipal WWTPs and were characterized in Table 5.2 according to wastewater type, plant type and characteristics, ash percentage, pH and sludge age.

Table 5.2 Characterization of the different sludges by wastewater type, plant type and characteristics, ash percentage (of TSS), pH and sludge age. Values are presented as averages with standard deviations. Ash percentages and pH values were determined at the start of each batch experiment. The sludge age was either calculated as an average during the total sampling period of the sludge involved or provided by the plant operators. Abbreviations used: Beer = Bavaria beer brewery in Lieshout, Bk = Bennekom, CAS = conventional activated sludge system, F = full-scale, P = pilot-scale, MBR = membrane bioreactor, N = nitrogen removal, Ni = nitrification, Nij = Nijmegen, Pb = biological phosphorus removal, Pc = chemical phosphorus removal, Pre = pre-settled wastewater, R = raw wastewater, Si = side stream membranes, Su = submerged membranes, Zoo = Noorder Dierenpark zoo in Emmen.

Sludge	Wastewater	Plant	Ash %	pH	Sludge age (days)
	Municipal				
R1	Bk, Pre	P, CAS, Ni	16 (±2)	6.7 (±0.4)	20 (±11)
R2	Bk, Pre	P, CAS, Ni	16 (±1)	6.7 (±0.6)	38 (±8)
M1	Bk, Pre	P, MBR, Su, Ni	20 (±2)	7.1 (±0.5)	18 (±3)
M2	Bk, Pre	P, MBR, Si, Ni	20	7.5	18 (±2)
Bk	Bk, R	F, CAS, N, Pb	25 (±4)	7.2 (±0.2)	40 (±6)
Nij	Nij, Pre	F, CAS, N, Pc	32	7.2	~16
	Non-municipal				
Zoo	Zoo	F, CAS	44	7.5	~400
Beer	Beer	F, CAS, Pc	43	8.4	~50

The WWTP at the Bavaria beer brewery included an anaerobic pre-treatment step in a UASB system. The sludge was taken from the aerobic part of the treatment plant. The WWTP at the Noorder Dierenpark zoo included a 'Living Machine', a water treatment system that employs constructed wetlands with plants. After this step, the water was led through a membrane filtration unit. It treated wastewater from zoo animals as well as from the visitors. The sludge was taken from the compartment with plants. It contained high aluminium concentrations (around 20 % of the TSS), dosed to improve the membrane filtration process.

Pre-treatment Sludges from reactors R1 and R2 and from WWTP Bennekom were pre-treated by means of the following methods:

Sludges were sieved using 75, 200 and 300 µm Retsch sieves, and 4.5 µm (Schleicher & Schuell) filters. This resulted in different sludge floc sizes of 0-4.5 µm, 0-75 µm, 0-200 µm, 0-300 µm, >300 µm, 75-200 µm and 200-300 µm. The sludge was sieved in portions to avoid the formation of a cake layer trapping smaller flocs. Sludge fractions were flushed with tap water to retain only flocs larger than the mesh size of the sieve. If necessary, fractions were diluted afterwards with tap water or effluent. Unfortunately, some re-aggregation of sludge flocs after this pre-treatment could not be avoided. In addition, we cannot rule out that the different size fractions were different in composition, e.g. the organic fraction.

Sludges were sterilized in an autoclave at 120 °C for 20 minutes. In some of the experiments the sludges were subsequently washed once (supernatant was replaced after centrifugation) with demineralised water and/or aerated for at least 1 hour. This was necessary to prevent accumulation of ammonia, which is highly toxic to aquatic organisms. The pH of the sterilized sludges was always between 6 and 8 and sterilizing did not change the pH to a great extent.

Sludges were incubated in aerated (oxic conditions: O_2 = 8-9 mg/ L) or closed bottles (anoxic conditions) for varying periods up to 217 days. Again, some of these sludges were washed and/or aerated to remove ammonia. Final pH values for the sludges that were pre-digested under anoxic conditions were between 6 and 8, and for those under oxic conditions between 4 and 7.

5.2.3 Worm properties

The influence of individual worm weight was tested in batch experiments with small (on average 5 and 8 mg wet weight) or large (on average 19 and 21 mg wet weight) specimens of *L. variegatus*. The influence of W/S ratios higher than 1.4 and population densities higher than 39,000 specimens per m^2 was tested in separate experiments.

5.2.4 Process conditions

The influence of iron (Fe^{3+}) addition or light/dark rhythm on digestion and growth was evaluated in batch experiments. To test the influence of iron, the consumption of sludge from WWTP Nijmegen, in which iron was dosed for phosphorus removal, was evaluated. In a further experiment, 19-23 mg $FeCl_3.6H_2O$ was added per litre sludge and fed to the worms. This concentration was based on influent quantities dosed in full-scale WWTPs. To test the influence of light/dark (LD) rhythm, batches were incubated under complete dark conditions, artificial complete light conditions or normal natural light/dark (16:8) rhythm.

5.2.5 Overview batch experiments

Table 5.3 gives an overview of sludge type, W/S ratio, sludge quantity, number of worms, experiment duration, number of batches and controls and temperatures for each batch experiment.

Table 5.3 Set-up of the individual batch experiments with *L. variegatus*. Abbreviations used: An = pre-digested under anoxic conditions, Beer = WWTP Bavaria beer brewery, Bk = WWTP Bennekom, Fr = fragmented, M1 = submerged membrane bioreactor, M2 = side-stream membrane bioreactor, na = not analysed, Nij = WWTP Nijmegen, Ox = pre-digested under oxic conditions, R1 = pilot-scale conventional activated sludge system 1 (sludge age ~ 20 days), R2 = pilot-scale conventional activated sludge system 2 (sludge age ~ 38 days), Sie = sieved, Sludge quantity at t_0 = sludge in worm batches at t_0 (TSS based), St = sterilized, Zoo = WWTP Noorder Dierenpark zoo, Δt = experiment duration, - = non-aerated control, + = aerated control.

Exp	Sludge	W/S ratio at t_0	Sludge quantity at t_0 (g)	Number of worms at t_0	Δt (d)	Number of batches	Number of controls	T (°C)
1	R1/R2	0.1-0.3	0.4-1.4	75	4	8	4-	18.7 (±0.5)
2	R1/R2	0.1-0.2	0.7-1.6	100-120	4	8	4-	20.0 (±0.5)
3	R1	0.6	0.7-0.9	200	5	9	4-, 1+	16.7 (±0.6)
4	R1	0.5	0.8-0.9	200-219	3	9	4-, 1+	16.2 (±0.8)
5	R1	0.5-0.6	0.8-0.9	150	6	10	2-, 2+	19.0 (±0.8)
6	R1	0.2-0.3	0.4	100	7	7	2-	18-22
7	R2	0.4	0.7-0.8	200-247	5	9	4-, 1+	16.6 (±0.8)
8	R2	0.3-0.5	0.4-0.5	166-167	5	5	2-	16-18
9	R2	0.2	0.9	100	6	6	2-	18-22
10	R1/R2/M1	0.2-0.4	0.3-0.4	100	3/6	12	6-	18-22
11	R2/M1	0.3	1.2-1.6	190	7	8	4-	16-18
12	Bk	0.4-0.5	0.6-0.7	200-206	5	9	4-, 1+	15.1 (±0.7)
13	Nij	0.3-0.4	0.4-0.6	75-203	4	4	1-, 1+	16.9 (±0.4)
14	Beer/R1	0.8-1.0	0.5-0.7	200	3	12	4-, 4 +	18.5 (±0.6)
15	Zoo/R2	0.2	0.8-0.9	100	6	8	4-	18-22
16	Sie/Bk	0.3-7.2	0.02-1.7	75-98	5	9	4-, 1+	16.3 (±1.0)
17	Sie/R1	0.4-0.9	0.02-0.7	10-132	3-8	10	0	16-18
18	Sie/Bk	na	na	variable	140	5	0	19.1 (±2.0)
19	R2/Fr/St	0.4-0.8	0.2-0.3	100-200	4	12	6-	16-18
20	Bk/R1/St	0.4-1.2	0.1-0.4	100-119	2	14	4-, 4+	18.8 (±1.2)
21	St	0.2-0.5	0.1-0.2	30	9	2	0	18-22
22	R1	0	0.6-0.7	0	9-120	12	12-	15
23	An/Ox	0.5-1.5	0.1-0.3	45-111	3-4	24	12-	18-22
24	R2/An	0.1-0.7	0.1-1.0	73-83	4	21	7-, 7+	17.6 (±0.5)
25	An	0.2-0.5	0.4-1.1	31-59	9	4	2-	18-22
26	An	0.8-0.9	0.1-0.2	100-108	4	4	1-, 1+	18-22
27	R1	0.1-0.2	1.0	54-105	5	5	2-, 1+	19.4 (±0.3)
28	M2	0.3-0.4	0.7-2.4	200-700	7	4	2+	18-22
29	R2	0.3	0.7-0.8	200	6	8	4 -	18-22
30	R1	1.0-1.7	0.4-0.6	150-152	4	15	6-, 3+	20

5.2.6 Calculations

Because we only determined sludge quantities, worm biomass and worm numbers at the start and end of the experiments, it is essentially unknown if they change linearly or non-linearly in time. However, we have indications from some earlier batch experiments (Experiment 1 in Table 4.1 of Chapter 4 plus two additional experiments) that sludge digestion by worms (after subtracting the endogenous digestion in the controls, which was on average 2 (±1) % of the TSS per day) and worm biomass increase approximately linear until the faeces percentage is 100 % and the maximum reduction percentage by worms only is reached (Appendix I, Figures A1 & A2). The increase in worm numbers (Appendix I, Figure A3) was variable in time (sometimes approximately linear and sometimes approximately exponential).

Given the fact that the batch experiments were mostly terminated before all the sludge was consumed (*Paragraph 5.2.1*), sludge digestion rates D (dry matter based) and biomass growth rates G (dry matter based) of *L. variegatus* could thus calculated with linear equations **(1)** and **(2)**. For number growth rates Gn, the linear approach was also chosen (equation **(3)**). The (worm) yield (dry matter based) was calculated from the biomass growth rate and the digestion rate with equation **(4)**.

Digestion rate:	$D = \Delta S\ W_0^{-1}\ \Delta t^{-1}$	(d^{-1})	**(1)**
Biomass growth rate :	$G = \Delta W\ W_0^{-1}\ \Delta t^{-1}$	(d^{-1})	**(2)**
Number growth rate:	$Gn = \Delta n\ n_0^{-1}\ \Delta t^{-1}$	(d^{-1})	**(3)**
Yield:	$Y = G\ D^{-1}$	(-)	**(4)**

Parameters:

ΔS	Sludge digestion by worms	(g TSS = g dry matter)
W_0	Worm weight at t_0	(g dry matter)
Δt	Experiment duration	(d)
ΔW	Worm weight change during experiment	(g dry matter)
Δn	Worm number change during experiment	(number)
n_0	Worm number at t_0	(number)

5.3 Results and discussion

5.3.1 Effect of sludges from different wastewater treatment plants

In short-term batch experiments randomly selected *L. variegatus* were fed with non-treated sludges from seven of the eight different WWTPs. Individual batches (some in duplicate) incubated under regular conditions and a normal 16/8 light/dark rhythm, were selected from Table 5.3. 'Regular conditions' refer to a duration between 2 and 8 days, a temperature between 15 and 20 °C, a population density between 2,000 and 11,000 specimens per m² and a W/S ratio between 0.1 and 1.0. Furthermore, sludges varied in pH and ash percentage. In Table 5.4, these variations are summarized for each sludge type.

Table 5.4 Variations in experiment characteristics within each of seven sludge types used in selected batch experiments with *L. variegatus*. For each characteristic the average value in bold with standard deviation in italic, minimum and maximum values are shown. Abbreviations used: N = no. of samples, P = population density of *L. variegatus*.

Sludge	Duration (d)	T (°C)	P (10^3 per m²)	W/S ratio	pH	Ash (% of TSS)
R1	**5** ±*2*	**19** ±*1*	**5** ±*2*	**0.4** ±*0.2*	**6.7** ±*0.4*	**16** ±*2*
(N = 17)	2-8	16-20	3-8	0.2-1.0	6.4-7.5	13-20
R2	**5** ±*1*	**19** ±*1*	**5** ±*2*	**0.3** ±*0.1*	**6.7** ±*0.6*	**16** ±*1*
(N = 24)	3-7	17-20	2-11	0.1-0.6	4.8-7.6	15-18
M1	**6** ±*2*	**19** ±*2*	**5** ±*2*	**0.3**	**7.1** ±*0.3*	**20** ±*3*
(N = 4)	3-7	17-20	4-7	0.3	6.8-7.4	17-22
Bk	**4** ±*2*	**17** ±*2*	**5** ±*2*	**0.4** ±*0.2*	**7.2** ±*0.2*	**25** ±*4*
(N = 3)	2-5	15-19	4-8	0.3-0.6	7.0-7.5	22-29
Nij	**4**	**17**	**5** ±*3*	**0.3**	**7.2**	**32**
(N = 2)	4	17	3-8	0.3-0.4	7.2	32
Zoo	**6**	**20**	**4**	**0.2**	**7.5**	**44**
(N = 2)	6	20	4	0.2	7.5	44
Beer	**3**	**19**	**7**	**1.0**	**8.4**	**43**
(N = 2)	3	19	7	1.0	8.4	43

Digestion rates (D), growth rates in biomass (G) or number (Gn) and yields (Y) for *L. variegatus* were calculated for the selected experiments. Figure 5.1 shows the minimum, average (with standard deviations) and maximum values for each sludge.

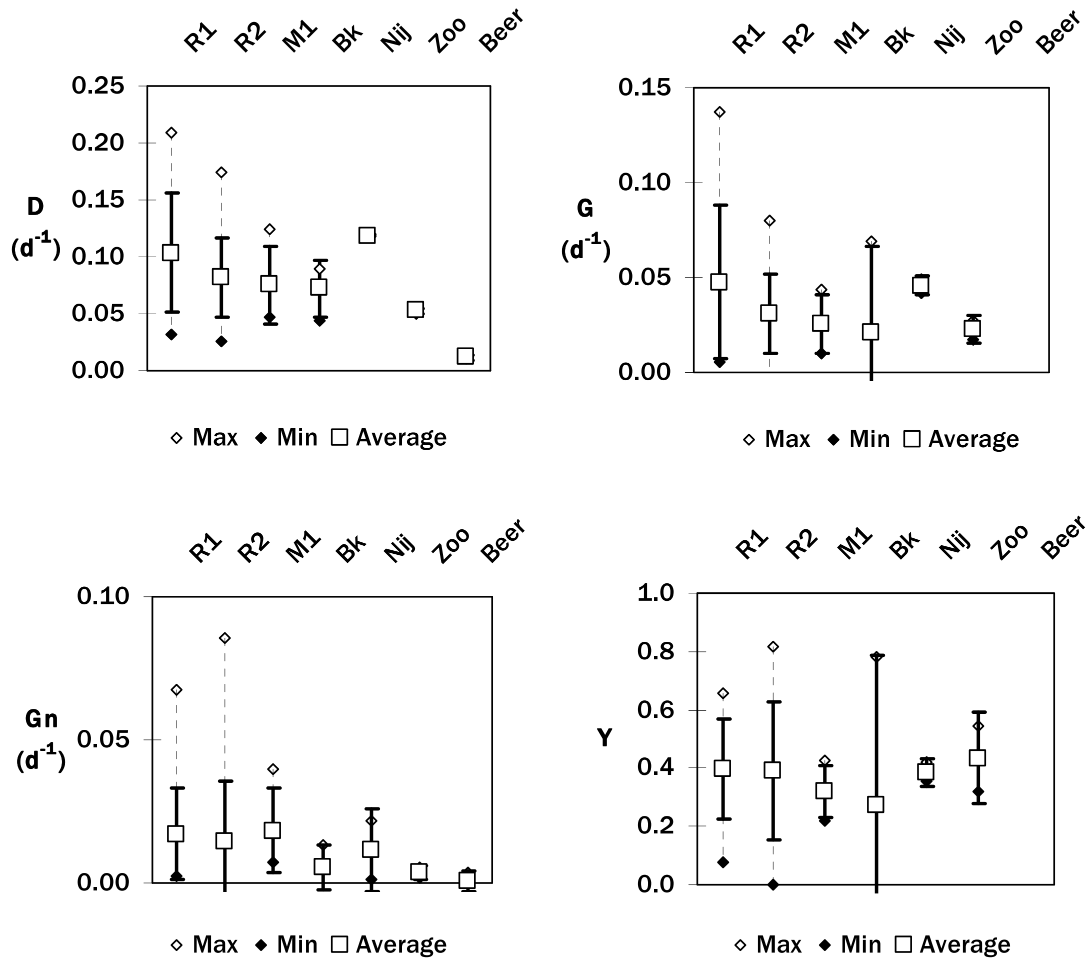


Figure 5.1 Average digestion rates (D in d^{-1}), growth rates (G and Gn in d^{-1}) and yields (Y) for *L. variegatus* feeding on sludges from reactors R1 (N = 17), R2 (N = 24) and M1 (N = 4), sludges from WWTPs Bennekom (N = 3) and Nijmegen (N = 2), Zoo sludge (N = 2) and Beer sludge (N = 2) in short-term batch experiments with standard deviations, minimum values and maximum values. Negative values, indicating worm death are included in the calculations but not shown in the graphs.

For each of the sludges in Figure 5.1 the variability in D, G, Gn and Y values was high, part of which may be explained by variations in experimental characteristics summarized in Table 5.4. Next to variations in experimental conditions, unknown discharges in the wastewaters that the sludges were fed with could have caused some variability. In addition, the average differences between duplicate batches within experiments were 0.02 (±0.02) d^{-1} for the digestion rates, 0.01 (±0.01) d^{-1} for the growth rates and 0.11 (±0.07) for the yields. For these reasons, apparent differences in rates and yields between and within batch experiments should be interpreted with caution.

To test the influence of the various experiment characteristics in Table 5.4 on D, G and Gn, their Spearman's rho correlation coefficients (since the data were not distributed normally) were calculated (SPSS 12.0.1) for R1 (N = 17) and R2 (N = 24) sludges, because these sludges were used in most batch experiments. The significant correlations are displayed in Table 5.5.

Table 5.5 Significant Spearman's rho correlation coefficients between pairs of variables in short-term batch experiments with R1 and R2 sludge. * = correlation is significant at the 0.05 level (2-tailed), ** = correlation is significant at the 0.01 level (2-tailed).

Sludge	Variable 1	Variable 2	Spearman's rho correlation coefficient	Variance explained (%)
R1	pH	G	-0.60*	36
R2	Duration	D	-0.62**	38
	pH	G	0.57**	32
	Ash%	G	0.44*	19
	P	Gn	-0.43*	18
	W/S ratio	Gn	-0.56**	31

The correlation coefficients indicated different and relatively small influences of variations in experiment characteristics on the average digestion and growth rates for both sludges. When a linear regression analysis (stepwise method) was carried out for the process characteristics and rates mentioned in Table 5.5, we found that for R2 sludge the digestion rate was influenced by duration, the biomass growth rate by pH and the average number growth rate by W/S ratio. However, none of the process characteristics explained more than 25 % of the variability in these rates.

In summary, experiment characteristics (individual or combined) could not sufficiently explain the variability in the digestion and growth rates within one sludge type. Therefore only very rough indications of differences in degradability between the different sludge types can be given.

Digestion rates Average digestion rates for most sludges, including Zoo sludge (that is partly of human origin), were in the same range (0.05-0.12 d^{-1}), with a total average of 0.09 (±0.04) d^{-1}, except for Beer sludge, which was lower (0.01 d^{-1}). Despite this low digestion rate 90 % of the Beer sludge had been converted into worm faeces at the end of the batch experiment. Beer sludge had a similarly high ash percentage as Zoo sludge (44 %) but their inorganic fractions contained different metals, iron and aluminium respectively. The high aluminium concentrations in Zoo sludge had no detrimental effects on the digestion rate and, as is proven in a further batch experiment (*Paragraph 5.3.5*), iron had no influence either. The cause for the low digestion rate of Beer sludge is unknown but could be due to toxic or refractory compounds.

Biomass growth rates The average biomass growth rates were usually positive and in the same range (0.02-0.05 d^{-1}), with a total average of 0.04 (±0.03) d^{-1}, except for the batches with Beer sludge. In these batches worm biomass decreased (data not shown), which was accompanied by low digestion rates and is a further indication that this sludge contains toxic or refractory compounds. In addition, in one batch with Bennekom sludge the worm biomass decreased for unknown reasons. In general, worm biomass always increased when feeding on this sludge, as shown in a long-term (140 days) sequencing batch growth experiment (*Paragraph 5.3.2*). In this experiment, normal average growth rates of 0.04 (± 0.02) d^{-1} on Bennekom sludge were found in 20 sequencing periods of 7 days.

Number growth rates The average number growth rates were mostly positive and in the same range (0.00-0.02 d^{-1}), with a total average of 0.01 (±0.02) d^{-1}. This time, the rate for Beer sludge was not substantially lower. In three batches with Beer sludge, Bennekom sludge and R2 sludge even a slight decrease in worm number was observed during the experiments. As mentioned before decreases in worm biomass do not necessarily coincide with decreases in numbers, i.e. worms usually keep reproducing even though they are starving (Buys, 2005), as was observed in one of the batches with Beer sludge.

Yields The average yields for *L. variegatus* for each sludge were between 0.27 and 0.43, with a total average of 0.38 (±0.22), except for Beer sludge, where the yield could not be calculated as worm biomass decreased. Zhang (1997) mentions a yield of 0.3 for the aquatic worms *Aeolosoma hemprichi*, *Nais* sp. and *Pristina* sp., which also feed on sludge.

In summary, average digestion and growth rates were highly variable between and within different sludges. This variability could not be explained by known variations in experiment characteristics. *L. variegatus* showed digestion and growth rates in the same variable range on sludges fed with municipal wastewater, regardless of the treatment system, from which they originated. The municipal sludges were better degradable than sludge fed with beer wastewater, on which worm biomass decreased.

5.3.2 Effect of pre-treated sludges

Sieving The influence of sludge floc size on sludge consumption was investigated in two short-term batch experiments (Experiments 16 & 17) with different size fractions (ranging from <75 μm to >300 μm). The digestion and growth rates in both experiments were similar for all fractions. To find out whether long-term growth on floc size fractions was different from that on non-sieved sludge, growth of *L. variegatus* on non-sieved Bennekom sludge and its fractions was also evaluated during a 140-days sequencing batch experiment (Experiment 18). The sludge was refreshed every 7 days and the population number was kept small by regular removal of worms. The size fractions were 0-4.5 μm, 0-75 μm, 75-200 μm and 200-300 μm. The growth rates over each 7-day period were calculated and the averages are shown in Figure 5.2.

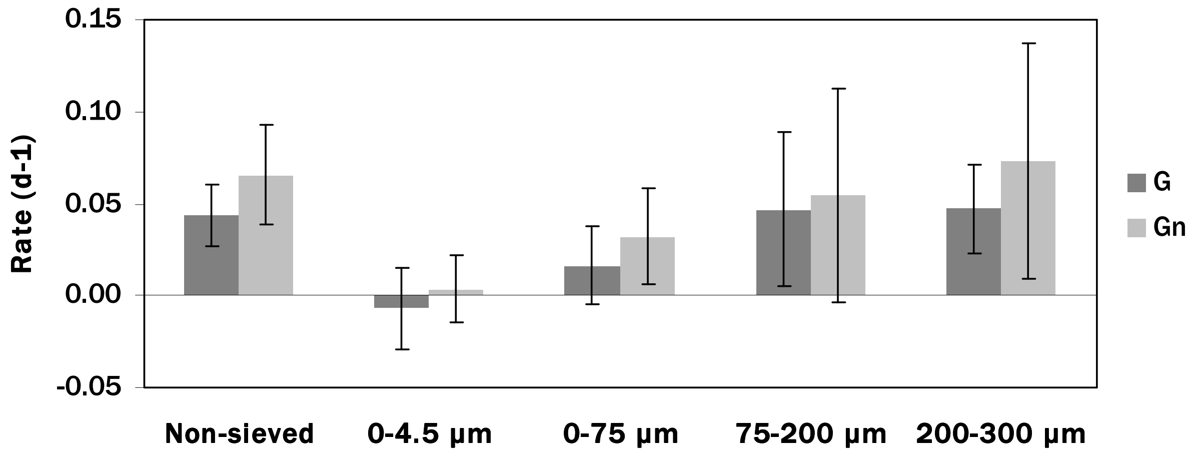


Figure 5.2 Average growth rates for biomass (G) and number (Gn) of *L. variegatus* feeding on different sludge fractions in Experiment 18 (N = 19 for each fraction). The water phase of the fractions consisted of tap water.

No significant differences in average growth rates for the fractions 0-75 µm, 75-200 µm and 200-300 µm were observed compared to the non-sieved sludge. Growth on the 0-4.5 µm fraction was very low in number and negative in biomass and significantly different from growth on floc sizes >75 µm. Even though the worms were able to compact even the small flocs from the 0-4.5 µm fraction into faeces, the fraction did not contain enough TSS to maintain a stable body weight or support biomass growth, in contrast to the larger fractions. *L. variegatus* can survive for months without food (Williams, 2005), while slowly decreasing in biomass. Buys (2005) found for example a biomass growth rate of -0.017 d^{-1} on tap water, but still a positive number growth rate of 0.006 d^{-1}. Interestingly, the numbers increased more slowly in the 0-4.5 µm fraction than in the tap water. This indicates again that division may be a survival mechanism under circumstances of food shortage, as was described in the *Introduction*.

Information on food particle size of *L. variegatus* is scarce, but there is some information on the ingestion of algae (Moore, 1978). He described that the maximum diameter of ingested algae is 150 µm for worms between 15 and 25 mm, and 200 µm for those between 35 and 50 mm. The smallest algae ingested for both size classes were 5-6 µm. There is no evidence from our experiments that the size of sludge flocs is limiting for uptake by *L. variegatus*, but this may be related to their soft structure, in contrast to the rigid cell walls of algae. Ratsak (2001) stated that the ingestion of sludge flocs by *N. elinguis* was limited by their mouth size and that very small or large flocs cannot be ingested but the above-described experiments proved this to be wrong. Sperber (1948) for example described that Naidinae, a subfamily of aquatic Oligochaeta, bite pieces of their food, when particle sizes are too large. As for the growth rates, Williams (2005) found number growth rates of 0.02-0.05 d^{-1} on Tetra Min fish food in aerated aquaria and the highest number growth rate was found for flow-through systems. In our stagnant systems with sludge, higher average number growth rates of 0.05-0.07 d^{-1} were found (Figure 5.2) and sludge apparently is a highly nutritious food source.

Sterilization Consumption of sterilized R2 sludge was compared to that of non-sterilized R2 sludge in Experiment 19. There was no digestion in the controls with sterilized sludge, which demonstrated the absence of bacterial activity. Sterilization of this sludge did not cause slower digestion and growth when compared to non-sterilized sludge (results not shown) and apparently, *L. variegatus* does not need living substrate.

During similar batch experiments (e.g. Experiment 20) worms often lost weight or died in fresh or stored sterilized R1, R2 or Bk sludges, even after washing and aerating the sludges prior to the experiments. Causes may have been the low oxygen concentrations often measured in these batches during the experiment (most likely because of increased bacterial activity) and/or the formation of unknown toxic compounds due to sterilization or loss of sterility during storage. The influence of autoclaving on sludge was illustrated by changes in pH, increased ash percentages and decreases in TSS and VSS concentrations in our experiments. In analogy, thermal pre-treatment of sludges is known to change the floc structure and enhance biological degradation by bacterial lysis with subsequent release of biopolymers and solubilization of COD (Neyens & Baeyens, 2003; Eskicioglu *et al.*, 2006).

To prevent detrimental side effects of sterilization on the worms, Experiment 21 was done (Figure 5.3), in which recently sterilized sludge in the batches was daily refreshed from separate closed bottles and washed prior to each addition.

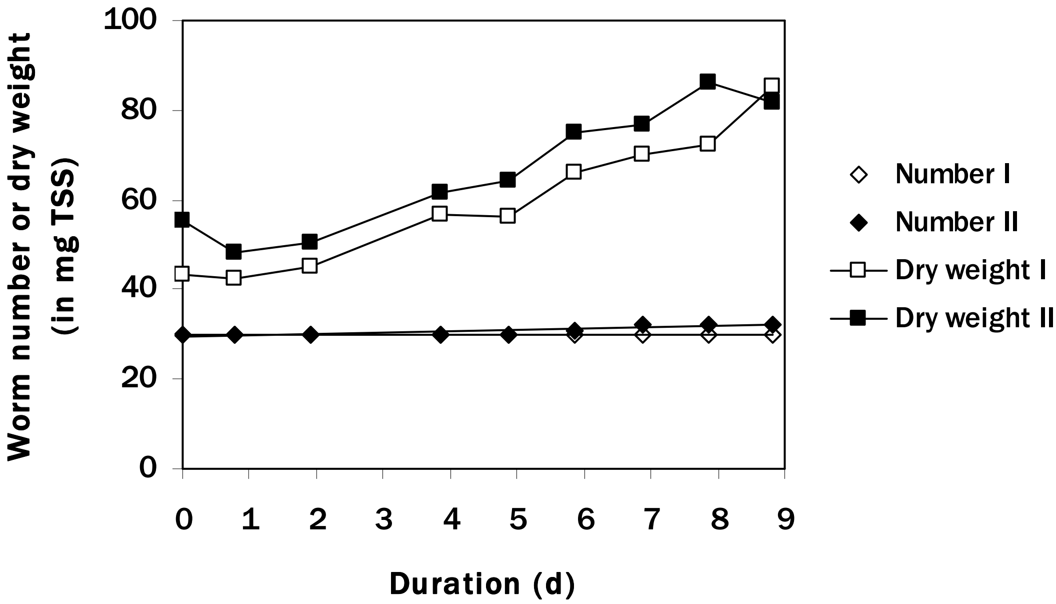


Figure 5.3 Number and total dry weight of *L. variegatus* feeding on daily refreshed sterilized R1 sludge in Experiment 21 in duplicate batches I and II. W/S ratios were between 0.2 and 0.5.

During the first day of the experiment, worms were immotile and irresponsive to tactile stimulations and the faeces percentage of the sludge was only 5 %, indicating adverse conditions. Thereafter they adapted with corresponding motility, touch-evoked reflexes and increased faeces percentages of 30-50 %. Feeding inhibition in *L. variegatus* could have been the result of exposure to toxic compounds, but also of an acclimatization period when transferred to a different substrate (Williams, 2005).

Worm numbers did hardly change but biomass (and as a result individual worm weight) increased gradually during the experiment with an occasional small decrease (Figure 5.3). The average biomass growth rate after the acclimatization period on sterilized R1 sludge was 0.08 (±0.07) d^{-1}. In contrast, the number growth rates were zero, since virtually no reproduction took place. Only during the last two days, digestion rates were determined, assuming endogenous digestion to be zero in sterile sludge. The rates were on average 0.13 (±0.04) d^{-1}. The yields were on average 0.4 (±0.2). The rates with sterilized sludges were similar to those with non-sterilized sludges (Figure 5.1). This is described by several authors for other aquatic worms (e.g., Liang *et al.*, 2006b). Digestion rates for *A. hemprichi* and *T. tubifex* were 0.80 and 0.54 d^{-1} respectively, which is extremely high compared to our results for *L. variegatus*, which were at most 0.17 d^{-1}.

It is generally accepted that the food of freshwater Oligochaeta consists almost exclusively of bacteria, which they extract from the sediment they ingest (Moore, 1978). The above experiments prove that *L. variegatus* could digest a sterile substrate and showed biomass growth. The rates on sterilized sludge were similar to those on non-sterilized sludges. However, the absence of live bacteria from the substrate seemed to suppress reproduction in *L. variegatus* and it also known to prevent biomass growth in sessile Tubificidae (Reynoldson, 1987). In addition, *L. variegatus* showed negative biomass and number growth rates or died on solutions of different single carbon sources (acetate, starch, tryptose, gelatine, tryptone, saccharose, glucose, yeast extract and casein) with added nutrients and trace elements (results not shown). This could also be due to their inability to take up dissolved organic material (Sedlmeier & Hoffmann, 1989).

Pre-digestion Activated sludge, when incubated for a long period without worms under oxic or anoxic conditions (Experiment 22) decreased gradually in dry weight due to endogenous digestion. A refractory portion however, of about 40-50 % of the TSS, was left in both cases (Table 5.6). Maximum endogenous digestion was reached considerably faster under oxic conditions than under anoxic conditions within 34 days and 114 days respectively.

For fresh sludge, the final maximum digestion percentage after consumption by *L. variegatus* is the same as without consumption (Chapter 4). To find out whether worms do increase the maximum digestion percentage of pre-digested sludges, Experiment 23 was carried out. Pre-digested sludges from Experiment 22 (Figure 5.4) were fed for an additional period of 3 to 4 days to *L. variegatus* in Petri dishes. Additional endogenous digestion in parallel control batch experiments was subtracted to calculate the digestion caused by the worms.

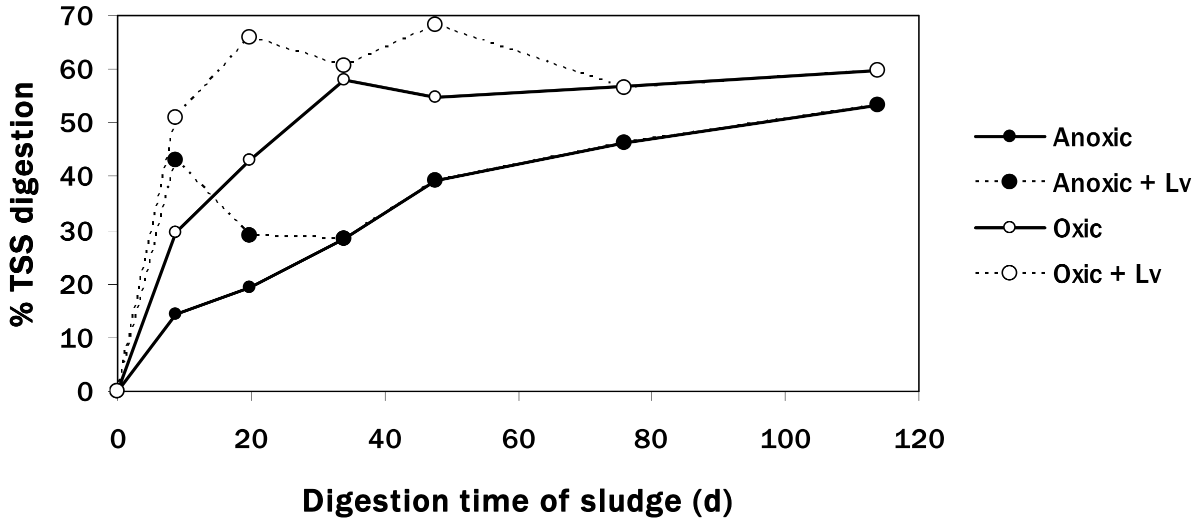


Figure 5.4 Digestion of sludge (R1) incubated under anoxic (in closed bottles) or oxic (aerated) conditions without worms during varying periods (digestion times) up to 114 days in Experiment 22 (solid lines) and **additional** digestion by *L. variegatus* after consumption of these pre-digested sludges during 3 to 4 days in Experiment 23 (dashed lines). W/S ratios were on average 0.8 (±0.3). pH under anoxic conditions was 6.3 ± 0.2 and under oxic conditions 4.6 ± 0.3 (with one exception of 6.6).

Figure 5.4 clearly demonstrates that the worms caused an additional digestion of 3-29 % with final percentages of more than 60 % of sludges that were pre-digested under oxic conditions for up to 48 days and of sludges that were pre-digested under anoxic conditions for up to 19 days. However, in the pre-digested sludges under (an)oxic conditions older than 20 days worm biomass decreased or worms even died. This can be due to low nutritional value of the oxic sludges or the accumulation of toxic compounds (such as ammonia) in the anoxic sludges.

To prevent the accumulation of toxic compounds other anoxic pre-digested R1 and R2 sludges (7, 121 and 217 days) were washed and aerated before feeding them to *L. variegatus* in Experiment 24. A small decrease in worm biomass was observed in two of the batches with 121 and 217 days old sludge. Table 5.6 shows an overview of digestion and biomass growth rates on pre-digested sludges in Experiments 23, 24 and in two additional Experiments 25 and 26. Batches in which worm biomass decreased or worms died were left out.

Table 5.6 Average digestion rates D, growth rates G and yields Y for *L. variegatus* feeding on (an)oxic pre-digested sludges of different ages in Experiments 23, 24, 25 and 26 (N = no. of samples).

Pre-digestion	Digestion time (days)	N	D (d^{-1})	G (d^{-1})	Y
Anoxic	6-20	5	0.06 (±0.03)	0.05 (±0.03)	0.78 (±0.52)
	120-121	3	0.06 (±0.02)	0.04	0.73 (±0.31)
	217	1	0.08	0.05	0.65
Oxic	9-20	2	0.06 (±0.03)	0.02 (±0.01)	0.29 (±0.02)

The average digestion rates on oxic and anoxic sludges were similar to those with non-treated reactor sludges (Figure 5.1). All number growth rates were close to zero, as was found previously for sterilized sludges and indicates suppressed reproduction for unknown reasons. Biomass growth rates were in the same range as for fresh sludges (Figure 5.1) but somewhat higher on anoxic sludges than on oxic sludges. Remarkably, there was still additional digestion and growth in sludges that were pre-digested under anoxic conditions for 120 days and longer. These washed and aerated pre-digested sludges obviously had some nutritional value left and were not toxic to the worms in contrast with oxic sludges older than 48 days and non-washed anoxic sludges older than 20 days respectively (Figure 5.4). The higher nutritional value of older sludges pre-digested under anoxic conditions can be explained by the fact that endogenous digestion under anoxic conditions is slower than that under oxic conditions (Figure 5.4). The average yields on anoxic sludges were higher than for most fresh sludges but with large standard deviations. Those for oxic sludges were the same as for fresh sludges. The exact reasons for these differences are unknown, but components formed by anoxic digestion are possibly more efficiently converted into worm biomass.

To find out which component was responsible for biomass decrease and worm death in several of the anoxic pre-digested sludges, nutrient concentrations ($N\text{-}NH_4^+$, $N\text{-}NO_3^-$, $N\text{-}NO_2^-$ and $P\text{-}PO_4^{2-}$) were measured in the supernatants of the sludges of Experiment 24, before and after aerating and washing (Table 5.7). The presence of toxic compounds could be determined by stress responses like autotomy and other lesions to the body of *L. variegatus* and finally, death. Stress responses are marked by the shaded areas in Table 5.7.

Table 5.7 Nutrient concentrations (in mg/ L) before and after washing and aerating in supernatants of R1 or R2 sludges that were anoxic pre-digested during 7, 121 and 217 days. Sludges that were lethal to the worms are shaded.

	Before washing and aerating				After washing and aerating			
Sludge	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	$N\text{-}NO_2^-$	$P\text{-}PO_4^{2-}$	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	$N\text{-}NO_2^-$	$P\text{-}PO_4^{2-}$
R1 7 days	48.7	0.6	0.1	14.2	8.1	10.2	2.2	3.3
R2 7 days	37.0	0.6	0.1	15.3	8.5	5.3	0.2	3.5
R1 121 days	175.6	0.9	0.2	32.3	29.0	3.2	0.1	5.1
R2 121 days	173.9	0.9	0.2	29.9	71.1	2.4	0.1	10.8
R1 217 days	167.2	0.8	7.3	32.5	49.5	3.2	2.1	10.2
R2 217 days	12.5	11.4	0.7	30.3	4.5	6.4	0.1	11.6

From Table 5.7 it is obvious that nitrate, nitrite and phosphate in these concentrations were not responsible for worm death, because higher concentrations of these compounds were also found in sludges that did not cause stress responses. Ammonia on the other hand had high values in all the sludges that were lethal. *L. variegatus* seemed to be able to survive concentrations of ammonia, nitrate, nitrite and phosphate (under these experimental conditions) of at least 49, 11, 2 and 30 mg/ L respectively. Schubauer-Berigan *et al.* (1995) have investigated the toxicity of ammonia to *L. variegatus* at different pH values. The toxicity of ammonia is dependent on pH, since at higher pH values its toxicity strongly increases by the formation of un-ionized ammonia (NH_3) from the relatively non-toxic ammonium ion (NH_4^+). The ratio of ammonia to ammonium will change by a factor of 10 with every unit change in the pH (Stephan *et al.*, 1999). The fact that concentrations of 49 mg/ L were non-toxic before washing and aerating, but toxic thereafter (Table 5.7) was most likely caused by the pH values of 5.9 and 8.1 that were measured in these respective sludges. At pH 5.9 0.03 % of this concentration was present as un-ionized ammonia but at pH 8.1 this concentration had increased to 5 % (2.5 mg/ L). The minimum concentration of un-ionized ammonia, which was lethal to the worms, was 0.2 mg/ L in this experiment.

