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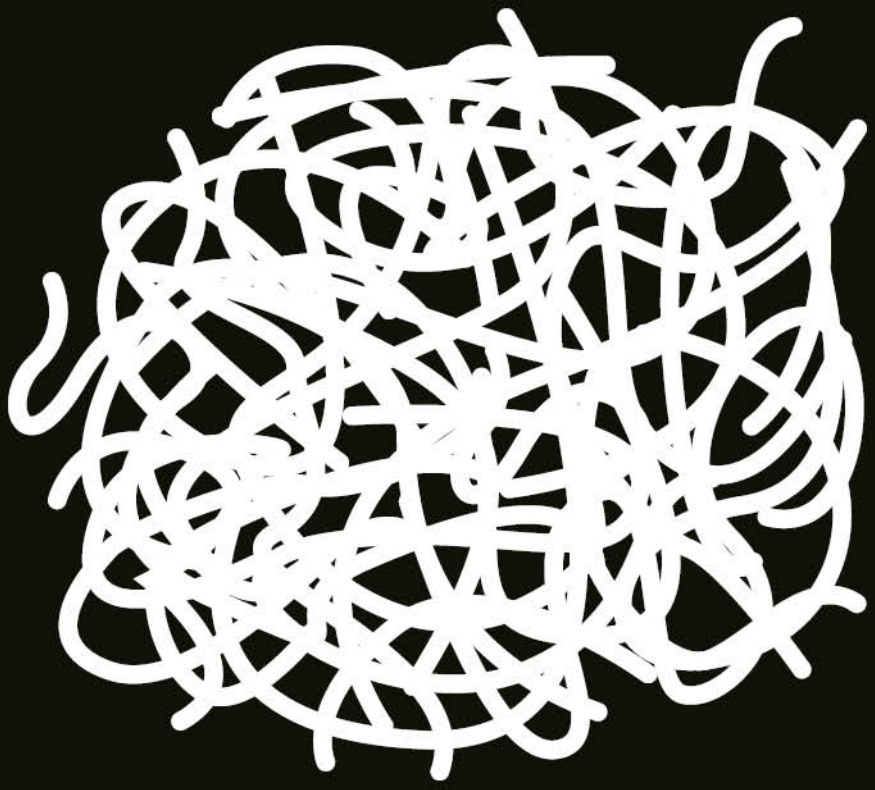

Unravelling the hydrolytic activity of waste activated sludge degrading aquatic worms

S.L. de Valk

Unravelling the hydrolytic activity of waste activated sludge degrading aquatic worms

Dissertation

For the purpose of obtaining the degree of doctor
at Delft University of Technology,
by the authority of the Rector Magnificus, Prof.dr.ir. T.H.J.J van der Hagen,
chair of the Board for Doctorates
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by

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Born in Amsterdam, the Netherlands

This dissertation has been approved by the promotors.

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Dedicated to all the people who, despite my thorough love for procrastination relentlessly and stubbornly pushed me forwards

PREFACE

Under the neon lights of the laboratory, the stage is set for a sterile theatre play. A bad piece of performance art is being applauded by dead specimens of *T. tubifex*. Millions cheered as the performing artists showed their tricks on stage. “I’m alive!!” the artists cheerfully shouted and the crowd wiggled their tails in anticipation. Not too long ago the cheering worms were part of the performance as well, alive and wriggling. Finally, the nostalgic smell of latex fills the theatre, signalling the start of the closing act. As the in latex wrapped hand descends from the heavens towards the stage, the final judgement is passed in the name of science.

PROPOSITIONS

1. Sludge predation by worms and extended aerobic and anaerobic bioconversion processes show high similarities in solids reduction potential; yet are worlds apart in process time. (This thesis)

2. Exopolymer hydrolysis, including polypeptides, is, as far as worms are concerned, the key to efficient waste activated sludge degradation. (This thesis)

3. Worm intestines are a breeding ground for proteolytic bacteria. (This thesis)

4. Waste activated sludge predation by worms, followed by anaerobic digestion is as effective in solids reduction as any state-of-the-art (pre-treatment) process. (This thesis)

5. Excess sludge production can be partly mitigated, simply by installing worm habitats in (existing) aerobic sludge processes.

6. Water treatment technologies can clean water only when the social construct of politics, policies and legislative enforcement allow for it.

7. Worm researchers, despite all their good intentions, are at some point mass (worm) murderers.

8. “For peace to reign on Earth, humans must evolve into new beings who have learned to see the whole first.” (Immanuel Kant, German Philosopher 1724 - 1804).

9. Stereotyping and prejudices are the result of the inheritability of social economic backgrounds including behaviour aspect typical to said social economic class, which further strengthens stereotypes and prejudice linked to this social economic background.

10. The affirmation of the cognitive status quo, trough social media algorithm generated social bubbles, leads to facts that are only true if you believe in them. (The social dilemma – Netflix)

These propositions, belonging to the thesis ‘Unravelling the hydrolytic activity of waste activated sludge degrading aquatic worms’ are regarded as opposable and defendable and have been approved as such by the promotors Prof.dr.ir. J.B. van Lier and Prof.dr.ir. M.K. de Kreuk.

Steef de Valk

Delft, 5th of July 2021

SUMMARY

The overall objective of this thesis was to investigate ways to improve the extent and rate of waste activated sludge (WAS) hydrolysis by researching the WAS degrading activities and mechanisms of the aquatic worm *Tubifex tubifex (T. tubifex)* as a starting point. The WAS degrading aquatic worms were taken as a model "biochemical reactor" of which its conversion processes still need to be unravelled. Because the worms are known for their excellent performance in WAS-solids reduction, i.e., up to 45% volatile solids (VS) reduction in 4 – 5 days, the focus was on worm-based enzymatic processes for improving WAS hydrolysis.

Generally, *T. tubifex* predation shows significantly higher WAS conversion rates compared to anaerobic and aerobic digestion processes. However, information on the effect of WAS predation on the overall WAS biodegradability was lacking. Hereto, experiments were conducted to assess the ultimate WAS biodegradability potential, after which results were used as a reference to compare the biodegradability potential of different combinations of worm predation and anaerobic digestion. Interestingly, worm predation combinations showed superior solids removal rates and superior overall conversion rates, compared to solely conventional anaerobic digestion. However, the overall WAS biodegradability potential was similar in both experimental set-ups, reaching 58% and 49% removal for chemical oxygen demand (COD) and VS respectively.

The improved WAS conversion rates during worm predation were related to the efficient removal of protein-like and, to a smaller extent, polysaccharide-like substances from the sludge matrix. Additionally, alginate-like exopolysaccharides (ALE), were partly consumed during worm treatment of WAS. The removal of protein, polysaccharide and ALE-like substances resulted in the disintegration of sludge flocs and the release of fulvic and humic substances as well as the cations Mg^{2+}, Al^{3+} and Fe^{3+} from the sludge matrix. The cations and the humic and fulvic substances have a known structural function in the extracellular polymeric substances (EPS) of sludge flocs and are therefore, most likely tightly associated with the removed protein-like fraction.

Corroborating with the removal of a protein-like fraction, an increased protease activity was observed in the predated WAS. The improved protease activity was likely related to *T. tubifex* based enzymes and/or the excretion of intestinal

proteolytic bacteria. More specifically, a maximum of 73% of the proteolytic activity, related to the conversion of the model substrate casein, was due to the activity of the worms, while the remaining activity could be linked to the intestinal proteolytic bacteria.

The synergy between bacteria and worms was further investigated using microbial community analysis. We showed that the worm faeces produced through WAS predation shared more similarities in microbial structure with predated protein rich substrates as compared to the WAS itself. The microbial change towards a microbiome, which was apparently related to protein degradation, was probably due to favourable conditions in the worm gut that facilitated a protein-degrading microbial community. It was further found that the genera *Burkholderiales*, *Chryseobacterium* and *Flavobacterium* were associated with predation by *T. tubifex* and were likely related to protein degradation.

Overall, the research demonstrated that the key aspects of efficient WAS hydrolysis are related to the removal and conversion of protein- and alginate-like substances as well as elevated protease activity. The type of proteases and possibly other mechanisms such as the lytic capabilities of the aquatic worms are yet to be investigated.

SAMENVATTING

De doelstelling van dit proefschrift was om onderzoek te doen naar de mogelijkheden om de mate en snelheid van hydrolyse van spuislib uit een communale zuivering te verbeteren, waarbij de nog onbekende slibreductiemechanismen van de aquatische worm *Tubifex tubifex* (*T. Tubifex*) als uitgangspunt dienden. De slib-afbrekende wormen, worden beschouwd als een 'biochemische reactor' waarvan de hydrolytisch processen nog verder ontrafeld moeten worden. Omdat de wormen bekend staan om hun uitstekende slibreductie prestaties (tot wel 45% van de organische slibfractie in 4 tot 5 dagen), ligt de nadruk van dit proefschrift op de enzymatische processen in de worm, die tot de verbeterde slibhydrolyse leiden.

In vergelijking met conventionele anaerobe en aerobe processen, geeft wormpredatie een significant hogere slibconversiesnelheid. Specifieke informatie over in hoeverre wormpredatie de mate van afbreekbaarheid van spuislib beïnvloedt, ontbrak echter. Om hier meer inzicht te krijgen is de slibafbraak tijdens wormpredatie en anaerobe gisting combinaties met elkaar vergeleken, waarbij de maximale afbreekbaarheid van spuislib als referentiekader diende. De wormpredatie en anaerobe gisting combinaties bleken superieur te zijn in zowel de mate van slibreductie als in de reductiesnelheid ten opzichte van enkelvoudige anaerobe gisting. Echter, de uiteindelijke hoeveelheid afgebroken spuislib bleef onveranderd op 58% en 49% voor wat betreft de respectievelijke parameters chemisch zuurstofverbruik en organische zwevende stof.

De verbeterde slibreductie-snelheid, valt samen met de verwijdering van eiwit-achtige en in mindere mate suikerachtige stoffen uit de slibmatrix. Ook worden alginaat-achtige suikerverbindingen gedeeltelijk geconsumeerd tijdens slibpredatie. De verwijdering van eiwitten, suikers en alginaat-achtige stoffen resulteert in het uiteenvallen van de slibvlokken en het vrijkomen van fulvine- en humus-achtige stoffen evenals de kationen Mg^{2+}, Al^{3+} en Fe^{3+} uit de slibmatrix. Deze vrijgekomen stoffen hebben een bekende structurele functie in de extracellulaire polymere substanties van de slibvlokken en zijn daarom hoogstwaarschijnlijk gekoppeld aan de verwijderde eiwit-achtige fractie.

Gelijktijdig met de verwijdering van een eiwit-achtige slibfractie, werd een verhoogde protease-activiteit waargenomen in het door wormen behandelde slib.

Deze verhoogde protease-activiteit kon worden gerelateerd aan de *T. tubifex*-eigen enzymen en/of aan de enzymen afkomstig van de darm-eigen eiwit-afbrekende bacteriën. Verder onderzoek wees uit dat maximaal 73% van de proteolytische activiteit, op het model eiwit caseïne, afkomstig is van de wormen en de resterende activiteit kon worden toegeschreven aan de darm-eigen eiwit-afbrekende bacteriën.

De synergie tussen de darm-eigen bacteriën en de wormen, in relatie tot de eiwitafbraak, werd verder onderzocht met behulp van microbiële gemeenschap analyse. Het bleek dat de microbiële structuur tussen de op spuislib gebaseerde wormuitwerpselen en de structuur na de predatie van de eiwitrijke substraten, meer gelijkenissen met elkaar vertoonden dan met de microbiële structuur van het spuislib zelf. Deze microbiële verandering kan mogelijk worden verklaard door de heersende omstandigheden in de wormdarm, die een eiwit afbrekende microbiële gemeenschap mogelijk maken.

Het onderhavige onderzoek laat zien dat de belangrijkste aspecten voor het ontwikkelen van een efficiënte hydrolyse van spuislib uit communale zuiveringen, zijn gerelateerd aan het afbreken van eiwit- en alginaat-achtige stoffen en de activiteit van proteasen. Het type protease en mogelijk andere actieve enzymen, zoals de lytische activiteit van de wormen, zijn onderwerpen die meer onderzoek behoeven.

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In 2012 I started as a PhD candidate with a freshly shaved buzz cut, borrowed white glasses and a bag full of cluelessness on how to start my adventure. Luckily for me, I was put in an office with senior PhD's to give me some advice and show me how to PhD. Thank you, Sandra, Ljiljana, Jingyi and Amir for showing me the ropes and Gang for showing me how to fold back my chair, hang back and relax. "No worries, it will be fine" he said. The rest is history.

As time went by, people moved on and the office gradually evolved into "4.41 and a dude": Eloise, Franca, Katie, Mona and me the dude. Dear ladies, thank you all for sharing the PhD life with me!

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To all my friends in the diaspora: Dr. Steefjesman in da house!
And to all those billions and billions and billions of worms: Your sacrifices shall not be forgotten! To their freed brothers and sisters in the canals and ditches around Delft: Live long and prosper!

Now a short decade later, my own glasses, a head full of (greying) dreadlocks and a bag full of wisdom, I've had a lot to be thankful about. There rests only one more thing: Showing my gratitude to my family: My wife Annemarie for loving me for who I am and supporting me trough and trough. My kids Luca and Lux who are perfectly oblivious of what their dad has accomplished. My dad Peter for believing and supporting me since for ever. My brother Elias for just letting me be me. My mother Ivy for always being that guiding force in my life: Mom, I did it: Bai di lanti!

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Wastewater treatment: The activated sludge process

The activated sludge process is the most used process to remove organic carbon and other pollutants from domestic and industrial waste waters. The organic fraction of wastewater is aerobically respirated and partly converted into bacterial biomass. This mixture of treated wastewater and bacterial biomass is called secondary sludge, or waste activated sludge (WAS) and can be considered a by-product of the activated sludge process.

The major problem associated with the activated sludge process is the costs for processing and disposal of the large amount of WAS. Annually 10 million tons of WAS is produced in the EU (2007 estimate) [1]. The processing of WAS mainly consists of concentrating the sludge solids by solids and liquid separation. After processing, the concentrated WAS is destined for disposal. In the Netherlands this merely means incineration, with the exception of some sewage treatment plants (STP) at the border that export the excess sludge to Belgium (Walloon region) or Germany for land application. The disposal of WAS can amount to 50% of the operational cost of a wastewater treatment plant (WWTP) [2].

1.2 Activated sludge treatment: Solids reduction by anaerobic digestion

In order to reduce the amount of surplus sludge that needs costly processing, anaerobic digestion (AD) of WAS is widely applied. This process reduces approximately 30 – 35% [3–5] of the organic sludge mass and partially recovers the biochemical energy contained in WAS in the form of biogas. In order to further reduce the amount of WAS, extensive research efforts have been made towards minimizing the amount of produced sludge [6,7] or increase the rate and extent of WAS degradation during anaerobic digestion [8–11].

AD is a complex microbial process in which organic matter is sequentially degraded to its mineralised compounds [12]. The first step in this conversion process is hydrolysis, which is a lumped term for the disintegration of the sludge flocs, the lysis of the bacterial biomass within the flocs and the hydrolysis of the (released) high molecular polymeric substances (e.g. fats, proteins and sugars)

into soluble monomers. These monomers (e.g. amino acids, simple carbohydrates and lipids) are subsequently converted into volatile fatty acids (VFA) and other products by acidogenic bacteria. The produced acids are used and converted into predominately acetate by acetogenic bacteria. In the final step of anaerobic digestion, the acetate is split into methane and carbon dioxide by methanogenic bacteria.

The hydrolysis step is generally rate-limiting for the entire AD process, directly influencing the rates of the subsequent processes [13] including biogas formation. Research on improving and understanding this first hydrolytic step is extensive. Research includes the development of kinetic models [14], localization and characterization of hydrolytic enzymes present in anaerobic digestion [15,16], microbial community characterization [17,18], physical and biochemical characteristics (e.g. the presence of inhibiting compounds, particle size and settleability) [19–22] and reactor design [6,23,24]. The results of these research efforts have given insight into the factors impacting hydrolysis but has, thus far, not yielded a method that increases the hydrolysis rate without negatively affecting the overall economics of wastewater treatment [25,26].

1.3 Project EnzyFOR

To address the limiting factors of hydrolysis and increase the overall value of wastewaters, the EnzyFOR project was commenced. EnzyFOR stands for the Enhanced Enzymatic Anaerobic Fermentation of Organic Residues. The main aim of EnzyFOR is to increase the valorisation of waste streams, such as agricultural and domestic waste by the utilization of enzymes to produce VFAs from the complex organic substrates present in the waste(water). VFAs are important precursors for biochemical processes in which biogas is the least valuable in terms of market price and environmental effects, but is the most produced, owing to its convenience to be locally used as energy source.

As a means to investigate methods for improving the extent and rate of hydrolysis, the efficient WAS degrading aquatic worms were taken as a model “biochemical reactor” of which its conversion processes still need to be unravelled. The main aim of the research presented in this thesis, is on improving our understanding of worm-based enzymatic processes that are responsible for improving WAS hydrolysis.

The EnzyFOR project was a collaboration between Delft University of Technology, University of Wageningen and the (research and corporate) partners: Delfluent Services BV, DSM, Royal Cosun, Tauw BV, STOWA and TTW (formerly known as STW).

1.4 Outline of this Thesis

During the past two decades, research concerning sludge predation has focused on developing worm-based reactor systems for treating WAS, in which worms are treated as a black box model. Due to this approach, detailed knowledge regarding the hydrolytic mechanisms of aquatic worms is lacking. To formulate a research approach, relevant literature regarding WAS and sludge predation by aquatic worms has been reviewed.

The review revealed relevant knowledge gaps by discussing several important aspects of worm predation. These aspects are: i) the general composition of WAS being the substrate of the aquatic worms; ii) the formation and disintegration of sludge flocs; iii) factors influencing hydrolysis rates, and iv) general information on the worms and how they interact with the WAS substrate. Based on the literature review, knowledge gaps were identified and research questions were formulated. These research questions are addressed in the research chapters presented in this thesis. The review, knowledge gaps and research questions are presented in **chapter 2**.

Worm activities require an aerobic environment and as such, the total sum of biochemical reactions that comprise worm predation in e.g. a worm reactors, is the sum of worm-induced activities and the respiration of the bacteria within the activated sludge floc. To better understand the role of aquatic worms, we should be able to differentiate between the hydrolytic effects of worm predation and the effects caused by solely heterotrophic respiration. Experiments were conducted to systematically research the effects of worm predation on sludge characteristics in comparison to extended aeration of the activated sludge. **Chapter 3** addresses this differentiation by presenting the physical and biochemical changes in WAS upon worm predation.

When worm predated sludge (WPS) is subsequently anaerobically digested, a larger percentage of the sludge solids are removed, compared to direct digestion

of the activated sludge. Because the worms already consumed solids prior to anaerobic digestion, it appears that the worms increase the extent of solids removal or, in other words, improve the overall biodegradability of activated sludge. **Chapter 4** investigates the apparent increase in the biodegradability of anaerobically digested worm predated sludge.

In general, hydrolysis is carried out by hydrolytic enzymes, which must be synthetized. Either worm associated bacteria and/or the worms themselves could be responsible for enzyme production. As such, the source of these enzymes remains elusive. The research on hydrolytic enzymes during aerobic (worm) treatment and insight into the origin of these enzymes are reported in **chapter 5**.

Micro-organisms play an essential role in WAS hydrolysis, therefore the interactions between worms and bacteria are important. A better understanding of the microbial community associated with worm predation will provide insight into the relations between the aquatic worms, their associated microbiome, and the efficient WAS reduction. **Chapter 6** describes the worm-associated microbiology.

Finally, a general overview of the work is presented in **chapter 7** which discusses the findings presented in this thesis into the perspective of potential applications of enzymatic enhancement of anaerobic fermentation of organic residues.

2 LITERATURE REVIEW

2.1 Introduction

There are numerous publications on the implementation of aquatic worms dealing with activated sludge reduction. However, research concerning the actual mechanisms behind sludge reduction is minimal. In order to gain a better understanding of the sludge eating worms, one must start with the basics: What are the main components in sludge that the worms eat? Where are these components located and what are their functionalities in the sludge flocs and how is this all related to sludge hydrolysis? And finally: What is known and more importantly, what is unknown about the worm predation process?

The worm predation process, in a technological setting, consists of several aspects that can have an influence on the overall sludge hydrolysis rates: a process vessel (e.g. a reactor), process conditions (e.g. dissolved oxygen concentration), sludge and worms. Therefore, this literature review addresses the main constituents or biopolymers in WAS and how the structural and functional features of these constituents relate to hydrolysis **(section 2.2)**.

Additionally, the interaction of these biopolymers in relation to floc formation is addressed. By understanding how flocs are formed and what parameters govern floc stability, more insight can be gained into floc disintegration, which has a positive effect on hydrolysis rates **(section 2.3)**. Furthermore, the literature concerning worm predation is reviewed and combined with the previous sections in order to formulate knowledge gaps **(section 2.4)**. The knowledge gaps and the research questions are finally presented in **sections 2.6 and 2.7**.

2.2 WAS composition

2.2.1 Extracellular polymeric substances

The bacterial mass in WAS is organised in flocs, which consists of bacterial cells and extracellular polymeric substances (EPS) and soluble microbial products (SMP). EPS can make up to 50-60% of the organic fraction of sludge while the SMPs, with a soluble COD of about 100 mg O_2/L make up a relatively small part of the total organics fraction. The biopolymers present in EPS from activated sludge consists for the majority of proteins, carbohydrates and humic substances [27].