In summary, worms could partly break down sludges that were pre-digested under oxic conditions for up to 50 days. The same was true for sludges that were pre-digested under anoxic conditions for up to 220 days if ammonia accumulation was prevented. The anoxic sludges seemed to have a higher nutritional value possibly resulting from slower digestion. Worm biomass growth was always observed in pre-digested sludges when younger than 20 days, but also in older anoxic sludges when ammonium accumulation was prevented. The worms were thus able to accelerate (an)oxic sludge digestion significantly. The final digestion percentage of oxic pre-digested sludges even seemed to increase somewhat by worm consumption (up to 68 % instead of 60 %) and the digestion rates on anoxic sludges of 120 days and older also suggested additional digestion.

Park *et al.* (2006) and Jung *et al.* (2006) described that alternating combinations of oxic and anoxic conditions could enhance the final endogenous sludge digestion percentage (up to 70 %) through bacterial selection. They stated that under intermittent aeration conditions sludge components were solubilized that were refractory under either oxic or anoxic conditions. In addition, Jung *et al.* (2006) suggested that a combination with consumption by higher organisms may be even more beneficial. Our experiments show there is potential for such a combination.

5.3.3 Effect of worm properties

Individual worm weight The influence of individual worm weight on sludge digestion and worm growth was tested in Experiments 13 and 27, in which small and large specimens were selected. In Experiment 13 with Nijmegen sludge, the digestion and biomass growth rates of small and large worms were the same (Figure 5.5), as well as the yields, which both were 0.4.

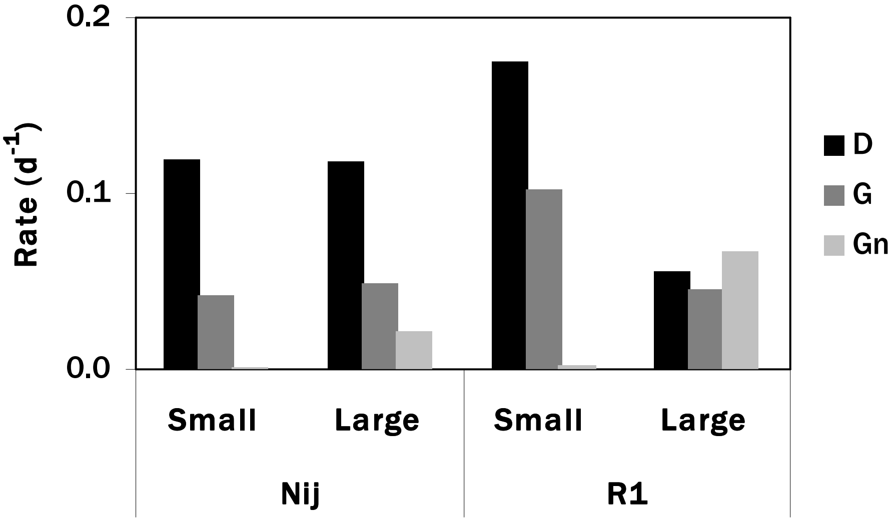


Figure 5.5 Digestion rates D, growth rates G and growth rates Gn for small and large *L. variegatus* specimens feeding on Nijmegen (Nij) or R1 sludge in Experiments 13 and 27. W/S ratios for Nij sludge 0.3 and 0.4, for R1 sludge 0.1 and 0.2. Duration = 4 and 5 days respectively.

In contrast, a big difference was found between the digestion and biomass growth rates of small and large worms with R1 sludge (Figure 5.5). On R1 sludge, small worms had a much higher digestion and biomass growth rate, but a somewhat smaller yield than the large worms; 0.6 and 0.8 respectively.

It seems plausible that small worms eat and grow faster, since reproduction followed by a non-feeding period is observed in large worms only. Leppänen & Kukkonen (1998b) even found a negative relationship between individual worm weight of *L. variegatus* and weight-corrected excretion rate. The results with R1 sludge support a similar conclusion. In contrast, Williams (2005) found a rather stable weight-corrected excretion rate as is supported by the results with Nijmegen sludge. The only similarity between both experiments were the higher number growth rates for large worms, especially in R1 sludge, indicating a positive correlation between individual worm weight and increase in numbers.

The influence of individual worm weight on digestion and biomass growth rates in sludge remains obscure. Number growth rates however seemed to be positively related to individual worm size.

Worm to sludge ratios and population densities When comparing data from different experiments for *L. variegatus* feeding on R1 or R2 sludge under regular conditions, slightly negative correlations were found between W/S ratios or population densities and number growth rates in R2 sludge (Table 5.5). No correlations with digestion rates or biomass growth rates were found.

The effect of very high W/S ratios (1.4-1.6) at normal worm population densities (5,600 specimens per m^2) was visible in Experiment 30, in which the influence of light and dark conditions was tested (Figure 5.8 'normal conditions'). Digestion rates were

low and biomass growth rates were negative or close to zero. At the same time, the worm numbers increased during the experiment, i.e. there was still reproduction but average individual worm weight decreased, as was also found under starvation conditions (*Paragraph 5.3.2*). This indicated food limitation.
The effect of very high population densities (>39,000 specimens per m^2) at normal W/S ratios (0.3 and 0.5) was tested in Experiment 28 (Figure 5.6).

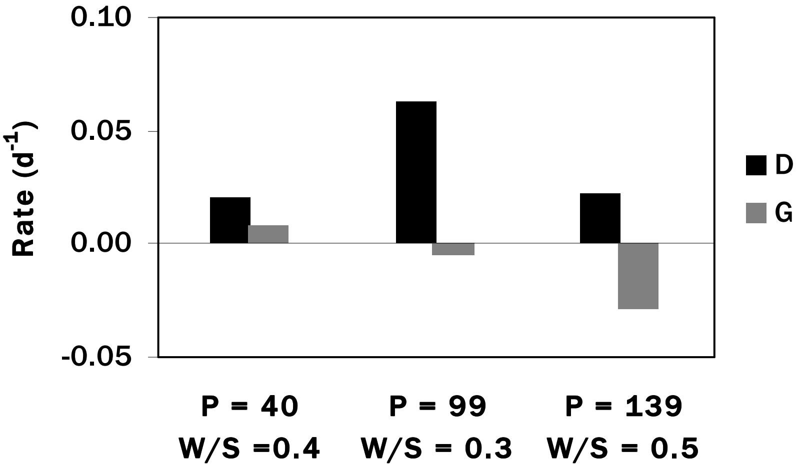


Figure 5.6 Digestion rates D and growth rates G for *L. variegatus* feeding on M2 sludge at high population densities (P in 10^3 specimens per m^2) and approximately equal W/S ratios (W/S) in Experiment 28. Duration = 7 days.

At high densities, the biomass growth rates were low or negative and the digestion rates were low, as was also found at high W/S ratios. Again there was reproduction (number growth rates were 0.01 d^{-1} in all the batches) while average worm weight decreased at the two highest densities. Since food was present in excess in this experiment, as was oxygen, other density effects like accumulation of excreted un-ionized ammonia may have been the cause. Related species like sessile Tubificidae are for example known to excrete ammonia at rates of 0.03-0.34 µg NH_4^+/ mg dry weight/ h (Postolache *et al.*, 2006).

Thus, extremely high values for population density and W/S ratio both lead to biomass decreases and lower digestion rates, most likely because of density effects.

5.3.4 Effect of process conditions

Addition Fe^{3+} Nijmegen sludge contained iron, which was dosed for phosphorus removal. The digestion rates of this sludge were quite high (Figure 5.1) but fall within the overall variability. Growth rates on Nijmegen sludge were not different from those on other municipal sludges.

The influence of $FeCl_3$ addition on sludge digestion and worm growth was tested in Experiment 29 (Figure 5.7). The addition of iron increased the ash percentage of the sludge with 1.2 % and decreased the pH from 7.3 to 6.5, but there were no indications that these factors could influence digestion or growth as was already shown in *Paragraph 5.3.1*. Endogenous digestion in the controls did not change after iron addition (results not shown). Digestion rates, growth rates and yields (0.6) for *L. variegatus* feeding on sludge did not change either after iron addition (Figure 5.7). The number growth rates were all close to zero.

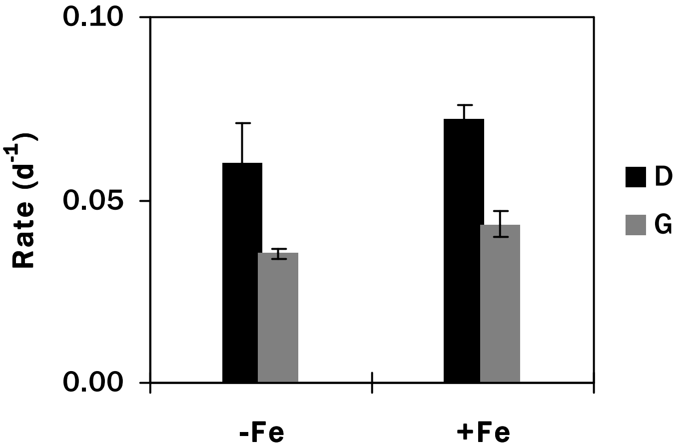


Figure 5.7 Average digestion rates D and growth rates G for *L. variegatus* feeding on R2 sludge with (+Fe) and without (-Fe) iron addition in Experiment 29 (N = 2 for all rates). W/S ratio was always 0.3. Duration = 6 days.

It is clear from both experiments that iron does not influence sludge digestion and worm growth.

Light/dark rhythm Figure 5.8 shows the digestion and growth rates under different light/dark (LD) rhythms in Experiment 30.

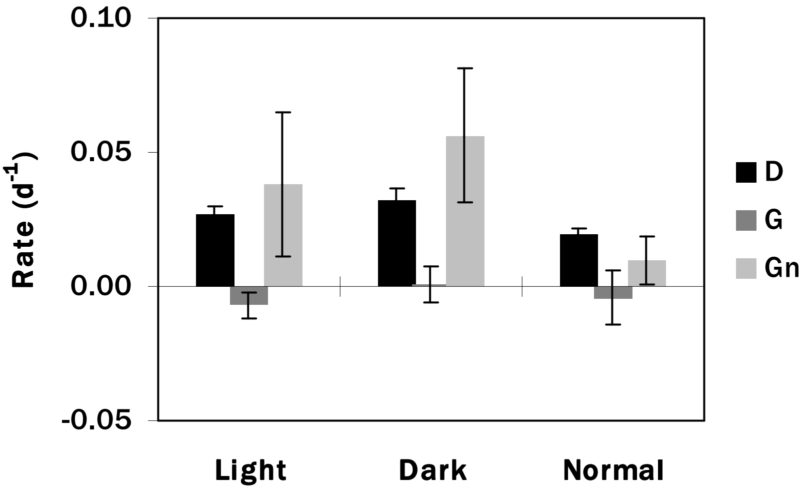


Figure 5.8 Average digestion rates D and growth rates G and Gn for *L. variegatus* feeding on R1 sludge under different light/dark (LD) rhythms: LD = 24:0 (light), LD = 0:24 (dark) and LD = 16:8 (normal) in Experiment 30 (N = 2 for all rates). Duration = 4 days. W/S ratios under dark, light and normal conditions where 1.0-1.7, 1.2-1.5 and 1.4-1.6 respectively.

Average digestion rates under the different conditions were similar. The average biomass growth rates (Figure 5.8) were usually negative. The decrease in worm biomass was probably related to the high W/S ratios of 1.0-1.7 that were applied in these experiments. More attention to this point was already given in *Paragraph 5.3.3*. The average yield could not be calculated since biomass growth rates were negative. The average number growth rate was somewhat higher under complete dark conditions than under normal conditions (Figure 5.8).

Stephenson (1930) mentioned that many Oligochaeta, avoid light (negative phototaxis) and are injured by direct sunlight. Therefore, dark conditions may be more favourable, because this mimics their natural habitat. In addition, Hendrickx *et al.* (2006) found slightly higher digestion rates of *L. variegatus* under dark conditions. They concluded that this may have been due to the phototoxicity of chemicals present in sewage sludge. It is therefore likely that *L. variegatus* can be influenced to some extent by light/dark rhythms, but the short-term influence on sludge digestion and biomass growth in this experiment was not evident.

5.4 Conclusions

In this chapter, short-term batch experiments were described that investigated the influence of different factors on waste sludge digestion by *Lumbriculus variegatus* and worm growth. Experiments on the influence of sludge properties showed that:

- The variability in sludge digestion rates, worm biomass growth rates, worm number growth rates and yields for each of seven tested municipal and non-municipal waste sludges was relatively high. This was caused to some extent by small variations in experimental conditions, pH and ash percentage of the sludge. Most of the variation could not be explained and was probably due to unknown differences in sludge composition.
- The average digestion rate (dry matter based) of *L. variegatus* on municipal sludges was 0.09 (±0.04) d^{-1}, while that on Beer sludge was lower.
- The average biomass growth rate (dry matter based) and number growth rate of *L. variegatus* on municipal sludges were 0.04 (±0.03) d^{-1} and 0.01 (±0.02) d^{-1} respectively, while these rates were again lower (or negative) for Beer sludge.
- The average yield (dry matter based) of *L. variegatus* on municipal sludges, except Beer sludge, was on average 0.38 (±0.22), which means that on average 38 (±22) % of the sludge digested by *L. variegatus* was converted into new worm biomass.
- Sludge floc size did not influence digestion and growth rates, unless the sludge concentrations of the used sludge fractions were too low. *L. variegatus* was able to ingest sludge flocs smaller than 4.5 and larger than 300 μm.
- The lack of live bacteria in sterile sludge did not affect digestion and biomass growth rates of *L. variegatus* (as long as no unknown toxic compounds were present). The

rates for sterilized sludges were similar to those for non-sterilized sludges. Reproduction in sterile sludges was however suppressed.

- The worms accelerated the digestion of sludges previously incubated under oxic and anoxic conditions as long as the presence of un-ionized ammonia was prevented in anoxic sludges and the nutritional value of the oxic sludges was still enough. In addition, the worms could enhance the final digestion percentage of some pre-digested sludges up to 68 %.

Experiments on the influence of worm properties showed that:

- There was no clear effect of individual worm weight of *L. variegatus* on digestion and biomass growth rates. Only the number growth rate slightly increased with individual worm weight.
- High population densities (>39,000 specimens per m^2) and W/S ratios (>1.4) caused lower digestion and biomass growth rates.

Experiments on the influence of process conditions showed that:

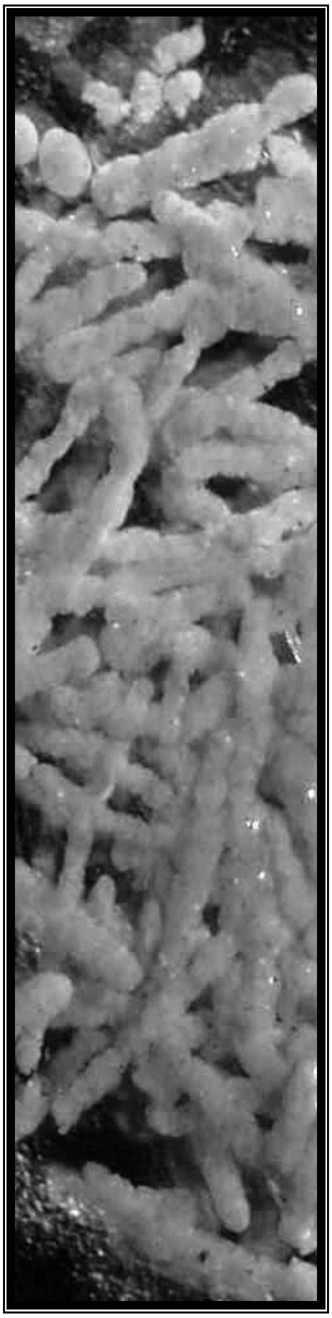
- The presence of ferric iron (Fe^{3+}) in sludge had no effect on digestion or worm growth. The same was true when iron was added to the sludge in the applied range, which is representative for municipal sludge with chemical phosphorus removal.
- Sludge digestion and biomass worm growth were not affected by light/dark rhythms. Only number growth rates were somewhat higher under complete dark conditions

Acknowledgments

We thank Bas Buys (Wageningen University) for co-designing and co-performing the batch experiments described in this chapter and Edwin Peeters (Wageningen University) for his help with the statistical analyses. In addition, we thank Dennis Piron (WWTP Nijmegen), Rob van Doorn (WWTP Bennekom), Hilde Prummel (Water Laboratorium Noord), and Manfred van den Heuvel (Bavaria beer brewery) for providing data, sludge and additional information.

╡Chapter 6╞

The effects of sludge consumption by *Lumbriculus variegatus* on sludge characteristics



Abstract

Waste sludge consumption by *L. variegatus* reduces the amount of waste sludge, but also changes the structure of the waste sludge, because the worm faeces are more compact. In addition, the composition of the worm faeces is likely to be different from that of the waste sludge. Therefore, the influence of sludge consumption on different sludge characteristics was investigated in short-term batch experiments. In these experiments, sludge consumption by *L. variegatus* always enhanced the initial settling rate of several municipal waste sludges and led to SVI_{30} values of around 60 mL/ g. Even though the dewaterability of worm faeces was expected to be higher than that of the waste sludge, this could not be demonstrated with the applied CST (capillary suction time) method. The turbidity of the water phase increased, due to the formation of more dissolved and/or colloidal materials. These materials possibly consisted of carbohydrates, but not of proteins, because a specific release of carbohydrates to the water phase was sometimes observed after sludge consumption. *L. variegatus* can specifically feed on the protein fraction of the organic matter in sludge, because next to a decrease in ash percentage, the total protein fraction as percentage of sludge TSS (total suspended solids) usually decreased. This effect was not observed for the total carbohydrate fraction. Sludge consumption by *L. variegatus* did not lead to bioaccumulation of heavy metals (cadmium, chromium, copper, lead, nickel and zinc) from sludge in worm biomass above concentrations already present in the worms and these concentrations were always substantially lower than in waste sludge. As a result, the heavy metal concentrations in worm faeces were higher than in the waste sludge. The distribution of the absolute heavy metal amounts over the sludge flocs, the water phase and the worms did not change after sludge consumption. Finally, worm faeces of *L. variegatus* were ingested by their own kind (conspecifics), but the digestion rates for this process were very low and worm biomass decreased.

The most important effects of sludge consumption by *L. variegatus* on sludge characteristics are thus the improved settleability, the decrease in protein content and the increase in heavy metals content. In addition, sludge digestion by *L. v*ariegatus takes place for the most part during the first gut passage.

6.1 Introduction

In municipal and non-municipal (industrial) wastewater treatment, large amounts of biological waste sludge are produced. Reducing this amount and improving sludge dewaterability are important issues for environmental and economical reasons (Neyens & Baeyens, 2003). Consumption of waste sludge by the aquatic worm *Lumbriculus variegatus* offers a possible solution.

L. variegatus actively gathers and ingests sludge flocs of variable sizes and converts them into uniform faecal pellets (Chapter 4). The worm faeces are around 1 mm long and 0.25 mm wide and can be easily discerned from waste sludge, even with the naked eye. They have a cylindrical compact shape and high density (Figure 6.1). Several other aquatic worms, like *Aeolosoma* sp. and *Nais* sp. (Figure 6.1), produce faeces with a similar yet smaller cylindrical shape, whereas faeces from different sessile

Tubificidae species have more variable shapes (Brinkhurst & Austin, 1979; own observations).

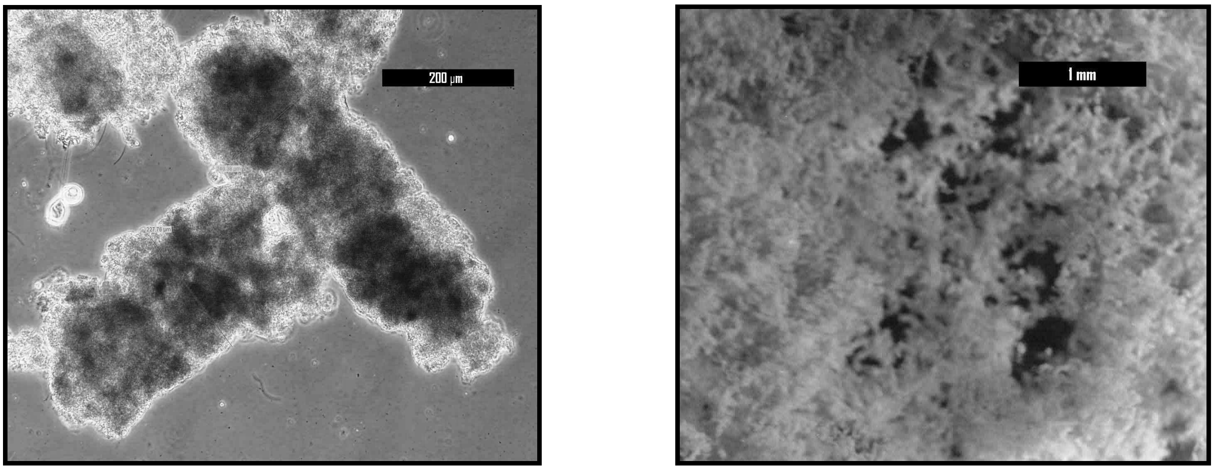


Figure 6.1 Left: Faeces of *L. variegatus* fed with activated sludge. Scale bar = 200 µm. Right: Faeces of *Nais* sp. fed with activated sludge. Scale bar = 1 mm.

Digestion of municipal waste sludge by *L. variegatus* is approximately twice as fast as the endogenous digestion rate of sludge and this digestion takes place at an average rate of 0.09 (±0.04) d^{-1} (dry matter based) (Chapters 4 & 5). Almost the entire reduction concerned the organic fraction of the sludge. It is likely that sludge consumption by *L. variegatus* not only changes the structure of the sludge flocs but also other characteristics like sludge settleability, sludge dewaterability, turbidity of the water phase, sludge composition in terms of proteins, carbohydrates, ash and heavy metals. In addition, the further degradability of sludge after consumption may change.

Settleability will be affected since this is largely dependent on floc size and density. Dewaterability of the sludge may also improve due to the compacting of the sludge flocs. Enhancement of both properties was observed after fungal treatment of sludge, which resulted in compact pellets of 2-5 mm (Alam & Fakhru'l-Razi, 2003), and consumption of sludge by *L. variegatus* may have the same effect. However, even though it is generally assumed that settleability and dewaterability are positively correlated, experimental results do not always confirm this and both characteristics should therefore be investigated (e.g. Hung *et al.*, 1996). The turbidity of the water phase may change after consumption because of the extra release of dissolved and colloidal COD (chemical oxygen demand) (Buys, 2005). This could have a negative effect on the quality of the effluent if this is directly discharged from WWTPs (wastewater treatment plants). After consumption, *L. variegatus* may specifically digest protein and/or carbohydrate fractions of the sludge and as a result, the ash content may increase. Related oligochaete species like earthworms are for example known to easily digest the proteins and carbohydrates in their substrate contrary to the more refractory compounds like cellulose (Curry & Schmidt, 2007). If consumption by *L. variegatus* has a similar effect, this could alter re-use options of the consumed sludge.

Waste sludge may also contain significant amounts of heavy metals. Heavy metals that end up in WWTPs mainly originate from three sources: municipal and non-municipal (industrial) effluents and runoff (Lester, 1987). Important sources of for example lead, zinc and copper in the Netherlands are roof materials, conduit-pipes and fireworks (Loeffen & Geraats, 2005). Table 6.1 shows total amounts and concentrations of heavy metals in waste sludge from Dutch WWTPs in 2005 (Statistics Netherlands (CBS), 2007) as well as maximum tolerated values for heavy metals in waste sludge according to Dutch BOOM regulations as from 1995. These regulations prevent the application of waste sludge with high heavy metal concentrations in agriculture. In 2005, copper, zinc, lead, cadmium and mercury did not meet these standards.

Table 6.1 Heavy metals in waste sludge produced in the Netherlands in 2005 and BOOM regulations.

Metal	Total (kg)	Concentration (mg/ kg sludge dry matter)	BOOM regulations[1)] (mg/ kg sludge dry matter)
Mercury	329	0.95	0.75
Cadmium	453	1.30	1.25
Arsenic	2,952	8	15
Nickel	8,967	26	30
Chromium	13,937	40	75
Lead	39,312	113	100
Copper	132,360	381	75
Zinc	372,960	1073	300
Total amount of sludge dry matter produced = 347,557 tonnes			

[1)] Source: http://www.eu-milieubeleid.nl/ch05s10.html

In a system with *L. variegatus* feeding on this waste sludge the distribution of the heavy metals between sludge flocs, sludge water phase, worms and worm faeces is dependent on several processes (Figure 6.2).

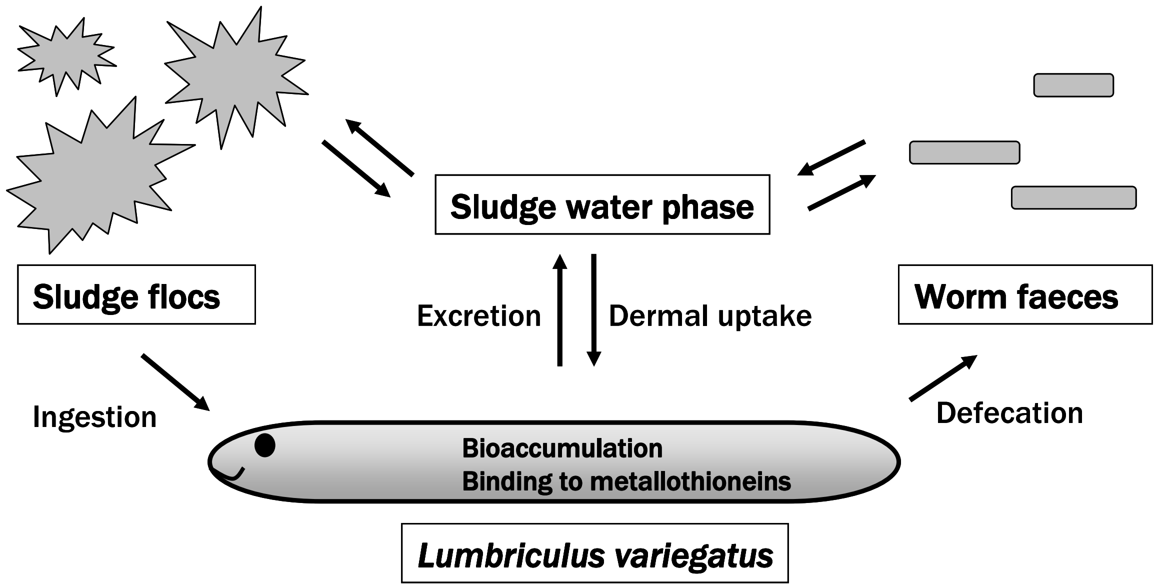


Figure 6.2 Processes influencing the heavy metal concentrations in worms and sludge (flocs and water phase) during sludge consumption by *L. variegatus*.

Several factors define the complex equilibrium between free and bound/absorbed metal forms in general: substrate type, organic matter content, pH values and AVS (acid-volatile sulphide) concentrations. In addition, free metals are easier bioaccumulated than bound forms and *L. variegatus* can bioaccumulate heavy metals to a great extent from sediments and water (Lester, 1987; Ankley *et al.*, 1994; Ankley, 1996; Liber *et al.*, 1996; Peterson *et al.*, 1996). Toxicity and bioaccumulation data for heavy metals in *L. variegatus* are thus dependent on the substrate used and can be highly variable (Williams, 2005). Several Oligochaeta species actively excrete accumulated metals. Well-known mechanisms for regulating heavy metal uptake include autotomy of tail regions, where metals are accumulated (Lucan-Bouché *et al.*, 1999; Bouché *et al.*, 2000; Vidal & Horne, 2003), and binding of metals to metallothionein-like proteins (e.g. Mosleh *et al.*, 2004), which are also possibly involved in metal excretion (Stürzenbaum *et al.*, 2001). Elevated tolerances in oligochaete worms against heavy metals might be accounted for by complexation by these proteins and, as a result, detoxification. After exposing *L. variegatus* in the lab to elevated cadmium concentrations, Bauer-Hilty *et al.* (1989) isolated a cadmium-binding metallothionein-like protein from the worms, but the exact mechanism remains obscure. Concentration of the heavy metals in the worm faeces would be more beneficial for further processing of the worm tissue, while bioaccumulation of metals in the worms would be a mechanism for removing heavy metals from waste sludge. We focused on the concentrations of Cd (cadmium), Cr (chromium), Cu (copper), Ni (nickel), Pb (lead) and Zn (zinc) in waste sludge before and after consumption by *L. variegatus*.

Not only the composition, but also the further degradability of sludge could change due to consumption. Earthworms for example ingest their faeces and this is supposed to stimulate organic matter assimilation (Curry & Schmidt, 2007). It is unknown whether *L. variegatus* ingests its own faeces with additional sludge digestion as a possible effect. It is also unknown if the faeces are further degradable by endogenous digestion.

In this chapter, experiments were done to test the influence of sludge consumption by *L. variegatus* on sludge characteristics. Sludge settleability, turbidity of the water phase, sludge dewaterability and sludge composition (in terms of proteins, carbohydrates, ashes and heavy metals) were determined before and after consumption of different municipal waste sludges. Subsequently we evaluated whether faeces of *L. variegatus* were further degradable by digestion by their own kind or by endogenous digestion. As controls, all analyses were also performed on the same sludges, incubated with as well as without aeration.

6.2 Materials and methods

6.2.1 General set-up

The experimental set-up was described in Chapter 4. Dry matter based sludge digestion rates D (d^{-1}), worm biomass growth rates G (d^{-1}) and yields Y (-) were calculated according to Chapter 5, as well as number growth rates Gn (d^{-1}). *Lumbriculus variegatus* specimens that were used in the experiments originated from a culture grown on effluent containing flushed out sludge flocs of a pilot-scale wastewater treatment system treating municipal wastewater from the village of Bennekom, the Netherlands. In Table 6.2 the set-up of the individual batch experiments is described.

Table 6.2 Set-up of the individual batch experiments with *L. variegatus*. Abbreviations used: Aer = aerated sludge, Bk = WWTP Bennekom, Fae = worm faeces, M1 = submerged membrane bioreactor, R1 = pilot-scale conventional activated sludge system 1 (sludge age ~ 20 days), R2 = pilot-scale conventional activated sludge system 2 (sludge age ~ 38 days), Sludge quantity at t_0 = sludge in worm batches at t_0 (TSS (total suspended solids) based), W/S ratio at t_0 = worm to sludge ratio at t_0 (dry matter based), Δt = experiment duration, - = non-aerated control, + = aerated control, * = sludge percentage <100 %.

Exp	Sludge	W/S ratio at t_0	Sludge quantity at t_0 (g)	Number of worms at t_0	Δt (d)	Number of batches	Number of controls	T (°C)
Settleability, dewaterability and composition of consumed sludge								
1	R2/M1*	0.3	1.2-1.6	190	7	8	4-	16-18
2	Bk	0.5	0.4-0.7	200-240	4	17	8-, 1+	16.2 (±0.7)
3	R1	0.6	0.7-0.9	200	5	9	4-, 1+	16.7 (±0.6)
4	R1*	0.5	0.8-0.9	200-219	3	9	4-, 1+	16.2 (±0.8)
5	R2*	0.4	0.7-0.8	200-247	5	9	4-, 1+	16.6 (±0.8)
6	Bk *	0.4-0.5	0.6-0.7	200-206	5	9	4-, 1+	15.1 (±0.7)
7	R2	0.4-0.7	0.5-0.6	203-210	7	4	1-	16-18
Degradability of *L. variegatus* faeces								
8	Fae	0.3-0.7	0.2-0.4	96-105	4	10	3-, 2+	16-18
9	Bk	0.7	0.1-0.2	167-171	5	7	1+	21.1 (±1.2)
	Fae/Aer	0.4	0.3-0.4	150-169	5	6	2+	20.6 (±1.6)

The experiments were usually terminated as soon as the faeces percentage in the worm batches was 100 %, but in Experiments 1, 4, 5 and 6 (marked with * in tables and figures of this chapter) faeces percentages (as visually observed) were only 70, 85, 85 and 35 %, respectively.

In addition, most analyses were performed for the total sludge mixture ('total sludge'), while some were performed separately for the sludge phase after centrifugation ('sludge pellet') and the water phase ('water phase'). This is indicated for each analysis.

6.2.2 Settleability and dewaterability of consumed sludge

The settleability and dewaterability of total sludges and the turbidity of the water phase before and after consumption by *L. variegatus* were tested. The sludges were taken from four batch experiments with three different sludges (Table 6.2, Experiments 1 & 2 for settleability, Experiments 2, 5 & 6 for dewaterability, Experiment 5 for water phase turbidity). Sludges originated from a pilot-scale aerobic activated sludge system R2, a pilot-scale aerobic submerged membrane bioreactor M1 and the WWTP of Bennekom. All systems were fed with the same municipal wastewater.

The settleability of consumed sludge was determined as SVI_{30} (sludge volume index after 30 minutes) (APHA, 1998). In addition, the sludge settling rate was evaluated by plotting the SVI in settling curves during these 30 minutes. The SVI_{30} and settling curves of the consumed sludges were compared to that of the non-treated sludges and of the controls.

Turbidity of the water phase i.e. after centrifugation for 10 min. at 3000 rpm was measured with a WTW Turb 550 turbidity meter before and after consumption.

The dewaterability of non-treated and consumed sludges was determined with the CST (capillary suction time) method (Vesilind, 1988; APHA, 1998). The CST in seconds was converted to the Sludge Filterability Constant χ (Cetin & Erdincler, 2004). According to this formula, lower CST values indicate higher filterability (χ) values and increasing sludge dewaterability.

Sludge Filterability Constant:	$\chi = \Phi(\mu * C)/CST$	($kg^2/m^4.s^2$)
Parameters:		
Φ	0.794 (dimensionless constant characteristic of the CST apparatus and paper used)	
μ	$1.002*10^{-3}$ kg/s.m (viscosity of water at the temperature of the sludge sample used in the CST test i.e. 20 °C)	
C	Sludge TSS concentration in kg/m^3	

6.2.3 Composition of consumed sludge

The composition of sludge before and after consumption by *L. variegatus* was determined in terms of TSS, VSS (volatile suspended solids), proteins and carbohydrates. The sludges were obtained from four batch experiments with three different sludges (Table 6.2, Experiments 3-6). In addition to sludges from reactor R2 and WWTP Bennekom (*Paragraph 6.2.2*), sludge was taken from reactor R1. This was a similar system as reactor R2, but reactor R2 had an average sludge age of 38 days, while reactor R1 had an average sludge age of 20 days.

TSS and VSS were determined for total sludge according to Standard Methods (APHA, 1998) as described in Chapter 4. Protein contents for total sludge and water phase (after 10 min. centrifugation at 3,500 rpm) were measured by the Biuret method (Boyer, 1993) and carbohydrate contents for total sludge and water phase (after 10 min. centrifugation at 3,500 rpm) by the phenol sulphuric acid method with glucose as a standard (Dubois *et al.*, 1956).

In order to study the effect of consumption on heavy metal concentrations in sludge a batch experiment was carried out with *L. variegatus* and R2 sludge (Experiment 7). At the start and end of the experiment, portions of worms and sludge (fractionated into sludge pellet and water phase by centrifuging for 20 min. at 3,500 rpm) were frozen in polypropylene bottles until analysed. The bottles and centrifuge tubes were rinsed with diluted HNO_3 before use. Metals (Cd, Cr, Cu, Ni, Pb and Zn) were extracted from the sludge pellets, water phases and worms with a microwave assisted aqua regia destruction step. Destruates were filled up to 100 ml with milliQ and filtered. 1 mL from each solution was dissolved in 9 mL milliQ and then analysed on an ICP-MS.

6.2.4 Degradability of consumed sludge

Initial visual inspection was performed with *L. variegatus* to prove ingestion of faeces by their own kind. The worms were fed with sludge from a WWTP treating wastewater from a paper factory. This sludge has a distinct green colour. The produced green faeces were then fed to a culture of *L. variegatus* that had been feeding on sludge from a municipal WWTP, which is characterized by a light brown colour. When these worms were taken out of the paper sludge, they produced green faeces, which proved that the faeces were ingested.

Sludge from reactor R1 was incubated with *L. variegatus* for 10 days in Petri dishes (Table 6.2, Experiment 8). After this period all the sludge was converted into faeces. The faeces were then fed to *L. variegatus* or incubated in non-aerated and aerated controls during 4 days. Part of the faeces was blendered and fed to *L. variegatus* or incubated in a non-aerated control, to check whether ingestion and degradability of these faeces were prevented by their structure.

In a second experiment (Table 6.2, Experiment 9), Bennekom sludge was incubated for 5 days with *L. variegatus* or in an aerated control. After this period, the faeces and the aerated sludge were incubated for 5 days with *L. variegatus* or in an aerated control.

6.3 Results and discussion

6.3.1 Settleability and dewaterability of consumed sludge

Table 6.3 shows the SVI_{30} values of different sludges before and after consumption by *L. variegatus*.

Table 6.3 SVI_{30} values of R1, R2 and Bennekom sludge before (Non-treated) and after two batch experiments with *L. variegatus* (Consumed). Averages and standard deviations represent duplicate measurements.

Sludge	Non-treated	Consumed
R2*	61 (±4)	55 (±3)
M1*	79 (±2)	55 (±2)
Bk	113	63

In addition, Figure 6.3 shows the settling curves of the Bennekom sludge before and after complete consumption.

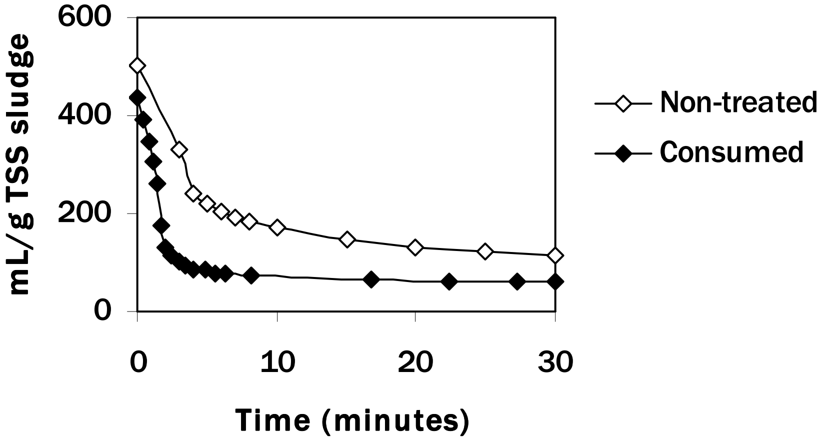


Figure 6.3 Settling curves (and SVI_{30} values) of Bennekom sludge before (Non-treated) and after a 4-day batch experiment with *L. variegatus* (Consumed).

The settleability of all three sludges clearly improved after consumption. The average SVI_{30} values for the different consumed sludges were between 55 and 63 mL/ g TSS and the initial sludge settling rate also increased. The faeces of *L. variegatus* have a uniform structure regardless of the non-treated sludge type, which explains these similar values. The sludges from the control batches (not shown) always displayed less steep settling curves and higher SVI_{30} values compared to the consumed sludges.

After the settleability tests a more turbid water phase was visually observed for the consumed sludges in comparison to the non-treated sludges. To quantify this effect, the turbidity of the water phase (after centrifugation) was determined before and after a batch experiment with R2 sludge. The turbidity in NTU was 6 before the experiment and 9 after consumption. This is in accordance with the release of dissolved and colloidal COD during consumption mentioned by Buys (2005). Turbidity of the water phase did not increase in the non-aerated control, but increased in the aerated control, possibly because of the shear forces on the sludge flocs.

Next to settleability, dewaterability of sludges before and after consumption was evaluated. Sludge Filterability Constants χ were determined for Bennekom sludges and R2 sludge before and after consumption (Figure 6.4).