The EPS biopolymeric compounds originate from high molecular weight excretion products of microorganisms, released due to cellular lysis and the hydrolysis products of other macro molecules [28]. Additionally, (in)organic compounds, present in wastewater can also adsorb to the EPS structure [29–31]. Comte et al. (2006) [32] found that soluble EPS might have larger adsorption strength towards heavy metals than bound EPS due to the higher fraction of proteins present in the soluble EPS.
Furthermore, lipids, nucleic acids, uronic acids and minerals (e.g. Na, K, Mg, Ca, Cu, Ag and As) have been extracted from EPS [33–35]. In pure cultures, polysaccharides are dominantly present [36], whereas in EPS extraction from activated sludge, proteins are the major constituents [37]. It has to be noted that the type and concentrations of the EPS constituents strongly depend on the growth conditions of the bacteria and also on the method and efficiency of the extraction procedure [29,34,35,38]. Generally, the amount of EPS components present in sludge is higher than in the extracted EPS fraction [39].

EPS can be subdivided into bound EPS and soluble EPS. Bound EPS has a strong attachment to the cell exterior and consists out of sheaths, capsular polymers, condensed gel, loosely bound polymers and attached organic material. Soluble EPS consists of colloids, slimes and soluble macromolecules, which are weakly bound and/or dissolved in the water present inside the EPS structure [40]. The distinction between bound and soluble EPS emerges from the separation by centrifugation; soluble EPS is found in the supernatant and bound EPS in the pellet [29,40]. Due to the strong attachment of EPS with bacterial cells, bacteria can aggregate into floc and biofilm structures, which are then further mechanically stabilised by other components within the EPS [41].

Besides this structural function, EPS also has a protective function for the floc-inhabiting bacteria. The EPS matrix protects bacteria against conditions such as dehydration, (in)organic toxic compounds but also acts as a feed source upon starvation [42]. Additionally, within the EPS matrix specific micro-environments are formed due to bacterial activity. The base of these micro-environments are diffusion gradients of, for example oxygen, pH, nitrogen and other substrates [42,43]. Moreover, substrate uptake is optimised due to the floc matrix that also entraps extra-cellular enzymes and retains lysis products that can then be recycled.

2.2.2 **Exo-polysaccharides**

Polysaccharides make up 10 to 15% of the organic content of sludge. [27,39]. Most of the polysaccharides are hetero-polysaccharides. They consist of neutral and charged sugar residues and can contain organic and inorganic substituents. Functional groups have a great influence on the properties of polysaccharides. The type of polysaccharide present in EPS depends on the type of microorganisms that are present. For instance, due to uronic acid substitutions the polysaccharides xanthan, alginate, and colanic acid are poly-anionic [22].

Polysaccharides play an important role in biofilm formation. For instance, polysaccharides are responsible for the formation and mechanical stability in the biofilm-model organism *Pseudomonas aeruginosa* [44]. Polysaccharides are closely linked with other constituents found in EPS. Additionally, carbohydrate extracted from EPS were associated with proteins. This finding indicates that the poly saccharides are sugar derivates such as lipopolysaccharides, glycoproteins or are linked to other EPS constituents [37,45].

As polysaccharides are a very diverse group of molecules, the glycosylases are a very diverse group of sugar degrading enzymes. Enzyme functionality depends on the position of the linkage between sugar subunits, the type of substitutions present, but also the endo-exo functionality of the enzyme. As such, bacteria can produce a large set of hydrolytic enzymes ranging from a few to up to 20 different enzymes for the efficient utilisation of a polysaccharide [46].

Alginate like exopolymers (ALE) are of special interest as this polysaccharide is produced by many different microorganisms and has a structural function in EPS. This main structural property comes from the distribution of D- mannopyranosyl and L- glucuronosyl sugars and the heavy acetylation of the D- subunits. Alginate is degraded by mannuronate and luronate lyases [47]. Additionally, ALE can be extracted from granular sludge as pre-cursor for potential industrial applications [48,49]. Furthermore, alginate-like has the ability to form gel-like properties by chelation of divalent ions such as Mg^{2+} and Ca^{2+} on the L- glucuronosyl residues [50].

2.2.3 **Proteins**

Proteins-like substances are the largest fraction in activated sludge. About 50% of the VS content of extracted EPS are protein-like substances [27]. Conrad et al. [51] found that 43% of the EPS extracted with cation exchange consisted of proteins. Some of these proteins are adsorbed from the waste water, others are excreted by the microorganisms present in the floc matrix or are released through cell lysis.

Proteins have an important role in maintaining floc stability by balancing the hydrophobic and hydrophilic interactions between EPS constituents [19]. Furthermore, some proteins are closely associated with polysaccharides [37]. These proteins, based on amino-acid sequencing results, appear to resemble lectins, which are carbohydrate binding proteins that can act as a link between the polysaccharides and the bacterial surface. These lectin-like proteins were found in industrial, domestic and synthetic sludge [45].

2.2.3.1 Enzymes

Other proteins are catalytic in nature and can be attached to bacterial membranes (ecto-enzymes) or released (exo-enzymes) into the medium [52]. The released exo-enzymes are readily adsorbed onto the EPS-matrix trough interactions with humic substances and other polymers [53]. As such, most of the enzymatic activity is associated with the sludge solids [54,55]. More specific, most of the protease (44%), L-Leu-aminopeptidase (5%), alpha- and beta-glucosidase (23%) activities are located in the easily extractable EPS fraction.

The in EPS entrapped extracellular enzymes can be liberated trough disruption of the sludge flocs by sonication [56]. The liberated enzymes come in contact with released EPS polymers, resulting in an increased aerobic degradation of the sonicated WAS. Interestingly, an innate high sludge hydrolysis rate, by the action of the embedded enzymes is generally not observed. This suggests that the sludge flocs are comprised of relatively stable compounds. It is highly likely that these stable compounds are in fact humic substances, which will be addressed in the next section.
The breakdown of complex organic matter largely depends on the enzymatic actions of the hydrolytic microorganisms. The efficiency of hydrolytic enzymes and the proliferation of hydrolytic bacteria depend on factors such as particle size,

process conditions (pH, temperature, mixing etc.) and the types of substrates that are to be hydrolysed. Pre-treatment methods and kinetic models revealed that disintegration, solubilisation and hence enlarged surface area, are key factors for improving WAS biodegradability and hydrolysis rates [14].

2.2.4 Humic substances

Humic substances are an important (structural) component of EPS. The amount of humic substances in EPS depends on the sludge condition and ranges from 10 – 20% [27,51]. Although, humics are considered as hardly biodegradable, fungal treatment was successful in treating humic rich waste waters [57]. Considering traditional wastewater treatment, the specialized fungal enzymes will not be present for the degradation of humics as the fungi are outgrown by bacteria [58].

Humic substances are heterogeneous acidic macromolecules and are generally negatively charged. Humic substances are usually divided in 3 classes: Humic acids, which are soluble at pH>2, Fulvic acids, which are soluble at any pH, and humins, which are insoluble at all pHs. There are different theories about how humic substances are formed and how their molecular structure looks like [59]. The consensus is that humic substances are composed of microbially and chemically (e.g. UV irradiation) degraded plant and animal material [60] that interacts by hydrophobic, hydrophilic and electrostatic interactions. The types of functional groups in humics depend on the source from which they originate [60,61].

The functional groups of humics (carboxylic, phenolic, ketonic, aromatic, aliphatic, quinone, amongst others) interact with inorganic and organic materials such as proteins, carbohydrates, ions etc. [59,62,63]. Enzymes can be entrapped [64] and pollutants and other soluble compounds can be adsorbed, which reduces the bioavailability of these components [65]. In contrast to the reduction in bioavailability of some compounds, the retention of minerals and nitrogen in soils has increased [66,67], which is a favourable aspect.

Fernandes et al. [68] demonstrated that the presence of humic substances has a negative impact on WAS hydrolysis rates, likely due to entrapment of enzymes by humics. It was postulated that hydrolysis is inhibited by a molecular binding between the reactive functional groups of humic substances and the hydrolytic

enzymes and that bivalent cations (such as Ca^{2+}) can mitigate the inhibiting effect [69,70]. In support to the hypothesis that describes the occurrence of stable enzyme-humic complexes, the mitigation of the inhibitory effect of humics on hydrolytic enzymes by the addition of divalent cations was eventually reported by Azman et al. (2015) [71].

Living organisms are affected by the presence of naturally occurring humic substances present in soil and (ground) water. The review by Steinberg et al. [66] focused entirely on the interaction between living organisms and humic substances. It has also been found that humic substances can be redox active and are utilized by some bacteria as electron shuttles, for anaerobic oxidation of organic and inorganic electron donors [72,73]. For instance, iron and sulphate reducing organisms and also fermentative bacteria utilize this electron mediating characteristic of humic matter [72,74,75]. From all functional groups, the quinone moieties are being recognised for having the highest electron mediating capacity in humic matter [76–78].

2.2.5 Bacterial cells

Bacteria are important constituent of WAS. Literature values for the amount of bacterial biomass in the sludge matrix range from 6-8% [27], 15% [26] to 23% [79] of the sludge volatile solids (VS). The bacterial biomass, consists mainly of gram-negative bacteria [80] and are organized in flocs in which EPS plays a dominant role.

Generally, bacterial biomass or more specifically the bacterial cell wall, is highly resistant to hydrolysis (e.g. cell lysis). The cell wall or the peptidoglycan layer of bacteria is surrounded by a second lipid membrane layer, containing lipopolysaccharides and lipo-proteins in case of gram-negative bacteria. Certain enzymes can hydrolyse cell wall constituents. These extracellular lysozymes, which are glycoside hydrolases can hydrolyse the β-(1,4)-bond in peptidoglycan, resulting in a loss of rigidity and rupture of the cell wall [81].
For gram-negative bacteria, the additional lipopolysaccharide layer has to be degraded first before lysozymes can attack the peptidoglycan layer. This lipopolysaccharide layer can be hydrolysed by synergetic efforts of proteases, glycosylases and lipases. In general, bacterial cells can remain intact for long periods of time, as long as the proton motive force remains functional. Dissipation

of the proton motive force due to lack of energy, can results in the inability to maintain cellular integrity, which eventually results in membrane instability and subsequent cell lysis [82,83].

2.3 Sludge properties

2.3.1 Flocculation

The ability of microorganisms to flocculate has an advantage in waste water treatment. Positive effects are mainly attributed to the settleability of the sludge flocs in waste water treatment systems, which results in effluents with a low turbidity. More recent technology developments focus on the development of more rigid types of flocs, i.e. granular sludge, with settling properties exceeding the more common waste activated sludge flocs [84].

Sludge granules and sludge flocs are characterised by structural and chemical elements, which hinder the enzymatic breakdown of EPS and lysis of the bacterial cells within the EPS structure. Therefore, bioconversion of excess sludge in e.g. anaerobic digesters is generally a slow process that is governed by the hydrolysis of the mentioned macromolecules. A better understanding of the basic principles of floc formation and disintegration is a prerequisite to advance in sludge hydrolysis.

The role of cations in flocculation has been described by various theories such as: the Double Layer Theory or DLVO Theory [85], the Divalent Cation Bridging (DCB) Theory and the Alginate Theory [86]. Sobeck & Higgins [87] concluded that the DCB theory best describes the role of divalent cations on floc stability. The DCB theory postulates those divalent cations bridge negatively charged functional groups within the EPS. This bridging stimulates flocculation by promoting aggregation and stabilization of the biopolymers and microbes

2.3.2 Floc stability: biopolymers and cations

The interaction between biopolymers such as proteins and carbohydrates, with cations are important for floc stability and formation. Important findings are mentioned in this section.

Divalent cations Ca^{2+} and Mg^{2+} have a positive effect on flocculation [87,88]. When divalent cations are replaced by monovalent cations ions, the floc structure deteriorates [89]. It has been shown that the removal of Ca^{2+} from the bulk liquid by cation exchange or by dilution with demineralised water, results in the release of Ca^{2+} and predominantly humics. Additionally, it is suggested that the actual calcium-ion is important and not the change in sludge surface charge [90,91].

The interaction of cations with proteins is also mentioned by several researchers. For instance, enzyme assisted degradation of EPS protein resulted in deflocculation and more release of Ca^{2+} and Mg^{2+} than when carbohydrates were degraded [45]. Deflocculation could be due to loss in EPS hydrophobicity and cellular surface charge (ζ-potential) when protein is removed which are deemed to be important parameters for flocculation [92]. Recent findings have shown that the tightly bound EPS fraction contains a higher fraction of hydrophobic protein related N-H groups compared to the loosely bound EPS fraction. It was concluded that hydrogen bonds are the dominant triggers that promote sludge aggregation [93].

EPS protein interaction with iron was also reported. Murthy & Novak [94] suggested that Fe^{3+} selectively binds to proteins released during anaerobic digestion of WAS and that iron retains part of the proteins in the floc structure. The removal of the EPS bound iron using reduced sulphur to reduce iron, resulted in deflocculation and the release of proteins [95]. Park & Novak [96] hypothesized that two types of biopolymer binding mechanisms are present in flocs. One fraction is associated with Ca^{2+} and Mg^{2+} and another fraction is associated with iron.

The Ca^{2+} and Mg^{2+} associated fraction (polysaccharides) is released from WAS under aerobic conditions. The release of these carbohydrates coincides with the decline in glycosidase activity, i.e. enzymes related to polysaccharides hydrolysis, under aerobic conditions [97]. The aerobically released fraction can be degraded in anaerobic conditions. The iron-associated fraction is released after iron is reduced from Fe^{3+} to Fe^{2+} in anaerobic conditions. The iron associated fractions, predominantly proteins degrade under anaerobic conditions.

2.4 Sludge reduction with the aquatic worm *T. tubifex*

Aquatic worms have been found to naturally inhabit the aerobic zones of WWTPs. Sudden worm growth or worm blooms, have been associated with improved sludge settling characteristics and a lower WAS production. These beneficial characteristics resulted in a large research interest in WAS reduction using sludge worms [98]. Sludge predation technologies are usually implemented after the activated sludge process and before anaerobic digestion. Although aquatic worms can consume anaerobically digested WAS [99], care has to be taken of the higher ammonium concentrations as ammonium can be toxic for aquatic worms [100,101].

Worm predation research is mostly concerned with the reduction of activated sludge and overcoming the main limitations of worm predation, namely maintaining a stable worm biomass. Different aquatic worms, such as *Tubifex tubifex*, *Lumbriculus variegatus*, *Aulophorus furcatus* and *Limnodrilus hoffmeisteri* have been researched and implemented in several different lab and pilot scale setups [102–107]. However, due to problems maintaining the worm biomass, the shift in the perception of sludge treatment (from waste to resource), and the inability to use the grown worms in the food chain, worm predation research has declined significantly during the past decade.

The aquatic oligochaete worm *T.Tubifex* was chosen as model organism for its high tolerance to harsh environments such as WAS, its high rate of sludge reduction [108] and the good availability in general pet shops. *T. tubifex* is a sessile hermaphroditic worm that reproduces sexually by laying eggs [109]. Additionally, these aerobic worms contains haemoglobin [110] and have the potential to survive extended anoxic periods [111,112]. These worms increase oxygen uptake by wiggling their tails that protrude the sediments the worms burrow in. Furthermore, due to the burrowing activities, aquatic worms play an essential role as bioturbators which affect the microbial activities in sediment they live in [113,114].

T. tubifex mainly ingests particles smaller than 63 µm [115] and which preferably contain a high clay and silt content [116]. The faecal pellets, produced by *T. tubifex* contain particles with a mean diameter below 63 µm [117] which suggest

that *T. tubifex* selectively ingests particle of certain sizes. Furthermore, about 75% (by volume) of the faecal matter was made up of particles with a mean diameter <25 µm [117]. Peristaltic movement transport the ingested particles through the worms intestines [118] in a similar fashion as terrestrial worms . These movements could exert a grinding effect on ingested particles and in turn affect particles sizes and thus hydrolysis rates.

Besides particle size selectivity, the organic content is also a criterion for tubifex worms. Conflicting studies show that *T. tubifex* prefers the fine grained nutrient rich fraction or course grained nutrient poor sediment [98]. Despite these conflicting results about the natural sediments these worms inhabit, the consensus is that activated sludge as the substrate induces increased growth rates of worms [119,120]. Possibly, the increased growth rates on WAS are due to the higher concentration of bacteria compared to natural sediments. Interaction with bacteria will be further discussed in section 2.4.3.

2.4.1 WAS solids removal

In general, worm predation results in WAS reductions of 8-40% (TSS based) depending on the type of worm, the type of sludge used, and the experimental setup used [102–106]. When these values are compared to the treatment efficiencies of common anaerobic digesters, which are in the range 30 to 40%, the overall treatment efficiencies are similar. The main difference between these two treatment methods is the process time or solids retention time (SRT). Worm predation has significantly shorter SRTs, which ranges from 2 to 4 days compared to the 20-30 days required for anaerobic digestion in completely stirred tank reactors.

Additionally, Tamis et al. [105] reported that the anaerobic storage of worm predated sludge (30 – 40 % solids removal due to predation), resulted in a higher degree of solids removal for the combined process (worm predation followed by anaerobic digestion) compared to only anaerobic digestion of raw activated sludge. About 60% of the original feed sludge was removed in the combined process compared to the 30-40% solids removal for solely anaerobic digestion. The authors suggested that worm predation increased the biodegradability of activated sludge solids.

Interestingly, a similar increase in biodegradability of activated sludge solids was also observed by Park et al. [121] using sequenced aerobic / anaerobic environments. They showed that the aerobic treatment (extensive aeration) followed by anaerobic treatment of WAS or vice versa, resulted in a solids removal of about 60% in 90 days. Additionally, Shao et al. [91] found comparable solids reduction levels as Tamis et al. [105] with aerobic or anaerobic treatment of WAS for 90 days. In contrast to the reported improvement in the biodegradability of sludge solids, Serrano et al. [122] showed that worm predation does not increase the methane potential of the processed sludge, i.e. the worm faeces.

2.4.2 Sludge predation characteristics

Sludge worms have been researched extensively as a potential technology to effectively reduce the activated sludge mass. Therefore, a high number of publications are available on the utilization and optimization of worm technology. In addition to the superior solids removal of the worm predation process, the conducted studies reveal various similar observations during worm predation, such as: i) increased $N\text{-}NH_4^+\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ release [104,105,123,124], ii) improved settling characteristics [99,125,126] and iii) sCOD release [127]. These similarities suggest that aquatic sludge degrading worms may use a common mechanism for sludge hydrolysis.

However, in contrast to the widely reported process parameters, limited information is available on how the worms actually degrade the sludge or which sludge components they consume. Important indications regarding the preferred substrate for aquatic worms are the preference for protein [99] and their interactions with bacteria [128–132]. Furthermore, an increased enzymatic activity and a reduction in particle size was observed in the predated sludge compared to the feed sludge, according to the MSc Thesis of Mooij et al. [133]. Unfortunately, enzymatic activities were not measured in a reference process without worms.

2.4.3 Common aspects of worm predation: Substrate specificity and sCOD release

2.4.3.1 Protein-like substances

L. Variegatus [99,134] specifically targets nitrogen compounds in sludge. Batch tests showed a preference for nitrogen compounds over carbohydrates. After predation and during endogenous digestion batch experiments, the amount of protein-like substances in the water phase increased. Carbohydrate-like substances also increased but only directly after worm predation [135]. The removal of proteins is accompanied by the release of inorganic nitrogen mainly in the form of NO_3^-. Liang et al. [102] observed improved growth rates of *Aeolosoma hemprichi* when grown on sludge with higher protein concentrations. This conclusion was reached by comparing *Aeolosoma* growth rates on sludges with different protein content. However, the used sludge originated from two different systems and were prepared differently. To conclude that the higher growth rate was due to the higher protein content is there for arguable.

Tian et al. [127] showed that worm predation by *Limnodrilus hoffmeisteri*, in a membrane bioreactor (MBR) coupled to a Static Sequencing Batch (SSB) worm reactor system, increased the amount of sCOD, whereas the amount of extractable EPS reduced. They linked the reduced amount of membrane fouling to the decrease in protein-like substances in EPS. Additionally, the functional groups of aromatic and tryptophan protein-like substances were altered in the EPS after a worm predation process. More specifically, the functional groups that became more apparent were carbonyl-, hydroxyl-, alkoxyl-, amino-, and carboxyl- groups.

The release of organic substances, such as carbohydrate- and protein-likes substances is generally measured as an increase in sCOD. A similar sCOD increase was reported by Tamis et al. (personal communication) using *A. furcatus* in a pilot worm reactor. The constituents of this increased sCOD were not specified. Possibly the increase in sCOD resulted in the observed improved solids removal during AD of predated sludge [105]. Additionally, Mooij et al. [133] found an increased protease and lipase activity in the supernatant of worm predated sludge. The origin of the increased enzymatic activity was not investigated but corroborated with the observed protein removal. However, the enzymatic activity

in a control process (i.e. without worms) was not determined, preventing to conclusively state that aquatic worms increased the enzymatic activity. Generally, enzymatic activity declines during aerobic sludge treatment [56,97].

2.4.3.2 Carbohydrate-like substances

Elvira et al. [136] found that the earthworm specie *Eisenia Andrei* accelerated the mineralisation of polysaccharides present in solid paper-pulp mill sludge. Aira et al. [137] found that some earth worm species show cellulolytic activity in cooperation with the microorganisms inside the intestines. Cellulose degradation in aquatic worms is to our knowledge not researched.

2.4.3.3 Bacterial interaction

It has been suggested that *T. tubifex* [129,131,138] and other aquatic worms [139–142] selectively consume bacteria as a food source. Ratsak et al. [98] commented that aquatic worms show a preference for gram-negative bacteria. Wavre & Brinkhurst [129], found that about 70% of the heterotrophic bacteria did not survive gut passage. Indications of bacteria consumption by aquatic worms was observed in oligochaetes species. This resulted in a concentration of certain bacterial species in the faecal pellets. Edwards & Fletcher [143] confirmed the up-concentration of certain microbial species in the worm intestines. Considering the consumption of bacteria, the increase in soluble TN and TP suggests an increased activity or selectivity towards proteins and/or bacterial hydrolysis [134]. More specifically, the increase in phosphate might be due to specific consumption of the gram-negative phosphate accumulating organisms (PAO's). This, however is speculative and requires further research.