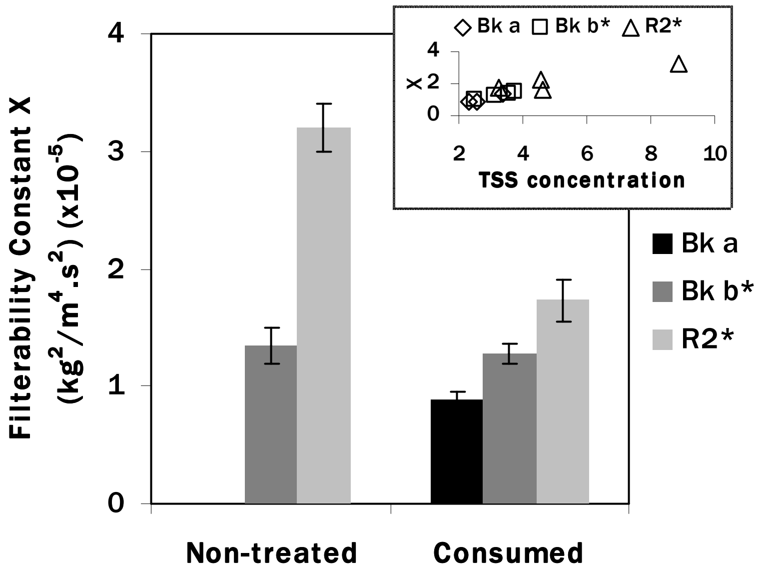


Figure 6.4 Sludge Filterability Constants χ (in 10^{-5} $kg^2/m^4.s^2$) before (Non-treated) and after (Consumed) 3 batch experiments with Bennekom (Bk, a and b are different batch experiments) and R2 sludge with *L. variegatus*. χ is displayed in the figure and the inset as X. CST measurements were repeated 4-6 times. The CST value of non-treated Bennekom sludge in the first batch experiment was not determined. **Inset**: χ-values as function of TSS concentrations in the sludges.

The results were not consistent. The χ-values of Bk b and R2 sludge after consumption were equal or lower than at the start of the experiment, which indicates dewaterability did not improve. The same was true for the controls (not shown). At the same time, even though the TSS concentrations of the sludges were corrected for in the calculation of χ-values, small differences between the TSS concentrations of the sludges did unexpectedly lead to proportional changes in the χ-values (inset Figure 6.4). This made the results unreliable even though other authors based conclusions regarding differences in dewaterability on similar experimental data (Cetin & Erdincler, 2004).

Consumption of sludge thus clearly improved initial settling rates and final SVI values, which is beneficial to further sludge processing. In addition, turbidity of the water phase increased, which means that direct release of this effluent from WWTPs should be prevented. A clear effect of consumption on sludge dewaterability could not be demonstrated.

6.3.2 Composition of consumed sludge

Proteins, carbohydrates and ash The composition of four sludges was determined before and after consumption. Table 6.4 shows an overview of digestion rates D, biomass growth rates G, yields Y (all dry matter based) and number growth rates Gn each experiment. Furthermore, the total digestion percentages plus faeces percentages for the worm batches are shown.

Table 6.4 Digestion rates D (d^{-1}), biomass growth rates G (d^{-1}), number growth rates Gn (d^{-1}) and yields Y (-) in four batch experiments with R1, R2 and Bennekom sludge. Total digestion percentages and faeces percentages of the consumed sludges are also shown.

Sludge	D	G	Gn	Y	Digestion %	Faeces %
R1a	0.07	0.03	0.00	0.5	25	100
R1b*	0.09	0.03	0.00	0.3	24	85
R2*	0.04	0.01	0.00	0.2	15	85
Bk*	0.04	0.01	0.00	0.3	17	35

The results for R1 sludge were similar—considering the relatively high overall variability found in Chapter 5— although the duration of the experiments was different; 5 and 3 days respectively. The digestion rates, biomass growth rates and yields on R2 and Bennekom sludge were somewhat lower than on R1 sludge, while the conditions in the 4 batch experiments were almost the same (Table 6.2). In addition, the digestion of R2 and Bennekom sludge was similar, while 65 % of the Bennekom sludge was not converted into faeces, compared to only 15 % of the R2 sludge.

Protein, carbohydrate and ash content of the total sludges were determined before and after each of the four batch experiments (Figure 6.5).

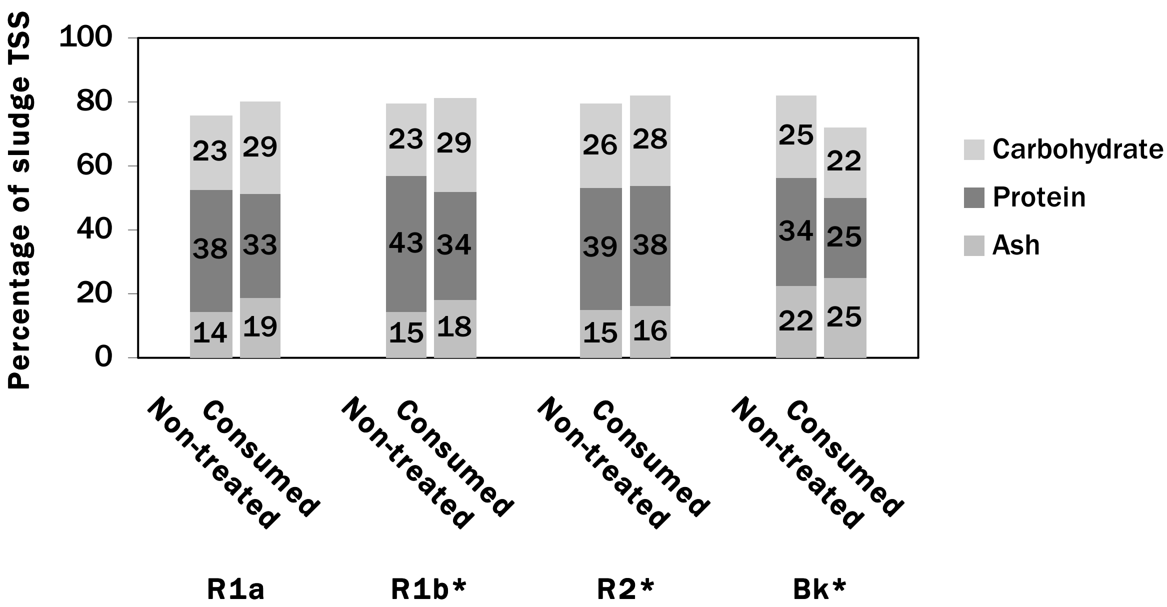


Figure 6.5 Total protein, carbohydrate and ash content of R1 (a and b are different batch experiments), R2 and Bennekom sludge (as percentage of TSS) before (Non-treated) and after four batch experiments with *L. variegatus* (Consumed). Numbers in the bars are the exact percentages of each component.

Proteins constituted the largest fraction of each sludge. The missing fraction of the sludges consisted of lipids, humic acids and other components. Figure 6.5 showed that consumption mainly affected the protein fractions of the sludges (as percentage of TSS). Consumption caused a 1-9 % decrease in the protein fractions of the sludges. This strongly suggests that *L. variegatus* specifically digested proteins from the organic fraction of sludge. However, Table 6.4 and Figure 6.5 did not show clear connections between sludge protein decrease and worm biomass increase or sludge protein content and digestion rate.

Next to the decreasing protein fractions, the ash fractions increased with 1-5 %. For R1 and Bk sludges, this was roughly proportional to the TSS digestion shown in Table 6.4, which is the result of specific digestion in the organic fraction of the sludge. Changes in carbohydrate fractions were not consistent. In R1 and R2 sludges, the fraction increased with 2-6 % but for Bk sludge decreased with 3 %. The changes in protein, ash and carbohydrate fractions were always smallest for R2 sludge. This is consistent with the overall low TSS digestion (Table 6.4) and confirms that this sludge was clearly less degradable because 85 % of the sludge had been already converted into faeces.

Typical values for protein, ash and carbohydrate content of activated sludge are respectively 32-41 %, 12-41 % (Tchobanoglous *et al.*, 2003) and 10-45 % (Forster, 1971) of the TSS.

Park *et al.* (2006) described that during aerobic digestion of sludge only proteins are completely degraded, while carbohydrate degradation is variable. Consumption of sludge by *L. variegatus* apparently displayed the same pattern as the worms fed more specifically on the protein fraction of sludge than on the carbohydrate fraction. During endogenous digestion in the control batches (results not shown) there were also decreases in the protein fractions (1-5 %) and increases in the ash fractions (up to 1 %), but to a lesser extent than in the consumed sludges. Again, in analogy with the consumed sludges, the carbohydrate fractions of the control sludges did not show any consistent trends. They decreased or increased as in the consumed sludges, but usually to a lesser extent.

The percentages shown in Figure 6.5 were determined in total sludge samples and as such did not give any information on changes of the distribution between sludge and water phase during consumption. Therefore, concentrations of carbohydrates and proteins were also determined in the water phase before and after consumption. Figure 6.6 shows the water phase contents as percentage of the total sludge contents.

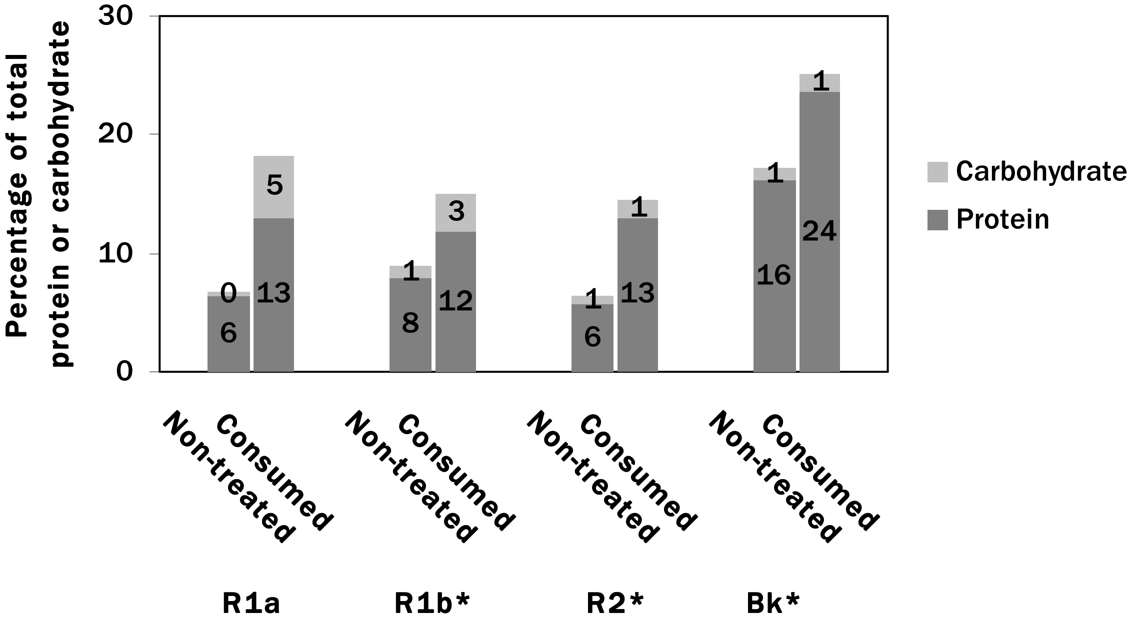


Figure 6.6 Protein and carbohydrate amounts in the water phase as percentage of total amounts in R1 (a and b are different batch experiments), R2 and Bennekom sludges before (Non-treated) and after four batch experiments with *L. variegatus* (Consumed). Numbers in the bars represent the exact percentage for each component.

Figure 6.6 indicates that sludge consumption was accompanied by protein release to the water phase, with increases of 4-8 %. This was also observed in most of the control batches with increases of 1-9 % (results not shown). Protein fractions in the water phase of Bk sludge were always higher than in the other sludges. In R1 sludges, the carbohydrate concentrations in the water phase concentrations increased after consumption with 2-5 %, but this was not the case for the other two sludges. The carbohydrate fractions in the water phases of the control batches did not change (results not shown).

L. variegatus specifically decreases the total protein fraction of waste sludge by consumption, but not the total carbohydrate fraction. Based on lower protein and higher ash contents of worm faeces, the energy content of waste sludge is thus lowered by sludge consumption. At the same time however, the protein content of worm biomass grown on waste sludge is around twice as high as that of the waste sludge (dry matter based). A release of proteins to the water phase was equally observed during digestion by worms and during endogenous digestion. However, a release of carbohydrates to the water phase was sometimes observed during digestion by worms only. This release could for example explain the increased turbidity of the water phase after sludge consumption.

Heavy metals Heavy metal concentrations were determined in R2 sludge before and after incubation with or without worms. The average total TSS digestion in 7 days was 35 (±1) % in the worm batches and in the control batches 12 (±0) % of the sludge was digested. The average total worm biomass increase was 13 (±10) %. The average digestion rate D was 0.06 (±0.02) d^{-1}, the average biomass growth rate G 0.02 (±0.02) d^{-1}, the average yield Y 0.32 (±0.20) (all dry matter based) and the average number growth rate Gn 0.00 (±0.00) d^{-1}. These rates were comparable to those found in other batch experiments (Table 6.4).

Table 6.5 shows the heavy metal concentrations (mg/ kg dry matter) in sludge (total of sludge pellet and water phase) and worms at the start and the end of the experiment. The average recovery of each heavy metal was 75 (±7) % at the end of the batch experiment (likely as a result of metal adsorption to the glass Petri dishes during the batch experiment), which means that concentrations should be interpreted with care.

Table 6.5 Average heavy metal concentrations with standard deviations between brackets in worms and total sludge (sludge pellet and water phase) at the start (N = 1) and end (N = 3) of Experiment 7. BOOM regulations are also shown for the analysed metals. All concentrations are in mg/ kg dry matter.

	Sludge start	Sludge end	Worms start	Worms end	BOOM
Cadmium	1.1	1.2 (±0.2)	0.5	0.3 (±0.1)	1.25
Nickel	15	17 (±1)	0.8	0.4 (±0.0)	30
Lead	37	43 (±1)	1.1	0.8 (±0.4)	100
Chromium	87	111 (±2)	4.7	3.7 (±1.1)	75
Copper	276	294 (±57)	35	30 (±3)	75
Zinc	758	774 (±57)	302	254 (±4)	300

Table 6.5 suggests that heavy metal concentrations in *L. variegatus* were always substantially lower than in sludge and meet the standards of the BOOM regulations in contrast to the waste sludge. In addition, the worms already contained heavy metals at the start of the batch experiment, because they originated from a ditch with effluent containing sludge flocs. During the batch experiment, the concentrations in the worms decreased slightly with on average 27 (±15) %, while those in the sludge increased somewhat with on average 13 (±9) %, but this was not proportional to respectively average percentage worm growth or sludge digestion. *L. variegatus* did not seem to accumulate metals from sludge.

Table 6.5 does not provide any information on the distribution of heavy metal amounts between sludge pellet, water phase and worms. Since metal recovery was not 100 %, the sums of the heavy metals amounts (in μg) in the three phases (worms, sludge pellet and water phase) at the start or end of the batch experiments were assumed 100 % in the calculations. Figure 6.7 shows the resulting distribution of Cd, Cr, Cu, Ni, Pb and Zn among these three phases.

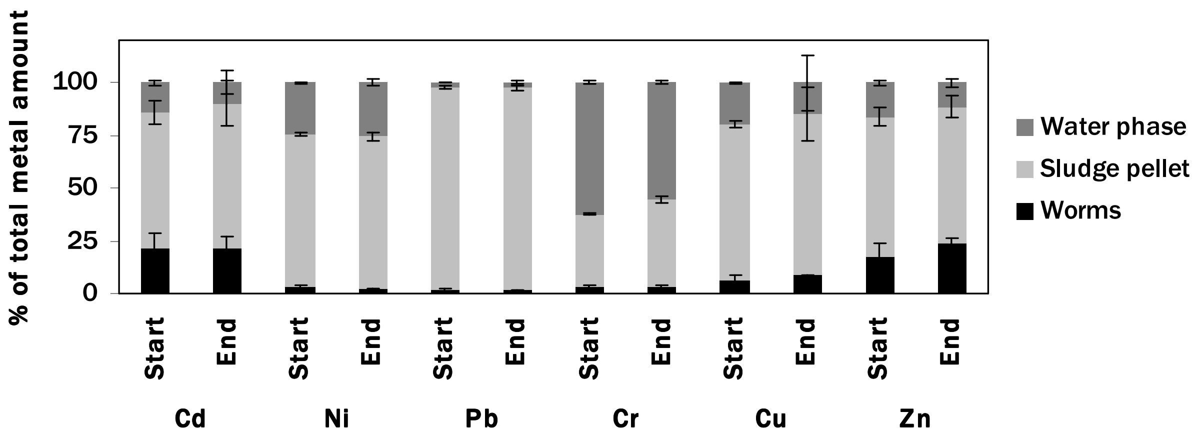


Figure 6.7 Relative heavy metal amounts in R2 sludge (sludge pellet and water phase) and worms before (start) and after (end) a 7-day batch experiment with *L. variegatus*. Standard deviations (N = 2) are given for each phase.

Figure 6.7 suggests that there was no redistribution of metals in sludge after consumption by *L. variegatus*. The largest fractions of all metals (except Cr, of which the largest fraction was found in the water phase) seemingly remained bound to sludge flocs. This can be explained by the high organic matter content of sludge because Chapman *et al.* (1999) mentioned that the bioaccumulation of heavy metals by *L. variegatus* from contaminated sediments is negatively correlated to the organic matter content of the substrate. Selck *et al.* (1999) also found that uptake of Cd from sediments by a polychaete decreased with increasing organic matter content (humic acids and exopolymers). Binding to organic matter prevents metals from entering the dissolved phase (Mahony *et al.,* 1996) and this probably decreases bioavailability. Usually less than 10 % of the metals in sludge are present as soluble and exchangeable species (Lake *et al.*, 1984). However, as Merrington *et al.* (2003) pointed out, fractionation and bioavailability of metals in sludge are complex issues and results vary widely, much in accordance with conflicting results for the influence of earthworms on the fractionation of heavy metals in soils (Liu *et al.*, 2005). In support of this, our results for Cr (Figure 6.7) show that metals mostly present in the water phase do not necessarily bioaccumulate to a high extent (Table 6.5). The bioavailability of Zn and Cd in our experiment was however clearly higher than that of the other metals, because for these two metals around 25 % of the total amount in the three phases was found in the worms. Alvarenga *et al.* (2007) found similar results with anaerobically digested sludge.

Another explanation for the overall lack of metal bioaccumulation from sludge in *L. variegatus* above initial concentrations in the worms is regulation of uptake by excretion. Excretion of metals from body tissues of *L. variegatus* is however a very slow process compared to experiment duration (Dawson *et al.*, 2003). Also, mechanisms for excretion are fairly unknown and there are only suggestions that metallothionein-like proteins may be involved (Protz *et al.*, 1993; Stürzenbaum *et al.*, 2001).

L. variegatus does not accumulate heavy metals during sludge consumption additional to initial worm concentrations. Metal concentration in the worms are usually very low and well below those in the waste sludge. Increases in worm biomass and decreases in sludge TSS during sludge consumption by worms will therefore lead to lower and higher metal concentrations in respectively worms and worm faeces. In addition, the average concentrations in the worms are below the maximum tolerated values according to BOOM regulations, in contrast to those in waste sludge. This broadens the re-use options in agriculture for worm biomass grown on waste sludge.

6.3.3 Degradability of faeces

In Experiment 8, sludge had been incubated for 10 days with *L. variegatus* until it was fully converted into faeces. These faeces (intact or blendered) were ingested by their own kind, which was confirmed by faeces production by worms that had been feeding on faeces. In contrast, Gnaiger & Staudigl (1987a) observed no reingestion of faeces. The large particle size of the faeces was not limiting for uptake by the worms. In Chapter 5, it was already shown that large sludge flocs are consumed to the same extent as small sludge flocs and the same seems to be true for the faeces. The worms were clearly able to bite pieces of the faeces, when they were too large to be ingested as a whole.

Furthermore, the faeces were hardly degradable under all applied conditions (aerated or non-aerated incubation, consumption by their own kind) since the digestion rates for *L. variegatus* feeding on their own faeces were zero and the control digestion was never higher than 2 % in 4 days. The worm biomass growth rates were all negative and number growth rates less than 0.01 d^{-1}. Even though we showed in *Paragraph 6.3.2* that worm faeces still contained a large amount of organic material, this organic material was not degradable by worms or endogenous digestion anymore, even when the faeces were blendered.

In a second experiment (Experiment 9), the age of the faeces that were fed to *L. variegatus* was 5 days instead of 10 days. Figure 6.8 shows the digestion and growth (in biomass and numbers) rates of *L. variegatus* feeding on these faeces next to those on the non-treated Bennekom sludge.

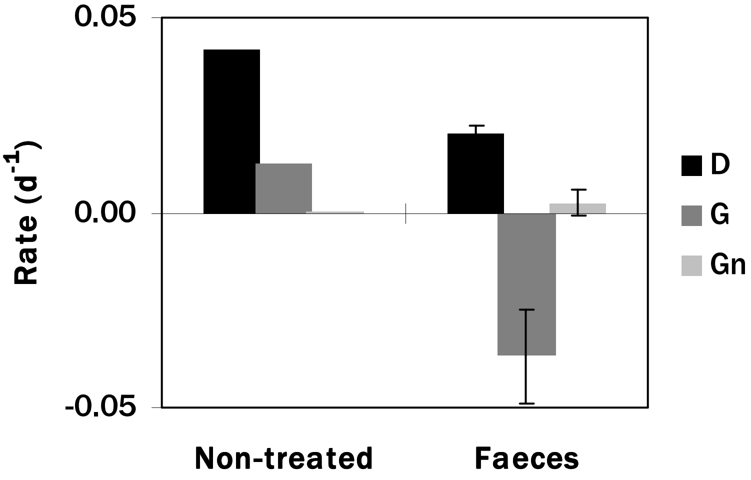


Figure 6.8 Average digestion rates D and growth rates G and Gn for *L. variegatus* feeding on non-treated Bennekom sludge (N = 1) and faeces from this sludge in Experiment 9 (5 days, N = 2).

In contrast with Experiment 8 there was still some additional digestion of the worm faeces, but the digestion rate of the faeces was low (0.02 d^{-1}) and worm biomass decreased. The low faeces digestion rates in Experiments 8 & 9 seemed to be inversely correlated to the age of the faeces and thus the extent to which endogenous sludge digestion had taken place.

This proved again that most sludge digestion took place during the first passage through the worm gut and that the faeces had little or no nutritional value anymore to the worms. Additional digestion of worm faeces seemed to be a very slow process.

.

6.4 Conclusions

In this chapter, batch experiments were described that investigated the influence of sludge consumption by *Lumbriculus variegatus* on sludge characteristics. These experiments showed that sludge consumption by *L. variegatus*:

- Enhanced the initial settling rate and the settleability of several sludges leading to SVI_{30} values of 55 to 63 mL/ g.
- Lead to an increase in water phase turbidity, due to the formation of more dissolved and/or colloidal materials. This material probably consisted of carbohydrates, which were sometimes released to the water phase. This release was not observed for proteins.
- Did not improve the dewaterability of sludges according to the CST method, but the results indicated that this method was unreliable.
- Decreased the protein fraction (and increased the ash fraction) as percentage of sludge TSS, but not the carbohydrate fraction.
- Did not cause bioaccumulation of the heavy metals cadmium, chromium, copper, lead, nickel and zinc from sludge in the worms above the concentrations already present in the worms. The concentrations in *L. variegatus* were always lower than in sludge and in addition, met the standards for BOOM regulations. The bioaccumulation of cadmium and zinc was relatively high.
- Did not seem to change the distribution of the absolute heavy metal amounts over the sludge flocs, the water phase and the worms.

Batch experiments that investigated the degradability of faeces of *L. variegatus* showed that:

- Worms ingested faeces of their own kind (conspecifics) when fed with waste sludge.
- Faeces of *L. variegatus* were hardly degradable by conspecifics or endogenous digestion and lead to decreases in worm biomass.

Acknowledgments

We thank Bas Buys (Wageningen University) and Tim Hendrickx (Wageningen University, Wetsus) for co-designing and co-performing many of the batch experiments described in this chapter.

╡Chapter 7╞

Comparison of sludge reduction between *Lumbriculus variegatus*, sessile Tubificidae and mixed cultures of both

Abstract

Sessile Tubificidae have been often used in sludge reduction research, with promising, but also variable results. To investigate if *L. variegatus* is more suitable for application in this research field several aspects of sludge reduction were compared in batch experiments with *L. variegatus* and sessile Tubificidae. In addition, it was investigated if the application of mixed cultures of both worm types is more advantageous than the application of monocultures of *L. variegatus* or 'monocultures' of Tubificidae.

Sludge digestion and worm biomass growth were usually higher and more stable in monocultures of *L. variegatus* than in monocultures of sessile Tubificidae. The number growth rates were comparable, which may have resulted from the hatching of sessile Tubificidae eggs already present. The results with concentrated sludges were similar, except for decreasing sessile Tubificidae numbers. The advantage from using mixed cultures of both worm types resulted mainly from increased biomass growth rates of *L. variegatus*. The (combined) sludge digestion rate of mixed cultures was however equal to that of the monocultures and it was not clear if mixed cultures enhanced the individual digestion rates. Both worm types were able to ingest worm faeces of conspecifics and intraspecifics, but digestion rates were low and biomass decreased. Digestion rates on intraspecific faeces were slightly higher, which suggested differences in digestion mechanism between both worm types.

When monocultures of *L. variegatus* and sessile Tubificidae were grown for more than half a year on sludge or the control substrate Tetra Min® fish food, concentrations of cadmium, chromium, copper, lead, nickel and zinc in both worm types were similar, regardless of the concentrations in these substrates. The bioaccumulation of cadmium and zinc was relatively high and concentrations in sludge and both worm types were similar. In addition, biomass concentrations of zinc in sessile Tubificidae and cadmium and zinc in *L. variegatus* grown on sludge were above the limits of the BOOM regulations for heavy metal concentrations in sludge.

In conclusion, from our batch experiments it seems that *L. variegatus* is more suitable for application in sludge reduction processes. The application of mixed cultures of *L. variegatus* and sessile Tubificidae may have beneficial effects, but not on overall sludge digestion rates.

7.1 Introduction

As was earlier discussed in the introduction of this thesis (Chapter 1), single species or mixtures of the sessile Tubificidae (mainly *Limnodrilus* spp. and *Tubifex tubifex*) are often used for sludge reduction research. These sessile Tubificidae resemble *Lumbriculus variegatus* (family Lumbriculidae) in appearance and basic feeding habits (Chapter 2), because they all are several cm long reddish aquatic worms that forage head-down in sediments with their tail protruded for oxygen uptake. *L. variegatus* can sometimes be found within commercially sold mixtures of sessile Tubificidae (Chapter 2).

Sessile Tubificidae are however essentially different from *L. variegatus* in their reproductive mode and (as most authors assume) in pollution tolerance (Chapter 2). While *L. variegatus* reproduces asexually by division and is often associated with an unpolluted habitat (Marshall & Winterbourn, 1979), sessile Tubificidae reproduce sexually through eggs and cocoons and are indicators of highly polluted areas (Finogenova & Lobasheva, 1987). Sessile Tubificidae need to reach a certain age, dependent on temperature and substrate, before they start the development of sexual organs and reproduction. The previous authors described the growth and reproduction of *T. tubifex* on activated sludge. Newly hatched specimens of *T. tubifex* started reproducing after 30 days, which was considerably faster than on sediments with a low organic content. Reproduction of *L. variegatus* was also positively correlated with organic matter content of sediments and it was found that it reproduces through division when a certain individual worm wet weight was reached (Leppänen & Kukkonen, 1998b; Lesiuk & Drewes, 1999), depending on the culture conditions (Chapter 2). With waste sludge as their substrate, there is also a trend that larger specimens divide more often than smaller specimens do. The average biomass and number growth rates of *L. variegatus* in several batch experiments with waste sludge were 0.04 (±0.03) and 0.01 (±0.02) d^{-1} (Chapter 5).

Some researchers found high sludge digestion and worm growth rates with sessile Tubificidae, even though results were also sometimes variable or unstable (Chapter 1). To find out whether *L. variegatus* is more suitable for sludge reduction, a direct comparison between sessile Tubificidae and *L. variegatus* was made in several short-term batch experiments, similar to those we used to demonstrate the sludge reduction potential of *L. variegatus*. In addition, combining different worm types in mixed cultures may enhance sludge digestion rates and/or worm growth. Several authors described that some species of sessile Tubificidae preferably feed on faeces of other aquatic worms, because each separate species contains different species-specific bacteria in their intestines and faeces (Brinkhurst *et al.*, 1972; Brinkhurst, 1974; Brinkhurst & Austin, 1979; Milbrink, 1987b; Mermillod-Blondin *et al.*, 2003). These authors reported increased growth and feeding rates and decreased respiration rates in mixed worm cultures as a result of this. Components that cannot be digested or bacteria that are excreted by one species may thus be degradable by another species. Therefore, we investigated whether sludge digestion and growth rates were enhanced in batch experiments with mixed cultures of sessile Tubificidae and *L. variegatus*. *L. variegatus* is able to ingest faeces of its own kind (conspecifics) but hardly any additional digestion takes place, as was shown in Chapter 6. In batch experiments, we investigated whether the same is true for sessile Tubificidae. In addition, cross-feeding batch experiments were done, in which cultures of sessile Tubificidae or *L. variegatus* were fed with faeces of the other species (intraspecifics).

When applying (mixtures of) worm species for sludge digestion, it is important to know to which extent the worms accumulate metals from the sludge. Sessile Tubificidae are known to bioaccumulate heavy metals (e.g. Patrick & Loutit, 1976), dependent on environmental conditions like organic matter concentrations (Bervoets *et al.*, 1997) and temperature (Back, 1990). They possess detoxification mechanisms for metals like internal compartmentalization (Ciutat *et al.*, 2005; Steen Redeker *et al.*, 2007), loss of their tail section (induced autotomy) (Lucan-Bouché *et al.*, 1999) and binding to metallothionein-proteins (Roesijadi, 1992; Mosleh *et al.*, 2006). These proteins possibly are also involved in excretion of the metals (Stürzenbaum *et al.*, 2001) and a similar protein was detected in *L. variegatus* (Bauer-Hilty *et al.*, 1989). The uptake of heavy metals from sludge was investigated in short batch experiments described in Chapter 6 for *L. variegatus* and this species contained lower concentrations of Cd (cadmium), Cu (copper), Cr (chromium), Ni (nickel), Pb (lead) and Zn (zinc) than the waste sludge (dry matter based). This was presumably due to strong binding of metals to the sludge flocs or to unknown detoxification mechanisms. Therefore, long-term bioaccumulation of the same metals in sessile Tubificidae was compared to that in *L. variegatus*. The bioaccumulation of heavy metals from sludge was compared to that from a control substrate —fish food— in both worm types.

7.2 Materials and methods

7.2.1 Organisms

Sessile Tubificidae originated from pet shops, where they are sold as fish food under the name 'Tubifex'. These mixtures contain mostly *Limnodrilus udekemianus*, *Limnodrilus hoffmeisteri* and *Tubifex tubifex*. This was regularly confirmed by identification of some adult specimens (recognizable by their clitellum i.e. reproductive organs) after mounting in polyvinyl lactophenol according to Brinkhurst (1971) and Timm (1999). The exact composition of the mixtures in each experiment was unknown, because identification with the naked eye (and without killing the worms) is impossible. *Lumbriculus variegatus* specimens that were used in the experiments originated from a culture grown on effluent containing flushed out sludge flocs of a pilot-scale municipal wastewater treatment system.

7.2.2 Sludge digestion by monocultures and mixed cultures of sessile Tubificidae and *L. variegatus*

The experimental set-up was described in Chapter 4. Dry matter based sludge digestion rates D (d^{-1}), worm biomass growth rates G (d^{-1}) and yields Y (-) were calculated for both worm types according to Chapter 5, as well as number growth rates Gn (d^{-1}). However, number growth rates for sessile Tubificidae were sometimes not calculated because of practical reasons. In Table 7.1, the set-up of the batch experiments is described.

Table 7.1 Set-up of the batch experiments with *L. variegatus* and sessile Tubificidae. Abbreviations used: Bk = WWTP Bennekom, Fae= faeces of sessile Tubificidae or *L. variegatus,* Lv = monoculture of *L. variegatus*, Mi = mixed culture of sessile Tubificidae and *L. variegatus* (1:1 dry weight based), na = not analysed, R1 = pilot-scale conventional activated sludge system 1 (sludge age ~ 20 days), R2 = pilot-scale conventional activated sludge system 2 (sludge age ~ 38 days), Sludge quantity at t_0 = sludge in worm batches at t_0 (TSS based), Tu = 'monoculture' of sessile Tubificidae, W/S ratio at t_0 = worm to sludge ratio at t_0 (dry matter based), Δt = experiment duration, - = non-aerated control, + = aerated control.

Exp	Sludge	W/S ratio at t_0	Sludge quantity at t_0 (g)	Number of worms at t_0	Δt (d)	Number of batches	Number of controls	T (°C)
1	R1	Tu 0.3-0.5 Lv 0.2-0.3	Tu 0.3 Lv 0.5-1.0	Tu 103-120 Lv 201-232	4	11	3-, 2+	18.7 (±0.6)
2	R2	Tu 0.2-0.3 Lv 0.2	0.9	Tu na Lv 100	6	6	2-	18-22
3	Bk	Tu 0.6-0.7 Lv 0.4-0.5	0.2	Tu 62-71 Lv 173-201	3	8	2-, 2+	17.5 (±0.8)
4	R1	Tu 0.3 Lv 0.5-0.6 Mi 0.4	Tu 0.8-1.0 Lv 0.8-0.9 Mi 0.9	Tu 300 Lv 150 Mi 375-425	6	10	2, 2+	16-18
5	Fae	Tu 0.4-0.6 Lv 0.3-0.7	Tu 0.1-0.2 Lv 0.2-0.4	Tu 150-160 Lv 96-105	4	10	3, 2+	16-18

Sludge digestion and worm growth rates were compared between sessile Tubificidae and *L. variegatus* in Experiments 1-3 with sludges from two pilot-scale systems (R1 and R2) and one full-scale WWTP (Bk) (Chapter 5). In Experiment 4, the sludge digestion and worm growth rates of mixed cultures of *L. variegatus* and sessile Tubificidae (1:1 dry weight based) were compared to those of *L. variegatus* monocultures and Tubificidae 'monocultures'. Process conditions, including W/S ratios, were kept similar for all worm batches. The average individual wet weight (5 mg) of sessile Tubificidae was lower than that of *L. variegatus* (14 mg) and therefore worm numbers in the batches with sessile Tubificidae were higher. The dry (to wet) weight percentage of sessile Tubificidae (17 %) is higher than that of *L. variegatus* (13 %) (Chapter 2).

Because sessile Tubificidae showed population decreases in the sludge concentrations that were applied in the above experiments (2-4 g TSS/ kg sludge), growth in biomass and numbers of *L. variegatus* or sessile Tubificidae in highly concentrated sludges (10-30 g TSS/ kg sludge) was evaluated in addition (experiment not shown in Table 7.1). These sludges were concentrated by centrifugation.

7.2.3 Faeces digestion by conspecific and intraspecific worms

In Experiment 5 (Table 7.1), the digestion of worm faeces by conspecifics and intraspecifis was investigated. Faeces were obtained by incubating sludge from reactor R1 for 10 days with *L. variegatus* or a mixture of sessile Tubificidae in Petri dishes. After this period, the faeces percentage was 100 %. The faeces of *L. variegatus* were then fed to their conspecifics and to intraspecifics (sessile Tubificidae) during 4 days. The same was done with sessile Tubificidae faeces.

7.2.4 Heavy metal bioaccumulation in sessile Tubificidae or *L. variegatus*

Worm tissue concentrations of Cd, Cr, Cu, Ni, Pb and Zn of long-term (> 6 months) cultures of sessile Tubificidae fed with activated sludge from pilot-scale reactors R1 and R2 were analysed as described in Chapter 6: a microwave assisted aqua regia destruction step followed by analysis on an ICP-MS. In addition, total sludge samples were analysed (i.e. the sludge was not fractionated prior to analysis as described in Chapter 6). For comparison, heavy metal concentrations were also analysed in long-term *L. variegatus* cultures on the same sludges and in long-term cultures of both worm types on Tetra Min® fish food. Worms grown on the former substrate were regarded as control group for worms grown on sludge. The heavy metal concentrations in the food sources were also analysed.

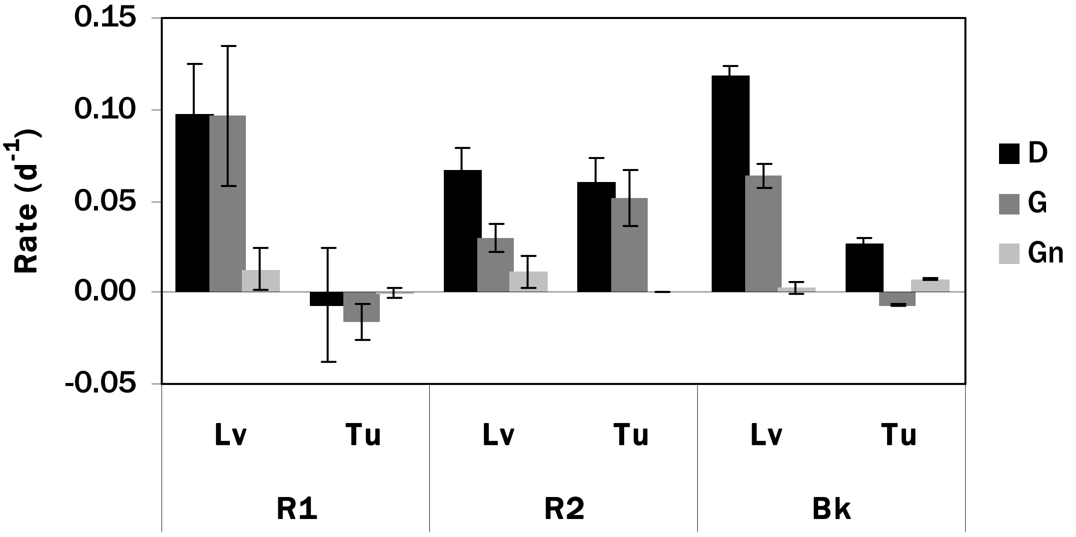
7.3 Results and discussion

7.3.1 Sludge digestion by monocultures and mixed cultures of sessile Tubificidae and *L. variegatus*

The faeces of *L. variegatus* could be easily discerned from the waste sludge by their cylindrical compact shape, even with the naked eye (Chapters 4 & 5), whereas those of sessile Tubificidae had more variable shapes. Brinkhurst & Austin (1979) described long and short cylindrical faeces for *Limnodrilus* sp. and *Tubifex* sp. respectively when feeding on sediment. In addition to these shapes, we found floc-like or round sessile Tubificidae faeces that were often indistinguishable from the waste sludge in our experiments.

Digestion and growth rates (in biomass and usually numbers, except in Experiment 2) for monocultures of *L. variegatus* or sessile Tubificidae feeding on R1, R2 and Bennekom sludge were calculated (Figure 7.1a). Subsequently, digestion and growth rates (in biomass and numbers) for monocultures of *L. variegatus* or sessile Tubificidae were compared to those of mixed cultures of both worm types feeding on R1 sludge (Figure 7.1b).

a)



b)

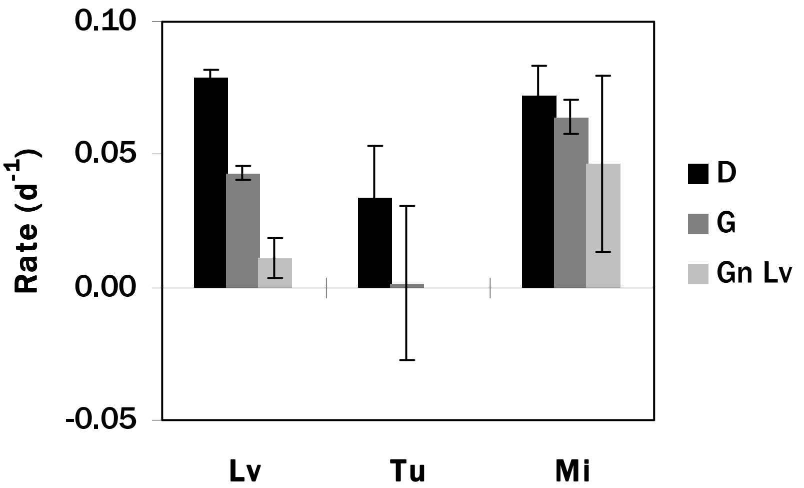


Figure 7.1 Comparison of sludge digestion and worm growth between monocultures and mixed cultures of *L. variegatus* and sessile Tubificidae. **a)** Average digestion rates D and growth rates G and Gn for monocultures of *L. variegatus* (Lv) or sessile Tubificidae (Tu) feeding on R1 (N = 3), R2 (N = 2) and Bennekom (Bk, N = 2) sludges in Experiments 1-3 (all in d^{-1}). The number growth rates Gn were not calculated for sessile Tubificidae in Experiment 2. **b)** Average digestion rates D and growth rates G and Gn for *L. variegatus* (Lv), sessile Tubificidae (Tu) or a mixture of both (Mi) feeding on R1 (N = 2) sludge in Experiment 4 (all in d^{-1}). The number growth rate Gn was only calculated for *L. variegatus* (Gn Lv), but not for sessile Tubificidae. The biomass growth rate G is displayed for the *total* biomass in each batch, but the average biomass growth rates G in the mixed cultures for the *separate* worm types were 0.04 (±0.01) d^{-1} for the sessile Tubificidae and 0.09 (±0.00) d^{-1} for *L. variegatus* (not shown).