In this perspective, the aforementioned removal of proteins from the EPS matrix, could be due to the consumption of bacteria residing in the EPS. The removal of bacteria or 'microbial stripping' [98], results in changes in microbial community of the natural sediments the worms inhabit [114]; or in case of sludge reduction, changes in the microbial community of the sludge reduction system the worms inhabit [132]. Changes in the microbial community of the worm gut and habitat for terrestrial oligochaete have also been observed [143–146] and is related to the type of substrate the worms consume [147]. In turn, these environmental changes could result in optimised growth conditions for specific bacterial species associated with the worms.

2.4.3.4 Heavy metals

Due to the high protein content, worms could serve as a feed stock. For this reason, the amount of metals in the worm predation system was investigated by several authors. Hendrickx et al. [134] reported that the metal content (Fe, Cu, and Zn) of worm faeces was higher than that of the consumed excess sewage sludge when expressed as g/kg TSS. When expressed as g/kg FSS the metal content was the same and the heavy metal accumulation in the worm biomass was negligible. Ratsak et al. [98] showed similar results with predation on metal contaminated sludge, where the heavy metals accumulated in the predated sludge. The release of heavy metals to the bulk liquid was not determined.

In partial contrast to these findings, Zhang et al. [148] found that the aquatic worm *Limnodrilus hoffmeisteri* accumulated Fe, Cu and Zn. They also reported an increase of 5-10% in soluble Fe, Cu, and Zn in the effluent, as well as an increase in metal concentration in the worm predated sludge. About 80% of the metals remained associated with the sludge. Tamis et al. (personal communication) observed a significant release of metals into the supernatant upon anaerobic digestion of worm-predated sludge. Whether this release of metals is due to the worm process or due to the anaerobic reactor conditions was not investigated.

As previously indicated, divalent cations play an important role in maintaining floc stability and thus, the release of these divalent cations could provide valuable information on the mechanisms of the sludge degradation process inside the worms. However, no relevant information is available regarding the release and role of divalent cations during worm predation.

2.5 Literature summary

Sludge contains a broad spectrum of different polymers, microorganisms and metals. These sludge constituents have been extensively documented but seldom in the perspective of worm predation. Standard parameters, such as N, P, COD and TSS are routinely measured and reported for sludge predation. However, rarely these parameters were measured in control systems without worms to distinguish between the effects of endogenous sludge respiration and worm predation.

In EPS extraction from activated sludge, proteins are the major constituents. Part of these proteins, in conjunction with divalent cations, have a structural property to maintain the floc structure. These ions together with specific protein and polysaccharide fractions are released when the EPS matrix is degraded. More specifically, aerobic degradation results in the release of polysaccharides and divalent calcium and magnesium, while during anaerobic degradation protein-like substances are released with iron. The release of cations during predation has not been reported. Other (heavy) metals are concentrated in worm faeces or released form the predated solids and do not seem, at moderate concentrations to have an influence on the worm predation process.

The increased nitrogen release during the worm predation process is due to the abundant protein availability and the preference of the aquatic worms for this protein-like fraction in sludge. The selectivity for proteinaceous matter could encompass the consumption of specific gram-negative bacteria. There is strong evidence for selective feeding on bacteria by aquatic worms. Bacterial consumption could explain the increased release of phosphate. Specific consumption of polysaccharides or lipids has not been reported. The increase in protease activity is therefore important. However, it remains unclear whether the increased enzyme activity resulted from the disintegration of flocs, the release of exo-enzymes through the activities of the aquatic worms and/or the influence of the worm's intestinal biome or the removal or inactivation of inhibiting compounds such as humics. Additionally, the mechanical grinding/mixing by peristaltic movement of the worm gut could also influence solids reduction. Nonetheless, these and other changes in the EPS matrix led to improved biodegradability of sludge solids.

2.6 Knowledge gaps

The worm predation process showcases efficient hydrolysis of proteinaceous matter and other sludge constituents as well as improved WAS biodegradability. The efficiency of sludge predation by aquatic worms is apparently dependent on different processes on both macro and micro level: i) the design and operation of the aerobic worm reactor, ii) the enzymatic, mechanical and biochemical conversions by the worm themselves, and/or iii) the catabolic activity of hydrolytic bacteria in the intestines of the worms.

The knowledge gaps, in relation to the goals of the EnzyFOR-project, are summarised as follows:

- ***An extensive comparison between the worm predation process and endogenous respiration/ extended aeration of activated sludge using the same process conditions is missing. Research should reveal the actual contribution of worms in the conversion process.***
  A systematic approach could thoroughly describe the various biochemical and physical changes in sludge during the predation process in comparison to a system without worms e.g. endogenous sludge respiration. Biochemical and physical changes that could influence hydrolysis rates are of key interest**.**

- ***The maximum biodegradability of (predated) WAS under aerobic and anaerobic conditions is unknown.***
  The reported biodegradability improvement of predated sludge solids requires validation and further investigation. Proper validation requires a comparison of the extent of the biodegradability improvement using worms against the maximum biodegradability achievable by bacteria and archaea only.

- ***The (increased) enzymatic activities in predated sludge have not been reported in scientific literature.***
  WAS hydrolysis is primarily determined by enzymatic activity. Further insight and proper description of the relevant enzymatic processes is of eminent importance to better understand the exact role of the worms in

the predation process. Especially protein removal deserves further attention.

- ***A clear distinction between activity of the worms and their intestinal bacterial community in relation to sludge biodegradation is unknown.*** As solids reduction is a result of enzymatic activity, the origin of these hydrolytic enzymes should be elucidated as they can originate either from the aquatic worms and/or from the intestinal bacterial community. A clear insight into the actual contribution of aquatic worms is necessary to further investigate worm-based enzymes and to propose possible full-scale applications.

- ***Information regarding the intestinal bacterial community of aquatic worms and the worm predation process is lacking.***
  Bacteria may play an important role in sludge reduction as hydrolytic enzyme producers. It is therefore important to further investigate the microbiology of the worm predation process to unravel the role of intestinal bacteria in the improved sludge reduction**.**

- ***The effect of mechanical (peristaltic movement) or bio-chemical activity (acidic or alkaline conditions) in the worm intestines on sludge solids is unknown.***
  From terrestrial worms it is known that peristaltic movement and changes in pH occur in the intestinal tract. However, it is unclear what the effect and relevance of these processes are on the efficient biodegradation of WAS solids.

- ***Genetic information regarding T. tubifex hydrolytic enzymes is non-existent.***
  To further explore the worm-based hydrolytic enzymes, the DNA sequences of these enzymes need to be unveiled. As such, genetic information of aquatic worms and identification markers for relevant hydrolytic enzymes, such as mRNA and protein sequence of enzymes, are required**.**

2.7 Research questions

After revealing these knowledge gaps, specific research questions were formulated in order to investigate the actual WAS conversion process inside the worm intestinal tract.
The main objective of the EnzyFOR project is to use these insights to propose or develop methods to enzymatically enhance the hydrolysis of WAS.
The knowledge gaps concerning the mechanical and chemical processes inside the worm intestinal tract, as well as a genomic analysis of the worm system are outside the scope of this thesis.

The research questions that will be addressed in this thesis are the following:

- What are the physical and biochemical changes in WAS upon predation? (**Chapter 3**)
- What is the maximally achievable biodegradability of WAS by worm predation compared to extended anaerobic and aerobic treatment? (**Chapter 4**)
- Does predation increase hydrolytic enzyme activities in WAS compared to endogenously respired sludge? (**Chapter 5**)
- To what extent are the worms and/or their intestinal bacterial community responsible for sludge hydrolysis? (**Chapter 5**)
- What is the structure of the microbial community, associated with the aquatic sludge worms? (**Chapter 6**)

To find answers to these questions, a batch worm reactor was designed in which worm predation and endogenous respiration could be studied in parallel, while operating the reactor modules under the same conditions, and feeding the modules with the same WAS. In order to answer the last two questions, the sludge worms were fed with different substrates and the microbial community was compared. Furthermore, antibiotics were used to suppress intestinal bacterial activity in order to make a distinction between the activity of the aquatic worms and their intestinal bacterial community.

2.8 References

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3 PHYSICAL AND BIOCHEMICAL CHANGES IN SLUDGE UPON *T. TUBIFEX* PREDATION

This chapter is based on:

Steef de Valk, Ahmad F. Khadem, Christine M. Foreman, Jules B. van Lier and Merle K. de Kreuk (**2017**) *Physical and biochemical changes in sludge upon Tubifex tubifex predation*. Environmental Technology, 38:12, 1524-1538

Abstract

Worm predation (WP) on activated sludge leads to increased sludge degradation rates, irrespective of the type of worm used or reactor conditions employed. However, the cause of the increased sludge degradation rates remains unknown. This paper presents a comparative analysis of the physical and biochemical aspects of predated sludge, providing insight into the hydrolytic mechanisms underlying WP. To this end, the sessile worm *T. tubifex* was used as a model oligochaete and was batch cultivated in an 18 L airlift reactor. Predation on activated sludge showed an average reduction rate of 12 ± 3.8 %/d versus 2 ± 1.3 %/d for endogenous respirated sludge. Sludge predation resulted in an increased release of inorganic nitrogen, phosphate and soluble chemical oxygen demand (sCOD). The sCOD consisted mainly of polysaccharides; however, fluorescence excitation emission matrix spectroscopy analysis also revealed the presence of Tryptophan-protein-like substances. Results suggest that the released polysaccharides contain a protein-like element. Additionally, soluble iron increased slightly in concentration after WP. The extent of hydrolysis seemed to reach an average plateau of about 40% volatile solids (VS) reduction after 4 days which is substantially higher than the 29% VS reduction for endogenous decay of activated sludge after 30 days. Furthermore, *T. tubifex* predominantly consumed the protein fraction of the extracellular polymeric substances. Results suggest that that the worms specifically target a fraction of the sludge that is predominantly biodegradable under aerobic conditions, albeit at significantly higher degradation rates when compared to the endogenous decay of waste activated sludge.

3.1 Introduction

Waste activated sludge (WAS) is a by-product of the aerobic treatment of sewage and industrial wastewater. Approximately 10 million ton of WAS is produced annually in Europe [1] (2007 estimate) and has to be disposed of according to the Council Directive of the Commission of European Communities [2]. Sludge disposal has been estimated to account for 50% of the total costs for waste water treatment plants (WWTPs) [3]. To lower these disposal costs, anaerobic digestion is used to reduce the amount of excess sludge and partially recover the biochemical energy stored in sludge as biogas. However, anaerobic digestion removes only about 30–40% of the organic component of biomass, which means that a large amount of undigested solids still needs costly processing.

These costs have stimulated many research projects that aim to minimize the amount of WAS that has to be disposed of. Some methods are aimed at increasing the rate and extent of hydrolysis during anaerobic digestion by physical and/or chemical methods or enzymatic pre-treatment of the sludge [4–6]. Other methods are aimed at reducing excess sludge production by increasing the sludge age [7–9] and thus increasing cell lyses and cryptic growth mechanics [10], which in turn results in a decrease in sludge production.

A special case of excess sludge reduction is by means of aquatic worms that naturally inhabit aerobic zones in WWTPs [11]. Several different sessile and free-swimming oligochaete worm species have been investigated with a variety of reactor designs for their ability to degrade sludge (Table 3.1).

Table 3.1: *Summary of oligochaete worm related research: Worm species, reactor design and important findings*

Worm species	**Reactor design and feed**	**Important results and remarks**	**References**
T. tubifex, Aeolosoma hemprichi	Plug flow reactors with sessile and free-swimming compartment (WAS)	Worms present in both control and experimental groups.	[12]
A. hemprichi	Continues conventional activated sludge system with different solid retention times to assess effect on worm growth. (WAS supplemented with artificial sludge)	Sludge protein content has a positive effect on growth. Chemical oxygen demand (COD) and N removal remained unchanged. Sludge volume index (SVI) decreased	[13]
Lumbriculus variegatus	Sequence batch reactor with carrier material and separate faeces collection. (WAS, BioP-WAS)	Compact faeces, protein preference of worms, low methane potential for worm faeces, SVI decreased, 16 – 42% Volatile suspended solids (VSS) reduction	[13–18]
Branchiua sowerbyi, Limnodrilns species	38 L Continuous vertical worm reactor coupled to a 5m^3 aerated ditch. (WAS)	±50% TS conversion in worm reactor. No effect on aeration ditch performance. No appropriate control.	[18]
T. tubifex	Continues worm reactor (Hydraulic retention time of 10h and fed artificial sludge) and 24h batch tests (sterile synthetic sludge). Both with varying worm densities.	No apparent effect on SVI, COD, or N-release regardless of worm density. 24h tests served as control.	[19]
Lumbriculida hoffmeisteri	Batch test nutrient release comparison. (Fresh and sterile sludge)	Due to low worm concentration, no difference in nutrient release.	[20]
Aulophorus furcatus	125m^3 continues flow worm reactor with carrier material (WAS)	Averaged ± 50% total suspended solids removal with release of mineralization products. No appropriate control.	[21]
Limnodrilus hoffmeisteri	Membrane reactor coupled with worm reactor (synthetic or WAS)	Less membrane fouling with WP. Soluble COD increased. No comparison made in regard to feed and no appropriate control.	[22–25]

As shown in Table 3.1, not all studies included appropriate control experiments, which are needed to properly relate worm predation (WP) to the change in sludge characteristics. However regardless of the variability in the different experiments, it is clear that the observed changes in sludge after WP have been similar, irrespective of the worm species and/or reactor setup used. Overall, these changes include increased sludge degradation accompanied by the release of mineralization products, an increased in soluble chemical oxygen demand (sCOD), improved settling characteristics and the removal of proteins. These similarities suggest a common mechanism of sludge hydrolysis employed by aquatic worms.

Worm characteristics that could influence hydrolysis rates are peristaltic movement and bioturbation [26–28], although the latter is expected to be of minor importance in a turbulent environment such as a WWTP. It has also been suggested that oligochaete worms consume bacteria [29–32] and change the microbial diversity in natural sediments [30] or the sludge [33] that the aquatic worms inhabit.

The mechanisms that worms use for hydrolysis deserve further attention because hydrolysis is considered to be the rate-limiting step in the degradation of excess sludge [34,35] and sludge predation increases this degradation rate. Even though knowledge regarding WP is steadily increasing, the underlying hydrolytic mechanism of predation has not been investigated specifically. The majority of research concerning aquatic worms has focused on implementation for sludge minimization with the worms depicted as a black-box model for sludge degradation or membrane fouling mitigation (Table 3.1). As a consequence, a complete data set to adequately research WP mechanics is lacking.

By using controlled reactor conditions, this paper presents a comparative analysis of the physical and biochemical components of initial feed WAS, worm predated sludge and endogenously respired sludge. By means of this method, a clear distinction can be made between the effects of the reactor conditions and sludge decay, on the one hand, and WP, on the other hand. With this analysis, further insights can be gained into the hydrolysis mechanics of aquatic worms as well as the general aspects of WP.

3.2 Materials and Methods

3.2.1 Lab-scale worm reactor

In this study, the sessile worm *T. tubifex* was chosen as a model oligochaete worm. The aquatic worms were batch-cultivated in a lab-scale reactor and fed with waste activated sludge (WAS) obtained from WWTP Harnaschpolder (Den Hoorn, The Netherlands), which treats municipal wastewater of 1.3 million population equivalents. The reactor was designed as a modified lab-scale version of the full-scale worm reactor that was used by Tamis et al. [21]. The reactor is composed of two identical 18 L compartments. One compartment was used as control to evaluate the conversion due to endogenous respiration (ER) and the effect of applying extended aeration on the structure of WAS. The second compartment was used for worm predation and contained approximately 40 ± 6 g/L wet weight worms. The amount of worms used was sufficient to make a clear distinction in volatile solids (VS) reduction between the endogenous respirated sludge and worm predation. The worms used in the experiments did not always originate from the same shipment of worms.

The worms were stored in an aerated vessel fed with WAS when not used in experiment. The intestines of the worms contain consumed sludge. Worms were not gut purged at the start of an experiment. This was done to compensate for the sludge solids that were ingested during experiments. These ingested solids would consequently result in an unwanted decrease in sludge solids at the end of an experiment when worms with purged guts would have been used. Both compartments were aerated and mixed by using an airlift system. The average dissolved oxygen concentration was ≥ 5 mg/L, as recommended by Cai et al. [36] and the temperature was maintained at 20 ± 1°C. The pH was left unaltered and was 7.4 ± 0.2 on average. Distilled water was used as make up water in case of evaporation.

3.2.2 Extended aeration

The ER sludge was transferred from the reactor to an aerated 5 L glass bottle. The sludge was aerated for an additional 30 days (ER-30) at room temperature.

3.2.3 **T. tubifex**

T. tubifex was bought at local wholesales (Aquadip B.V. and De Maanvis B.V., The Netherlands). Upon arrival, the worms were thoroughly rinsed to remove dead specimens and other contaminants. Worms were then transferred to and stored in the aforementioned aerated vessel containing WAS. Fresh worms were stored for at least 1 week in order to adapt to the sludge, before use.

It was observed that clumps of worms tend to concentrate sludge particles around their bodies. To remove these particles, the worms were thoroughly rinsed with tap water in a large beaker. In the beaker, a vortex was created by hand to remove attached particles. The worms were then left to settle for ± 1 minute and the upper water phase was discarded. This process was repeated until the water layer was clear and the worms were clean. Worms were spread out and weighed, after most of the adhered water was removed using paper tissues.

3.2.4 **Analytical methods**

Total solids (TS) and volatile solids (VS) were measured in triplicate. For dissolved compounds determination, mixed liquor samples were filtered over 0.45-μm glass fibre membrane filters prior to analysis. Dissolved nitrate, ammonia and phosphate were measured in duplicate, while COD was measured in triplicate, using Spectroquant photometric test kits (Merck Millipore, Darmstadt, Germany). Analytical methods were in accordance with the standard methods [37].

3.2.5 **Sludge dewaterability**

The SVI, the zone settling velocity (ZSV) and capillary suction time (CST) were determined according to the standard methods [37]. For the turbidity analysis, the sludge was left to settle for 30 minutes and subsequently, the water phase was analysed with a HACH 2100N Turbidimeter (Hach, Loveland – Colorado, USA). CST measurements were performed using a Type 304M CST apparatus (Triton Electronics Ltd., Essex, England).

3.2.6 **Particle size distribution**

The particle size distribution (PSD) was performed using liquid particle counting device (model HIAC 9703 Hach, Loveland – Colorado, USA) equipped with a HRLD400/HC sensor (Hach Ultra, Grants Pass – Oregon, USA). The operational principle is based on the light-blockage method in the size range of 2–400 µm. Particles are counted in fixed size increments of 0.78 µm. The flow was set to 100 mL/min. Samples were diluted 1000× in demineralized water and subsequently sieved (400 µm mesh) prior to analysis. The presented data was not multiplied with the dilution factor. Data were recorded with WGS Software (version 2.4), which was supplied with the particle counting device, and analysed by using the normalized data, as recommended by APHA [37].

Particle counting data require to be normalized to avoid apparent peaks, due to the variability of the size increments [37]. The obtained normalized PSD was then compared with the theoretically defined PSD of Lawler [38]. A PSD can be presented as a power-law function where the slope of the distribution, referred to as β in literature, can either be constant or variable according to theoretical models. Normalization of the data consists of dividing the particle count, in a given size range, by the size interval and presenting the data on a logarithmic scale.

An example of the difference between raw counts and normalised data is given in Figure 3-1.

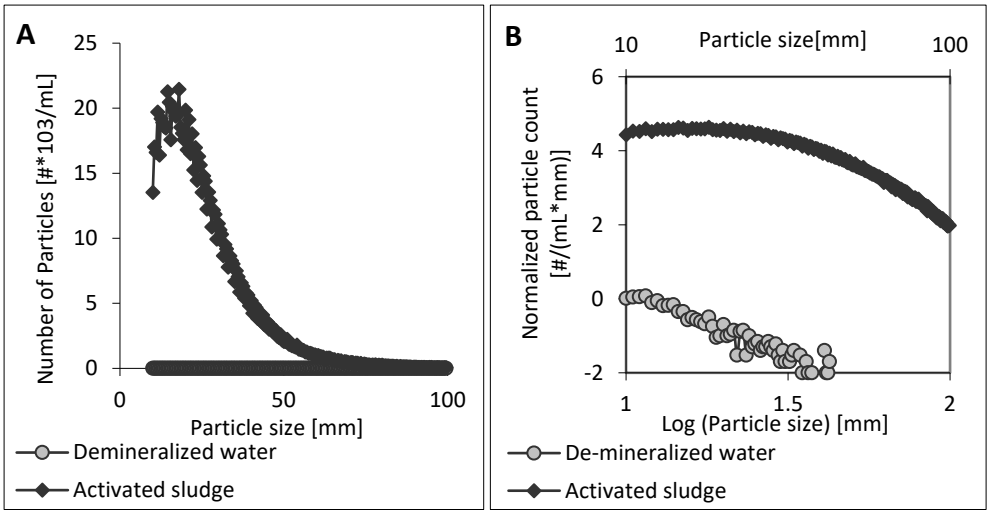

Figure 3-1: *An example for the normalization of particle size distributions. Number of particles/mL per particle size (A) and normalised particle counting data (B) from activated sludge and demineralized water samples. Figure adapted from Lousada-Ferreira [85].*

3.2.7 **sCOD: quantitative protein and carbohydrate determination.**

Dissolved carbohydrate-like and protein-like substances in the sCOD fraction were obtained by filtering the mixed liquor using 0.45-µm glass fibre filters. The sCOD fraction was then quantified by using the phenol-sulphuric acid method for carbohydrate determination, with D-glucose as a standard [39]. For protein determination the Lowry method [40], with Bovine Serum Albumin (BSA) as a standard, was used. The classical Lowry method was chosen, instead of the modified version of Frølund et al. [41], due to the low absorbance values (absorption around 0.1) as proposed by Avella et al. [42].