Figure 7.1 (a & b) shows that the digestion and biomass growth rates for sessile Tubificidae were significantly lower than for *L. variegatus* under similar conditions in three of the four experiments (with R1 and Bk sludges). The biomass of sessile Tubificidae often even decreased. However, the number growth rates of sessile Tubificidae (determined in Experiments 1 & 3) were comparable to those of *L. variegatus*. Because of the different reproductive strategies and growth patterns of sessile Tubificidae and *L. variegatus*, care should be taken when comparing their growth rates, especially since the formation of cocoons by sessile Tubificidae during the experiments was not taken into consideration. The digestion and growth rates for *L. variegatus* displayed the usual variability, as was described in Chapter 5 of this thesis. The average yield in the batches where sessile Tubificidae biomass did increase was 0.7 (±0.2), while in the parallel batches with *L. variegatus* the average yield was 0.5 (±0.1). This difference was however not significant.

In the mixed cultures (Figure 7.1b), the average combined digestion rate was as high as in the *L. variegatus* cultures, but lower than in the sessile Tubificidae cultures. The influence on individual digestion rates was not clear, because we could not conclude which part of the breakdown could be attributed to the separate worm types, as it was impossible to discern sessile Tubificidae faeces from the waste sludge. In addition, it could not be ruled out that the worms had ingested each other's faeces. Figure 7.1b shows that the average total biomass growth rate of the mixed cultures was also relatively high compared to that of the monocultures. This resulted from average separate biomass growth rates (not shown) of 0.04 (±0.01) d^{-1} for the sessile Tubificidae and 0.09 (±0.00) d^{-1} for *L. variegatus*, which were both high in comparison to monocultures. In addition, the separate number growth rate of *L. variegatus* ('Gn Lv') in the mixed cultures was also high (0.05 (±0.03) d^{-1}) compared to that in the monocultures, while that of the sessile Tubificidae was not determined.

It seems that especially the sessile Tubificidae profit from mixed cultures. This is in accordance with the enhanced feeding and biomass growth rates in mixed cultures found by several authors (e.g. Brinkhurst *et al.*, 1972; Milbrink, 1987b) as a result of selective feeding on bacteria associated with the faeces of other worm species (intraspecifics). In addition, Chapman *et al.* (1982b) mentioned that mixed cultures of sessile Tubificidae are more tolerant to toxic compounds than monocultures, which may, next to increased biomass growth rates, be a further advantage for the application of mixed cultures of *L. variegatus* and sessile Tubificidae.

The low sludge digestion and growth rates in the monocultures of sessile Tubificidae in comparison with those of *L. variegatus* may have been caused by their reproductive behaviour in combination with experiment duration. Even though *L. variegatus* does not feed for up to 7 days after reproduction (Leppänen & Kukkonen, 1998b), their numbers and biomass increased steadily in our batches, because the populations consisted of random mixtures of reproducing (dividing) and non-reproducing (non-dividing) specimens. At the same time, the biomass of sessile Tubificidae decreased in more than half of the batches for unknown reasons, even though there was sludge digestion in addition to endogenous sludge digestion in most cases. In three of the five batches in which the sessile Tubificidae were counted, there was a small increase in numbers and in the remaining two batches a small decrease. The duration of the batch experiments was however too short for a full developmental cycle of the sessile Tubificidae, because that of *T. tubifex* takes for example 30 days and embryonic development at least 7 days (Finogenova & Lobasheva, 1987; Marchese & Brinkhurst, 1996). Therefore, the only explanation for the increasing numbers would be hatching of eggs that were already present in the batches. When assuming the growth rate for juvenile *T. tubifex* on waste sludge calculated by Finogenova & Lobasheva (1987), the populations in our batch experiments could have increased 250 % in biomass in 6 days, a substantial higher biomass increase than with *L. variegatus*. However, they also mentioned decreasing biomass growth rates and death in older cultures, which resembles our results better. The negative results with sessile Tubificidae could thus have been caused by the short duration of the batch experiments, in combination with their reproductive stage. Therefore, worm growth of sessile Tubificidae would probably be more stable in long-term experiments, in which all reproductive stages are represented and full reproductive cycles can take place and this was confirmed by Buys (2005).

Another factor, which may have affected sessile Tubificidae growth rates and also the sludge digestion rates (which were highly unstable and usually low) in combination with the short experiment duration, could be the nature of the substrate. It is known that sessile Tubificidae species like *T. tubifex* and *L. hoffmeisteri* are linked to highly polluted habitats and display reduced growth and reproduction rates in 'clean' waters (Finogenova & Lobasheva, 1987). Brinkhurst & Austin (1979) and McMurtry *et al.* (1983) suggested that sessile Tubificidae specifically ingest organically rich fractions of sediments. It is possible that sessile Tubificidae needed highly concentrated sludges and could not thrive in the concentrations that were applied in the described batch experiments (2-4 g TSS/ kg sludge). Therefore, growth of *L. variegatus* or sessile Tubificidae was compared at increased sludge concentrations of 10 to 30 g TSS/ kg sludge during 25 days. Unexpectedly, growth rates for *L. variegatus* were positive in biomass and numbers (both ranging from 0.02 to 0.05 d^{-1}) for all concentrations, while numbers and biomass of sessile Tubificidae still remained constant or decreased. In analogy, Densem (1982) found that *T. tubifex* populations decreased sharply in numbers and biomass when grown on concentrated activated sludge (around 70 g TSS/ kg sludge) for 40 days. He explains this by anaerobiosis of the substrate, leading to the formation of unknown toxic compounds and higher expenditures by the worms for maintaining aerobic conditions. Again in analogy with our batch experiments, the duration of Densems' experiments (< 40 days) may not have been long enough for a significant increase in sessile Tubificidae numbers, but this cannot explain the simultaneous decrease in biomass. It remains unexplained as well why *L. variegatus* can cope with these seemingly adverse conditions and why some authors reported growth of sessile Tubificidae on activated sludge (also on low concentrations as Buys (2005) did) while others did not. Results for sludge digestion rates of sessile Tubificidae are also contradictory: Liang *et al.* (2006b) found 0.54 d^{-1} for *T. tubifex*, while we found only

0.02 (±0.03) d^{-1} on average for sessile Tubificidae (Figure 7.1). In conclusion, the results with *L variegatus* are much more stable than with sessile Tubificidae. The exact reasons for the variable results with sessile Tubificidae remain unknown. Nevertheless, the application of mixed cultures of both worm types could have some advantages, but not for the sludge digestion rates.

7.3.2 Faeces digestion by conspecific and intraspecific worms

Conspecific faeces ingestion of sessile Tubificidae (in analogy with *L. variegatus*) was confirmed by faeces production by worms that had been feeding on faeces. Intraspecific faeces ingestion (or cross-feeding) was confirmed by the conversion of *L. variegatus* faeces into sessile Tubificidae faeces and vice versa. The increased particle size of the faeces (in comparison to that of the waste sludge) was therefore not limiting for uptake by the worms. This was also observed in experiments with different sludge floc size fractions, described in Chapter 5, and it is likely that the worms bite pieces of the sludge flocs, when these are too large to be ingested as a whole.

Following ingestion, the faeces of sessile Tubificidae (in analogy with those of *L. variegatus*) were hardly degradable and digestion rates were very low, when compared to the average rate on municipal sludge of 0.09 (±0.04) d^{-1} (Chapter 5). These digestion rates were zero when feeding on their own faeces, and very low in the cross-feeding experiments: 0.02 d^{-1} for *L. variegatus* fed with sessile Tubificidae faeces and 0.01 d^{-1} for sessile Tubificidae fed with *L. variegatus* faeces. The biomass growth rates were all negative and number growth rates less than 0.01 d^{-1}. Apparently, the worm faeces had no nutritional value to the worms since there was no biomass increase, as was also found in Chapter 6. The slightly higher digestion rates in these cross-feeding experiments (compared to experiments where the worms were fed with faeces of conspecifics) may indicate differences in digestion mechanisms between *L. variegatus* and sessile Tubificidae and could result from a different faeces composition (e.g. Milbrink, 1987b). They are possibly capable of breaking down a small fraction of the sludge that the other species is incapable of and this could be a further indication that mixed cultures may be more advantageous.

7.3.3 Heavy metal bioaccumulation in sessile Tubificidae or *L. variegatus*

Average concentrations (in mg/ kg dry matter) of Cu, Zn, Cd, Ni, Pb and Cr in cultures of sessile Tubificidae or *L. variegatus* and their food sources (sludges from reactors R1 and R2 or Tetra Min® fish food) are shown in Figure 7.2.

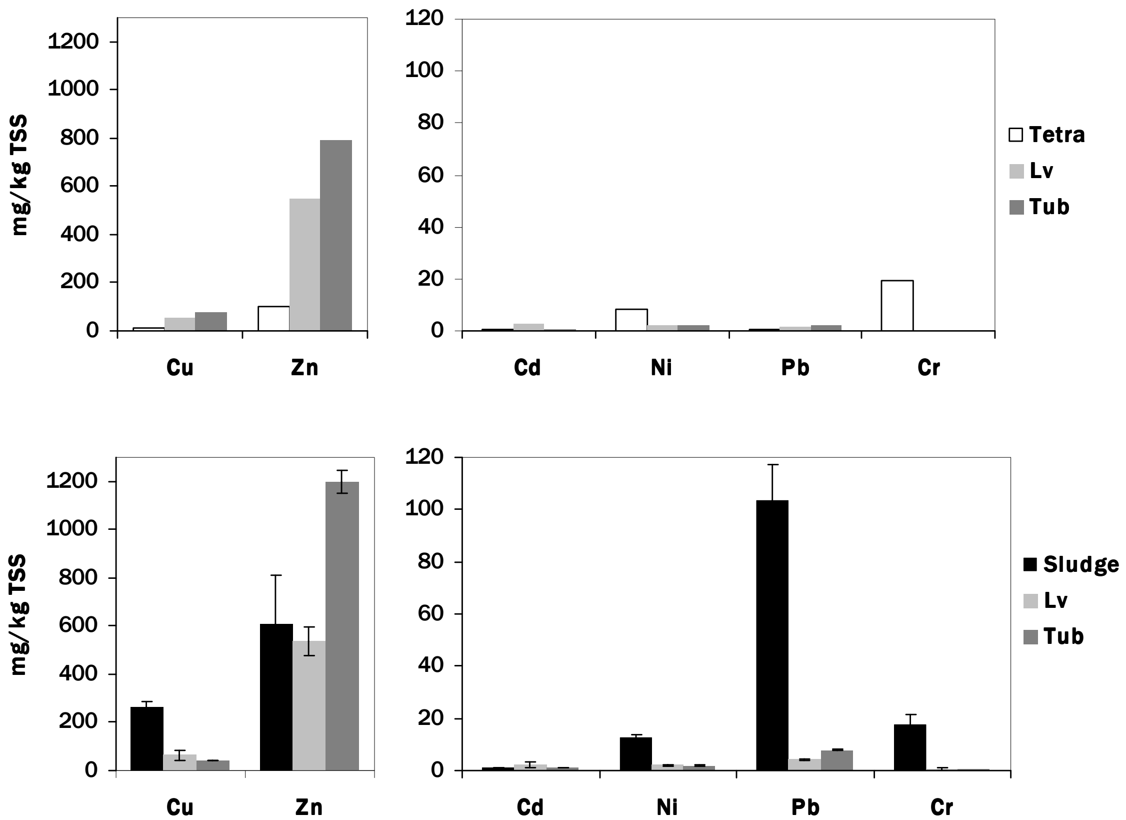


Figure 7.2 Average heavy metal concentrations (in mg/ kg dry matter) with standard deviations in cultures of *L. variegatus* (Lv) or sessile Tubificidae (Tub) on Tetra Min® fish food (**upper** graphs) or sludge (**lower** graphs). For comparing the results between the two food sources, the scales of the Y-axes are kept similar in the upper and lower graphs.

As expected, the heavy metal concentrations in sludge— except for Ni and Cr, which were almost equal in both food sources— were higher than in fish food. The heavy metal concentrations in both worm types were very similar, except for higher Zn concentrations in the sessile Tubificidae. Table 7.2 shows the dry matter based average biota 'sediment' accumulation factors (BSAFs) for the six heavy metals in *L. variegatus* or sessile Tubificidae feeding on sludge or fish food were calculated according to Williams (2005) as [*tissue concentration of metal/ food source concentration of metal*].

Table 7.2 Average BSAFs for six heavy metals in *L. variegatus* or sessile Tubificidae feeding on sludge or Tetra Min® fish food. Bold numbers indicate BSAFs higher than 1.

	Sludge		Tetra Min®	
	L. variegatus	Sessile Tubificidae	*L. variegatus*	Sessile Tubificidae
Cu	0.2	0.2	**4.9**	**7.1**
Zn	0.9	**2.0**	**5.5**	**7.9**
Cd	**2.0**	0.8	**8.8**	**1.2**
Ni	0.2	0.1	0.2	0.3
Pb	0.0	0.1	**3.6**	**5.8**
Cr	0.0	0.0	0.0	0.0

Table 7.2 shows that the BSAFs for both worm types were similar for each different substrate. Heavy metal concentrations in both worm types were always lower than in waste sludge, except for Cd in *L. variegatus* and Zn in sessile Tubificidae. Both worm types accumulated metals up to concentrations a factor 9 higher than in fish food, except for Ni and Cr, which were hardly accumulated. In Chapter 6, the heavy metal concentrations in L. *variegatus* were always lower than in sludge, but Cd and Zn concentrations were relatively high to those in sludge (Table 6.5). This may have been due to higher bioavailability of Cd and Zn, but the differences in bioavailability and bioaccumulation for the different metals could not be related to their partitioning between sludge flocs and water phase (Chapter 6).

Most importantly, the absolute metal concentrations in the worms were rather constant in spite of the different metal concentrations in the substrates. Gunn *et al.* (1989) also found that concentrations of Zn in sessile Tubificidae were independent on the substrate concentration and they proposed that this was due to uptake regulation. The worms possibly excreted heavy metals above a certain concentration by an unknown mechanism, for example related to metallothionein-like proteins (Bauer-Hilty *et al.*, 1989; Stürzenbaum *et al.*, 2001), but these mechanisms are virtually unknown and seem slow (Chapter 6). Densem (1982) showed that sessile Tubificidae grown on sludge of similar composition to that in our experiments contained much higher Cu concentrations (285 mg/ kg dry matter) than we found, while they contained similar Cd and Zn concentrations to the worms in our experiment. Therefore, it is likely that metal concentrations in worms are also determined by other chemical, biological and physical characteristics of the substrate.

Zn and Cd seem to be the metals of most concern for both sessile Tubificidae and *L. variegatus*, because their bioaccumulation factors were highest. In addition, the average concentrations of Zn in sessile Tubificidae and Zn and Cd in *L. variegatus* in this experiment (Figure 7.2) were above the BOOM regulations in contrast to the concentrations in *L. variegatus* found in an earlier experiment (Chapter 6). This would prevent agricultural re-use of the worm biomass in the Netherlands. Because the sludge metal concentrations in the current experiment were equal to or lower than those in the earlier experiment, the higher worm metal concentrations are possibly caused by unknown differences in sludge characteristics or by the difference in experiment set-up. The worms described in this chapter were exposed for a long time to sludge only, while the worms described in Chapter 6 were first exposed for a long time to effluent containing sludge flocs, followed by a short-term exposure to sludge only in a batch experiment.

7.4 Conclusions

In this chapter, batch experiments were described that compared the sludge reduction capacity of *Lumbriculus variegatus* and sessile Tubificidae. Short-term batch experiments with *L. variegatus*, sessile Tubificidae or mixed cultures of both showed that:

- Digestion rates and growth rates in biomass of *L. variegatus* were more stable and usually higher than those of sessile Tubificidae in low sludge concentrations. Growth rates in numbers were comparable for both worm types in these experiments.
- Biomass and numbers of sessile Tubificidae often decreased when the sludge was highly concentrated, in contrast to those of *L. variegatus*.
- Mixed cultures enhanced biomass and number growth rates of *L. variegatus* and biomass growth rates of sessile Tubificidae. The (combined) sludge digestion rate of mixed cultures was equal to that of monocultures of *L. variegatus*, but higher than that of 'monocultures' of sessile Tubificidae. Therefore, the only clear advantage for *L. variegatus*, resulting from the use of mixed cultures with sessile Tubificidae, was the increase in biomass growth rates of *L. variegatus*.

Short-term batch experiments that investigated the ingestion and digestion of faeces of conspecifics and intraspecifics showed that:

- Worms ingest faeces of conspecifics and intraspecifics when fed with activated sludge but digestion rates and number growth rates were low and biomass growth rates were always negative.
- The digestion rates were slightly higher when faeces were fed to intraspecifics, which suggests differences in digestion mechanisms.

Experiments that investigated the long-term heavy metal bioaccumulation in sessile Tubificidae from sludge in comparison to that in *L. variegatus* showed that:

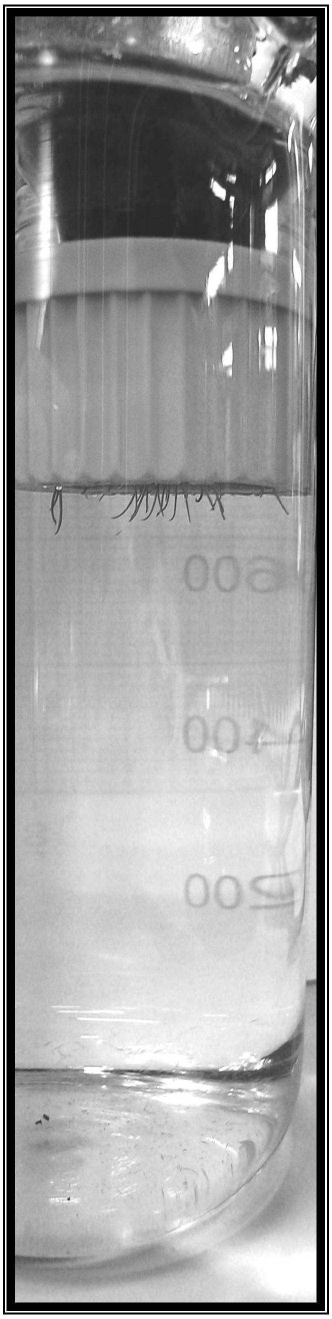
- Heavy metal concentrations in both worm types grown either on sludge or the control substrate Tetra Min® fish food were similar, regardless of the concentrations in these substrates.
- Bioaccumulation factors of cadmium and zinc were relatively high and concentrations in sludge and both worm types were similar. In addition, the biomass concentrations of zinc in sessile Tubificidae and cadmium and zinc in *L. variegatus* were above the limits of the BOOM regulations for heavy metal concentrations in sludge.

Acknowledgments

We thank Bas Buys (Wageningen University) and Tim Hendrickx (Wageningen University, Wetsus) for co-designing and co-performing many of the batch experiments described in this chapter.

╡Chapter 8╞

A new reactor concept for sludge reduction using *Lumbriculus variegatus*



Based on paper in Water Research (Elissen, Hendrickx, Temmink & Buisman, 2006)

Abstract

Biological wastewater treatment results in the production of waste sludge. The final treatment option in the Netherlands for this waste sludge is usually incineration. A biological approach to reduce the amount of waste sludge is through consumption by aquatic worms. In this chapter, we tested the applicability of a new reactor concept for sludge reduction by the aquatic worm *Lumbriculus variegatus*. In this reactor concept, the worms are immobilized in a carrier material. In a sequencing batch experiment, the sludge reduction in the worm reactor was compared to sludge reduction in a control reactor (i.e. without worms). Consumption by the worms results in a distinct sludge reduction, which is almost three times higher than in the control experiment. Due to the configuration of the worm reactor, waste sludge, worm faeces and worms are separated, which is beneficial to further processing. The obtained results show that the proposed reactor concept has a high potential for use in full-scale sludge processing. However, comparisons with the results of other experiments shows that sludge reduction percentages and other parameters of the process can be variable, dependent on the process conditions.

8.1 Introduction

Both municipal and non-municipal wastewaters are often treated by the (aerobic) activated sludge process. This results in the production of large amounts of waste sludge, consisting of biomass and (in)organic material. This waste sludge needs to be processed and disposed of. Regulations for its disposal are becoming more stringent, as it often contains contaminants such as heavy metals and organic micropollutants. Usually incineration is the final option for sludge treatment in the Netherlands. Since sludge consists mainly of water, with only a small percentage of solids, incineration is preceded by dewatering and thickening. In particular, at small WWTPs (wastewater treatment plants), transport of the thickened sludge to central sludge processing installations is required. This increases both the environmental burden and the total sludge processing costs. The latter may be as high as 50–60 % of the total operational costs of WWTPs (Wei *et al.*, 2003a). A reduction in the amount of waste sludge is therefore attractive from both an environmental and an economical point of view. This can be accomplished by mechanical, chemical, physical and biological methods (Ødegaard, 2004). The main disadvantage of most of these techniques is a high energy input and/or the use of chemicals. A biological approach is consumption of waste sludge by higher organisms, such as protozoans and metazoans. The idea is to extend the food chain, which is accompanied by a decrease in the total amount of biomass. Several researchers have proposed to apply higher organisms that naturally occur in wastewater treatment processes (Wei *et al.*, 2003a). In particular, aquatic 'bristle worms' (Oligochaeta and Aphanoneura) have received a lot of attention, such as the free-swimming species *Aeolosoma* spp., *Nais* spp. and the sessile Tubificidae. The free-swimming worms can appear in high densities—during peak periods (Chapter 3)—in the aeration tanks or sludge basins of WWTPs. The peak periods are reported to be accompanied by lower sludge production rates. However, Wei *et al.* (2003b) mentioned that a practical application is still uncontrollable as there is no clear relationship between process conditions (e.g. retention times, temperature, sludge loading rates and shear forces) and worm growth. They state that one of the challenges is to maintain high densities of worms for a long time, in particular in full-scale applications. However, conditions beneficial to growth of the worms or other sludge consumers may not be optimal for bacterial processes and overall treatment efficiency. To overcome this problem, Lee & Welander (1996) applied a two-stage system in which the first reactor favoured bacterial growth, whereas the second step was optimized for sludge consumer growth. Although Protozoa were used for sludge consumption, the same principle could also be applied with aquatic worms. The introduction of the consumption step resulted in lower apparent sludge yields compared to systems without sludge consumers.

Several aquatic worm species were investigated for their sludge reduction ability (Buys, 2005; own data). We concluded that the sessile species *Lumbriculus variegatus* (Oligochaeta; Lumbriculidae) showed high potential for waste sludge reduction in a separate reactor. *L. variegatus* rarely occurs in wastewater treatment processes, but is found widely throughout Europe and North America in natural water bodies. Specimens can be up to 10 cm long and 1.5 mm thick. In its natural habitat *L. variegatus* uses its head to forage in sediments and debris, while its tail end— specialized for gas exchange—typically projects upwards (Drewes & Fourtner, 1989). As reproduction takes place through fragmentation (autotomy), *L. variegatus* has a clear advantage over sexually reproducing aquatic worms such as sessile Tubificidae, which need a 'breeding' stage. It has been shown in batch experiments that *L. variegatus* can double the reduction rate of activated sludge (Chapter 4). This reduction is the sum of sludge digestion by the worms and natural sludge digestion by several microbial processes that take place in activated sludge such as maintenance and endogenous respiration (van Loosdrecht & Henze, 1999). Initial experiments also showed that separation of waste sludge and worm faeces is possible with a new reactor concept in which *L. variegatus* is immobilized in a carrier material. This also eliminates the need to separate the worms from the sludge. This chapter describes the results of a sequencing batch experiment in which the feasibility of this reactor concept for sludge reduction was investigated.

8.2 Materials and methods

8.2.1 Reactor concept

An outline of the reactor concept is presented in Figure 8.1. It consists of a beaker (sludge compartment) containing both waste sludge and worms. The open side of the beaker is covered with a carrier material, through which the worms can protrude their tails. The beaker is placed in the water compartment (partially submerged) with the carrier material facing downwards. By aerating the water compartment, the worms position themselves in the carrier material since *L. variegatus* feeds with its head, but respires and defecates via its tail. As a result, the worms keep their heads in the sludge compartment and protrude their tails into the water compartment. The carrier material, therefore, acts as both a support material for the worms and a separation layer between the waste sludge and the worm faeces. The feasibility of this reactor concept was investigated with a sequencing batch experiment.

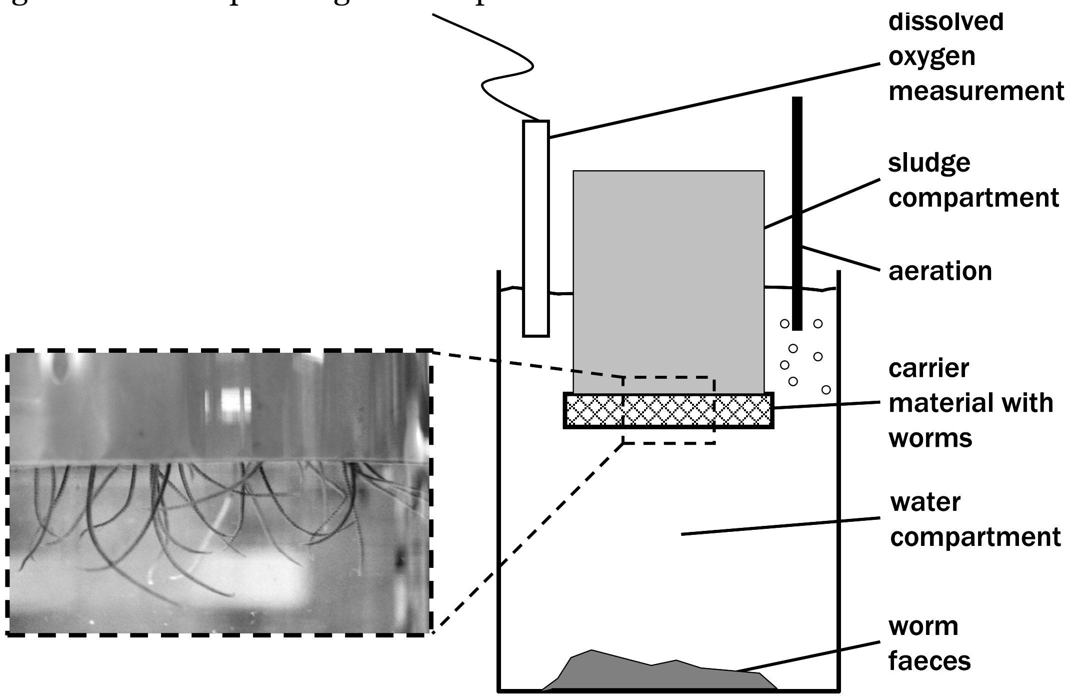


Figure 8.1 Experimental set-up for the sequencing batch experiment.

8.2.2 General analyses

TSS (total suspended solids) of sludge and worm faeces in the experiment were determined according to Standard Methods (APHA, 1998) using Schleicher & Schuell 589[1] black ribbon ash-free filters (retention >12-25 µm). Possible errors, as a result of sample handling, were checked by filling the sludge compartment and then immediately emptying it for TSS analysis. On average 99 % of the TSS was recovered, demonstrating the accuracy of the applied method. The wet weight of the worms was determined by placing the worms on a perforated piece of aluminium foil. Adhering water was removed by pushing the back of the foil against dry tissue paper and gently squeezing the worms. Dry weight of *L. variegatus* is 13 % of its wet weight (Chapter 2).

8.2.3 Sequencing batch experiment

The set-up shown in Figure 8.1 was used for the sequencing batch experiment. Daily, the contents of the water and the sludge compartment were replaced. The sludge compartment was filled with 100 mL of activated sludge (nitrifying sludge, ± 4 g TSS/ kg sludge) from the municipal WWTP of the city of Leeuwarden, the Netherlands. Sludge was provided in excess to the worms, to ensure that sludge availability was not a limiting factor. To remove coarse material from the sludge, it was first sieved using a 1 mm mesh. The water compartment was filled with effluent from the same treatment plant. This effluent was first filtered using Schleicher & Schuell 589[1] black ribbon ash-free filters (retention >12-25 µm) to remove any suspended material that could interfere with the accuracy of the TSS measurements. At the end of each step (24 h) in the batch sequence, the sludge compartment was taken away from the water compartment. The worms were separated from the remaining sludge, counted, weighed and used in the next step in the batch sequence. TSS of the remaining sludge in the sludge compartment and of the worm faeces in the water compartment were determined. As a carrier material, a polyamide mesh (300 µm; SEFAR) with a surface area of 7.5 cm^2 was used.

The water compartment was aerated to maintain the DO (dissolved oxygen) concentration between 8 and 9 mg/ L, which was checked using a Hach® LDO (luminescent dissolved oxygen) meter. This ensured that the process was not limited by oxygen availability. Hendrickx *et al.* (2006) showed that a lower DO (~2.5 mg/ L) indeed results in a lower sludge consumption rate. Together with the sequencing batch experiment with worms, a control sequencing batch experiment without worms was run under the same conditions. In these control tests, only the TSS of the sludge in the sludge compartment was determined.

8.3 Results

Within a few minutes after the start of each step in the batch sequence, the worms protruded their tails through the carrier material (as shown in Figure 8.1). During the experiment, a maximum of 5 % of the worms fell from the carrier material into the water compartment. The sludge within the sludge compartment settled onto the carrier material, forming a sludge blanket that did not settle through the mesh openings.

8.3.1 Sequencing batch experiment

Figure 8.2 compares the cumulative sludge reduction in the worm experiment and the control experiment. As sludge had been provided in excess, the sludge was never completely consumed at the end of each run. The sludge reduction rates were approximately constant, with 77 mg TSS/ d in the worm experiment and 28 mg TSS/d in the control experiment. If we assume that the natural sludge reduction/digestion takes place to the same extent in both experiments, the difference of 49 mg TSS/ d can be attributed to consumption by the worms, which equals 0.48 mg sludge/ mg worm/ d (dry matter based).

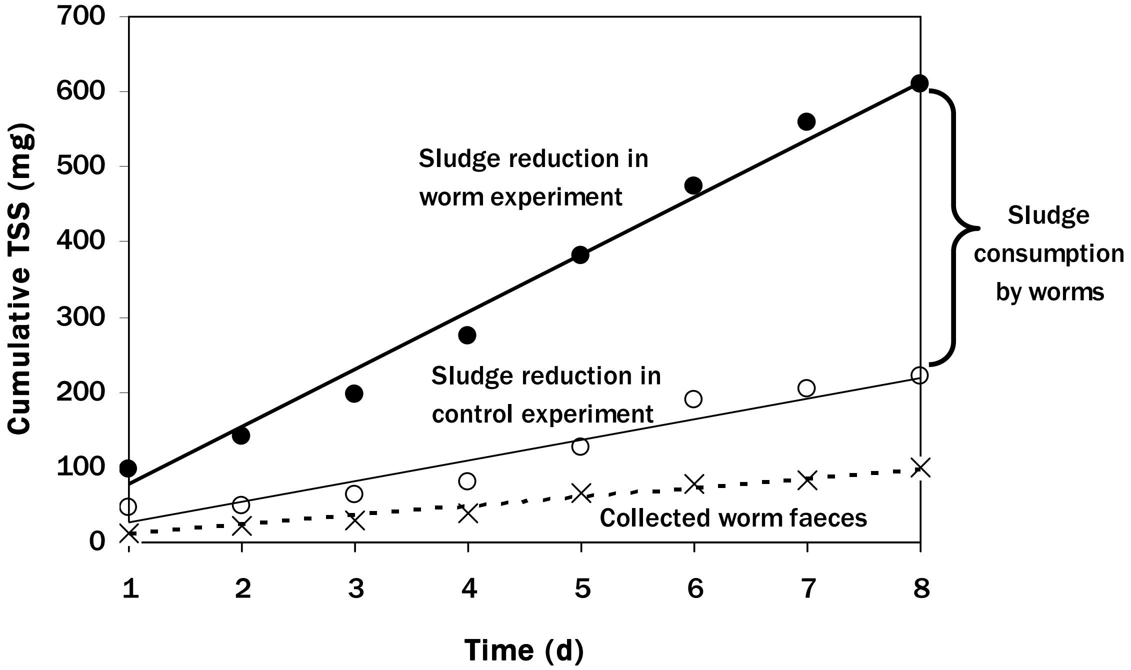


Figure 8.2 Cumulative sludge reduction in the sludge compartments from the control (○) and worm (●) sequencing batch experiments and faeces production (×) in the worm sequencing batch experiment. T = 22.9 ± 1.2 °C. DO concentration in the water phase = 8.4 ± 0.4 mg O_2/ L. Initial weight of 77 worms: 0.79 ± 0.04 g wet weight (~0.10 g dry weight).

Also shown in Figure 8.2 is the amount of produced worm faeces in the worm experiment. Comparing sludge consumption by the worms with produced worm faeces shows that only 25 % of the consumed sludge was converted into worm faeces (based on TSS). Under the conditions of this experiment, this means that the worms have digested 75 % of the consumed sludge. Figure 8.3 shows a dry matter based mass balance for the sludge that was consumed by the worms.

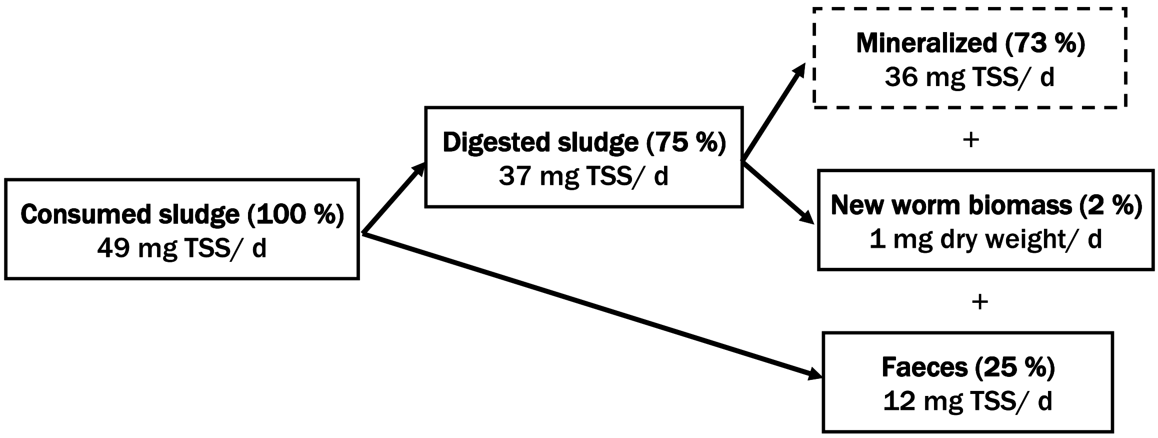


Figure 8.3 Dry matter based mass balance for the sludge that was consumed by the worms per day in the sequencing batch experiment.

Based on a total worm dry weight of around 0.1 g, this mass balance resulted in consumption, digestion and defecation rates of 0.48, 0.36 and 0.12 d^{-1} respectively (dry matter based). During the experiment changes in worm biomass varied between -8 and 7 mg dry weight/ d, with an average of 1 mg dry weight/ d (~ 8 mg wet weight/ d), which resulted in an average worm biomass yield of 0.03 g dry weight / g digested TSS (3 %). However, it should be noted that the daily worm growth rates are in the same order as the experimental error of the wet weight determination.

8.3.2 Worm faeces

As mentioned earlier, the proposed reactor concept makes it possible to separate the waste sludge from the worm faeces. Figure 8.4 shows the distinct compact structure of the collected worm faeces from the set-up in comparison to the sludge flocs of the waste sludge. As described in Chapter 6, the faeces settle much faster than the waste sludge and the final SVI values are also lower.

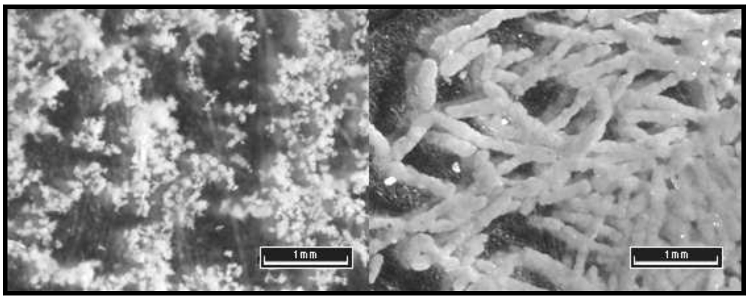


Figure 8.4 Waste sludge (left) versus worm faeces (right) in the reactor set-up. Scale bar = 1 mm.

8.4 Discussion

8.4.1 Sludge reduction rate

The rate of sludge reduction in the worm experiment was significantly higher than the sludge reduction rate in the absence of worms. Under the conditions described in this chapter, a single-layer surface area of 81,000 m^2 would be required to deal with a waste sludge production of 5,300 kg TSS/ d (from a 100,000 p.e. (population equivalent) WWTP) (Statistics Netherlands (CBS), 2007). However, we used a worm density of 1 kg wet weight per m^2 (~100,000 specimens per m^2), which was not yet optimized. In practice, much higher worm densities with a higher sludge reduction rate can possibly be obtained. This is determined by the available sludge and the maximum possible worm density per surface area. Especially the latter factor will determine the economic feasibility of the reactor concept.

8.4.2 Sludge reduction percentage

A 75 % reduction in the TSS amount of consumed waste sludge was observed in addition to natural sludge reduction/digestion. Not only would this substantially reduce the amount of waste sludge that needs to be disposed of, but it can also lead to a decrease in the associated sludge processing costs and environmental burden. By way of precaution, we can however not rule out that the high reduction percentage could partly be due to defecation of the worms in the sludge compartment. This means that not all worm faeces were collected in the water compartment and accounted for and, therefore, a higher apparent sludge reduction efficiency was observed.

8.4.3 Worm faeces

Worm faeces and waste sludge were separated by the carrier material. As was shown in Chapter 6, the worm faeces settled much faster than the waste sludge and final SVI values were lower than those of the waste sludge. These improved settling characteristics of the final waste product will contribute towards a decrease in sludge processing costs if dewaterability characteristics will improve accordingly. Even though we found no improvement of the dewaterability with the CST method (Chapter 6), this should be investigated further, preferably on a large scale.

8.4.4 Worm biomass

The worm yield of 3 % in the sequencing batch experiment was low, when compared to the yields mentioned elsewhere in this thesis (e.g. Chapter 5). This could be due to the immobilization and inverted positioning of the worms in the carrier material, which could restrain the worms in their feeding behaviour. Additionally, the daily worm growth was in the same order as the experimental error and only small in relation to the average total wet weight of 790 mg. To accurately determine the growth rate of the worms in this reactor set-up, long-term experiments with larger amounts of sludge and worms will have to be carried out. It will be important to consider the fate of the worm biomass, as we have partially converted the waste sludge into worm biomass. The high protein content of the worms, 60 % of their dry weight (Hansen *et al.*, 2004), makes re-use an attractive option, for example as live fish food or as slow fertilizer in agriculture (Winters *et al.*, 2004). However, care should be taken regarding the fate of some heavy metals (Chapters 6 & 7) and organic micropollutants originating from the waste sludge, as these possibly accumulate in the worms. This should be further investigated depending on the application.

8.4.5 Comparison with other experiments

To estimate the feasibility of the reactor set-up, the data from the sequencing batch experiment were compared to those from the batch experiments elsewhere in this thesis (Table 8.1). The average consumption rate and digestion percentage in the batch experiments with municipal waste sludge described in Table 5.4 (Chapter 5) could be calculated after subtraction of endogenous sludge digestion and by taking into account the faeces percentage in these batch experiments. Table 8.1 also shows the average digestion rate calculated in Chapter 5 and the resulting approximate defecation rate.

Table 8.1 Comparison of sludge consumption and digestion rates (in d^{-1}), digestion percentages of the consumed sludge by worms only (in %) and worm yields based on the amount of sludge digested by worms only (in %) in batch experiments and the reactor set-up (all dry matter based).

Parameter	Batch experiments	Reactor set-up
Consumption rate	0.46 (±0.23)	0.48
Digestion rate	0.09 (±0.04)	0.36
Defecation rate	~0.3	0.12
Digestion percentage	17 (±6)	75
Worm yield percentage	38 (±22)	3

Table 8.1 shows that only the consumption rates were similar, while the digestion rates and digestion percentages were much lower and defecation rates and worm yield were much higher in the batch experiments. This suggests a higher sludge reduction efficiency in the reactor set-up. However, further experiments with this set-up under varying conditions showed usually lower, but variable reduction percentages between 15 and 80 % and lower consumption, digestion and defecation rates than in the initial experiment (Hendrickx *et al.*, 2006; Hendrickx, 2007). The worm yield was usually equally low.

The variable results indicate that the performance of the process is strongly dependent on process operation and conditions, such as the immobilization and inverted positioning of the worms, the type of sludge and oxygen concentration. Kaster *et al.* (1984) for example found that sessile Tubificidae have lower defecation rates when their position is inverted and the same seems to apply to *L. variegatus* in the reactor set-up, with possibly higher sludge reduction percentages as a result due to longer gut retention times. In addition, the defecation rates of *L. variegatus* in waste sludge are relatively low. In sediments for example, Leppänen (1999) and Williams (2005) found much higher defecation rates for *L. variegatus* of around 4-11 d^{-1} (dry matter based). They found that substrates with a low organic content (i.e. less nutritious value) are processed at much higher rates than those with a high organic content and this may be the cause of the overall lower rates on sludge. This is also supported by the results of Gaskell *et al.* (2007) and Gnaiger & Staudigl (1987a) who found average gut retention times of 3 and 6 hours in sediments and sand with spinach respectively.

8.5 Conclusions

In this chapter, a new reactor concept for sludge reduction by *L. variegatus* is described. A sequencing batch experiment with this reactor concept showed that:

- A 75 % reduction in the amount of waste sludge could be achieved, because the sum of worm faeces and produced worm biomass was much lower than the amount of waste sludge that the worms consumed.
- *L. variegatus* could be immobilized in a mesh-like carrier material and a complete separation between waste sludge and worm faeces with highly improved settling characteristics could be achieved.

Even though the reactor concept is not optimized yet, and sludge digestion and worm growth parameters seem to be dependent on process conditions, it seems to have potential for decreasing the environmental burden and costs of waste sludge processing at WWTPs.

Acknowledgments

We thank Bas Buys (Wageningen University) for his valuable contribution to the research presented in this article. We also thank the operators of WWTP Leeuwarden for their assistance in obtaining the sludge and effluent used in our experiment.

╡Chapter 9╞

General discussion and outlook towards application of *Lumbriculus variegatus* in wastewater treatment

9.1 Introduction

Sludge reduction by aquatic worms The overview in Chapter 2 showed that several species of sessile Tubificidae and Lumbriculidae and free-swimming Aeolosomatidae and Naidinae have specific characteristics, which could make them suitable to reduce the amount of sludge that is produced in wastewater treatment. These characteristics include — next to the consumption and digestion of waste sludge — their high pollution tolerance, potential to reach very high population densities or natural presence in WWTPs (wastewater treatment plants). This initiated several authors to investigate the application of Naidinae, Aeolosomatidae and sessile Tubificidae in sludge reduction processes and they concluded that this technology has potential. However, their results were highly variable and to maintain a stable continuous system for sludge reduction with aquatic worms was almost impossible (Chapter 1). The reasons for the unstable (and uncontrollable) growth of especially the free-swimming species are unknown. Our own, long-term survey of four Dutch WWTPs (Chapter 3) showed similar unstable population densities of free-swimming species. Furthermore, a multivariate analysis did not demonstrate a distinct relation between these densities and process characteristics or process performance (e.g. waste sludge production). Based on these results and those found by the other authors, the application of free-swimming species will not be an option, until their growth can be controlled and their influence on process performance is clear.

In contrast, the sessile species *Lumbriculus variegatus* however did demonstrate a clear reduction in the amount of waste sludge, a stable growth and other positive effects on sludge characteristics (Buys, 2005; own data) and it was selected for further research (Chapters 4, 5, 6 & 8). With this species, a distinction can be made between sludge reduction by worms, sludge reduction by endogenous processes and worm growth in batch experiments. The importance of this distinction has been overlooked in many previous researches and it cannot be made with free-swimming worms, because of their size (Chapters 1 & 2). A direct experimental comparison with other sessile worms of the family Tubificidae suggested that sludge reduction and worm growth were more stable with *L. variegatus* (Chapter 7).

The main results of our research thus indicated that *L. variegatus* in general is a more suitable candidate for sludge reduction than Aeolosomatidae, Naidinae and sessile Tubificidae. The further discussion therefore focuses on the application of *L. variegatus* in wastewater treatment.

Sludge reduction by *L. variegatus* Five factors that are considered especially important for the application of *L. variegatus* in wastewater treatment are the effect of waste sludge consumption by *L. variegatus* on total TSS (total suspended solids) reduction, the TSS reduction rate, the settleability and dewaterability of the remaining sludge, and the production of worm biomass resulting from TSS consumption. A short overview of the main results is presented below.

To assess the sludge reduction potential of *Lumbriculus variegatus*, we distinguished between sludge reduction by worms and sludge reduction by endogenous processes (Chapters 4 & 5). Figure 9.1 shows an overview of the main results of such a typical batch experiment with municipal waste sludge.

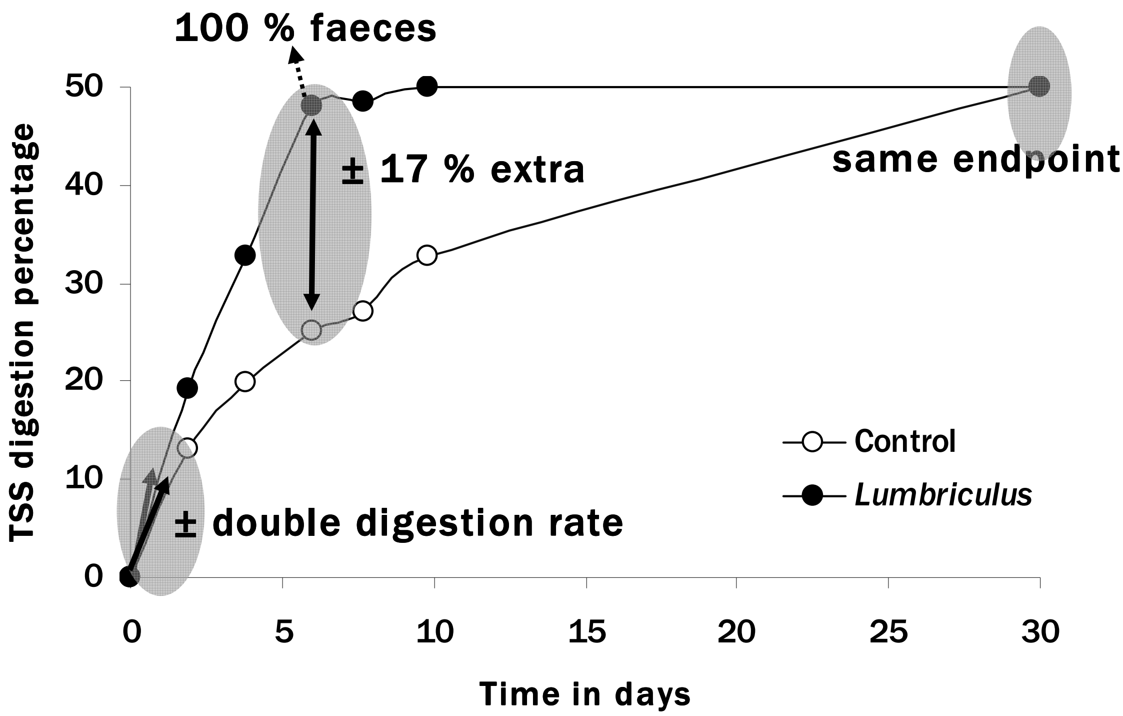


Figure 9.1 Overview of the main results of a typical batch experiment with *Lumbriculus variegatus* feeding on municipal waste sludge.

Most importantly, Figure 9.1 shows that *L. variegatus* accelerated the digestion of municipal waste sludge with at least a factor 2. After complete consumption of the waste sludge by *L. variegatus* (i.e. a faeces percentage of 100 %), the maximum reduction percentage of waste sludge was around 50 %. Only 17 % was due to digestion by *L. variegatus* (Chapter 8). The remaining part of the 50 % sludge reduction could be attributed to endogenous sludge digestion. In addition, the final sludge reduction percentage in the presence of *L. variegatus* proved to be no different from that in the absence of *L. variegatus*. A mass balance for the above-mentioned processes and worm growth, based on dry matter, is shown in Figure 9.2.

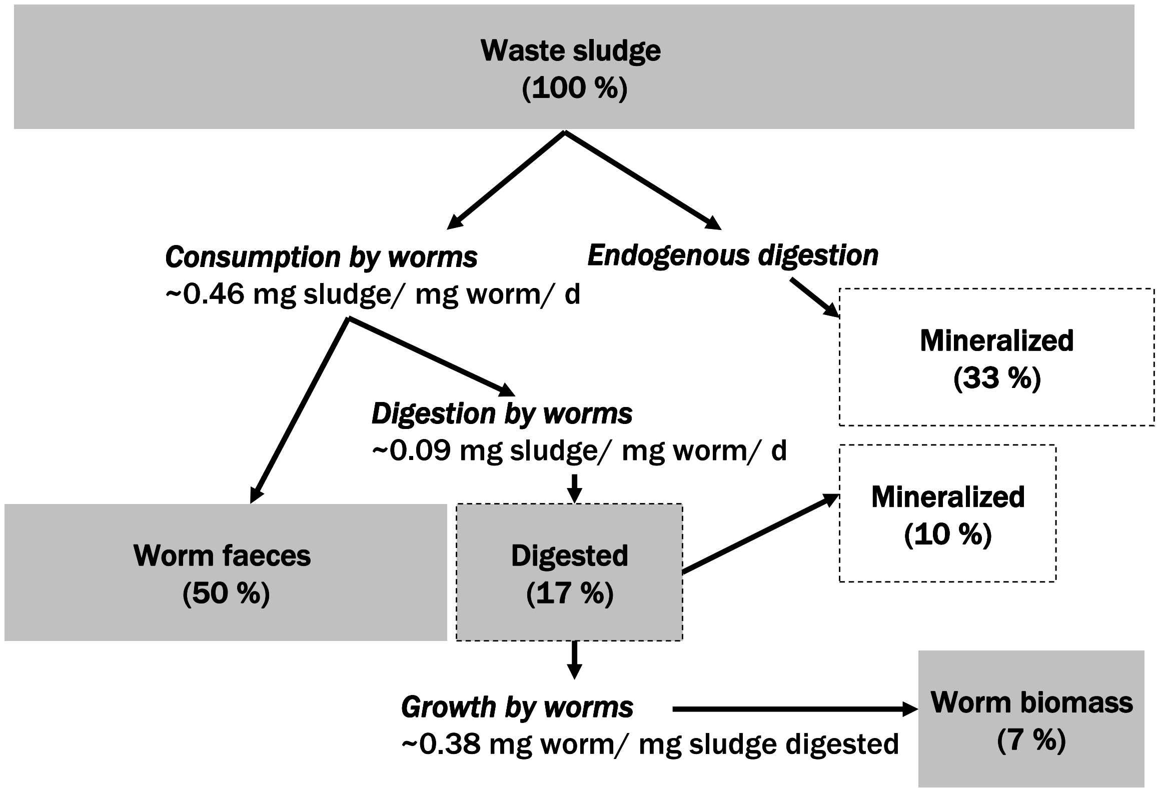


Figure 9.2 Dry matter based average mass balance for sludge digestion in batch experiments with *L. variegatus* and municipal waste sludge.

Figure 9.2 shows that digestion of municipal waste sludge by *L. variegatus* took place at average consumption (Chapter 8) and digestion rates of 0.46 and 0.09 mg sludge/ mg worm/ d respectively. The yield for this process was on average 38 %, i.e. 0.38 mg worm biomass was formed when 1 mg of waste sludge was digested by the worms. As a result, 7 % of the waste sludge was converted into a protein-rich resource with re-use potential (*Paragraph 9.2.5*).

The worm faeces settled much faster than the waste sludge due to their compact shape and higher density. The final SVI (sludge volume index) of the faeces was always low, around 60 mL/ g (Chapter 6). The worm faeces did not show improved dewaterability according to the CST method, but our results indicated that this method was not reliable (Chapter 6).

However, a sequencing batch experiment (Chapter 8) and further experiments with a reactor set-up (Hendrickx *et al.*, 2006; Hendrickx, 2007), in which the worms were immobilized, often demonstrated higher sludge reduction percentages (15-80 %), but also lower consumption, digestion and defecation rates as well as a lower worm yield. It is therefore likely that immobilization of *L. variegatus* in a continuous system for sludge reduction will influence sludge digestion and worm growth.

Full-scale application For a continuous full-scale system based on *L. variegatus*, six aspects should be considered: the waste sludge, the worm population, the reactor set-up, the worm faeces, the effluent, and finally, the produced worm biomass. Figure 9.3 shows an overview of the considerations for each of the six aspects, which will be discussed in the indicated paragraphs.

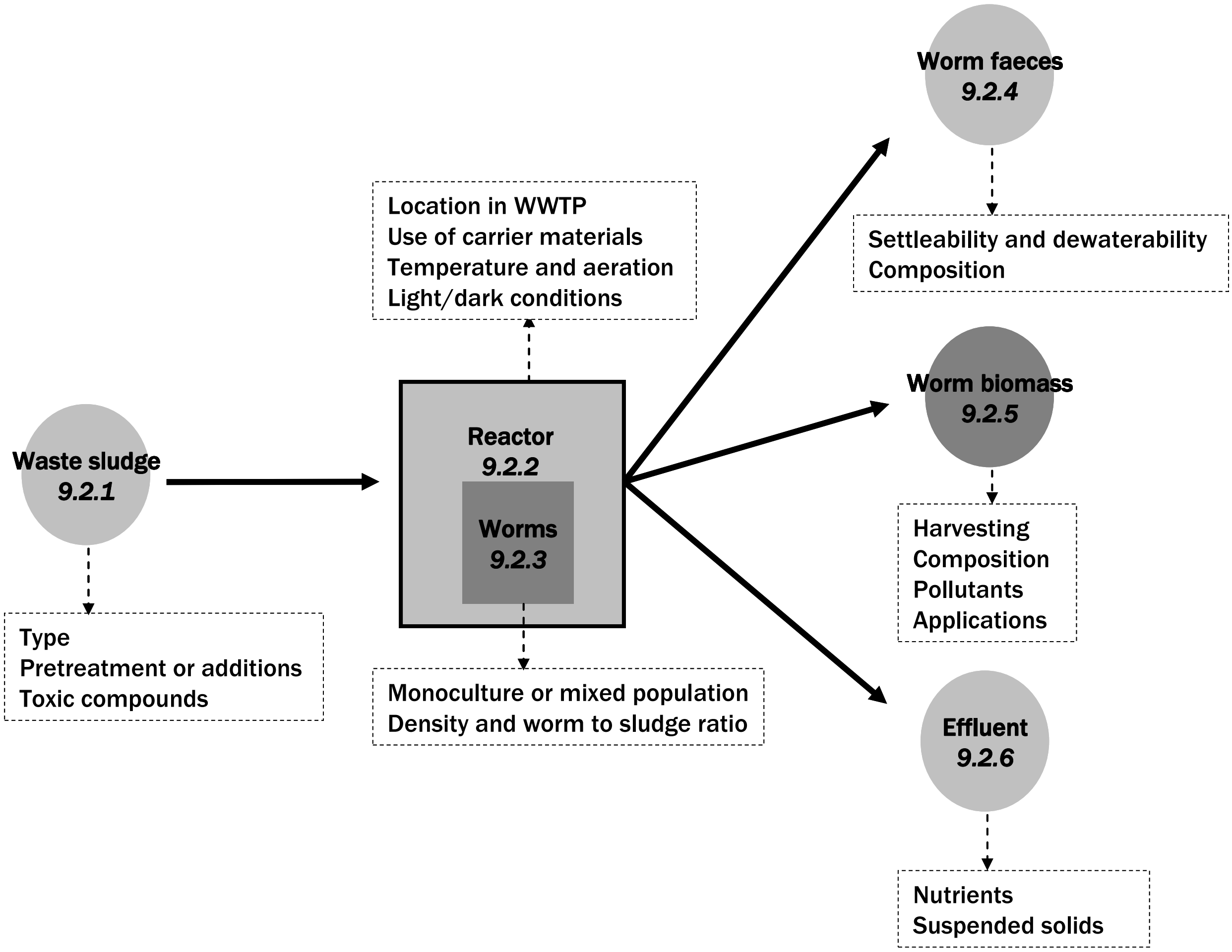


Figure 9.3 Overview of a continuous full-scale system with *L. variegatus* as sludge consumer with considerations for each aspect.

Finally, the overall feasibility of a continuous full-scale system with *L. variegatus* will be discussed and recommendations will be given for future research.

9.2 Considerations for a full-scale system for sludge reduction with *L. variegatus*

9.2.1 Waste sludge

Sludge type, pre-treatment and Fe^{3+}-addition In our batch experiments, *L. variegatus* was fed with various waste sludges from municipal and non-municipal WWTPs (Chapters 4 & 5). The latter sludge originated from a plant treating beer wastewater. The worms were able to consume all the sludge types. The sludge floc size was not limiting for uptake and did not influence sludge digestion or worm growth. However, digestion and growth rates (in worm biomass and numbers) were higher on municipal sludges than on beer sludge. The beer sludge was hardly digested and led to low or even negative growth rates. The variability of the rates was high for each sludge type. A linear regression analysis with the two most common municipal sludge types indicated that this variability was almost independent of variations in experiment duration (2-8 days), temperature (16-20 °C), worm density (2,000-11,000 m^2), W/S ratio (0.1-1.0), pH (4.8-7.6) and ash percentage of the waste sludge (13-20 %). Apparently, the variability between and within sludge types seemed to be caused by an unknown sludge composition in terms of digestible, refractory and toxic compounds.

The influence of waste sludge composition was investigated in several experiments. Most reduction concerned the organic fraction of the sludge (Chapter 4), which increases the ash fraction of the worm faeces. Batch experiments on the influence of sludge protein content suggested that *L. variegatus* specifically digests part of the protein fraction. A higher protein content however did not necessarily lead to higher digestion rates (Chapter 6). The absence of live bacteria in waste sludge suppressed reproduction, but not sludge digestion and biomass growth (Chapter 5). This was also observed in batch experiments with sludges that were pre-digested under oxic conditions for periods up to 114 days and most likely had a low content of live bacteria (Chapter 5). Even though these experiments suggested that sludges with a high protein content (e.g. sludges from the food industry) or large fraction of live bacteria (e.g. sludges with short sludge ages) may be more suitable for digestion by *L. variegatus*, further research on which specific components of the sludge are digested and to what extent can be useful for predicting the suitability of different sludges.

Although the maximum digestion percentage of waste sludge after endogenous digestion was equal to that after a *simultaneous* combination of endogenous and worm digestion (Chapter 4), one of our experiments suggested that a *successive* combination of endogenous digestion under oxic conditions (up to 48 days) with worm digestion sometimes resulted in an increase of the total maximum digestion percentage from 60 to 68 % (Chapter 5). This may be due to the development of different bacterial populations and decomposition products during the successive digestion stages, which are then degraded during the following digestion stage. Park *et al.* (2006) and Jung *et al.* (2006) found similar increased digestion percentages by successive combinations of oxic and anoxic digestion and the latter authors suggested that a combination with sludge-consuming higher organisms may lead to even higher digestion percentages. In theory, feeding worms with pre-digested sludges (e.g. after composting or anaerobic digestion) or with sludges treated by for example ultra-sound (Khanal *et al.*, 2007) thus could be advantageous for reducing the total amount of sludge produced in a WWTP. In practice, the applicability of this variant of waste sludge digestion will however be limited for anaerobic sludge, unless ammonia, of which the un-ionized form is toxic to *L. variegatus* (Chapter 5), is stripped. As mentioned before, sludges with a low fraction of live bacteria may also be unsuitable.

Starting from these observations, *L. variegatus* has most potential for digesting activated sludge directly wasted from the aeration tanks of municipal WWTPs. A further investigation of different sludge types, e.g. from food-related industries or from different process stages in WWTPs, is necessary to assess the complete potential of this technology. Other substrates may also be suitable for digestion by *L. variegatus*. For example, several authors (e.g. Garg *et al.*, 2006; Turnell *et al.*, 2007) described the consumption of related waste substrates with high organic contents (e.g. manure, kitchen waste, vegetable material) or mixtures thereof with waste sludge by earthworms. In addition, other authors (e.g. Landesman, 1996; Marsh *et al.*, 2005; Schneider, 2006; Bischoff, 2007) described the culture of different detritivores (e.g. *L. variegatus*, *Nereis diversicolor*) on aquaculture waste products and their subsequent re-use as consumption fish food.

Toxic compounds in sludge Waste sludge can contain many toxic compounds. At certain concentrations, these toxic compounds can form a threat to the viability or even survival of the worm population feeding on this sludge, since they are known to bioaccumulate toxic compounds through their food, and, to lesser extent, through their skin (Leppänen, 1999). Toxicity data for *L. variegatus* are abundantly available (e.g. United States Environmental Protection Agency, 2007; Pesticide Action Network North America, 2007), since it is a standard test organism for toxicity and bioaccumulation assays (United States Environmental Protection Agency, 2000). Toxic effects in *L. variegatus* are for example evaluated by changes in growth, mortality, reproduction, feeding, clumping, burrowing, colour and motility but also by swellings and mucus production (e.g. Bailey & Liu, 1980; Dermott & Munawar, 1992; Williams, 2005).

So far, we identified only ammonia —and especially its un-ionized form— as important toxic compound in experiments with *L. variegatus* in sludge that was pre-incubated under anoxic conditions. It could cause massive mortality in *L. variegatus* populations at concentrations as low as 2 mg/ L un-ionized ammonia at pH 8 (~70 mg/ L total ammonia; Chapter 5). This species is however not more vulnerable than other aquatic worm species, since the toxicity of un-ionized ammonia is approximately equal for *L. variegatus* and sessile Tubificidae (Schubauer-Berigan *et al.*, 1995). In aerobic WWTPs with N removal, ammonia concentrations should not exceed those toxic to *L. variegatus*, but process failures (e.g. interrupted aeration) that lead to high ammonia concentrations can endanger the entire worm population. In addition, high pH values and temperatures increase the ratio of toxic un-ionized ammonia to total ammonia in waste sludge.

In general, worm mortality was not often observed and apparently, *L. variegatus* survives most toxic compounds present in sludge. However, the presence of unknown toxic compounds and sublethal toxicity effects may have been reflected by the observed variabilities in sludge digestion and worm growth rates (Chapter 5).

Simple toxicity, digestion and growth tests should be done with small worm populations and the applied waste sludge. Furthermore, a superficial risk analysis of the toxic compounds that could harm the worm population should be made based on the composition of the wastewater, from which the sludge is produced. In addition, an early warning system for high ammonia concentrations is necessary.

9.2.2 Reactor

Location in WWTPs and use of carrier materials *L. variegatus* can be applied in a separate reactor fed with waste sludge. Direct application in the aeration tank is probably less suitable, because good access of the worms to the waste sludge is essential and too much turbulence —and wash-out of worms— should be avoided, because *L. variegatus* is a sessile species. Carriers can be applied, e.g. mesh-like (Chapter 8) but also sponge-like (e.g. Recticel®) materials. The use of these carrier materials also facilitates concentrating and harvesting of worm biomass. To optimize the worm/sludge contact area and limit the size of a reactor, stacked layers of carrier material (racks) can be applied at an intermediate distance of several cm. There should be a constant flow of sludge or effluent to maintain a healthy worm population. Several authors (Leppänen & Kukkonen, 1998b; Williams, 2005) have found that the growth rates of *L. variegatus* are higher in systems where the water is constantly renewed than in stagnant systems, due to the removal of impurities and enhanced aeration.

Temperature, aeration and light/dark conditions Based on our experiments and literature data, *L. variegatus* is specifically adapted to temperate regions and its optimal temperature lies around 15-25 °C. Temperatures approaching 30 °C should definitely be avoided, because they are harmful to the worms. Temperatures towards 5 °C should also be avoided, because at this temperature growth and feeding almost stops (Chapter 5). The average temperature in the aeration tanks of several Dutch WWTPs was around 16 °C (Chapter 3) with minimum and maximum values of 7 and 25 °C. Additional heating may therefore be necessary in cold periods to maintain high digestion and growth rates by the worms, but also endogenous digestion. This option however requires the input of a substantial extra amount of energy. This could for example be provided by excess heat from a nearby industry (e.g. the influent of WWTP Nijmegen is heated with cooling water from the nearby waste incinerator, Chapter 3). Alternatively, the reactor dimensions could be enlarged.

The respiration rate of *L.* variegatus under aerobic conditions is 1.2-2.7 g O_2/ kg worm dry weight/ h at temperatures of 10-20 °C (Kaufmann, 1983; Gnaiger & Staudigl, 1987a; Brodersen *et al.*, 2004). During normal activity *L. variegatus* is an oxy-conformer, which means that higher oxygen concentrations lead to higher oxygen uptake rates (Gnaiger *et al.*, 1987b). Sludge digestion rates also increase with oxygen concentration (Hendrickx *et al.*, 2006). Even though *L. variegatus* can survive periods of anoxia and is regarded as a facultative anaerobe (Putzer *et al.*, 1990), anoxic conditions slow down their feeding rates and in addition lead to toxic ammonia built-up in the sludge. Therefore, in a reactor, access of the worm tails to oxygen in a liquid or gas phase is crucial and oxygen supply to the worms should be as high as possible. Oxygen can be supplied by aerating waste sludge or effluent (e.g. Chapter 8), but optimal use should also be made of the oxygen naturally present in air or flowing effluent, for example by making use of the before-mentioned thin layers. Not much is known about the optimal oxygen concentration for *L. variegatus*, but usually, researchers keep their populations in aquaria with sediment and water at concentrations of at least 6 mg/ L (e.g. Williams, 2005). When *L. variegatus* feeds on sludge and has no direct access to air, a tentative minimum of 3-4 mg O_2/ L sludge is advisable, based on our own observations and those of Hendrickx *et al.* (2006). When there is direct access to air, minimum concentrations in the sludge can be lower (e.g. 1-2 mg O_2/ L) according to our own observations. However, the effect of anoxia and possible toxic compound formation in the sludge surrounding their head part should be determined in more detail.

Due to the natural characteristics of *L variegatus*, a reactor for sludge reduction with this species does not require a substantial amount of extra energy input in the form of constant high temperatures or light (Chapter 5). Ratsak (1994) found that the energy consumption for oxygen supply in a municipal WWTP decreased with increasing numbers of (free-swimming) *Nais* sp. without a clear reason. An explanation would be that the net oxygen consumption of worms plus the reduced sludge amount is less than that of the sludge without worms. Spanjers (1993) for example mentions an endogenous respiration rate of 4-8 g/ kg TSS/ h for activated sludge. Because *L. variegatus* has a substantially lower respiration rate, this would explain a decreased oxygen demand as a result of sludge digestion by these worms.

9.2.3 Worms

Monoculture or mixed culture with other species In polluted habitats, aquatic worms are often present in mixed cultures. A mixed culture of aquatic worms may have advantages over a monoculture. Even though digestion rates of *L. variegatus* did not increase in a mixed culture with sessile Tubificidae, growth rates did increase (Chapter 7). Their sensitivity to toxic compounds may also decrease (Chapman *et al.*, 1982b). Furthermore, detrimental conditions may not affect the complete worm population, when it consists of different species with different characteristics.

Regardless of the use of a mono- or mixed culture, aquatic worms are a natural food source for many organisms. Some of these organisms (e.g. leeches, flatworms, mosquito larvae) are known to inhabit activated sludge systems (Curds & Hawkes, 1975).

Several authors mention predation of Oligochaeta by these organisms (e.g. Cross, 1976; Young, 1978; Marian & Pandian, 1985; Young & Procter, 1985) and individual leeches can for example consume around 1 worm per day (Cross, 1976). A worm population in a confined reactor set-up could thus be seriously endangered by a massive invasion of any of these predators.

Worm growth rates, density and worm to sludge ratio In batch experiments, we found average biomass growth rates of around 2-5 % per day and average number growth rates of 1-2 % per day. In a reactor with continuous sludge supply, the worm population will grow until equilibrium is reached between worm density and food (sludge) supply. Worm to sludge ratios and population densities can be regulated to some extent by frequent harvesting and adjusting the sludge amount that they are fed with. Both should be as high as possible without causing negative effects (on sludge digestion and also worm growth rates) like competition for food or oxygen and the accumulation of excretion products from the worms. In natural sediments, densities of *L. variegatus* hardly exceed 12,000 specimens per m^2, which corresponds to around 0.1 kg wet weight per m^2. However, tests with the sponge-like carrier material Recticel® in sludge showed that it could contain densities of 120,000-132,000 specimens per m^2, which corresponds to 1.0-1.1 kg wet weight per m^2 (own data). This is similar to the density in the mesh-like carrier of the reactor set-up (Chapter 8), but Hendrickx (2007) mentioned higher densities of 217,000 specimens per m^2 with this set-up, which corresponds to 0.32 kg wet weight per m^2. Batch experiments described in Chapter 5 indicated that densities of 39,000-139,000 specimens per m^2 and worm to sludge ratios of around 1.5 negatively affected sludge digestion and worm growth rates, respectively most likely as a result of accumulation of excretion products, like un-ionized ammonia, and food limitation. However, in continuous systems with high population densities, this may be prevented by providing sufficient sludge to keep the worm to sludge ratios below 1, sufficient oxygen and flow-through.

It was also found that a minimum worm to sludge ratio was required for a measurable effect on sludge digestion, dependent on the endogenous activity of the sludge used (Chapter 4). Therefore, a sufficient stock of worm biomass is necessary when a reactor should be started up fast. This can either be obtained by waiting until the worms have colonized the reactor or by creating a breeding facility, which serves as a central stock for supplying new reactors and in case of calamities. *L. variegatus* is however not bred commercially like in the US, where they are fed with fish food in large outdoor ponds and are sold for around 10 € per kg wet weight (Aquatic Foods, 2007). In the Netherlands, only earthworms are bred on a large scale for commercial purposes.

9.2.4 Worm faeces

Settleability and dewaterability The increased settleability of worm faeces will result in a decreased volume of sludge that has to be disposed of. The dewaterability of the faeces should however be investigated further with other methods (e.g. specific resistance to filtration (SRF) or compacting), because this is one of the most important issues in sludge treatment.

Composition As mentioned before, worm faeces contain more inorganic material than the waste sludge. We have also indications that their protein fraction is smaller. The effect of sludge consumption by *L. variegatus* on carbohydrates remains unclear. Worm faeces still contain a relatively high amount of organic matter (typically around 70 %, when the ash fraction before sludge consumption by worms was around 20 %, Chapter 4), but the worm faeces seem refractory to further digestion. Returning the worm faeces to the aeration tanks therefore seems pointless. Furthermore, we found that cadmium, copper, chromium, nickel, lead and zinc in waste sludge were not accumulated to a large extent by *L. variegatus* during sludge consumption and thus end up in the worm faeces, most likely because they remain bound to the large organic fraction of sludge. Sludge consumption did not seem to influence the distribution of metals between the sludge and the supernatant phase. However, because the metal recovery in the experiment described in Chapter 6 was not 100 %, a closed metal mass balance including sludge, worms and supernatant before and after consumption deserves further investigation. The overall low metal concentrations in worm biomass increases the re-use possibilities as described in *Paragraph 9.2.5*, but decreases those of the worm faeces and the latter will have to be processed according to existing methods, e.g. incineration. Apart from the concentrations of the six metals mentioned before, those of arsenic and mercury should also be determined in worm faeces, waste sludge and worms.

9.2.5 Worm biomass

Harvesting In contrast to the smaller free-swimming species like Aeolosomatidae and Naidinae, *L. variegatus* can easily be separated from waste sludge. Using 300 μm sieves already leads to complete separation, as applied in our experiments, but using bigger mesh sizes may also have the desired effect, with less clogging of filter materials. However, harvesting of worms from a sponge-like carrier (e.g. Recticel®) by applying mechanical techniques, low oxygen concentrations, light sources or flushing the carrier with water seems ineffective. Recently, a method has been developed for harvesting of pure and live worm biomass from a carrier (Elissen *et al.*, 2007).

Composition Waste sludge typically contains 32-41 % protein and 12-41 % ash (dry matter based) (Tchobanoglous *et al.*, 2003). Sludge consumption by *L. variegatus* therefore results in a more valuable resource in this respect, since worm biomass has a higher protein content and lower ash content (Appendix II, Table A1). However, it also leads to losses in phosphorus content (dry matter based), because waste sludge contains 3-11 % (Tchobanoglous *et al.*, 2003), while *L. variegatus* contains only 2 % (Appendix II, Table A1). The caloric values of waste sludge and worms are the same; around 5 kcal/ g dry matter. The composition of *L. variegatus* roughly resembles the composition of other aquatic Oligochaeta like *T. tubifex* and *L. hoffmeisteri* (e.g. Whitten & Goodnight, 1966b), but also terrestrial Oligochaeta like *Enchytraeus albidus* (Ivleva, 1973) and other earthworms (de Boer & Sova, 1998). For one species, percentages are quite variable between authors, which could be due to different food sources (Ivleva, 1973), but also to different analytical methods. Mulder & Beelen (2007) determined the monosaccharide and amino acid composition of the carbohydrate and protein fraction of *L. variegatus* (Appendix II, Table A2). The amino acid composition of *L. variegatus* again roughly resembles that of sessile Tubificidae (Yanar *et al*., 2003; Mulder & Beelen, 2007), as do the molecular weights of the proteins, except for a heavier fraction in *L. variegatus* (Mulder & Beelen, 2007). Based on *L. variegatus* biomass composition, new applications can be thought of, but also existing applications for worm species with a similar composition should be considered (section *Applications*). However, because *L. variegatus* is grown on a waste product, the nature and importance of pollutant content of the biomass for each application should also be considered (section *Pollutants)*.

Pollutants Because waste sludge contains pollutants, *L. variegatus* biomass possibly also contains pollutants after sludge consumption, such as heavy metals, micropollutants and parasites.

The concentrations of copper, chromium, nickel and lead in *L. variegatus* biomass were well below those in waste sludge (Chapter 6 & 7). Only cadmium and zinc sometimes bioaccumulated to concentrations respectively higher (around 2 times) or equal to those in waste sludge. The overall low bioaccumulation of heavy metals by the worms was probably caused by metal binding to the organic fraction of sludge that was excreted by the worms. Metal concentrations in worms grown on Tetra Min® fish food that contained much lower metal concentrations than waste sludge were similar. Next to the basic composition of the substrate, unknown characteristics of the substrate and possibly regulation mechanisms in the worms may therefore also determine the concentrations in the worms (Chapters 6 & 7). For predicting metal concentrations on different kinds of sludges, further research on determinants for uptake is necessary. Storage of heavy metals in certain worm tissues is another interesting topic for further investigation. Heavy metals are often stored in chloragogenous tissues of worms (e.g. Back & Prosi, 1985), which are also involved in lipid storage (Morgan & Winters, 1991). Selective storage of metals in extractable fat fractions would for example enable selective removal of contaminated fractions from the worm biomass.

Concentrations of micropollutants in waste sludge (and effluents of WWTPs) are a reason for growing concern and currently legislation is prepared in the European Framework Directive. Because *L. variegatus* is used as a standard organism for toxicity and bioaccumulation tests, information is available on many of these compounds in more than 100 peer-reviewed papers. Examples are hormones (Liebig *et al*., 2005), PAHs (e.g. Ankley *et al*., 1997; Leppänen & Kukkonen, 2000; Ingersoll *et al*., 2003), PCBs (e.g. Kukkonen & Landrum, 1995; Fisher *et al*., 1999; Sun & Ghosh, 2007), insecticides/herbicides (e.g. Mäenpää *et al*., 2003; Wiegand *et al*., 2007), drugs (e.g. Oetken *et al*., 2005; Nentwig, 2007). Extensive internet databases contain most of these data (United States Environmental Protection Agency, 2007; Pesticide Action Network North America, 2007). *L. variegatus* is able to bioaccumulate many of these compounds and some of them are toxic. *L. variegatus* presumably possesses mechanisms for organic micropollutants, in analogy with those for metals, which involve storage in certain body tissues like chloragocytes and decrease the toxicity of these compounds. This was found for hormones by Liebig *et al.* (2005). However, as with metals, bioaccumulation and toxicity of these compounds will be dependent on binding to components in the sludge and can be much lower than from the standard test substrates (sediments and water). This deserves further investigation.

Several Oligochaeta like sessile Tubificidae can harbour myxozoan parasites (Lowers & Bartholomew, 2003). These parasites can cause fish diseases in for example trout (Brinkhurst, 1996; Morris & Adams, 2006) and Oligochaeta thus serve as an intermediate host for transfer. *L. variegatus* has also been mentioned as an intermediate host for these parasites (Morris & Adams, 2006), but it is unknown to which extent these parasites occur in WWTPs.

Depending on the anticipated application of *L. variegatus* biomass grown on waste sludge, a thorough screening of some of the abovementioned unwanted compounds in the biomass will be necessary.

Applications *L. variegatus* biomass in theory can be used alive or dead (whole or fractions). One of the most important considerations for re-use, next to the composition and produced amount, is the pollutant content of the biomass, because they are fed with waste sludge.

Stephenson (1930) already mentioned the use of live sessile Tubificidae as aquarium fish food as early as 1910. In Europe, sessile Tubificidae are still often used as aquarium fish food, while in the US, *L. variegatus* ('blackworms') is a more preferred and popular live food for aquarium fish (Aquatic Foods, 2007). Some other aquatic animals that can be fed with *L. variegatus* are flatworms, crayfish, leeches, shrimps, insect larvae, reptiles and fiddler crabs (e.g. Drewes, 2005). Many authors (e.g. Timm, 1980; Lietz, 1987; de Boer & Sova, 1998) describe the potential of aquatic and terrestrial Oligochaeta (sessile Tubificidae, *L. variegatus* and earthworms) as a live or dried food source for consumption fish. According to them and other authors (e.g. Krishnan & Reddy, 1989; Evangelista *et al.*, 2005), both applications have high potential, considering the low-maintenance culturing of worms as well as increased appetite and growth rates of fishes. However, pollutants from the substrate that is used for feeding the worms can be transferred to fish (e.g. Egeler *et al.*, 2001; Hansen *et al.*, 2004) with sometimes detrimental effects when concentrations are high enough (Stafford & Tacon, 1984; Hansen *et al.*, 2004). Therefore, as long as pollutant concentrations are low enough and do not affect fish health, as described for metals in *L. variegatus* in concentrations similar to those in our experiments (Hansen *et al.*, 2004), worm biomass grown on waste sludge may be a suitable food source for aquarium fish and other animals not used for human consumption. This is illustrated by the fact that sessile Tubificidae used for aquarium fish food usually originate from polluted rivers in Eastern Europe. However, it is questionable whether the application of worm biomass grown on waste sludge for consumption fish food (or any livestock feed) will be accepted, because of the risk of pollutant transfer to humans or for ethical reasons. To a lesser extent, this is also true for applications of worm biomass in agriculture, for example as fertilizer or as carrier for agro-chemicals (Winters *et al.*, 2004). Based on heavy metal concentrations in sludge, the Netherlands currently have the most stringent legislation (BOOM) for the application of sludge in agriculture in the European Union, together with Denmark, Finland and Sweden. Even though the current and future EU requirements are far less stringent (Appendix II, Table A3), the possible re-use of worm biomass in agriculture will depend on the regulations applied in each individual country.

Alternatively, live worms can be applied as toxicity test organism, if they comply with requirements, such as a constant worm composition and a well-defined food source (United States Environmental Protection Agency, 2000). In analogy, they can be used as prey organism for conducting dietary exposure studies with fish (Mount *et al.*, 2006) or as biocarriers for drugs delivery to fish as was described for *Nereis virens* eggs by Katharios *et al.* (2005). .

Instead of using the bulk material, fractions of worm biomass may also be used, if economically feasible. Winters *et al.* (2004) tested the applicability of proteins from sessile Tubificidae as coatings, surfactants and glues and concluded that the last option had most potential. They also mentioned a possible application as carrier for agro-chemicals. Because the composition of sessile Tubificidae resembles that of *L. variegatus*, it is likely that the qualities of the biomass and its derived products are also alike. Another application for the proteins may be use in bioplastics, as is for example done with proteins in egg white (Jerez *et al.*, 2007). Amino acids (Table 9.3) and possibly enzymes may be commercially interesting. Amino acids can be applied in nutrition or medicine, but they can also be converted to products for the petrochemical industry, to decrease the use of fossil fuels (Scott *et al.*, 2007). De Boer & Sova (1998) described a technique for isolating enzyme mixtures, which can be applied as biodegradable detergents, from earthworms grown on waste materials. The earthworms adapted their enzymes to the applied substrate.