Within the modified Lowry method, the measured absorbance without using the $CuSO_4$ reagent is a measure for interfering substances for the protein determination. These interfering substances are ascribed to the humic-like fraction [41]. However, it remains questionable whether these interfering compounds are indeed exclusively humic substances. When interfering humic-like substances were intended to be measured, the modified Lowry method [41] was used with humic-acid sodium salts (H16752) as a standard. All the reagents were purchased at Sigma-Aldrich.

3.2.8 sCOD qualitative: Fluorescence excitation emission matrix spectroscopy.

Fluorescence excitation emission matrix spectroscopy (FEEMS) can be used to probe the composition, concentration, and dynamics of organic matter from various source materials [43–45]. Sludge mixed liquor samples were filtered over 0.2-µm glass fibre filters and stored at 4°C in glass vials prior to analysis. FEEMS were measured on a Horiba Jobin Yvon Fluoromax-4 Spectrofluorometer equipped with a Xenon lamp light source and a 1-cm path length quartz cuvette following D'Andrilli et al. [46].

Samples were analysed for UV absorbance with a Thermo Scientific Genesys 10 scanning UV spectrophotometer with a 1-cm path length, from 190 to 1100 nm on optically dilutes samples (absorbance values < 0.3 at 254 nm). Samples with absorbance values >0.3 at 254nm were diluted with nanopure water until they were below 0.3 in order to reduce inner filter effects during post processing of the FEEMS [44].

Post-processing of the fluorescence data was completed in MATLAB to generate 3D FEEMS data, which included sample corrections for inner filter effects, Raman scattering and blank water subtraction. Positions and intensities (Excitation and Emission maxima values) for individual fluorophores were determined to gain more information on the composition of the material. Samples were compared to each other for the different wavelength regions. These regions are related to the composition of different substances (Table 3.2).

Table 3.2: *FEEMS excitation and emission wave length regions with the associated substances.*

Substance	**Excitation (nm)**	**Emission (nm)**	**Reference**
Tryptophan protein like substances	270-280	320-350	[43]
Aromatic protein like substances	220-240	320-350	[47]
Humic like substances	330-350	420-480	[48]
Fulvic acid-like substances	250-260	380-480	[44]

3.2.9 Divalent and trivalent cations

Total and soluble Al^{3+}, Ca^{2+}, Mg^{2+}, Fe^{3+} and Na^{+} were determined using the digestion method described by van Langerak et al. [49]. Samples were analysed using an ICP-MS Xseries II (Thermo Fisher Scientific Carlsbad – California, USA) except soluble Al^{3+}, which was determined using Spectroquant photometric test kits (Merck Millipore, Darmstadt, Germany). Cation measurements were performed in triplicate.

3.2.10 Extraction of EPS and ALE

Extracellular polymeric substances (EPS) were extracted according to the method used by Frølund et al. [50], using Dowex marathon C cation exchange resin. Extraction was carried out with magnetic stirring at 350 rpm for 17h at 4°C. An amount of 0.5 g sludge was used per extraction. The EPS extracts were analysed for protein and humic-like content using the modified Lowry method as proposed by Frølund et al. [41] with BSA and humic sodium salts (H16752) as standards. Carbohydrate content was determined using the aforementioned method. Reagents were obtained from Sigma-Aldrich.

Alginate-like exopolysaccharides (ALE) were extracted using the method described by Lin et al. [51]. About 1 L of feed and processed sludge was used in the extraction. Extractions were performed in triplicate. After extraction, the supernatant was obtained by centrifugation (3500 RCF, room temperature, 20 min) and subsequently filtered over a 0.45-µm glass fibre membrane filter to obtain the ALE extract. The carbohydrate content was determined using the method described earlier.

3.3 Results and discussion

3.3.1 Taxonomy of worms

To make sure that the worms used in the experiments were indeed of the tubificid genus, the taxus of 100 individuals were determined. Almost all individuals were of the *Tubifex* genus with a sporadic presence of *L. variegatus*. Reproductive organs were not observed. Egg sacs attached to the worms were observed as white/pink perturbations on the segments of the worms. Dispersed cocoons

(white/pink) were found throughout the sludge (Appendix Figure 3-10). External stimuli resulted in 'curling up' of the worms, which is distinctly different compared to its similar looking counterpart *L. variegatus*, who shows a 'corkscrew' escape movement. The apparent healthiness of the worms was visually assessed by evaluating the response of a clump of worms after touching. A healthy response is the formation of a firm clump. If the worms were not healthy, the clump was fluffy with an open structure.

3.3.2 Sludge degradation and physical characteristics.

The difference in VS reduction between WP sludge and ER sludge was researched using batch incubations of 4 days. In agreement with the recommendations by Buys et al. [52] regarding WP, approximately 45 g/L

wet weight worms were used, which was indeed sufficient to give a clear distinction between the WP sludge and the ER sludge within the duration of the batch experiment.

The presence of *T. tubifex*, during the aerobic stabilization of WAS had a significant impact on the extent and rate of WAS degradation (Table 3.3).

Table 3.3: *Solids reduction of activated sludge for worm predated (WP) and endogenous respirated (ER) sludges. Average results of 10 different 4-day batch tests.*
Values are expressed as percentage difference compared to the feed sludge.
Errors are expressed as percent point standard deviations.

	ER	WP
TS reduction (%/d)	2% ± 1	10% ± 3
TS reduction totals (%)	8% ± 4	41% ± 13
VS reduction (%/d)	2% ± 1	12% ± 4
VS reduction totals (%)	9% ± 5	47% ± 15

On average, 47% ± 15 of the initially present VS was converted upon WP versus 9% for the endogenous respired sludge. The corresponding averaged VS reduction rates were 12 %/d and 2 %/d for WP and ER, respectively. Interestingly, extended aeration of WAS for duration of 30 days (ER-30) resulted in a similar 29% ± 1.9 reductions in VS. The large difference in incubation time clearly demonstrates the increased VS reduction rate in the presence of worms.

The VS reduction was accompanied by an increased release rate of the soluble organic fraction (sCOD) and inorganic material, predominantly $N–NH_4^+$, $N–NO_3^-$ and $P–PO_4^{3-}$, as is presented in Table 3.4.

Table 3.4: *Average rates for worm predation (WP) and endogenous respiration (ER). Average results of 10 different 4 – day batch tests, except for N, P and sCOD release, which were measured in 6 different 4 – day batches. Errors are expressed as standard deviations.*

Parameter	**Units**	**ER**	**WP**
Ratio Worms / VS	g Worms /g VS	-	14.1 ± 1.4
Concentration Worms	g Worms/L	-	40.2 ± 6.0
TS Removal rate	g TS/d	1.4 ± 0.9	8.2 ± 2.0
VS Removal rate	g VS/d	1.3 ± 0.7	6.2 ± 1.5
COD Removal rate	g COD/d	1.7 ± 1.8	7.2 ± 4.4
$N–NH_4^+–NO_3^-$ Release rate	mg N/d	5.7 ± 3.1	10.5 ± 0.9
$P–PO_4^{3-}$ Release rate	mg P/d	2.10 ± 1.4	3.6 ± 1.1
sCOD Release rate	mg sCOD/d	0.06 ± 0.06	0.30 ± 0.2

These increased release rates upon WP are in line with other studies. For example, Hendrickx et al. [16] found similar values for nitrogen and phosphorus compounds, with 55 mg N/g TSS removed and 17 mg P/g TSS removed versus 30.8 ± 17.6 mg N/g TS removed and 10.5 ± 5.7 mg P/g TS removed in this study. Worm-specific removal rates can be found in the Appendix Table 3.9.

3.3.3 Changes in EPS upon WP

The changes in EPS composition before and after treatment are shown in Table 3.5.

***Table 3.5**: EPS extraction data of waste activated (WAS), endogenous respirated (ER) and worm predated (WP) sludges, after a 4-day batch incubation. Values were obtained from the 0.5 g VS used for the extraction. Extraction was performed in triplicate. Error values are expressed as standard deviations.*

	Proteins (mg/gVS)	Carbohydrates (mg/gVS)	Humic-like (mg/gVS)	VS Reduction (%)
WAS	17.6 ± 2.4	17.0 ± 3.0	45.9 ± 6.3	-
ER	17.6 ± 2.3	17.8 ± 2.9	52.5 ± 7.0	5%
WP	6.7 ± 1.6	12.9 ± 2.5	49.2 ± 6.6	29%

The high release of inorganic nitrogen was accompanied by a large decrease in the protein fraction of the EPS of worm-predated sludge. In contrast, the protein and carbohydrate fraction of WAS and ER remained similar. The EPS-carbohydrate component of WP sludge also decreased, but to a lesser extent than the protein fraction. The increased N release coupled with a decrease in the protein EPS fraction indicates that the aquatic worms predominantly target the protein fraction of the polymers in the sludge. These results are in line with the results of Hendrickx et al. [15], who reported a 35% decrease in worm predated sludge's nitrogen content.

The humic-like fraction remained relatively stable upon ER and WP treatment (Table 3.5), reflecting the inert behavior of humic-like substances. Although within the error margins, the average humic fraction slightly increased. This increase might be well ascribed to an increased extraction efficiency, due to an increase in the number of small particles (which will be further discussed in section 3.3.5). The average humic-like fraction was slightly higher in ER compared to WP, which might suggest that part of the humic-like substances was removed or altered.

Electrostatic interaction between humic-like compounds and proteins have been reported by multiple authors [53–55]. They showed that electrostatic interactions are responsible for protein and humic substance complexation. Additionally, humic substances can contain protein-like elements [56–58]. Shan et al. [59] showed the removal of protein-like elements during vermicomposting, using ^{14}C-

labelled proteinaceous components bound to the humic substances. Possibly, during WP, a similar disruption of the electrostatic interactions and subsequent conversion of the humic-protein complexes occurred.

3.3.4 Changes in ALE upon WP

As alginate is an important structural component in (granulated) activated sludge [51], the ALE fractions of the sludge, before and after pretreatment, were compared (Table 3.6).

Table 3.6: *ALE polysaccharide concentrations for WAS, endogenous respirated (ER) and worm predated (WP) sludges after 4-day batch incubation of 2 separate batches. Extractions were performed on 1 L of (treated) sludge in triplicate. Error values are expressed as standard deviations.*

	mg ALE/L	mg ALE/g VS	% VS reduction
WAS	177 ± 15	58 ± 3	-
ER	212 ± 4	71 ± 2	5%
WP	102 ± 9	49 ± 1	43%

The amount of ALE that could be extracted from WAS and ER were in a similar range, i.e. 72 ± 6 mg/gVSS, as was found by Lin et al. [60] for suspended activated sludge. ALE concentrations increased for ER and decreased for WP compared to WAS. It seems that aeration and the associated shear forces resulted in smaller particles with a larger total surface area, thus increasing the extractability of ALE.