9.2.6 Effluent

Nutrients and suspended solids A batch experiment described in Chapter 4 suggested that digestion of sludge by *L. variegatus* mostly led to a higher ammonium release (0.002-0.07 µg N/ mg dry worm weight/ h) than was expected base on the TSS digestion percentage, possibly as a result of consuming the nitrifying bacteria population or the extra release of ammonium by the worms. Gardner *et al.* (1981) and Postolache *et al.* (2006) found that sessile Tubificidae in sediments with full or empty guts excreted ammonium and also phosphate (inorganic and organic) at rates of 0.03-0.27 µg N and 0.002-0.01 µg P/ mg worm dry weight/ h respectively. The extra release of ammonium is however in contradiction with the higher protein content of worms in comparison to waste sludge and the observed specific digestion of the protein fraction of waste sludge.

A more turbid water phase was also observed after sludge consumption by worms (Chapter 6). Discharging these extra suspended solids and nutrients from a worm reactor into surface waters should be prevented, because maximum permitted concentrations for N_{total}, P_{total}, BOD, COD and TSS are 10-15, 1-2, 20, 125 and 30 mg/ L respectively in effluents of Dutch WWTPs (Loeffen & Geraats, 2005). These regulations are expected to become stricter as a result of the European Framework Directive. Therefore, the exact release of these compounds in a continuous system should be further investigated or, in case of a separate worm reactor, the effluent can be discharged in the WWTP.

9.3 Feasibility of a worm reactor for waste sludge reduction with *L. variegatus* at a 100,000 p.e. WWTP

Even though the data from our batch experiments cannot be directly extrapolated to a continuous system, they can give some indications of the feasibility of a reactor set-up. A 100.000 p.e. (person equivalent) WWTP has a daily waste sludge production of around 5,300 kg TSS (Statistics Netherlands (CBS), 2007). Based on Figure 9.2, a worm population of 11,520 kg (~ 88,615 kg wet weight) would be needed to digest 50 % of this sludge. From the digested sludge, 371 kg dry worm biomass (~ 2,854 kg wet weight) could be produced. The optimal worm density and the number of layers that can be applied will determine the volume of a reactor. For example, a worm density of 100,000 specimens per m^2 (~ 1 kg wet weight per m^2) would result in a required surface area of around 88.615 m^2. Thirty layers at an intermediate distance of 5 cm would result in a reactor volume of 4431 m^3 and a reactor surface of 2954 m^2, which is roughly equal to 30 % of the aeration tank surface in these plants. Because these results are based on batch experiments and an assumed population density, we can only give rough indications of the economic feasibility, based on approximate sludge processing costs of 300 € per ton TSS (Wiegant *et al.*, 2005) and approximate reactor investment costs of 300 € per m^3 reactor volume (Janssen, 2007). If the produced worm biomass could be sold for around 2 € per kg dry weight, the resulting period for return of investment would be 2.4 years, which is acceptable.

The feasibility of this concept in continuous applications will thus not only be dependent on the reduced sludge processing costs, but most likely even more on both the investment costs of a reactor (based on the maximum population densities that can be maintained at high sludge reduction rates) and the production and sale of worm biomass. However, results in a continuous system may be entirely different, as was already shown by the comparison between data of the batch experiments and data of the sequencing batch experiments with the reactor set-up (Chapter 8). Currently, the reactor set-up described in Chapter 8 is under investigation by Hendrickx (2007), who already found densities of around 200,000 specimens per m^2.

Other profits were not considered at this stage. These are possible economical profits from improved sludge settleability and dewaterability and from reduced dimensions of mechanical installations in the WWTPs. In addition, environmental profits, resulting from the decrease in CO_2-emissions associated with a decreased sludge transport, were not considered. However, methane production in anaerobic waste sludge digesters probably is lower from worm faeces than from waste sludge, due to the reduction of its organic content, and possible costs for aerating and heating a worm reactor should also be taken into account.

9.4 Overall feasibility of sludge reduction with *L. variegatus* and recommendations for further research

Based on the above information, the application of *L. variegatus* in wastewater treatment has potential. This aquatic worm can reduce the amount and volume of different municipal waste sludges. It seems possible to maintain a stable population of *L. variegatus* with steady biomass and number growth rates in a continuous set-up for sludge reduction in WWTPs when the above-mentioned conditions are met. These conditions largely resemble the conditions in the aeration tanks of municipal WWTPs. In addition, the protein-rich worm biomass that is produced from the waste sludge has potential for re-use, because it can easily be separated from the sludge and contains low levels of copper, chromium, lead and nickel in comparison to the waste sludge. Finally, *L. variegatus* can be immobilized in a reactor set-up for sludge reduction with a complete separation between waste sludge and worm faeces. However, the feasibility of this process cannot be well assessed based on the data currently available. To be able to make a correct assessment is it necessary to get more detailed information about the following aspects:

- The dependence of sludge digestion rates, worm growth rates and worm yield on process conditions in a continuous set-up should be further investigated, as well as the maximum effective worm population density that can be maintained, the re-use possibilities for worm biomass and profits from it, the dewaterability of worm faeces, and the reduction of waste sludges from different origins and pre-digested waste sludges.
- In a feasibility study based on data from a continuous system, a comparison with other technologies for sludge reduction and energy recovery must be made. It is likely that sludge reduction with worms is especially suitable for smaller WWTPs without anaerobic sludge digesters, which have to transport their waste sludges for further processing.

╡Summary and conclusions╞

In WWTPs (wastewater treatment plants), large amounts of waste sludge are produced (**Chapter 1**). The costs for processing this sludge are estimated to be around half of the total costs of wastewater treatment. In the Netherlands, the final option is usually incineration, mainly because the application of sludge in agriculture or disposal in landfills is no longer allowed due to its high heavy metal content. Current technologies for sludge minimization involve chemical, physical, mechanical and biological technologies and combinations thereof. In addition, the recovery of materials and energy from sludge has a high priority. A biological technology for reducing the amount of produced sludge and simultaneous conversion into protein-rich biomass is sludge consumption by aquatic worms. Experimental results with this technology published by several research groups in the Netherlands, Japan and China are promising, but also highly variable. This was mainly caused by uncontrollable worm population growth. An additional problem was the lack of a reliable experimental set-up, which could exactly quantify sludge reduction by aquatic worms.

Both problems were addressed in the research described in this thesis, by studying the population dynamics of free-swimming aquatic worms Naidinae and Aeolosomatidae in WWTPs and by studying sludge reduction and worm growth in batch experiments with the sessile aquatic worms *Lumbriculus variegatus* and Tubificidae. Important factors for the application of worms (especially *L. variegatus*) in wastewater treatment were identified.

In **Chapter 2** an overview of the aquatic worms investigated in this thesis is presented, which includes their appearance, natural habitat, food, reproduction and use in sludge reduction research. Sessile Tubificidae and free-swimming Aeolosomatidae and Naidinae are common in WWTPs and are therefore commonly used in sludge reduction research, in contrast with *L. variegatus*. This overview shows that each of the described (sub)families has characteristics that can be advantageous or disadvantageous for application in sludge reduction processes. Logically, species with the most optimal combination of these characteristics under the conditions in WWTPs should therefore be applied. Alternatively, specific conditions in a separate worm reactor can also be optimized for the selected species.

To get more insight in the population dynamics of free-swimming worms in WWTPs, and to relate their presence to process characteristics and performance, these worms were sampled regularly over a 2.5-year period in the ATs (aeration tanks) of four Dutch WWTPs (**Chapter 3**). The species composition was limited. The most abundant worms were *Aeolosoma hemprichi*, *A. tenebrarum*, *A. variegatum*, *Chaetogaster diastrophus*, *Nais* spp., and *Pristina aequiseta*. Worms were present all year round, even in winter. The worm populations displayed peak periods, which lasted 2-3 months and were similar between the ATs of each WWTP, but no yearly recurrences of these periods were observed. The population doubling times in these periods were short, around 3-6 days, probably as a result of stable food supply and temperature, and the absence of predation from the WWTPs. The disappearance of worm populations from the WWTPs was presumably caused by declining asexual reproduction and subsequent removal with the sludge. Multivariate analysis indicated that 36 % of the variability in worm populations was only due to variations in sampled WWTP, sampling year and month. No more than 4 % of the variability in worm populations was related to variations in the available process characteristics. This dataset suggests that population growth of free-swimming aquatic worms is rather uncontrollable and that their effects on treatment performance (e.g. sludge settleability and production, nutrient removal) are unclear, which makes stable application in wastewater treatment for sludge reduction difficult.

The sessile species *L. variegatus* was selected for further investigation, because initial experiments with this species showed stable sludge reduction and worm growth (Buys, 2005; own data). Other advantages are its asexual reproduction by division (architomy) and the compact shape of its faeces, which can be distinguished from sludge with the naked eye.

In **Chapter 4**, a simple batch test with *L. variegatus* is described, that can accurately distinguish between sludge reduction by worms, sludge reduction by endogenous processes and worm growth. Sludge reduction by *L. variegatus* was approximately twice as fast as endogenous reduction of sludge, but the final sludge reduction percentage, which was usually around 50 %, was not affected. Around 16 % of the sludge TSS (total suspended solids) was digested by *L. variegatus* next to endogenous digestion. This equals around 19 % of the sludge VSS (volatile suspended solids), because most reduction concerned the organic fraction of the sludge. The test also showed that a minimum initial W/S ratio (ratio of worm to sludge dry matter) will be required for a noticeable effect of worms on sludge reduction. The exact ratio is however dependent on the endogenous activity of the sludge and was 0.4 in this case. Under the test conditions, 20-40 % of the total amount of digested sludge —the initial sludge amount minus the amount of worm faeces and possible residual sludge—was converted into worm biomass (organic matter based). Based on its sludge reduction rates and growth, *L. variegatus* shows high potential for application in wastewater treatment.

For effective application, it is essential to know if and how several sludge properties, worm properties and process conditions influence sludge digestion by *L. variegatus* and resulting worm growth. Therefore, sludge digestion, worm biomass growth and worm number growth rates, as well as the worm growth yield, were calculated in different short-term batch experiments, described in **Chapter 5**. In these experiments, *L. variegatus* consumed a variety of municipal sludges as well as non-municipal beer sludge. The municipal sludges from WWTPs and pilot-scale conventional and membrane bioreactor systems were digested at average rates of 0.09 (±0.04) d^{-1} (dry matter based) and worm biomass and number growth rates were on average 0.04 (±0.03) d^{-1} (dry matter based) and 0.01 (±0.02) d^{-1} respectively. On average 38 (±22) % of the sludge digested by worms was converted into worm biomass (dry matter based). The rates and yield on beer sludge were substantially lower. Moreover, for both municipal and non-municipal sludges, the overall rates and yields showed a high variability. However, a statistical analysis of the results with two of the most frequently used municipal sludges showed that this was only caused to a small extent by variations in experimental conditions, pH and ash percentage of the sludge. Therefore, most of the variability seemed to be caused by unknown differences in sludge composition. Experiments with different sludge floc size fractions indicated that *L. variegatus* is able to consume all floc sizes, even < 4.5 μm and > 300 μm, at comparable digestion and growth rates, as long as the sludge concentrations are high enough. Experiments with sterilized and pre-digested sludges suggested that a low content of live bacteria in the substrate suppresses worm reproduction. The experiments with pre-digested sludges (under oxic or anoxic conditions) also suggested that *L. variegatus* is able to increase the final reduction percentage (from 60 % to 68 %) of sludges that were pre-digested for at most 48 days under oxic conditions. However, this effect was not observed with older sludges or sludges that were pre-digested under anoxic conditions. In this latter experiment, worm growth was often negatively affected, most likely because of the presence of toxic un-ionized ammonia. Larger worm sizes seemed to enhance reproduction, as was expected, but the effect on sludge digestion and biomass growth was not clear. High population densities (> 39,000 specimens per m^2) and W/S ratios (> 1.4) negatively affected sludge digestion and worm growth in the batch experiments. The effects of ferric iron addition and complete darkness were tested, because literature data suggest that both factors possibly have a positive influence on worm growth and sludge digestion. The addition of ferric iron did not have an influence and incubation under complete dark conditions only seemed to enhance worm number growth slightly. Most importantly, *L. variegatus* thus seems applicable for different non-treated municipal sludges.

Sludge consumption by *L. variegatus* not only reduces the amount of sludge, but also changes the physical structure and most likely the composition of the sludge. Therefore, the influence of sludge consumption on different sludge characteristics was investigated in short-term batch experiments, described in **Chapter 6**. Sludge consumption by *L. variegatus* always enhanced the initial settling rate of several municipal sludges and led to SVI_{30} (sludge volume index after 30 min.) values of around 60 mL/ g. Even though the dewaterability of worm faeces was expected to be higher than that of the sludge, the CST (capillary suction time) method did not reveal any changes, but the results indicated that this method is not reliable. The turbidity of the sludge water phase increased, due to the formation of colloidal and/or dissolved materials. The experiments indicated that these materials possibly consist of carbohydrates, but not of proteins. After sludge consumption, the total protein fraction as percentage of sludge TSS usually decreased, which indicates specific feeding by *L. variegatus* on the protein fraction of sludge. This was not found for the carbohydrate fraction. Heavy metals (cadmium, chromium, copper, lead, nickel and zinc) from sludge were not bioaccumulated above concentrations already present in the worms and the latter concentrations were always substantially lower than in sludge. The heavy metal concentrations in worm faeces in contrast were higher than in the sludge, in this experiment 13(±9) %. The distribution of absolute heavy metal amounts over sludge flocs, water phase and worms did not seem to change after sludge consumption. Finally, faeces of *L. variegatus* were ingested by their own kind (conspecifics), but the digestion rates for this process were very low and worm biomass decreased. The most pronounced effects of sludge consumption by *L. variegatus* on sludge characteristics were thus the increased settling rates and settleability, an increase in heavy metal content and usually a decrease in protein content. The worm faeces seem refractive to further digestion.

To find out whether *L. variegatus* has more potential than sessile Tubificidae for application in sludge reduction processes, sludge digestion was compared between both worm types in batch experiments described in **Chapter 7**. It was also investigated if the application of mixed cultures of both worm types can be more advantageous than the application of monocultures. The experiments showed that sludge digestion and worm biomass growth were usually higher and more stable in monocultures of *L variegatus* than in ‘monocultures’ of sessile Tubificidae, but the number growth rates were comparable. Most of the latter results were also found with sludges containing higher TSS concentrations, except for decreasing sessile Tubificidae numbers. The advantage from using mixed cultures of both worm types resulted mainly from increased biomass growth rates of *L. variegatus*. The (combined) sludge digestion rate of mixed cultures was however equal to that of the monocultures of *L. variegatus* and it was not clear if mixed cultures enhanced the individual digestion rates. Both worm types were able to ingest worm faeces of conspecifics (which was already shown in Chapter 6 for *L. variegatus*) and intraspecifics, but digestion rates were low and biomass decreased. Digestion rates on intraspecific faeces were slightly higher, which suggests differences in digestive mechanisms between both worm types. Long-term monocultures of *L. variegatus* and sessile Tubificidae on sludges or on the control substrate Tetra Min® fish food contained similar concentrations of the six heavy metals mentioned above, regardless of the concentrations in these substrates. The bioaccumulation of cadmium and zinc was relatively high and concentrations in sludge and both worm types were similar for these two metals in contrast to the results of Chapter 6 for *L. variegatus*. This was possibly due to unknown differences in sludge composition or to differences in experiment duration. Most importantly, *L. variegatus* seems to have more potential for application in sludge reduction processes than sessile Tubificidae, but mixed cultures may have some advantages for worm growth. Besides, literature data indicate that mixed cultures also enhance the stability of worm populations.

For the application of *L. variegatus* for sludge reduction in wastewater treatment, a reactor set-up has to be developed. **Chapter 8** describes the results of an initial sequencing batch experiment in a reactor set-up, in which *L. variegatus* was immobilized in a mesh-like carrier material and sludge and worm faeces were separated.

A set-up with worms was compared to a control set-up. As in the batch experiments from the previous chapters, sludge consumption by *L. variegatus* led to a distinct decrease in the amount of sludge and increased settleability of the worm faeces. The obtained results show that the proposed reactor concept has a high potential for use in full-scale sludge processing. The reactor concept should be further optimized as comparisons with the results of other experiments show that sludge reduction percentages (on average 17 (±6) % for experiments in Chapter 5, but 75 % in this chapter) and other parameters of the process can be variable. This is probably dependent on the process conditions, for example the immobilization of the worms.

In **Chapter 9**, the results of the previous chapters are discussed with emphasis on the implications for the application of *L. variegatus* in wastewater treatment. Figures 9.1 & 9.2 summarize the results of a typical batch experiment with *L. variegatus* feeding on municipal sludge. These results (together with literature data) are discussed for each section of full-scale sludge treatment system with *L. variegatus* (Figure 9.3): sludge, reactor, worms, worm faeces, worm biomass and effluent. Finally, the overall feasibility of such a system is discussed and recommendations for further research are given.

As mentioned above, municipal sludges directly wasted from the ATs of WWTPs seem most suitable for digestion by *L. variegatus*, but sludges with a high protein content (e.g. from food-related industries) or certain pre-treated sludges (e.g. pre-digested) may also be suitable. The concentration of un-ionized ammonia may however never exceed 2 mg/ L and therefore anoxic conditions or high pH values should be prevented. An early warning system for this toxic compound is therefore necessary. *L. variegatus* should preferably be applied in a separate reactor with layers of carrier material, with a continuous flow of sludge or effluent and sufficient aeration to keep the oxygen concentrations in the medium surrounding their tails above 3-4 mg/ L. Additional heating may be necessary in cold periods, to maintain high digestion rates. Temperatures above 25 °C should be avoided. The application of a mixed culture with sessile Tubificidae may increase growth rates of *L. variegatus* and the overall stability of the worm population. The presence of large populations of predators (e.g. leeches) should be prevented. The W/S ratio will be dependent on the conditions (e.g. sludge supply) in a reactor, but can be regulated to some extent by frequent harvesting of part of the worms. A minimum initial W/S ratio will be required for a measurable effect on sludge digestion, dependent on the endogenous activity of the sludge used. The maximum and/or optimal worm densities that can be maintained in a continuous system are however still unknown parameters. The main characteristics of the structure and composition of the worm faeces that have to be disposed of are their decreased volume and their increased ash and heavy metal concentrations. In addition, the protein content is often lower. The dewaterability of the faeces is still to be determined, but is expected to be better than that of the sludge. The main characteristics of the produced worm biomass are its high protein content and the low concentrations of chromium, copper, lead and nickel in comparison to the sludge. The concentrations of cadmium and zinc are however sometimes similar to those in sludge. The content of these metals and other pollutants (e.g. organic micropollutants) has to be determined dependent on the anticipated application of *L. variegatus* biomass. Possible options for the whole biomass are aquarium fish food and fertilizer in agriculture. Alternatively, fractions of the biomass (e.g. amino acids or enzymes) can be recovered and re-used. The effluent of a worm reactor must probably be discharged in the WWTP, because it most likely contains higher concentrations of nutrients and suspended solids than regular effluents.

Calculations with the data obtained from this research indicate that a continuous system with *L. variegatus* has high potential for sludge reduction in wastewater treatment and the recovery of valuable materials. The ultimate feasibility will however not only be dependent on the reduced sludge processing costs. More important factors are the maximum effective worm population density that can be maintained (which determines the reactor costs), and the production, re-use possibilities and value of the produced worm biomass. These are all points for further investigation.

╡Samenvatting en conclusies╞

In RWZI's (rioolwaterzuiveringsinstallaties) worden grote hoeveelheden zuiveringsslib geproduceerd (**Hoofdstuk 1**). De verwerkingskosten voor dit slib bedragen ongeveer de helft van de totale kosten van het zuiveringsproces. In Nederland wordt het meeste slib uiteindelijk verbrand, omdat de toepassing als meststof in de landbouw en het storten niet meer zijn toegestaan vanwege een te hoog gehalte aan zware metalen. Om de productie van slib te verminderen worden chemische, fysische, mechanische, biologische of gecombineerde methodes ingezet. Ook het terugwinnen van materialen en energie uit slib heeft een hoge prioriteit. Een biologische methode om de slibproductie te reduceren en gelijktijdig het slib om te zetten in eiwitrijke biomassa is slibconsumptie door aquatische wormen. Resultaten van onderzoeksgroepen in Nederland, Japan en China met deze technologie zijn veelbelovend, maar ook erg variabel. Dit werd grotendeels veroorzaakt doordat de populatiegroei van de wormen niet gestuurd kon worden. Een bijkomend probleem was het ontbreken van een betrouwbare proefopzet waarmee de slibvertering door de wormen gekwantificeerd kon worden.

De experimenten die beschreven worden in dit proefschrift besteedden aandacht aan beide problemen: in RWZI's werd de populatiedynamica van vrijzwemmende aquatische wormen (Naidinae en Aeolosomatidae) bestudeerd en in batchexperimenten de slibvertering door sessiele aquatische wormen (*Lumbriculus variegatus* en Tubificidae). Tevens werd de groei van deze soorten bepaald. Belangrijke factoren voor het toepassen van de wormen in de afvalwaterzuivering werden met name voor de soort *L. variegatus* geïdentificeerd.

Hoofdstuk 2 beschrijft de morfologie, de habitat, het voedsel en de voortplanting van verschillende soorten aquatische wormen uit dit proefschrift evenals hun gebruik in slibreductie-onderzoek. Sessiele Tubificidae en vrijzwemmende Aeolosomatidae en Naidinae komen vaak voor in RWZI's en worden daarom het meest gebruikt voor onderzoek naar slibreductie in tegenstelling tot *L. variegatus*. Het overzicht liet zien dat elke (sub)familie kenmerken heeft die voordelig of nadelig kunnen zijn voor slibreductie. Logischerwijs zouden soorten met de meest optimale combinatie van deze kenmerken onder de specifieke condities in RWZI's toegepast moeten worden. Echter, de specifieke condities in een losstaande wormenreactor zouden ook geoptimaliseerd kunnen worden voor een geselecteerde wormensoort.

Om meer inzicht te krijgen in de populatiedynamica van vrijzwemmende wormen in RWZI's werden deze gedurende tweeënhalf jaar regelmatig geteld in de ATs (aëratietanks) van vier Nederlandse RWZI's (**Hoofdstuk 3**) en werden hun aantallen gekoppeld aan de proceskarakteristieken (procescondities en effectiviteit van het zuiveringsproces). De soortendiversiteit was beperkt. De meest voorkomende vrijzwemmende wormen waren *Aeolosoma hemprichi*, *A. tenebrarum*, *A. variegatum*, *Chaetogaster diastrophus*, *Nais* spp. en *Pristina aequiseta*. De wormen waren het hele jaar aanwezig, zelfs in de winter. De populaties vertoonden groeipieken van 2 tot 3 maanden, die vaak synchroon verliepen in de verschillende ATs van elke RWZI. Er werd geen jaarlijks patroon gevonden. De korte populatieverdubbelingstijden in deze periodes (ca. 3 tot 6 dagen) waren waarschijnlijk het gevolg van de stabiele voedselvoorziening en temperatuur en de afwezigheid van natuurlijke vijanden in de RWZI's. De oorzaak van het verdwijnen van de populaties uit de RWZI's was waarschijnlijk een afnemende ongeslachtelijke voortplanting, gevolgd door afvoer met het slib. Een multivariate analyse liet zien dat 36 % van de variatie in wormenpopulaties uitsluitend samenhing met de locatie van de RWZI, monsterjaar en -maand. Slechts 4 % van de variatie hing samen met de proceskarakteristieken, die door medewerkers van de RWZI's ter beschikking waren gesteld. De conclusie uit deze dataset is dat populatiegroei van vrijzwemmende aquatische wormen vrijwel oncontroleerbaar is en hun invloed op effectiviteit van het zuiveringsproces (zoals slibbezinking en –productie of nutriëntenverwijdering) onduidelijk, wat een stabiele toepassing voor slibreductie in afvalwaterzuivering bemoeilijkt.

De sessiele soort *L. variegatus* werd geselecteerd voor verder onderzoek, omdat deze in oriënterende experimenten een stabiele slibvertering en wormengroei vertoonde (Buys, 2005; eigen data). Verdere voordelen van deze soort zijn de ongeslachtelijke voortplanting door deling (architomie) en de compacte feces, die met het blote oog van slibvlokken onderscheiden kunnen worden.

Hoofdstuk 4 beschrijft een eenvoudig batchexperiment met *L. variegatus*, waarmee slibvertering door wormen, slibreductie door endogene processen in het slib en wormengroei onderscheiden kunnen wormen. Slibvertering door *L. variegatus* was in dit experiment ruwweg twee keer zo snel als de endogene slibverteringssnelheid, maar er was geen effect op het uiteindelijke reductiepercentage, dat meestal ca. 50 % van de droge stof was. *L. variegatus* verteerde ongeveer 16 % van de droge stof. De meeste vertering vindt plaats in de organische fractie van het slib, hetgeen resulteert in een hoger verteringspercentage op organische stof basis, ongeveer 19 %. Het experiment liet ook zien dat een minimale worm/slib verhouding (op droge stof basis) nodig is voor een merkbaar effect op de slibvertering. De precieze verhouding is echter afhankelijk van de endogene activiteit van het slib en was 0.4 in dit experiment. Ongeveer 20-40 % van het totaal verteerde slib —de beginhoeveelheid slib minus de wormenfeces en het eventuele restslib— werd omgezet in wormenbiomassa op organische stof basis. Gebaseerd op de versnelling van de slibvertering en de groeisnelheden, is *L. variegatus* een veelbelovende soort voor toepassing in afvalwaterzuivering .

Voor een effectieve toepassing van *L. variegatus* is het noodzakelijk te weten of en hoe slibvertering en wormengroei beïnvloed worden door verschillende slibeigenschappen, wormeigenschappen en procescondities. **Hoofdstuk 5** beschrijft daarom verschillende kortdurende batchexperimenten, waaruit snelheden van slibvertering, wormengroei (in biomassa en aantallen) en wormenopbrengst berekend werden. *L. variegatus* consumeerde diverse slibsoorten, zowel van communale (uit

RWZI's of conventionele en membraan bioreactorsystemen op laboratoriumschaal) als industriële oorsprong (uit een zuivering van een bierbrouwerij). De communale slibsoorten werden verteerd met een gemiddelde snelheid van 0.09 (±0.04) d^{-1} (op droge stof basis). Wormenbiomassa en –aantal namen gemiddeld toe met respectievelijk 0.04 (±0.03) d^{-1} (op droge stof basis) en 0.01 (±0.02) d^{-1}. Gemiddeld werd 38 (±22) % van het door wormen verteerde slib omgezet in wormenbiomassa (op droge stof basis). Voor het bierslib waren deze getallen aanmerkelijk lager. Voor alle slibsoorten waren de snelheden en wormenopbrengsten bovendien erg variabel. Een statistische analyse van de resultaten met de twee meest gebruikte slibsoorten gaf aan dat deze variatie maar voor een klein deel samenhing met verschillen in de experimentele condities, pH en asgehalte van het slib. Het merendeel van de variatie leek veroorzaakt te worden door onbekende verschillen in slibsamenstelling. Experimenten met slibfracties van verschillende vlokgroottes lieten zien dat *L. variegatus* alle vlokgroottes (zelfs < 4.5 μm en >300 μm) kan opnemen. Vlokgrootte lijkt geen invloed te hebben op de verterings- en groeisnelheden, mits de slibconcentraties hoog genoeg zijn. Experimenten met gesteriliseerde en voorverteerde slibsoorten deden vermoeden dat een lage concentratie levende bacteriën in het voedsel de voortplanting van de wormen onderdrukt. De experimenten met voorverteerde slibsoorten gaven verder aan dat *L. variegatus* het uiteindelijke reductiepercentage (van 60 % tot 68 %) kan verhogen indien het slib maximaal 48 dagen voorverteerd was onder beluchte condities. Bij langere periodes was er geen verhoging meer en het werd ook niet waargenomen bij anoxisch voorverteerde slibsoorten. In dit laatste experiment werd de wormengroei negatief beïnvloed, waarschijnlijk door de aanwezigheid van het toxische ongeïoniseerde ammonia. Zoals verwacht leek de voortplantingsnelheid toe te nemen met de wormengrootte, maar het effect hiervan op slibvertering en biomassagroei was niet duidelijk. Hoge populatiedichtheden (> 39.000 wormen per m^2) en worm/slib verhoudingen (> 1.4) hadden een negatief effect op de slibvertering en wormengroei. De effecten van Fe^{3+} toevoeging en volledige duisternis werden getest, aangezien literatuurgegevens suggereren dat beiden een positieve invloed zouden kunnen hebben op wormengroei en slibvertering. Het toevoegen van Fe^{3+} had geen invloed en volledige duisternis leek alleen de voortplantingsnelheid licht te verhogen. De hoofdconclusie is dat *L. variegatus* toepasbaar lijkt voor een brede variatie van onbehandelde communale slibsoorten.

Consumptie van slib door *L. variegatus* vermindert niet alleen de hoeveelheid slib, maar verandert ook de structuur en hoogstwaarschijnlijk ook de samenstelling van het slib. **Hoofdstuk 6** beschrijft kortdurende batchexperimenten, waarin de invloed van slibconsumptie op verschillende slibeigenschappen onderzocht werd. Slibconsumptie door *L. variegatus* verhoogde altijd de initiële bezinkingssnelheid van verschillende communale slibsoorten en resulteerde in SVI_{30} (slib volume index na 30 minuten) waarden van ongeveer 60 mL/g. Hoewel verwacht werd dat de ontwaterbaarheid van wormenfeces beter was dan die van slib, liet de CST (capillaire suctie tijd) methode geen veranderingen zien. De resultaten gaven echter aan dat deze methode onbetrouwbaar is. De troebelheid van de waterfase van het slib nam toe door het vrijkomen van zwevend en/of opgelost materiaal, dat mogelijk uit koolhydraten, maar niet uit eiwitten bestaat. Meestal daalde het totale eiwit als percentage van de droge stof na slibconsumptie, wat erop zou kunnen wijzen dat *L. variegatus* zich specifiek voedt met de eiwitfractie van slib. Dit werd niet gevonden voor de koolhydraatfractie. Zware metalen (cadmium, chroom, koper, lood, nikkel en zink) uit slib werden niet opgehoopt boven concentraties die reeds aanwezig waren in de wormen. Laatstgenoemde concentraties waren altijd veel lager dan in slib. De concentraties metalen in de wormenfeces waren daarentegen hoger dan in het slib, in dit experiment 13(±9) %. De verdeling van de absolute hoeveelheden metalen over slibvlokken, waterfase en wormen leek niet te veranderen na slibconsumptie. Ten slotte werden feces van *L. variegatus* weliswaar opgenomen door soortgenoten, maar de verteringssnelheden tijdens dit proces waren erg laag en de biomassa van de wormen nam af. De belangrijkste effecten van slibconsumptie door *L. variegatus* op slibeigenschappen zijn dus de verhoogde bezinkingssnelheden en bezinkbaarheid, verhoogde concentraties zware metalen en meestal verlaagde eiwitconcentraties. De wormenfeces lijken amper verder verteerbaar te zijn.

Hoofdstuk 7 beschrijft batchexperimenten, waarin de slibvertering vergeleken werd tussen *L. variegatus* en sessiele Tubificidae om vast te stellen welke soort meer potentie heeft voor slibreductie. Ook werd onderzocht of de toepassing van mengpopulaties van beide typen wormen voordelen zou kunnen hebben boven de toepassing van monoculturen. De slibvertering en wormenbiomassagroei waren meestal hoger en stabieler in monoculturen van *L. variegatus* dan in 'monoculturen' van sessiele Tubificidae. De groei in aantallen wormen was vergelijkbaar voor beide types. Voorgaande resultaten waren vrijwel identiek voor slibsoorten met hogere concentraties droge stof, behalve dat de aantallen Tubificidae afnamen. Het voordeel van mengpopulaties uitte zich voornamelijk in de toegenomen biomassagroei van *L. variegatus*. De (gecombineerde) slibverteringssnelheid van mengpopulaties was echter gelijk aan die van monoculturen van *L. variegatus* en het was niet duidelijk of gemengde populaties de individuele verteringssnelheden bevorderden. Beide typen wormen waren in staat om wormenfeces van hun soortgenoten (wat al eerder beschreven was voor *L. variegatus* in Hoofdstuk 6) en het andere type worm op te nemen, maar de verteringssnelheden tijdens dit proces waren laag en de wormenbiomassa nam af. Verteringssnelheden op feces van het andere type worm waren iets hoger, wat zou kunnen duiden op verschillen in verteringmechanismen van beide typen wormen. Monoculturen van *L. variegatus* en sessiele Tubificidae die langdurig met slib of het controlevoedsel Tetra Min® visvoer waren gevoed bevatten vergelijkbare concentraties van de zes eerder genoemde zware metalen, ongeacht de concentraties in deze twee voedselbronnen. In tegenstelling tot de resultaten met *L. variegatus* in Hoofdstuk 6, was de ophoping van cadmium en zink relatief hoog en de concentraties in slib en beide typen wormen waren vergelijkbaar. Dit kwam mogelijk door onbekende verschillen in slibsamenstelling of verschillen in experimentele duur.

De hoofdconclusie is dat *L. variegatus* meer potentieel lijkt te hebben voor toepassing in slibreductie dan sessiele Tubificidae, maar mengpopulaties hebben mogelijk voordelen voor groei van de wormenpopulatie. Literatuurdata geven bovendien aan dat mengcultures ook de stabiliteit van de populatie kunnen bevorderen.

Om *L. variegatus* te kunnen toepassen voor slibreductie in de afvalwaterzuivering is een reactorconfiguratie nodig. **Hoofdstuk 8** beschrijft de resultaten van een oriënterend serieel batchexperiment met een prototype voor een reactorconfiguratie, waarin *L. variegatus* gepositioneerd is in een net-achtige drager, en slib en wormenfeces gescheiden worden. Een prototype met wormen werd vergeleken met een prototype zonder wormen. Zoals in de batchexperimenten uit de voorgaande hoofdstukken leidde slibconsumptie door *L. variegatus* duidelijk tot een afname van de hoeveelheid slib en een toegenomen bezinkbaarheid van de wormenfeces. Dit geeft aan dat het prototype veelbelovend is voor slibreductie. Dit prototype zal geoptimaliseerd moeten worden, omdat vergelijkingen met andere experimentele resultaten aangaven dat slibverteringspercentages (gemiddeld 17 (±6) % in Hoofdstuk 5 t.o.v. 75 % in dit hoofdstuk) en andere parameters van het proces variabel zijn. Dit is waarschijnlijk afhankelijk van procescondities zoals het positioneren van de wormen.

In **Hoofdstuk 9** worden de resultaten van de voorgaande hoofdstukken besproken met nadruk op de gevolgen voor de toepassing van *L. variegatus* in afvalwaterzuivering. Figuren 9.1 & 9.2 vatten de resultaten samen van een typisch batchexperiment waarin *L. variegatus* gevoed wordt met communaal slib. Deze resultaten (samen met literatuurgegevens) worden besproken voor elke sectie van een grootschalig systeem voor slibreductie met *L. variegatus* (Figuur 9.3): slib, reactor, wormen, wormenfeces, wormenbiomassa en effluent. Ten slotte wordt de haalbaarheid van een dergelijk systeem besproken en worden aanbevelingen voor verder onderzoek gedaan.

Zoals boven vermeld, lijken communale slibsoorten rechtstreeks afkomstig uit de ATs van RWZI's het meest geschikt voor vertering door *L. variegatus*, maar slibsoorten met een hoog eiwitgehalte (zoals van voedselverwerkende industrieën) of voorbehandelde slibsoorten (bijv. voorverteerd) zouden ook geschikt kunnen zijn. De concentratie ongeïoniseerd ammonia mag echter nooit hoger zijn dan 2 mg/L en mede daarom moeten anoxische condities en hoge pH waarden vermeden worden. Een waarschuwingssysteem voor deze verbinding is daarom noodzakelijk. *L. variegatus* kan het best toegepast worden in een afzonderlijke reactor met lagen dragermateriaal, een voortdurende stroom slib of effluent en voldoende beluchting om de zuurstofconcentratie rond hun staarten boven 3 tot 4 mg/L te houden. In koudere perioden zou verwarming nodig kunnen zijn om hoge slibverteringssnelheden te handhaven, maar temperaturen boven 25 °C moeten vermeden worden. De toepassing van een mengpopulatie met sessiele Tubificidae zou de groeisnelheden van *L. variegatus* kunnen verhogen, evenals de totale stabiliteit van de wormenpopulatie. De aanwezigheid van grote populaties natuurlijke vijanden, zoals bloedzuigers, moet voorkomen worden. De worm/slib verhouding zal afhankelijk zijn van de condities in een reactor (zoals slibtoevoer), maar zou wellicht bijgestuurd kunnen worden door regelmatig een deel van de wormen te oogsten. Een minimale initiële worm/slib verhouding is nodig voor een meetbaar effect op slibreductie, afhankelijk van de endogene activiteit van het gebruikte slib. De maximale en/of optimale wormendichtheden die in een continu systeem gehouden kunnen worden zijn echter nog onbekende parameters. De belangrijkste eigenschappen van de structuur en samenstelling van de wormenfeces zijn het afgenomen volume, de toegenomen concentraties as en zware metalen en waarschijnlijk ook een lagere eiwitconcentratie. De ontwaterbaarheid van de feces moet nog bepaald worden maar het vermoeden bestaat dat deze beter is dan die van het slib. De belangrijkste eigenschappen van de geproduceerde wormenbiomassa zijn de hoge eiwitconcentraties en de lage concentraties chroom, koper, lood en nikkel in vergelijking met slib. De concentraties cadmium en zink zijn echter soms vergelijkbaar met die in slib. Afhankelijk van de verwachte toepassing van de wormenbiomassa zullen de concentraties van deze metalen en andere verontreinigingen zoals organische microverontreinigingen bepaald moeten worden. Mogelijke opties voor het totale wormenmateriaal zijn aquariumvisvoer en meststof in de landbouw. Anderzijds kunnen bepaalde fracties uit het materiaal (zoals aminozuren of enzymen) geïsoleerd en hergebruikt worden. Het effluent van een wormenreactor moet waarschijnlijk geloosd worden in de RWZI, aangezien het waarschijnlijk hogere concentraties nutriënten en zwevende stof bevat dan reguliere effluenten.

Berekeningen met de data uit dit onderzoek laten zien dat de toepassing van de aquatische wormensoort *L. variegatus* in afvalwaterzuivering voor het verminderen van de slibproductie en het terugwinnen van waardevolle componenten potentie heeft. De uiteindelijke haalbaarheid zal echter niet alleen afhankelijk zijn van de lagere slibverwerkingskosten. Minstens zo belangrijke factoren zijn de maximaal effectieve wormendichtheid die aan te houden is (welke de reactorkosten bepaalt), evenals de productie, hergebruiksmogelijkheden en waarde van de geproduceerde wormenbiomassa. Dit zijn alle punten voor verder onderzoek.

A

Alam, M. Z. & A. Fakhru'l-Razi (2003). Enhanced settleability and dewaterability of fungal treated domestic wastewater sludge by liquid state bioconversion process. *Water Research* **37**: 1118-1124.

Alsterberg, G. (1922). Die respiratorischen Mechanismen der Tubificiden. Eine experimentell-physiologische Untersuchung auf ökologischer Grundlage. Lund, Hăkan Ohlssons Buchdrückerei (in German).

Alsterberg, G. (1924). Die Sinnesphysiologie der Tubificiden. Eine experimentelle Untersuchung mit besonderer Berücksichtigung der respiratorischen Mechanismen. Lund, Hăkan Ohlssons Buchdrückerei (in German).

Alvarenga, P., P. Palma, A. P. Gonçalves, R. M. Fernandes, A. C. Cunha-Queda, E. Duarte & G. Vallini (2007). Evaluation of chemical and ecotoxicological characteristics of biodegradable organic residues for application to agricultural land. *Environment International* **33**: 505-513.

Andreottola, G. & P. Foladori (2006). A review and assessment of emerging technologies for the minimization of excess sludge production in wastewater treatment plants. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering* **41**: 1853-1872.

Ankley, G. T. (1996). Evaluation of metal/acid-volatile sulfide relationships in the prediction of metal bioaccumulation by benthic macroinvertebrates. *Environmental Toxicology and Chemistry* **15**: 2138-2146.

Ankley, G. T., R. J. Erickson, B. R. Sheedy, P. A. Kosian, V. R. Mattson & J. S. Cox (1997). Evaluation of models for predicting the phototoxic potency of polycyclic aromatic hydrocarbons. *Aquatic Toxicology* **37**: 37-50.

Ankley, G. T., E. N. Leonard & V. R. Mattson (1994). Prediction of bioaccumulation of metals from contaminated sediments by the oligochaete *Lumbriculus variegatus*. *Water Research* **28**: 1071-1076.

APHA (1998). Standard methods for the examination of water and wastewater. 20th edition. Washington, DC, American Public Health Association.

Appleby, A. G. & R. O. Brinkhurst (1970). Defecation rate of three tubificid oligochaetes found in the sediment of Toronto Harbour, Ontario. *Journal of the Fisheries Research Board of Canada* **27**: 1971-1982.

Aquatic Foods (2007). 'Aquatic Foods' webpage, 2007. www.aquaticfoods.com.

Aston, R. J. (1973). Tubificids and water quality: a review. *Environmental Pollution* **5**: 1-10.

Aston, R. J. & A. G. P. Milner (1981). Conditions required for the culture of *Branchiura sowerbyi* (Oligochaeta: Tubificidae) in activated sludge. *Aquaculture* **26**: 155-160.

B

Back, H. (1990). Epidermal uptake of lead cadmium and zinc in tubificid worms. *Oecologia Berlin* **85**: 226-232.

Back, H. & F. Prosi (1985). Distribution of inorganic cations In *Limnodrilus udekemianus* Oligochaeta Tubificidae using laser induced microprobe mass analysis with special emphasis on heavy metals. *Micron and Microscopica Acta* **16**: 145-150.

Bailey, H. C. & D. H. W. Liu (1980). *Lumbriculus variegatus*, a benthic oligochaete, as a bioassay organism. In Proceedings of: Aquatic Toxicology and Hazard Assessment, 3rd Symposium, ASTM STP 707, Philadelphia,

Bajsa, O., J. Nair, K. Mathew & G. E. Ho (2003). Vermiculture as a tool for domestic wastewater management. *Water Science and Technology* **48**: 125-132.

Bauer-Hilty, A., R. Dallinger & B. Berger (1989). Isolation and partial characterization of a cadmium-binding protein from *Lumbriculus variegatus* (Oligochaeta, Annelida). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* **94**: 373-379.

Bell, G. (1984). Evolutionary and nonevolutionary theories of senescence. *American Naturalist* **124**: 600-603.

Berezina, N. A. (2001). Influence of ambient pH on freshwater invertebrates under experimental conditions. *Russian Journal of Ecology* **32**: 343-351.

Bervoets, L., R. Blust, M. de Wit & R. Verheyen (1997). Relationships between river sediment characteristics and trace metal concentrations in tubificid worms and chironomid larvae. *Environmental Pollution* **95**: 345-356.

Bischoff, A. A. (2007). Solid waste reduction of closed recirculated aquaculture systems by secondary culture of detritivorous organisms. PhD thesis, Christian Albrechts University, Kiel, Germany.

Boehler, M. & H. Siegrist (2006). Potential of activated sludge disintegration. *Water Science and Technology* **53**: 207-216.

Borcard, D., P. Legendre & P. Drapeau (1992). Partialling out the spatial component of ecological variation. *Ecology* **73**: 1045-1055.

Bouché, M.-L., F. Habets, S. Biagianti-Risbourg & G. Vernet (2000). Toxic effects and bioaccumulation of cadmium in the aquatic oligochaete *Tubifex tubifex*. *Ecotoxicology and Environmental Safety* **46**: 246-251.

Bowker, D. W., M. T. Wareham & M. A. Learner (1985). The ingestion and assimilation of algae by *Nais elinguis* (Oligochaeta: Naididae). *Oecologia* **67**: 282-285.

Boyer, R. F. (1993). Modern experimental biochemistry. 2nd edition. Redwood City, California, The Benjamin/Cumming Publishing Co. Inc.

Brinkhurst, R. O. (1971). A guide for the identification of British Aquatic Oligochaeta. 2nd revised edition. Ambleside, Freshwater Biological Association.

Brinkhurst, R. O. (1974). Factors mediating interspecific aggregation of tubificid oligochaetes. *Journal of the Fisheries Research Board of Canada* **31**: 460-462.

Brinkhurst, R. O. (1996). On the role of tubificid oligochaetes in relation to fish disease with special reference to the myxozoa. *Annual Review of Fish Diseases* **6**: 29-40.

Brinkhurst, R. O. & M. J. Austin (1979). Assimilation by aquatic Oligochaeta. *Internationale Revue der gesamten Hydrobiologie* **64**: 245-250.

Brinkhurst, R. O. & K. E. Chua (1969). Preliminary investigation of the exploitation of some potential nutritional resources by three sympatric tubificid oligochaetes. *Journal of the Fisheries Research Board of Canada* **26**: 2659-2668.

Brinkhurst, R. O., K. E. Chua & N. K. Kaushik (1972). Interspecific interactions and selective feeding by tubificid oligochaetes. *Limnology and Oceanography* **17**: 122-133.

Brinkhurst, R. O. & B. G. M. Jamieson (1971). Aquatic Oligochaeta of the world. Edinburgh, Oliver and Boyd.

Brinkhurst, R. O. & C. R. Kennedy (1965). Studies on the biology of the Tubificidae (Annelida, Oligochaeta) in a polluted stream. *Jounal of Animal Ecology* **34**: 429-443.

Brodersen, K. P., O. Pedersen, C. Lindegaard & K. Hamburger (2004). Chironomids (Diptera) and oxy-regulatory capacity: An experimental approach to paleolimnological interpretation. *Limnology and Oceanography* **49**: 1549-1559.

Bukuru, G. & Y. Jian (2005). Synchronous municipal sewerage-sludge stabilization. *Journal of Environmental Sciences* **17**: 59-61.

Buys, B. R. (2005). Personal communication. Sub-department of Environmental Technology, Wageningen University, the Netherlands.

C

Cardosa, V. L. & C. E. Ramirez (2002). Vermicomposting of sewage sludge: A new technology for Mexico. *Water Science and Technology* **46**: 153-158.

Cetin, S. & A. Erdincler (2004). The role of carbohydrate and protein parts of extracellular polymeric substances on the dewaterability of biological sludges. *Water Science and Technology* **50**: 49-56.

Chapman, K. K., M. J. Benton, R. O. Brinkhurst & P. R. Scheuerman (1999). Use of the aquatic oligochaetes *Lumbriculus variegatus* and *Tubifex tubifex* for assessing the toxicity of copper and cadmium in a spiked-artificial-sediment toxicity test. *Environmental Toxicology* **14**: 271-278.

Chapman, P. M. & R. O. Brinkhurst (1984). Lethal and sublethal tolerances of aquatic oligochaetes with reference to their use as a biotic index of pollution. *Hydrobiologia* **115**: 139-144.

Chapman, P. M., M. A. Farrell & R. O. Brinkhurst (1982a). Relative tolerances of selected aquatic oligochaetes to combinations of pollutants and environmental factors. *Aquatic Toxicology* **2**: 69-78.

Chapman, P. M., M. A. Farrell & R. O. Brinkhurst (1982b). Effects of species interactions on the survival and respiration of *Limnodrilus hoffmeisteri* and *Tubifex tubifex* (Oligochaeta, Tubificidae) exposed to various pollutants and environmental factors. *Water Research* **16**: 1405-1408.

Christensen, B. (1980). Animal Cytogenetics. Volume 2. Annelida. Berlin, Stuttgart, Gebrueder Borntraeger.

Christensen, B. (1984). Asexual propagation and reproductive strategies in aquatic Oligochaeta. *Hydrobiologia* **115**: 91-95.

Ciutat, A., M. Gerino, N. Mesmer-Dudons, P. Anschutz & A. Boudou (2005). Cadmium bioaccumulation in Tubificidae from the overlying water source and effects on bioturbation. *Ecotoxicology and Environmental Safety* **60**: 237-246.

Coenen, J., M. van Gastel & K. de Jong (2005). Potentieel voor duurzame energie met biogas uit rioolwaterzuiveringen. STOWA, the Netherlands. Report 2005-wO3.

Conrad, A. U., S. D. Comber & K. Simkiss (2002). Pyrene bioavailability; effect of sediment-chemical contact time on routes of uptake in an oligochaete worm. *Chemosphere* **49**: 447-454.

Cook, D. G. (1969). Observations on the life history and ecology of some Lumbriculidae (Annelida, Oligochaeta). *Hydrobiologia* **34**: 561-574.

Cross, W. H. (1976). A study of predation rates of leeches on tubificid worms under laboratory conditions. *Ohio Journal of Science* **76**: 164-166.

Curds, C. R. & H. A. Hawkes (1975). Ecological aspects of used-water treatment. Volume 1 - The organisms and their ecology. London, Academic Press Inc.

Curry, J. P. & O. Schmidt (2007). The feeding ecology of earthworms - A review. *Pedobiologia* **50**: 463-477.

D

Dajoz, R. (1977). Introduction to ecology. London, Hodder and Stoughton.

Dawson, T. D., K. G. Lott, E. N. Leonard & D. R. Mount (2003). Time course of metal loss in *Lumbriculus variegatus* following sediment exposure. *Environmental Toxicology and Chemistry* **22**: 886-889.

De Boer, G. F. & O. Sova (1998). Vermi-composting as a resource for biodegradable detergents. *Concept article for the 4th International Congress on Zero Emissions Research and Initiatives (ZERI), Namibia, October 14-17, 1998.*

De Jong, K. (2007). Zelf zuiveringsslib verwerken wordt aantrekkelijk. *Utilities* **4**: 20-23 (in Dutch).

Degn, H. & B. Kristensen (1981). Low sensitivity of *Tubifex* sp respiration to hydrogen sulfide and other inhibitors. *Comparative Biochemistry and Physiology B* **69**: 809-818.

Delmotte, S., F. J. R. Meysman, A. Ciutat, A. Boudou, S. Sauvage & M. Gerino (2007). Cadmium transport in sediments by tubificid bioturbation: An assessment of model complexity. *Geochimica et Cosmochimica Acta* **71**: 844-862.

Densem, J. W. (1982). Growth studies of *Tubifex tubifex* (Oligochaeta: Tubificidae) on sewage sludge. PhD thesis, University of Wales, UK.

Dermott, R. & M. Munawar (1992). A simple and sensitive assay for evaluation of sediment toxicity using *Lumbriculus variegatus* (Müller). *Hydrobiologia* **235-236**: 407-414.

Drewes, C. D. (1999). Helical swimming and body reversal behaviors in *Lumbriculus variegatus* (Annelida: Clitellata: Lumbriculidae). *Hydrobiologia* **406**: 263-269.

Drewes, C. D. (2005). 'Invertebate biology, *Lumbriculus variegatus*, Neurobiology: Educational materials and resources' web page, 2007. http://www.eeob.iastate.edu/faculty/DrewesC/htdocs.

Drewes, C. D. & C. R. Fourtner (1989). Hindsight and rapid escape reflex in a freshwater oligochaete. *Biological Bulletin* **177**: 363-371.

Drewes, C. D. (2004). Personal communication. Iowa State University, US.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers & E. Swith (1956). Colorimetric method for determination of sugar and related substances. *Analytical Chemistry* **28**: 350-356.

E

Egeler, P., M. Meller, J. Roembke, P. Spoerlein, B. Streit & R. Nagel (2001). *Tubifex tubifex* as a link in food chain transfer of hexachlorobenzene from contaminated sediment to fish. *Hydrobiologia* **463**: 171-184.

Elissen, H. J. H., H. Temmink & C. J. N. Buisman (2007). Werkwijze voor het vrijmaken van aquatische wormen uit een drager en predatiereactor. Wetsus, Centre for Sustainable Water Technology, the Netherlands. *Patent application* NL1032621 *to be published in April 2008* (in Dutch).

Erseus, C., M. Kallersjo, M. Ekman & R. Hovmoller (2002). 18S rDNA phylogeny of the Tubificidae (Clitellata) and its constituent taxa: Dismissal of the Naididae. *Molecular Phylogenetics and Evolution* **22**: 414-422.

Eskicioglu, C., K. J. Kennedy & R. L. Droste (2006). Characterization of soluble organic matter of waste activated sludge before and after thermal pretreatment. *Water Research* **40**: 3725-3736.

Evangelista, A. D., N. R. Fortes & C. B. Santiago (2005). Comparison of some live organisms and artificial diet as feed for Asian catfish *Clarias macrocephalus* (Günther) larvae. *Journal of Applied Ichthyology* **21**: 437-443.

F

Finogenova, N. P. & T. M. Lobasheva (1987). Growth of *Tubifex tubifex* Müller (Oligochaeta, Tubificidae) under various trophic conditions. *Internationale Revue der gesamten Hydrobiologie* **72**: 709-726.

Fisher, S. W., S. W. Chordas III & P. F. Landrum (1999). Lethal and sublethal body residues for PCB intoxication in the oligochaete, *Lumbriculus variegatus*. *Aquatic Toxicology* **45**: 115-126.

Forster, C. F. (1971). Activated sludge surfaces in relation to the sludge volume index. *Water Research* **5**: 861-870.

Frederickson, J. & G. Howell (2003). Large-scale vermicomposting: emission of nitrous oxide and effects of temperature on earthworm populations. *Pedobiologia* **47**: 724-730.

Frossard, P. (1982). The erythrocruorin of *Eisenia fetida*. I. Properties and subunit structure. *Biochimica et Biophysica Acta* **704**: 524-534.

G

Gardner, W. S., T. F. Nalepa, M. A. Quigley & J. M. Malczyk (1981). Release of phosphorus by certain benthic invertebrates. *Canadian Journal of Fisheries and Aquatic Sciences* **38**: 978-981.

Garg, P., A. Gupta & S. Satya (2006). Vermicomposting of different types of waste using *Eisenia foetida*: A comparative study. *Bioresource Technology* **97**: 391-395.

Gaskell, P. N., A. C. Brooks & L. Maltby (2007). Variation in the bioaccumulation of a sediment-sorbed hydrophobic compound by benthic macroinvertebrates: Patterns and mechanisms. *Environmental Science and Technology* **41**: 1783-1789.

Gnaiger, E. & I. Staudigl (1987a). Aerobic metabolism and physiological responses of aquatic oligochaetes to environmental anoxia: heat dissipation, oxygen consumption, feeding and defecation. *Physiological Zoology* **60**: 659-677.

Gnaiger, E., R. Kaufmann & I. Staudigl (1987b). Physiological reactions of aquatic oligochaetes to environmental anoxia. *Hydrobiologia* **155**: 155.

Gujer, W., M. Henze, T. Mino & M. van Loosdrecht (1999). Activated sludge model no. 3. *Water Science and Technology* **39**: 183-193.

Gunn, A. M., D. T. E. Hunt & D. A. Winnard (1989). The effect of heavy metal speciation in sediment on bioavailability to tubificid worms. *Hydrobiologia* **188-189**: 487-496.

Guo, X.-s., J.-x. Liu, Y.-s. Wei & L. Li (2007). Sludge reduction with Tubificidae and the impact on the performance of the wastewater treatment process. *Journal of Environmental Sciences* **19**: 257-263. *Full article not yet available.*

H

Hansen, J. A., J. Lipton, P. G. Welsh, D. Cacela & B. MacConnell (2004). Reduced growth of rainbow trout (*Oncorhynchus mykiss*) fed a live invertebrate diet pre-exposed to metal-contaminated sediments. *Environmental Toxicology and Chemistry* **23**: 1902-1911.

Harper, R. M., J. C. Fry & M. A. Learner (1981a). A bacteriological investigation to elucidate the feeding biology of *Nais variabilis* (Oligochaeta: Naididae). *Freshwater Biology* **11**: 227-236.

Harper, R. M., J. C. Fry & M. A. Learner (1981b). Digestion of bacteria by *Nais variabilis* (Oligochaeta) as established by autoradiography. *Oikos* **36**: 211-218.

Hartenstein, R., D. L. Kaplan & E. F. Neuhauser (1984). Earthworms and trickling filters. *Journal of the Water Pollution Control Federation* **56**: 294-298.

Hawkes, H. A. & M. R. N. Shephard (1972). The effect of dosing frequency on the seasonal fluctuations and vertical distribution of solids and grazing fauna in sewage percolating filters. *Water Research* **6**: 721-730.

Hendrickx, T. L. G. (2007). Personal communication. Sub-department of Environmental Technology, Wageningen University, the Netherlands.

Hendrickx, T. L. G., H. Temmink, C. J. N. Buisman & H. J. H. Elissen (2006). Sludge predation using aquatic worms. In Proceedings of: IWA Specialized Conference - Sustainable Sludge Management: State of the Art, Challenges and Perspectives, Moscow, Russia, 2006.

Hessling, R. & G. Purschke (2000). Immunohistochemical (cLSM) and ultrastructural analysis of the central nervous system and sense organs in *Aeolosoma hemprichi* (Annelida, Aeolosomatidae). *Zoomorphology* **120**: 65-78.

Hornor, S. G. & M. J. Mitchell (1981). Effect of the earthworm, *Eisenia foetida* (Oligochaeta), on fluxes of volatile carbon and sulfur compounds from sewage sludge. *Soil Biology & Biochemistry* **13**:

Hospido, A., M. T. Moreira, M. Martín, M. Rigola & G. Feijoo (2005). Environmental evaluation of different treatment processes for sludge from urban wastewater treatments: Anaerobic digestion versus thermal processes. *International Journal of Life Cycle Assessment* **10**: 336-345.

Huang, X., P. Liang & Y. Qian (2007). Excess sludge reduction induced by *Tubifex tubifex* in a recycled sludge reactor. *Journal of Biotechnology* **127**: 443-451.

Hung, W. T., I. L. Chang, W. W. Lin & D. J. Lee (1996). Unidirectional freezing of waste-activated sludges: Effects of freezing speed. *Environmental Science and Technology* **30**: 2391-2396.

I

Inamori, Y., Y. Kuniyasu, N. Hayashi, H. Ohtake & R. Sudo (1990). Monoxenic and mixed cultures of the small metazoa *Philodina erythrophthalma* and *Aeolosoma hemprichi* isolated from a waste-water treatment process. *Applied Microbiology and Biotechnology* **34**: 404-407.

Inamori, Y., Y. Kuniyasu & R. Sudo (1987). Role of smaller metazoa in water purification and sludge reduction. *Japanese Journal of Water Treatment Biology* **23**: 12-23 (in Japanese with English abstract).

Inamori, Y., R. Suzuki & R. Sudo (1983). Mass culture of small aquatic oligochaeta. *Research report from the National Institute for Environmental Studies* **47**: 125-137 (translated from Japanese).

Ingersoll, C. G., E. L. Brunson, N. Wang, F. J. Dwyer, G. T. Ankley, D. R. Mount, J. Huckins, J. Petty & P. F. Landrum (2003). Uptake and depuration of nonionic organic contaminants from sediment by the oligochaete *Lumbriculus variegatus*. *Environmental Toxicology and Chemistry* **22**: 872-885.

Ingersoll, C. G., G. A. Burton, T. D. Dawson, F. J. Dwyer, D. S. Ireland, N. E. Kemble, D. R. Mount, T. J. Norberg-King, P. K. Sibley & L. Stahl (2000). Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. 2nd edition. United States Environmental Protection Agency, US. Report EPA 600/R-99/064.

Ivleva, I. V. (1973). Mass cultivation of invertebrates. Biology and methods. Jerusalem, Keter Press.

J

Janssen, P. M. J. (2007). Personal communication. DHV Water, the Netherlands.

Janssen, P. M. J., W. H. Rulkens, J. H. Rensink & H. F. van der Roest (1998). The potential for metazoa in biological wastewater treatment. *Sludge Management. WQI* 25-27.

Janssen, P. M. J., J. Verkuijlen & H. F. van der Roest (2002). Slibpredatie door inzet van oligochaete wormen. Pilotonderzoek naar slibreductie op de rwzi Bennekom. STOWA, the Netherlands. Report 2002-17 (in Dutch with English abstract).

Jerez, A., P. Partal, I. Martínez, C. Gallegos & A. Guerrero (2007). Egg white-based bioplastics developed by thermomechanical processing. *Journal of Food Engineering* **82**: 608-617.

Jongman, R. H., C. J. F. ter Braak & O. F. R. van Tongeren (1987). Data analysis in community and landscape ecology. Wageningen, the Netherlands, Pudoc.

Jørgensen, K. F. & K. Jensen (1978). Mass occurrence of the oligochaete *Aeolosoma hemprichi* Ehrenberg in activated sludge. *Biokon Rep.* **7**: 9-11.

Juget, J., V. Goubier & D. Barthélémy (1989). Intrinsic and extrinsic variables controlling the productivity of asexual populations of *Nais* spp. (Naididae, Oligochaeta). *Hydrobiologia* **180**: 177-184.

Jung, S.-J., K. Miyanaga, Y. Tanji & H. Unno (2006). Effect of intermittent aeration on the decrease of biological sludge amount. *Biochemical Engineering Journal* **27**: 246-251.

K

Kamemoto, F. I. & C. J. Goodnight (1956). The effects of various concentrations of ions on the asexual reproduction of the oligochaete *Aeolosoma hemprichi*. *Transactions of the American Microscopical Society Invertebrate Biology* **75**: 219-228.

Kaster, J. L., J. V. Klump, J. Meyer, J. Krezoski & M. E. Smith (1984). Comparison of defecation rates of *Limnodrilus hoffmeisteri* claparede (Tubificidae) using two different methods. *Hydrobiologia* **111**: 181-184.

Katharios, P., R. P. Smullen & V. Inglis (2005). The use of the polychaete worm *Nereis virens* eggs as vehicle for the delivery of oxytetracycline in *Solea solea* larvae. *Aquaculture* **243**: 1-7.

Kaufmann, R. (1983). VAMP: a video activity monitoring processor for the registration of animal locomotor activity. *Journal of Experimental Biology* **104**: 295-298.

Khanal, S. K., D. Grewell, S. Sung & J. van Leeuwen (2007). Ultrasound applications in wastewater sludge pretreatment: A review. *Critical Reviews in Environmental Science and Technology* **37**: 277-313.

Kim, M. H. & J. H. Hao (1990). Comparison of activated sludge decay under aerobic and anoxic conditions. *Research Journal of the Water Pollution Control Federation* **62**: 160-168.

Kizilkaya, R. & S. Hepsen (2004). Effect of biosolid amendment on enzyme activities in earthworm (*Lumbricus terrestris*) casts. *Journal of Plant Nutrition and Soil Science* **167**: 202-208.

Kosiorek, D. (1974). Development cycle of *Tubifex tubifex* Müll. in experimental culture. *Polish archives of hydrobiology/Polskie archiwum hydrobiologii* **21**: 411-422.

Krishnan, N. & S. R. Reddy (1989). Combined effects of quality and quantity of food on growth and body composition of the air-breathing fish *Channa gachua* (Ham.). *Aquaculture* **76**: 79-96.

Kroiss, H. (2004). What is the potential for utilizing the resources in sludge? *Water Science and Technology* **49**: 1-10.

Kukkonen, J. & P. F. Landrum (1995). Measuring assimilation efficiencies for sediment-bound PAH and PCB congeners by benthic organisms. *Aquatic Toxicology* **32**: 75-92.

Kuniyasu, K., N. Hayashi, Y. Inamori & R. Sudo (1997). Effect of environmental factors on growth characteristics of Oligochaeta **33**: 207-214 (translated from Japanese).

Kuwahara, M. & T. Yamamoto (1981). Culture of *Aeolosoma* spp Archioligochaeta found in activated sludge. *Kagawa Daigaku Nogakubu Gakujutsu Hokoku* **33**: 75-80 (in Japanese with English abstract).

L

Lake, D. L., P. W. W. Kirk & J. N. Lester (1984). Fractionation, characterization, and speciation of heavy metals in sewage sludge and sludge-amended soils: A review. *Journal of Environmental Quality* **13**: 175-183.

Landesman, L. (1996). 'Blackworms (*Lumbriculus variegatus*), a second crop from trout farms' webpage, 2007. http://us.share.geocities.com/landesman.geo/Blackworm.ppt.

Learner, M. A. (1979). The distribution and ecology of the Naididae (Oligochaeta) which inhabit the filter-beds of sewage-works in Britain. *Water Research* **13**: 1291-1299.

Learner, M. A. & H. A. Chawner (1998). Macro-invertebrate associations in sewage filter-beds and their relationship to operational practice. *Journal of Applied Ecology* **35**: 720-747.

Learner, M. A., G. Lochhead & B. D. Hughes (1978). A review of the biology of British Naididae (Oligochaeta) with emphasis on the lotic environment. *Freshwater Biology* **8**: 357-375.

Lee, N. M. & T. Welander (1996). Reducing sludge production in aerobic wastewater treatment through manipulation of the ecosystem. *Water Research* **30**: 1781-1790.

Lee, S., S. Basu, C. W. Tyler & I. W. Wei (2004). Ciliate populations as bio-indicators at Deer Island Treatment Plant. *Advances in Environmental Research* **8**: 371-378.

Leppänen, M. T. (1999). Bioaccumulation of sediment-associated polycyclic aromatic hydrocarbons in the freshwater oligochaete *Lumbriculus variegatus* (Müller). PhD thesis, University of Joensuu, Finland.

Leppänen, M. T. & J. V. K. Kukkonen (1998a). Factors affecting feeding rate, reproduction and growth of an oligochaete *Lumbriculus variegatus* (Müller). *Hydrobiologia* **377**: 183-194.

Leppänen, M. T. & J. V. K. Kukkonen (1998b). Relationship between reproduction, sediment type and feeding activity of *Lumbriculus variegatus* (Müller): implications for sediment toxicity testing. *Environmental Toxicology and Chemistry* **17**: 2196-2202.

Leppänen, M. T. & J. V. K. Kukkonen (2000). Fate of sediment-associated pyrene and benzo[a]pyrene in the freshwater oligochaete *Lumbriculus variegatus* (Müller). *Aquatic Toxicology* **49**: 199-212.

Lesiuk, N. M. & C. D. Drewes (1999). Autotomy reflex in a freshwater oligochaete, *Lumbriculus variegatus* (Clitellata: Lumbriculidae). *Hydrobiologia* **406**: 253-261.

Lester, J. N. (1987). Heavy metals in wastewater and sludge treatment processes. Volume 1. Sources, analysis and legislation. Boca Raton, Florida, CRC Press, Inc.

Liang, P., X. Huang & Y. Qian (2006a). Excess sludge reduction in activated sludge process through predation of *Aeolosoma hemprichi*. *Biochemical Engineering Journal* **28**: 117-122.

Liang, P., X. Huang, Y. Qian, Y. Wei & G. Ding (2006b). Determination and comparison of sludge reduction rates caused by microfaunas' predation. *Bioresource Technology* **97**: 854-861.

Liber, K., D. J. Call, T. P. Markee, K. L. Schmude, M. D. Balcer, F. W. Whiteman & G. T. Ankley (1996). Effects of acid-volatile sulfide on zinc bioavailability and toxicity to benthic macroinvertebrates: a spiked-sediment field experiment. *Environmental Toxicology and Chemistry* **15**: 2113-2125.

Liebig, M., P. Egeler, J. Oehlmann & T. Knacker (2005). Bioaccumulation of 14C-17[alpha]-ethinylestradiol by the aquatic oligochaete *Lumbriculus variegatus* in spiked artificial sediment. *Chemosphere* **59**: 271-280.

Lietz, D. M. (1987). Potential for aquatic oligochaetes as live food in commercial aquaculture. *Hydrobiologia* **155**: 309-310.

Little, C. (1984). Ecophysiology of *Nais elinguis* (Oligochaeta) in a brackish-water lagoon. *Estuarine, Coastal and Shelf science* **18**: 231-244.

Liu, X., C. Hu & S. Zhang (2005). Effects of earthworm activity on fertility and heavy metal bioavailability in sewage sludge. *Environment International* **31**: 874-879.

Lochhead, G. & M. A. Learner (1983). The effect of temperature on asexual population growth of three species of Naididae (Oligochaeta). *Hydrobiologia* **98**: 107-112.

Loden, M. S. (1981). Reproductive ecology of Naididae (Oligochaeta). *Hydrobiologia* **83**: 115-123.

Loeffen, P. & B. Geraats (2005). Toekomstige kwantiteit en kwaliteit van zuiveringsslib. STOWA, the Netherlands. Report 2005-06 (in Dutch).

Lotzof, M. (1999). The wonder of worms for sludge stabilisation. *Journal of the Australian Water & Wastewater Association* **26**: 38-42.

Low, E. W. & H. A. Chase (1999). Reducing production of excess biomass during wastewater treatment. *Water Research* **33**: 1119-1132.

Lowers, J. M. & J. L. Bartholomew (2003). Detection of myxozoan parasites in oligochaetes imported as food for ornamental fish. *Journal of Parasitology* **89**: 84-91.

Lucan-Bouché, M.-L., S. Biagianti-Risbourg, F. Arsac & G. Vernet (1999). An original decontamination process developed by the aquatic oligochaete *Tubifex tubifex* exposed to copper and lead. *Aquatic Toxicology* **45**: 9-17.

Luxmy, B. S., T. Kubo & K. Yamamoto (2001). Sludge reduction potential of metazoa in membrane bioreactors. *Water Science and Technology* **44**: 197-202.

M

MacMichael, G. J., L. R. Brown & C. M. Ladner (1988). An oligochaete as a potential food source for fish in aquaculture. *The Progressive Fish-culturist* **50**: 31-35.

Madoni, P., D. Davoli & E. Chierici (1993). Comparative analysis of the activated sludge microfauna in several sewage treatment works. *Water Research* **27**: 1485-1491.

Mäenpää, K. A., A. J. Sormunen & J. V. K. Kukkonen (2003). Bioaccumulation and toxicity of sediment associated herbicides (ioxynil, pendimethalin, and bentazone) in *Lumbriculus variegatus* (Oligochaeta) and *Chironomus riparius* (Insecta). *Ecotoxicology and Environmental Safety* **56**: 398-410.

Mahony, J. D., D. M. Di Toro, A. M. Gonzalez, M. Curto, M. Dilg, L. D. De Rosa & L. A. Sparrow (1996). Partitioning of metals to sediment organic carbon. *Environmental Toxicology and Chemistry* **15**: 2187-2197.

Marchese, M. R. & R. O. Brinkhurst (1996). A comparison of two tubificid oligochaete species as candidates for sublethal bioassay tests relevant to subtropical and tropical regions. *Hydrobiologia* **334**: 163-168.

Marian, M. P. & T. J. Pandian (1985). Interference of *Chironomus* in an open culture system for *Tubifex tubifex*. *Aquaculture* **44**: 249-251.

Marsh, L., S. Subler, S. Mishra & M. Marini (2005). Suitability of aquaculture effluent solids mixed with cardboard as a feedstock for vermicomposting. *Bioresource Technology* **96**: 413-418.

Marshall, J. W. & M. J. Winterbourn (1979). An ecological study of a small New-Zealand stream with particular reference to the Oligochaeta. *Hydrobiologia* **65**:

Martinez, D. E. & J. S. Levinton (1992). Asexual metazoans undergo senescence. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 9920-9923.

McElhone, M. J. (1978). A population study of littoral dwelling Naididae (Oligochaeta) in a shallow mesotrophic lake in North Wales. *Journal of Animal Ecology* **47**: 615-626.

McElhone, M. J. (1982). The distribution of Naididae (Oligochaeta) in the littoral zone of selected lakes in North Wales and Shropshire. *Freshwater Biology* **12**: 421-425.

McMurtry, M. J., D. J. Rapport & K. E. Chua (1983). Substrate selection by tubificid oligochaetes. *Canadian Journal of Fisheries and Aquatic Sciences* **40**: 1639-1646.

Mermillod-Blondin, F., C. M. C. Des & M. Gerino (2003). Effects of the interaction between tubificid worms on the functioning of hyporheic sediments: An experimental study in sediment columns. *Archiv für Hydrobiologie* **156**: 203-223.

Merrington, G., I. Oliver, R. J. Smernik & M. J. McLaughlin (2003). The influence of sewage sludge properties on sludge-borne metal availability. *Advances in Environmental Research* **8**: 21-36.

Michiels, I. C. & W. Traunspurger (2004). A three year study of seasonal dynamics of a zoobenthos community in a eutrophic lake. *Nematology* **6**: 655-669.

Milbrink, G. (1987a). Biological characterization of sediments by standarized tubificid bioassays. *Hydrobiologia* **155**: 267-275.

Milbrink, G. (1987b). Mutualistic relationships between cohabiting tubificid species. *Hydrobiologia* **155**: 193.

Milbrink, G. & T. Timm (2001). Distribution and dispersal capacity of the Ponto-Caspian tubificid oligochaete *Potamothrix moldaviensis* Vejdovský et Mrázek, 1903 in the Baltic Sea Region. *Hydrobiologia* **463**: 93-102.

Millward, R. N., J. W. Fleeger, D. D. Reible, K. A. Keteles, B. P. Cunningham & L. Zhang (2001). Pyrene bioaccumulation, effects of pyrene exposure on particle-size selection, and fecal pyrene content in the oligochaete *Limnodrilus hoffmeisteri* (Tubificidae, Oligochaeta). *Environmental Toxicology and Chemistry* **20**: 1359-1366.

Moore, J. W. (1978). Importance of algae in the diet of the oligochaetes *Lumbriculus variegatus* (Müller) and *Rhyacodrilus sodalis* (Eisen). *Oecologia* **35**: 357-363.

Morgan, A. J. & C. Winters (1991). Diapause in the earthworm, *Aporrectodea longa*: Morphological and quantitative x-ray microanalysis of cryosectioned chloragogenous tissue. *Scanning Microscopy* **5**: 219-227.

Morris, D. J. & A. Adams (2006). Transmission of freshwater myxozoans during the asexual propagation of invertebrate hosts. *International Journal for Parasitology* **36**: 371-377.

Mosleh, Y. Y., S. Paris-Palacios, F. Arnoult, M. Couderchet, S. Biagianti-Risbourg & G. Vernet (2004). Metallothionein induction in the aquatic oligochaete *Tubifex tubifex* exposed to the herbicide isoproturon. *Environmental Toxicology* **19**: 88-93.

Mosleh, Y. Y., S. Paris-Palacios & S. Biagianti-Risbourg (2006). Metallothioneins induction and antioxidative response in aquatic worms *Tubifex tubifex* (Oligochaeta, Tubificidae) exposed to copper. *Chemosphere* **64**: 121-128.

Mosleh, Y. Y., S. v. Paris-Palacios, M. Couderchet, S. Biagianti-Risbourg & G. Vernet (2005). Effects of the herbicide isoproturon on metallothioneins, growth, and antioxidative defenses in the aquatic worm *Tubifex tubifex* (Oligochaeta, Tubificidae). *Ecotoxicology* 1-13.

Mount, D. R., T. D. Dawson & L. P. Burkhard (1999). Implications of gut purging for tissue residues determined in bioaccumulation testing of sediment with *Lumbriculus variegatus*. *Environmental Toxicology and Chemistry* **18**: 1244-1249.

Mount, D. R., T. L. Highland, V. R. Mattson, T. D. Dawson, K. G. Lott & C. G. Ingersoll (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.

Mulder, W. J. & B. Beelen (2007). Personal communication. Agrotechnology & Food Sciences Group, Wageningen University and Research Centre, the Netherlands.

N

Nandini, S. & S. S. S. Sarma (2004). Effect of *Aeolosoma* sp. (Aphanoneura: Aeolosomatidae) on the population dynamics of selected cladoceran species. *Hydrobiologia* **526**: 157-163.

Ndegwa, P. M. & S. A. Thompson (2001). Integrating composting and vermicomposting in the treatment and bioconversion of biosolids. *Bioresource Technology* **76**: 107-112.

Nentwig, G. (2007). Effects of pharmaceuticals on aquatic invertebrates. Part II: The antidepressant drug fluoxetine. *Archives of Environmental Contamination and Toxicology* **52**: 163-170.

Neyens, E. & J. Baeyens (2003). A review of thermal sludge pre-treatment processes to improve dewaterability. *Journal of Hazardous Materials* **98**: 51-67.

Neyens, E., J. Baeyens, B. de Heyder & M. Weemaes (2004). The potential of advanced treatment methods for sewage sludge. *Management of Environmental Quality* **15**: 9-16.

Niederlehner, B. R., A. L. Buikema jr., C. A. Pittinger & J. Cairns jr. (1984). Effects of cadmium on the population growth of a benthic invertebrate *Aeolosoma headleyi* (Oligochaeta). *Environmental Toxicology and Chemistry* **3**: 255-262.

Nowak, O. (2006). Optimizing the use of sludge treatment facilities at municipal WWTPs. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering* **41**: 1807-1817.

O

Ødegaard, H. (2004). Sludge minimization technologies - an overview. *Water Science and Technology* **49**: 1-10.

Ødegaard, H., B. Paulsrud & I. Karlsson (2002). Wastewater sludge as a resource: Sludge disposal strategies and corresponding treatment technologies aimed at sustainable handling of wastewater sludge. *Water Science and Technology* **46**: 295-303.

Oetken, M., G. Nentwig, D. Löffler, T. Ternes & J. Oehlmann (2005). Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. *Archives of Environmental Contamination and Toxicology* **49**: 353-361.

P

Park, C., M. M. Abu-Orf & J. T. Novak (2006). The digestibility of waste activated sludges. *Water Environment Research* **78**: 59-68.

Patrick, F. M. & M. Loutit (1976). Passage of metals in effluents, through bacteria to higher organisms. *Water Research* **10**: 333-335.

Peeters, E. T. H. M., A. Dewitte, A. A. Koelmans, J. A. van der Velden & P. J. den Besten (2001). Evaluation of bioassays versus contaminant concentrations in explaining the macroinvertebrate community structure in the rhine-meuse delta, The Netherlands. *Environmental Toxicology and Chemistry* **20**: 2883-2891.

Pelegrí, S. P. & T. H. Blackburn (1995). Effects of *Tubifex tubifex* (Oligochaeta: Tubificidae) on N-mineralization in freshwater sediments, measured with 15N isotopes. *Aquatic Microbial Ecology* **9**: 289-294.

Penttinen, O.-P. (1997). Effects of anoxia and xenobiotics on the metabolic heat dissipation by freshwater benthic invertebrates. PhD thesis, University of Joensuu, Finland.

Pérez-Elvira, S. I., P. Nieto Diez & F. Fdz-Polanco (2006). Sludge minimisation technologies. *Re-views in Environmental Science and Biotechnology* **5**: 375-398.

Pesticide Action Network North America (2007). 'PAN Pesticides database' webpage, 2007. http://www.pesticideinfo.org.

Petersen, S., G. Arlt, A. Faubel & K. R. Carman (1998). On the nutritive significance of dissolved free amino acid uptake for the cosmopolitan oligochaete *Nais elinguis* Müller (Naididae). *Estuarine, Coastal and Shelf Science* **46**: 85-91.

Peterson, G. S., G. T. Ankley & E. N. Leonard (1996). Effect of bioturbation on metal-sulfide oxidation in surficial freshwater sediments. *Environmental Toxicology and Chemistry* **15**: 2147-2155.

Phipps, G. L., G. T. Ankley, D. A. Benoit & V. R. Mattson (1993). Use of the aquatic oligochaete *Lumbriculus variegatus* for assessing the toxicity and bioaccumulation of sediment-associated contaminants. *Environmental Toxicology and Chemistry* **12**: 269-279.

Pickavance, J. R. (1971). The ecology of *Lumbriculus variegatus* (Müller) (Oligochaeta, Lumbriculidae) in Newfoundland. *Canadian Journal of Zoology* **49**: 337-342.

Poole, J. E. P. & J. C. Fry (1980). A study of the protozoan and metazoan populations of three oxidation ditches. *Water Pollution Control* **79**: 19-27.

Postolache, C., G. Risnoveanu & A. Vadineanu (2006). Nitrogen and phosphorous excretion rates by tubificids from the Prahova River (Romania). *Hydrobiologia* **553**: 121-127.

Protz, R., W. J. Teesdale, J. A. Maxwell, J. L. Campbell & C. Duke (1993). Earthworm transport of heavy metals from sewage sludge: a micro-PIXE application in soil science. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms* **77**: 509-516.

Putzer, V. M., A. Zwaan & W. Wieser (1990). Anaerobic energy metabolism in the oligochaete *Lumbriculus variegatus* Müller. *Journal of Comparative Physiology B* **159**: 707-715.

Q

Quinn, J. M., G. L. Steele, C. W. Hickey & M. L. Vickers (1994). Upper thermal tolerances of twelve New Zealand stream invertebrate species. *New Zealand Journal of Marine and Freshwater Research* **28**: 391-397.

R

Ramakrishna, D. M. & T. Viraraghavan (2005). Strategies for sludge minimization in activated sludge process - A review. *Fresenius Environmental Bulletin* **14**: 2-12.

Rao, S. R. (2002). Applied numerical methods for engineers and scientists. Upper Saddle River, New Jersey, Prentice Hall.

Ratsak, C. H. (1994). Grazer induced sludge reduction in wastewater treatment. PhD thesis, Vrije Universiteit, the Netherlands.

Ratsak, C. H. (2001). Effects of *Nais elinguis* on the performance of an activated sludge plant. *Hydrobiologia* **463**: 217-222.

Ratsak, C. H., S. A. L. M. Koouman & B. W. Kooi (1993). Modelling the growth of an oligochaete on activated sludge. *Water Research* **27**: 739-747.

Ratsak, C. H., K. A. Maarsen & S. A. L. M. Kooijman (1996). Effects of protozoa on carbon mineralization in activated sludge. *Water Research* **30**: 1-12.

Ratsak, C. H. & J. Verkuijlen (2006). Sludge reduction by predatory activity of aquatic oligochaetes in wastewater treatment plants: science or fiction? A review. *Hydrobiologia* **564**: 197-211.

Reh, U. (1991). Calorimetry in ecology. *Thermochimica Acta* **193**: 107-124.

Reible, D. D., V. Popov, K. T. Valsaraj, L. J. Thibodeaux, M. D. F. Lin, M. A. Todaro & J. W. Fleeger (1996). Contaminant fluxes from sediment due to tubificid oligochaete bioturbation. *Water Research* **30**: 704-714.

Rensink, J. H. & W. H. Rulkens (1997). Using metazoa to reduce sludge production. *Water Science and Technology* **36**: 171-179.

Reynoldson, T. B. (1939a). On the life-history and ecology of *Lumbricullus lineatus* Müll. (Oligochaeta). *Annals of Applied Biology* **26**: 782-799.

Reynoldson, T. B. (1939b). Enchytraeid worms and the bacteria bed method of sewage treatment. *Annals of Applied Biology* **26**: 138-164.

Reynoldson, T. B. (1948). An ecological study of the enchytraeid worm population of sewage bacterial beds. Synthesis of field and laboratory data. *Journal of Animal Ecology* **17**: 27-38.

Reynoldson, T. B. (1987). The role of environmental factors in the ecology of tubificid oligochaetes- an experimental study. *Holarctic Ecology* **10**: 241-248.

Reynoldson, T. B., S. P. Thompson & J. L. Bamsey (1991). A sediment bioassay using the tubificid oligochaete worm *Tubifex tubifex*. *Environmental Toxicology and Chemistry* **10**: 1061-1072.

Roesijadi, G. (1992). Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology* **22**: 81-114.

Roman, H. J., J. E. Burgess & B. I. Pletschke (2006). Enzyme treatment to decrease solids and improve digestion of primary sewage sludge. *African Journal of Biotechnology* **5**: 963-967.

Rulkens, W. H. (2004). Sustainable sludge management - What are the challenges for the future? *Water Science and Technology* **49**: 11-19.

Rulkens, W. H. & J. D. Bien (2004). Recovery of energy from sludge - Comparison of the various options. *Water Science and Technology* **50**: 213-221.

S

Schneider, O. (2006). Fish waste management by conversion into heterotrophic bacteria biomass. PhD thesis, Wageningen University, the Netherlands.

Schönborn, W. (1984). The annual energy transfer from the communities of Ciliata to the population of *Chaetogaster diastrophus* (Gruithuizen) in the river Saale. *Limnologica* **16**: 15-23.

Schönborn, W. (1985). Die ökologische Rolle der Gattung *Nais* (Oligochaeta) in der Saale. *Zoologischer Anzeiger Jena* **215**: 311-328.

Schubauer-Berigan, M. K., P. D. Monson, C. W. West & G. T. Ankley (1995). Influence of pH on the toxicity of ammonia to *Chironomus tentans* and *Lumbriculus variegatus*. *Environmental Toxicology and Chemistry* **14**: 713-717.

Scott, E., F. Peter & J. Sanders (2007). Biomass in the manufacture of industrial products- the use of proteins and amino acids. *Applied Microbiology and Biotechnology* **75**: 751-762.

Sedlmeier, U. A. & K. H. Hoffmann (1989). Integumentary uptake of short-chain carboxylic acids by two freshwater oligochaetes *Tubifex tubifex* and *Lumbriculus variegatus* Specificity of uptake and characterization of transport carrier. *Journal of Experimental Zoology* **250**: 128-134.

Selck, H., A. W. Decho & V. E. Forbes (1999). Effects of chronic metal exposure and sediment organic matter on digestive absorption efficiency of cadmium by the deposit-feeding polychaete *Capitella* species 1. *Environmental Toxicology and Chemistry* **18**: 1289-1297.

Singer, R. (1978). Suction feeding in *Aeolosoma (*Annelida). *Transactions of the American Microscopical Society* **97**: 105-111.

Singh, N. B., A. K. Khare, D. S. Bhargava & S. Bhattacharya (2004). Optimum moisture requirement during vermicomposting using *Perionyx excavatus*. *Applied Ecology and Environmental Research* **2**: 53-62.

Smith, M. E. (1985). Population and reproductive dynamics of *Nais communis* (Oligochaeta: Naididae) from a Wisconsin limnocrene. *American Midland Naturalist* **114**: 152-158.

Smith, M. E. (1986). Ecology of Naididae (Oligochaeta) from an alkaline bog stream: life history patterns and community structure. *Hydrobiologia* **133**: 79-90.

Solowiew, M. M. (1924). Uber die Rolle der *Tubifex tubifex* in der Schlammerzeugung. *Internationale Revue der gesamten Hydrobiologie und Hydrographie* **12**: 90-101.

Spanjers, H. (1993). Respirometry in activated sludge. PhD thesis, Wageningen University, the Netherlands.

Sperber, C. (1948). A taxonomical study of the Naididae. Uppsala, Almqvist & Wiksells Boktryckeri Ab.

Sperber, C. (1950). A guide for the determination of European Naididae. In Zoologiska bidrag från Uppsala 29, p. 46-85.

Sperry, W. A. (1943). Aurora (Illinois) sanitary district -1942. *Sewage Works Journal* **15**: 949-953.

Spinosa, L. (2001). Evolution of sewage sludge regulations in Europe. *Water Science and Technology* **44**: 1-8.

Spinosa, L. (2004). From sludge to resources through biosolids. *Water Science and Technology* **50**: 1-8.

Spinosa, L. (2007). Wastewater sludge: A global overview of the current status and future prospects. London, IWA Publishing Ltd.

Stafford, E. A. & A. G. J. Tacon (1984). Nutritive value of the earthworm, *Dendrodrilus subrubicundus*, grown on domestic sewage, in trout diets. *Agricultural Wastes* **9**: 249-266.

Statistics Netherlands (CBS) (2007). 'CBS Statline' webpage, http://statline.cbs.nl.

Steen Redeker, E., K. van Campenhout, L. Bervoets, H. Reijnders & R. Blust (2007). Subcellular distribution of Cd in the aquatic oligochaete *Tubifex tubifex*, implications for trophic availability and toxicity. *Environmental Pollution* **148**: 166-175.

Stephan, C., R. Erickson, C. Delos, T. Willingham, K. Ballentine & R. Pepin (1999). 1999 Update of ambient water quality criteria for ammonia. United States Environmental Protection Agency, US. Report EPA-822-R-99-014.

Stephenson, J. (1930). The Oligochaeta. Oxford, Clarendon Press.

Stürzenbaum, S. R., C. Winters, M. Galay, A. J. Morgan & P. Kille (2001). Metal ion trafficking in earthworms. Identification of a cadmium-specific metallothionein. *Journal of Biological Chemistry* **276**: 34013-34018.

Sun, X. & U. Ghosh (2007). PCB bioavailability control in *Lumbriculus variegatus* through different modes of activated carbon addition to sediments. *Environmental Science and Technology*

Szczesny, B. (1974). The effect of sewage from the town of Krynica on the benthic invertebrate communities of the Kryniczanka stream. *Acta Hydrobiologica* **16**: 1-29.

T

Tchobanoglous, G., F. L. Burton & H. D. Stensel (2003). Wastewater engineering: treatment and reuse. New York, McGraw-Hill.

Ter Braak, C. J. F. (1986). Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology* **67**: 1167-1179.

Ter Braak, C. J. F. (1990). Update notes: CANOCO, Version 3.1. Wageningen, Wageningen University, Agricultural Mathematics Group.

Ter Braak, C. J. F. & P. Smilauer (1998). CANOCO reference manual and user's guide to Canoco for Windows: Software for canonical community ordination (version 4.0). Ithaca, NY, Microcomputer Power.

Timm, T. (1980). Methods of culturing aquatic oligochaeta. In Proceedings of: Aquatic Oligochaeta Worms. Taxonomy, ecology and faunistic studies in the USSR. Proceedings of the symposium on aquatic oligochaeta held in Tartu, 1967, New Delhi.

Timm, T. (1999). A guide to the Estonian Annelida. Volume 1. Tallinn, Estonian Academy Publishers.

Trodd, W. R., Mary Kelly-Quinn, P. Sweeney & B. Quirke (2005). A review of the status and distribution of the free-living freshwater Oligochaeta of Ireland. *Biology and Environment: Proceedings of the Royal Irish Academy* **105B**: 59-64.

Turnell, J. R., R. D. Faulkner & G. N. Hinch (2007). Recent advances in Australian broiler litter utilisation. *World's Poultry Science Journal* **63**: 223-231.

U

United States Environmental Protection Agency (2000). Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. 2nd edition. EPA 600/R-99/064.

United States Environmental Protection Agency (2007). 'ECOTOX database' webpage, 2007. http://cfpub.epa.gov/ecotox/.

V

Van Loosdrecht, M. C. M. & M. Henze (1999). Maintenance, endogeneous respiration, lysis, decay and predation. *Water Science and Technology* **39**: 107-117.

Van Rens, M., L. Luning & P. Loeffen (2005). Literatuurstudie slibdesintegratie. STOWA, the Netherlands. Report 2005-w04.

Vesilind, P. A. (1988). Capillary suction time as a fundamental measure of sludge dewaterability. *Journal of the Water Pollution Control Federation* **60**: 215-220.

Vidal, D. E. & A. J. Horne (2003). Mercury toxicity in the aquatic oligochaete *Sparganophilus pearsei* II: Autotomy as a novel form of protection. *Archives of Environmental Contamination and Toxicology* **45**: 462-467.

W

Wachs, B. (1967). Die Oligochaeten-Fauna der Fliessgewässer unter besonderer Berücksichtigung der Beziehungen zwischen der Tubificiden-Besiedlung und dem Substrat. *Archiv für Hydrobiologie* **63**: 310-386 (in German with English abstract).

Wallace, J. B. & J. R. Webster (1996). The role of macroinvertebrates in stream ecosystem function. *Annual Review of Entomology* **41**: 115-139.

Wang, X. & G. Matisoff (1997). Solute transport in sediments by a large freshwater oligochaete, *Branchiura sowerbyi*. *Environmental Science and Technology* **31**: 1926-1933.

Wavre, M. & R. O. Brinkhurst (1971). Interactions between some tubificid oligochaetes and bacteria found in the sediments of Toronto Harbour, Ontario. *Journal of the Fisheries Research Board of Canada* **28**: 335-341.

Wegener, J. W. M., G. A. van de Berg, G. J. Stroomberg & M. J. M. van Velzen (2002). The role of sediment-feeding oligochaete *Tubifex* on the availability of trace metals in sediment pore waters as determined by diffusive gradients in thin films (DGT). *Journal of Soils and Sediments* **2**: 71-76.

Wei, Y. & J. Liu (2005). The discharged excess sludge treated by Oligochaeta. *Water Science and Technology* **52**: 265-272.

Wei, Y. & J. Liu (2006). Sludge reduction with a novel combined worm-reactor. *Hydrobiologia* **564**: 213-222.

Wei, Y., R. T. van Houten, A. R. Borger, D. H. Eikelboom & Y. Fan (2003a). Comparison performances of membrane bioreactor and conventional activated sludge processes on sludge reduction induced by Oligochaete. *Environmental Science and Technology* **37**: 3171-3180.

Wei, Y., R. T. van Houten, A. R. Borger, D. H. Eikelboom & Y. Fan (2003b). Minimization of excess sludge production for biological wastewater treatment. *Water Research* **37**: 4453-4467.

Whitley, L. S. (1968). The resistance of tubificid worms to 3 common pollutants. *Japanese Journal of Zoology* **32**: 193-205.

Whitten, B. K. & C. J. Goodnight (1966a). Toxicity of some common insecticides to tubificids. *Journal of the Water Pollution Control Federation* **38**: 227-235.

Whitten, B. K. & C. J. Goodnight (1966b). The comparative chemical composition of two aquatic oligochaetes. *Comparative Biochemistry and Physiology* **17**: 1205.

Wiederholm, T., A.-M. Wiederholm & G. Milbrink (1987). Bulk sediment bioassays with five species of fresh-water oligochaetes. *Water, Air and Soil Pollution* **36**: 131-154.

Wiegand, C., S. Pehkonen, J. Akkanen, O. P. Penttinen & J. V. K. Kukkonen (2007). Bioaccumulation of paraquat by *Lumbriculus variegatus* in the presence of dissolved natural organic matter and impact on energy costs, biotransformation and antioxidative enzymes. *Chemosphere* **66**: 558-566.

Wiegant, W. M., M. Würdemann, H. E. Kamphuis, J. van de Marel & W. F. Koopmans (2005). Slibketenstudie. Onderzoek naar de energie- en kostenaspecten in de water- en slibketen. STOWA, the Netherlands. Report 2005-26 (in Dutch).

Williams, N. V., J. F. de L. G. Solbé & R. W. Edwards (1968). Aspects of the distribution, life history and metabolism of the enchytraeid worms *Lumbricullus rivalis* (Levinsen) and *Enchytraeus coronatus* (N. & C.) in a percolating filter. *Journal of Applied Ecology* **6**: 171-183.

Williams, P. M. (2005). Feeding behaviour of *Lumbriculus variegatus* as an ecological indicator of *in situ* sediment contamination. PhD thesis, University of Stirling, UK.

Winters *et al.*, R. O. (2004). Substantial reduction of organic waste streams using the natural food chain. Public report. Wageningen University and Research Centre, DHV Water, Gemeentelijk Waterbedrijf Amsterdam, Norit Membrane Technology, the Netherlands. Report 235.

Y

Yanar, M., Y. Yanar & M. A. Genç (2003). Nutritional composition of *Tubifex tubifex* Müller, 1774 (Annelida). *E. U. Journal of Fisheries and Aquatic Sciences* **20**: 103-110.

Young, J. O. (1978). An ecological study of *Phaenocora unipunctata* (Örsted) (Turbellaria; Rhabdocoela): predator-prey interrelationships. *Hydrobiologia* **60**: 145-149.

Young, J. O. & R. M. Procter (1985). Oligochaetes as a food resource of lake-dwelling leeches. *Journal of Freshwater Ecology* **3**: 181-187.

Z

Zhang, B. (1997). A study on microbial activities and the role of predators in membrane separation activated sludge process. PhD thesis, University of Tokyo, Japan.

Summary of sludge TSS (total suspended solids) reduction and growth (in biomass and numbers) in time by *Lumbriculus variegatus* in 3 typical batch experiments. The experiments were done in aerated Erlenmeyer flasks (Experiment 1) or non-aerated Petri dishes (Experiments 2 & 3) at 15-20 °C, at W/S (worm to sludge) ratios of 0.2-0.3 (dry matter based) with municipal sludges. Experiment 1 was also described in Chapter 4 (Figure 4.3). In this experiment, all the sludge was consumed (faeces percentage = 100 %) after 6 days. Faeces percentages in the other two experiments were not determined.

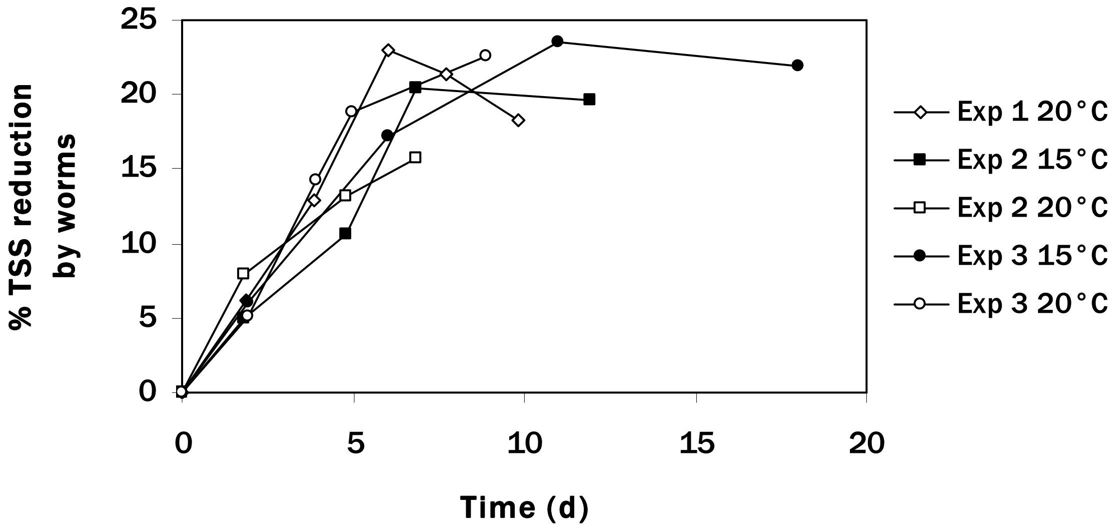


Figure A1 Percentage TSS reduction in time by *L. variegatus* only (i.e. after subtraction of TSS reduction in the control experiments).

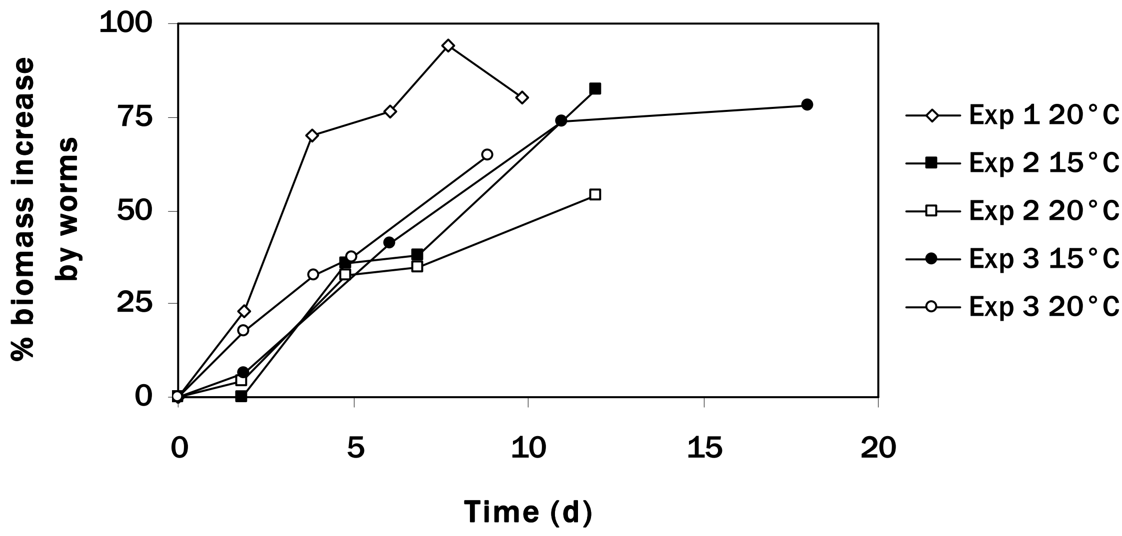


Figure A2 Percentage biomass increase in time of *L. variegatus*.

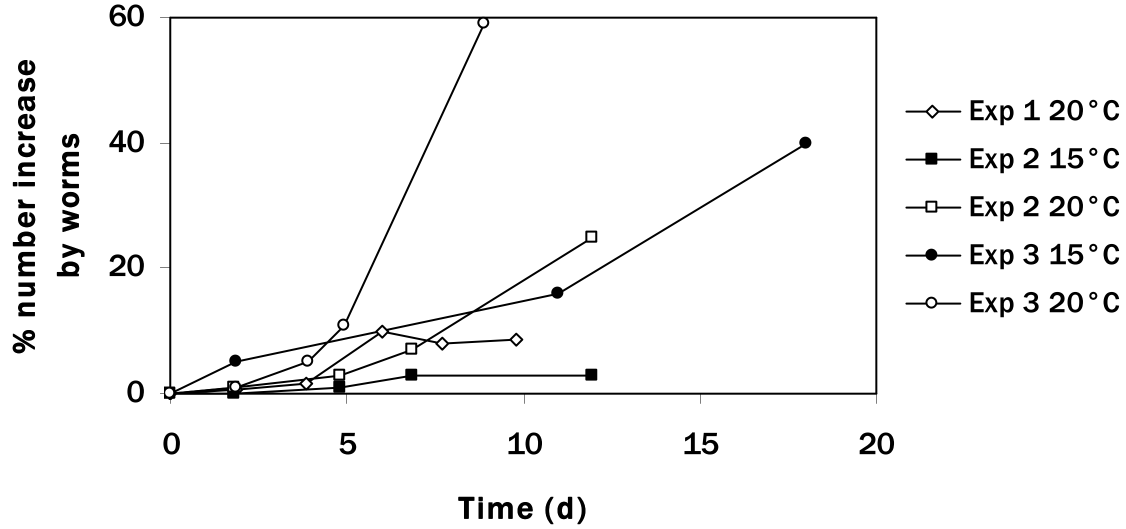


Figure A3 Percentage number increase in time of *L. variegatus*.

Table A1 Basic composition of *Lumbriculus variegatus* (in % of dry weight) on different food sources. Worm dry weight is 13-15 % of the wet weight.

	Sludge[1)]	Sediments[2)]
Proteins	63	62-66
Carbohydrates	5-9	13-18
Lipids	24-26	11-12
Ash	6-7	9-11
Calcium	-	0.2-0.3
Phosphorus	1.6	1.4-2.1
Calories (kcal/ g dry weight)	-	4.76-4.88

[1)] Mulder & Beelen, 2007, own data [2)] Hansen *et al.*, 2004)

Table A2 Carbohydrate (monosaccharide) and amino acid composition of *L. variegatus* (Mulder & Beelen, 2007).

Percentage of total	Component
	Monosaccharides
10-20	Ara Gal Man Rha Xyl
>30	Glu
	Amino acids
<1	Cys Gln Hyp
1-5	Asn His Met Phe Pro Ser Trp Tyr
5-10	Ala Arg Asp Glu Gly Ile Leu Lys Thr Val

Table A3 Concentrations of heavy metals in *L. variegatus* biomass (average of experiments in Chapter 6 & 7) and limit values for heavy metals in sludge (all in mg/ kg dry matter) according to current and future Dutch and European legislations.

		Limit values		
		Current		***Future***
Metal	***L. variegatus***	**the Netherlands[1)]**	**EU[2)]**	**EU[3)]**
Cd	1.3 (±0.6)	1.25	20-40	2
Ni	1.2 (±0.2)	30	300-400	100
Cr	2.1 (±0.6)	75	-	-
Pb	2.6 (±0.2)	100	750-1200	200
Cu	47 (±10)	75	1000-1750	600
Zn	395 (±29)	300	2500-4000	1500

[1)]BOOM regulations [2)]European Directive 86/278/EEC [3)]European Directive in preparation, final values Source: http://www.eu-milieubeleid.nl/ch05s10.html

About the author

Hellen Johannes Hubertina Elissen was born on the 14th of July in 1975 in Heerlen, the Netherlands. After her pre-university education ('Gymnasium B') at the Grotius College in Heerlen, she studied Biology at the University of Groningen from 1993 on. She focused on Microbial Ecology (biodegradation of chloroethenes), Population Genetics (genetic variation in pine martens) and Animal Ecology (ecology and behaviour of black crowned cranes in Cameroon). In 1998, she obtained her MSc degree and worked at TNO-MEP in Apeldoorn as analyst on the biodegradation of HCH in soils. In 2000, she started the PhD research described in this thesis at the Sub-Department of Environmental Technology at the Wageningen University. Since 2006, she works at Wetsus Centre for Sustainable Water Technology in Leeuwarden as researcher.

╡ Publications ╞

Scientific publications

- Drzyzga, O., J. Gerritse, J. A. Dijk, H. Elissen & J. C. Gottschal (2001). Coexistence of a sulphate-reducing *Desulfovibrio* species and the dehalo-respiring *Desulfitobacterium frappieri* TCE1 in defined chemostat cultures grown with various combinations of sulphate and tetrachloroethene. *Environmental Microbiology* **3**: 92-99.
- Elissen, H. J. H., T. L. G. Hendrickx, H. Temmink & C. J. N. Buisman (2006). A new reactor concept for sludge reduction using aquatic worms. *Water Research* **40**: 3713 – 3718.
- Hendrickx, T. L. G., H. Temmink, C. J. N. Buisman & H. J. H. Elissen (2006). Sludge predation using aquatic worms. In Proceedings of the IWA Specialized Conference - Sustainable Sludge Management: State of the Art, Challenges and Perspectives, Moscow, Russia, 2006.
- Elissen, H. J. H., E. T. H. M. Peeters, B. R. Buys, A. Klapwijk & W. H. Rulkens (submitted). Population dynamics of free-swimming Annelida in four Dutch wastewater treatment plants in relation to process characteristics. *Conditionally accepted for Hydrobiologia*.
- Buys, B. R., H. J. H. Elissen, A. Klapwijk & W. H. Rulkens (submitted). Development of a test to assess the sludge reduction potential of *Lumbriculus variegatus* in waste sludge. *Submitted for Bioresource Technology*.
- Elissen, H. J. H., Mulder, W. J., Hendrickx, T. L. G., Buys, B. R., Temmink, H. (in preparation). Production of aquatic worms from waste sludge: biomass composition and applications.

Patents

- Elissen, H. J. H., H. Temmink & C. J. N. Buisman (2007). Werkwijze voor het vrijmaken van aquatische wormen uit een drager en predatiereactor. Wetsus, Centre for Sustainable Water Technology, the Netherlands. *Patent application* NL1032621 *to be published in April 2008* (in Dutch).
- Elissen, H. J. H., B. R. Buys, W. H. Rulkens & H. Temmink (2006). Werkwijze en inrichting voor het zuiveren van een waterige afvalstroom/ Method and plant for the treatment of an aqueous waste stream. Wageningen University and Research Centre, the Netherlands. *Patent* NL1029052C (in Dutch)/ WO2007040397 (in English).

Conferences

- Elissen, H. J. H., B. R. Buys, C. R. Ratsak (2003). Application of oligochaetes in wastewater treatment. Part B: Growth of free-swimming oligochaetes in wastewater treatment plants. Presentation at the 9th International Symposium on Aquatic Oligochaete Biology, Wageningen, the Netherlands, 2003.

Reports and non-refereed publications

- Langenhoff, A., H. Elissen, H. Rijnaarts, C. Pijls, A. Alphenaar, J. Boode, C. te Stroet & M. van Zutphen (2000). Bioremediatie van HCH locaties. Eindrapportage fase 1. TNO, the Netherlands. Report TNO-MEP – R99/445 (in Dutch).
- Winters *et al.*, R. O. (2004). Substantial reduction of organic waste streams using the natural food chain. Public report. Wageningen University and Research Centre, DHV Water, Gemeentelijk Waterbedrijf Amsterdam, Norit Membrane Technology, the Netherlands. Report 235.
- Wormen als schoonmakers. *EOS* (in Dutch)
- Vuil slib? Neem wormen! *Nederlands Dagblad, Utrechts Nieuwsblad* (in Dutch)
- Zwemmende darmkanalen lusten wel pap van afvalslib. *Volkskrant* (in Dutch)

Supervised MSc theses

- Jansen, A. (2002). The possibilities for application of oligochaete worms in wastewater treatment with a MBR.
- Nogaj, K. (2003). The application of oligochaete worms in wastewater treatment. Sludge reduction by using aquatic worms (*Lumbriculus variegatus*).
- Lei, K. (2004). Sludge stabilization by aquatic worms.
- Gustavo, J. G. (2006). Predation of activated sludge by Oligochaeta.

╡Acknowledgments/Dankwoord╞

Zo! Dat was het dan. En achteraf gezien had het eigenlijk heel simpel kunnen zijn. Je neemt de volgende twee zinnen in het achterhoofd:

You can't milk a cow with your hands in your pants (Admiral Freebee)

The Truth is out there…. (The X Files)

Je combineert dit met het schrijven van nog geen tiende pagina per dag gedurende een aantal jaren en ziedaar: Het '(afstudeer)verslag', de 'scriptie' of hoe mensen het ook plachten te noemen is klaar en je bent de 's' uit je titel kwijt.

Na een korte tijd als analiste gewerkt te hebben realiseerde ik me dat ik meer verdieping wilde en een onderzoek dat meer 'van mezelf' was. Het gekozen onderwerp 'Slibreductie met aquatische wormen' was gericht op het oplossen van een milieuprobleem en combineerde ook nog eens meerdere vakgebieden: milieutechnologie, aquatische ecologie en microbiologie. Dit geheel was erg aantrekkelijk, ook al kwam het niet helemaal overeen met 'werken voor Greenpeace op Antarctica' zoals ik op de lagere school altijd wilde. Ik moet zeggen dat ik nog nooit niet-begrijpende blikken (wel veel geamuseerde!) gezien heb als ik uitlegde waar ik mee bezig was: Wormen en poep. Tot op de dag van vandaag kan ik zelf dan ook nog steeds gefascineerd zijn als ik zo'n 'zwemmende darm' onder de stereomicroscoop zie, ook al blijken ze dan niet 93 % van het slib (zie Hoofdstuk 1) als sneeuw voor de zon te kunnen laten verdwijnen…
Enfin, er waren goede en niet zulke goede momenten de afgelopen jaren en ik wil graag de mensen bedanken die erbij waren.

Bram en Wim, mijn co-promotor en promotor, het is me helaas niet meer gelukt om het compleet af te ronden voor jullie afscheid van de vakgroep, maar dat heeft jullie er niet van weerhouden om bijna alle versies van mijn hoofdstukken met zorg na te kijken. Het begeleiden van dit als vrij curieus te boek staande onderzoek, uitgevoerd door twee eigenwijze promovendi (die steeds fundamenteler gingen werken ipv een reactor uit de grond te stampen), zal niet altijd meegevallen zijn maar er is een proefschrift verschenen over het onderwerp waar Henk† al die jaren in geloofde.

En natuurlijk Bas, met wie ik de 'wurmen' vormde. Onze samenwerking was niet altijd even gemakkelijk en ik had het graag samen met jou af willen ronden. We hebben echter het grootste deel van het onderzoek samen gedaan ('nog even 5000 wormen tellen vanavond en dan zijn we al klaar en nemen we een wijntje') en ik heb ontzettend veel van je geleerd. Mijn dank daarvoor en ik hoop dat ik over een tijdje jouw dankwoord zal lezen.

Hardy, ook al ben je officieel niet als begeleider of co-promotor aan dit onderzoek verbonden, je hebt een enorme bijdrage geleverd in de laatste fase van mijn promotie. Ik heb erg veel aan je supersnelle commentaar en opbeurende woorden gehad, ook al was het af en toe even schrikken van termen als 'dit lijkt wel een roman' of 'dit kan toch helemaal niet' of 'HUH?' en lopen onze discussies wel eens uit op een wederzijds 'nou ja laat ook maar, ik weet het toch beter'.

Christa, alias 'opperwurm', onze begeleidster in de eerste jaren. Je presteerde het om altijd enthousiast en gefascineerd door het onderzoek te blijven. Ik wil je bedanken en ik hoop dat we binnenkort elkaar een goede live update van de afgelopen jaren gaan geven.

Tim, dit onderzoek trekt blijkbaar mensen aan die op 1 dag na even oud zijn en geen rijbewijs hebben. Ook jij bedankt voor al je experimentele hulp, je nuttige commentaren en zeker ook voor de rust die je uitstraalt. Arie, Katarzyna, Lei en Jesús: dank je en thank you for contributing to this thesis through your MSc researches. Arie, misschien hadden we vantevoren kunnen bedenken dat wormen een side-stream membraan niet overleven. Katarzyna, I am glad I did not understand the Polish cursing when there were another 1500 worms to count. Lei, I still can't answer your essential question 'Why do worms divide?'. Jesús, I still think you should publish your story about the Proefhal-symposium and the sludge flood...

Titia, je noemde ons in je eigen dankwoord al 'partners in crime' en ik wil me daar onorigineel bij aansluiten. Je bent zelfs meer vriendin dan collega, maar het besef dat er op zaterdagavond om 10 uur nog iemand 20 km verderop aan het werk was die bijvoorbeeld ook opeens data kwijt was ('aaaargh het is NIET te geloven') gaf veel steun. Na deze zoveelste laatste loodjes (waarbij je me als paranimf nog even fanatiek op Wim's rails houdt) beloof ik je dat onze nachtelijke 'dansen op verantwoorde muziek' hobby heel binnenkort weer opgepikt gaat worden.

Ik heb het geluk gehad op een bijzonder mooi afgelegen (en soms bijzonder stinkend of stikheet of beide) plekje tussen de weilanden te mogen werken: de voormalige Proefhal Milieutechnologie in Bennekom, en dit bleek vooral op zomeravonden tijdens borrels en (halal) bbq's met de bovengenoemde Bennekommers en oa Adriaan, Bert, Claudia, Darja, Eunhee, Ghada, Grietje, Hamed, Kasia, Katja, Lina, Lucas, Maha, Nedal, Renato, Rob, Tania, Tarek, Vinnie en Wendy. De overgang naar het bordes van het Biotechnion was dan ook groot, maar de weinige keren dat ik er was had ik wel een 'nice roommate' Sonia. Liesbeth, Gusta, Anita en Heleen; bedankt voor het beantwoorden van al mijn praktische vragen.

Buiten de vakgroep wil ik een aantal mensen bedanken: Edwin van Aquatische Ecologie, je hebt me een inkijkje gegeven (en veel hulp) in de complexe wereld van de multivariate analyse en stelde me een beetje gerust door het terug te brengen tot 'data in de computer stoppen tot hij begint te roken'. Antoon† en Wilma van Microbiologie voor jullie gastvrijheid en geduldige hulp bij onze uitstapjes in de wereld van PCR en DGGE. Ook de begeleidingcommissie (oa Paul en Jaap) en projectmedewerkers van de STW en EET projecten wil ik hierbij bedanken voor hun inbreng. Cees, door jou kon ik het onderwerp waar ik over aan het schrijven was verder bestuderen bij Wetsus. Alle andere 'Wetsianen' wil ik bedanken voor de goede atmosfeer en zeker Lucia 'who provided my second home with *glovely* music in the North'.

En natuurlijk lieve familie en vrienden: er leek geen einde aan te komen (vond ik zelf ook wel hoor), maar jullie hebben me altijd een riem onder het hart gestoken (zoals Juul zou zeggen), het is AF en ik zal jullie ongetwijfeld vaker komen opzoeken... Roy zeker ook jij bedankt voor je liefde en steun de eerste jaren.

Marc, het leven met een AIO valt inderdaad niet altijd mee (waar heb ik dat eerder gelezen), maar je weet zelf ook hoe lastig de wormen kunnen zijn uit ervaring. Mijn dank voor al je liefde, relativeringsvermogen, positiviteit ('ik haal fluitend mijn startmotor voor de zesde keer die dag uit elkaar'), ontbijtjes en hartige flapjes... we moesten maar eens op vakantie wat jij?

En toch.. valt er nog veel meer uit te zoeken..

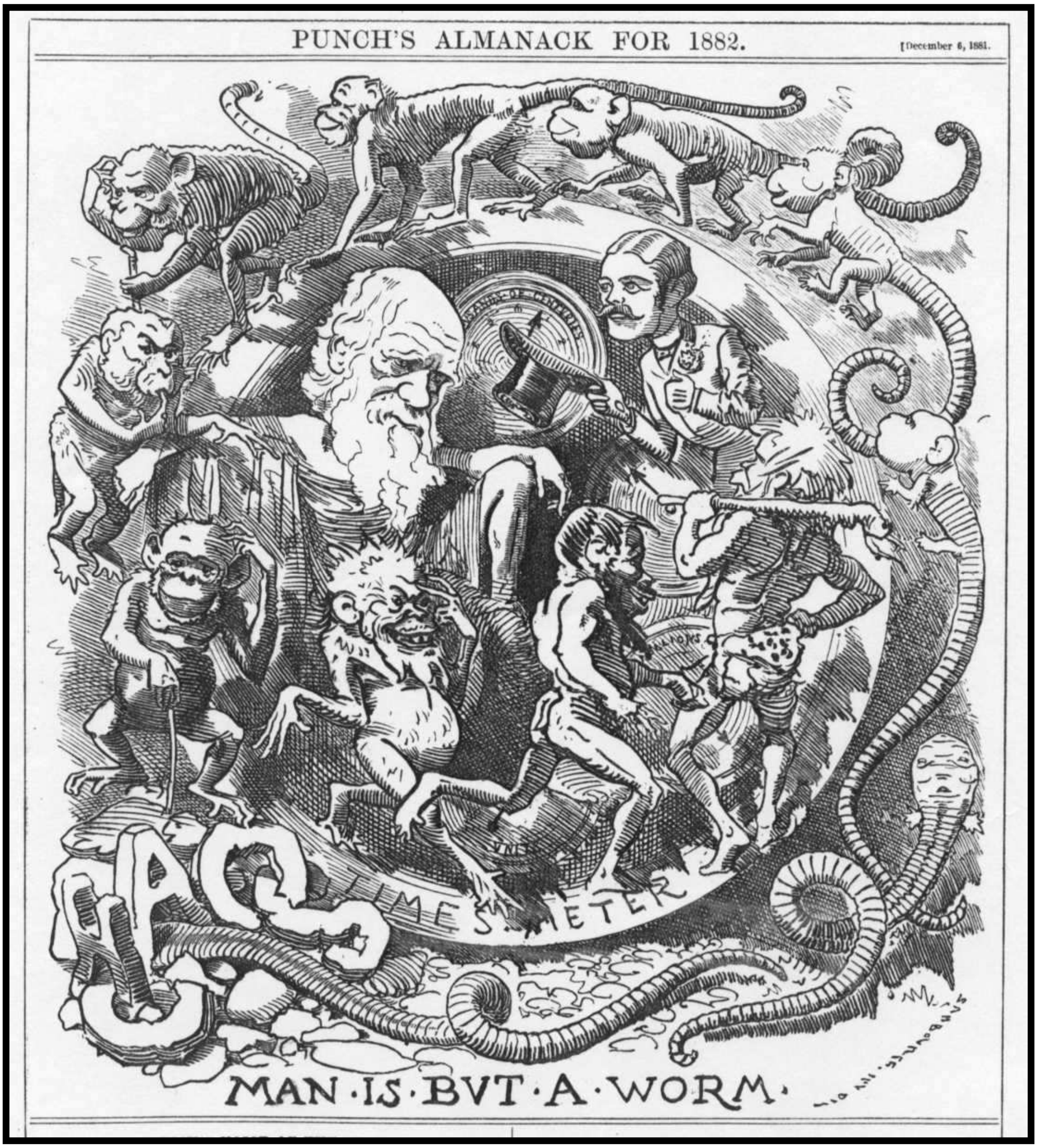
Hellen, 21 november 2007, Randwijk

⫫

As one blackworm once said to another
I am sure we're both sister and brother
Now, if parents we're seeking
Fragmentally speaking
Our head end's our father AND mother

Charlie Drewes†, 1997

╥



Caricature of Darwin's theory by Linley Sambourne in the Punch almanac for 1882 when Charles Darwin had recently published his last book 'The Formation of Vegetable Mould Through the Action of Worms'.

The research described in this thesis was supported by the Dutch Economy, Ecology and Technology (EET) programme in the project 'Substantial reduction of organic waste streams using the natural food chain' and by the Dutch Technology Foundation STW.

Images on cover and title pages (by author, unless mentioned otherwise)

Cover	*Lumbriculus variegatus* specimen with sludge in its gut
p. 11	Sludge flocs
p. 19	Male reproductive organs of *Limnodrilus* sp.
p. 31	Chaetae ('bristles') of *Nais* sp.
p. 51	*Lumbriculus variegatus* tail producing faeces after feeding on sludge
p. 65	Groups of *L. variegatus* in Petri dish collecting sludge
p. 91	Faeces of *L. variegatus* after feeding on sludge
p. 111	Cocoon with unidentified juvenile Tubificidae (original image by Katarzyna Nogaj)
p. 125	Detail of set-up used for testing the reactor concept with *L. variegatus* (original image by Tim Hendrickx)
p. 135	Mucus bubbles produced by *L. variegatus* during stress situations