Irrespective of the increased extractability due to aeration, the concentration of ALE extracted from worm-predated sludge decreased by roughly 40%. Around 62 ± 15 mg ALE per gram of degraded sludge was removed in the presence of worms. It seems that the worms consume part of the extractable ALE. Whether these extracted carbohydrates contain a protein element, which would support the reduced EPS protein fraction, is unknown, as the protein component of the ALE extract was not measured.

3.3.5 PSD and turbidity

The effect of aeration and predation on the particle size distribution of the treated sludge is shown in Figure 3-2.

It can be seen that the number of large particles, in the range of 30 – 200 µm, decreased upon treatment of WAS. Shear forces introduced by aeration and sludge decay (e.g. endogenous respiration) are known to break up sludge flocs in smaller particles [61,62]. The difference between ER-30 and the other samples mostly reflects the effect of long-term aeration on particle size reduction. The difference in the number of particles, between WAS, on the one hand, and ER and WP, on the other, reflects the difference in VS removal because both samples were maintained under the same aeration conditions.

The breakdown of large particles results in an increase in the 2 – 30 µm range, when comparing the samples to WAS (Figure 3-2B). This increase can be seen clearly for the PSD of ER-30, which shows more small particles and fewer big flocs. Extended aeration clearly breaks up sludge flocs predominantly by prolonged exposure due to mechanical shear. ER and WP were aerated and thus exposed to the same mechanical shear for the same amount of time; the PSD of ER and WP almost overlap and are clearly different from the PSD of WAS, showing higher amounts of small particles.

The original WAS is altered by the activity of the worms that apparently reduced the size of the flocs (Figure 3-2A) due to their degradation activities, and simultaneously produced a higher amount of small particles (Figure 3-3). However, the specific size fraction that the worms consume and excrete cannot be determined from the data due to the large effect that aeration has on floc size. In future work this could be compared to sludge treated in a passively aerated environment.

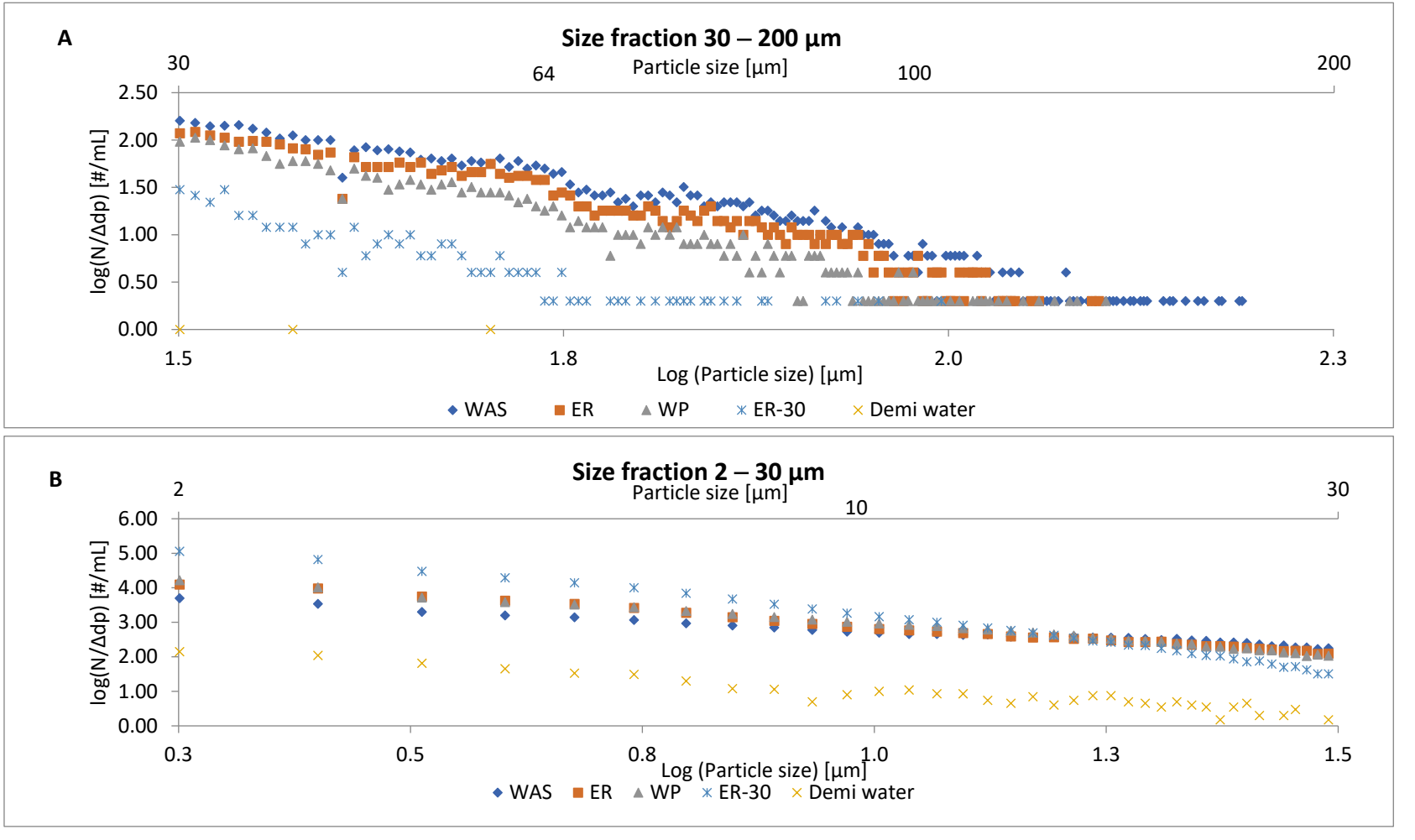

Figure 3-2: *Normalized particle size distribution of treated sludges, showing the averages of triplicate measurements. Graph split in two parts:* ***A****: 30 – 200 µm;* ***B****: 2 – 30 µm. Error bars are omitted for clarity. Horizontal line around 0.5 counts/mL*µm is due to low particle counts (1 -2 counts/mL) in the measurements. The outlier at 1.6µm* is probably due to a fault in the machine as it is consistent in all the measurements. Particles larger than 200 µm were not observed in the sludges. Batch VS reduction was for WP 42%, ER 18% and ER-30 29%. For visualization, the values plotted were not multiplied by the dilution factor.*

The increase in smaller particles also becomes apparent in the turbidity measurements presented in Table 3.7 and the normalised PSD of the supernatant of settled sludge shown in Figure 3-3.

Table 3.7: *Turbidity and triplicate particle counting measurements of water phase of 30 minutes settled waste activated (WAS), endogenous respirated (ER), worm predated (WP), 30- day extended aerated (ER-30) sludges and demineralised water (demi water). Errors expressed as standard deviations.*

	Turbidity (NTU)	2 µm fraction (counts/mL)
Demi water	0	70 ± 10
WAS	8	2485 ± 126
ER	15	6205 ± 313
WP	103	8264 ± 119
ER-30	330	57091 ± 1799

It can be seen that with increasing aeration times, the turbidity increases with the amount of particles in the 2 – 30 µm size fraction (Figure 3-3).

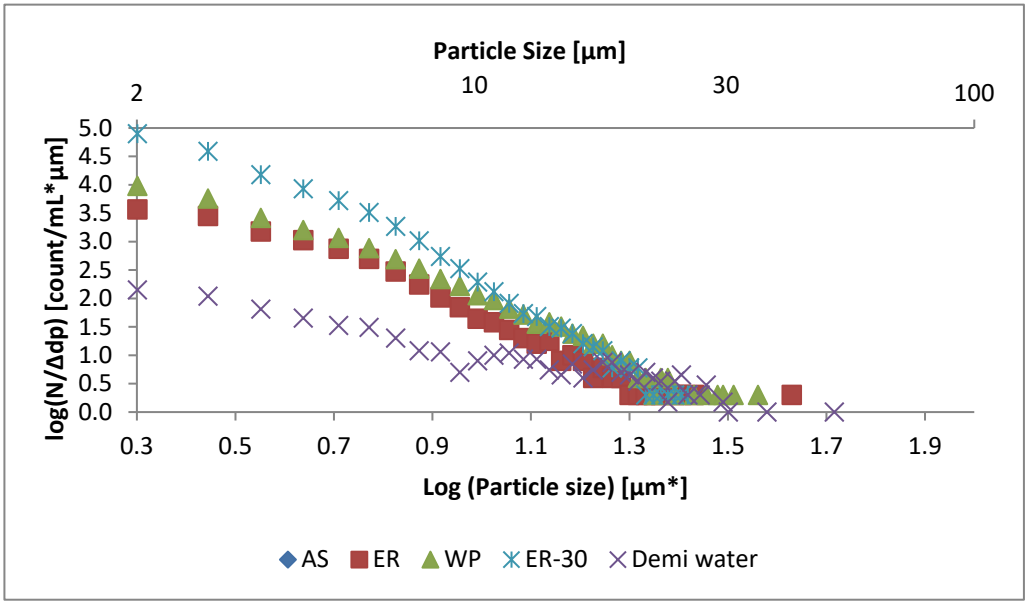

Figure 3-3: *Particle number distribution of the supernatants of WAS (AS), Worm Predated (WP), Endogenous Respirated (ER), Extended aerated (ER-30) and demi water after 30 minutes of settling. Averages are shown from triplicate particle size measurements.*

Furthermore, the presence of worms increases the number of small particles compared to ER. Figure 3-4 visually shows the difference in turbidity and settleability between the sludges.

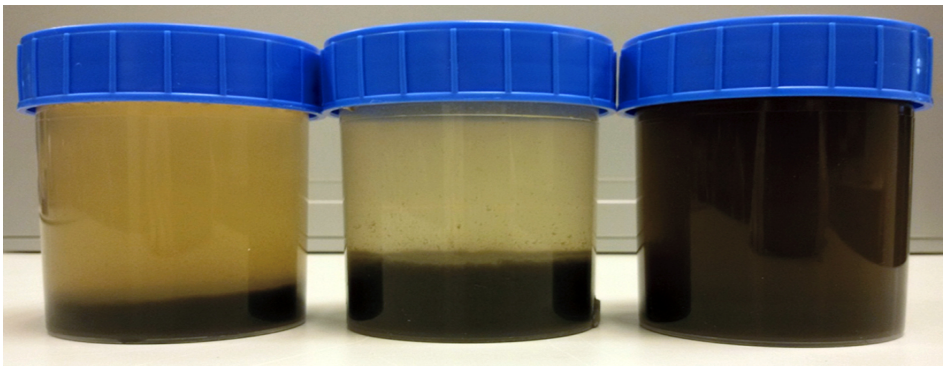

Figure 3-4: *Images of 30 minutes settled sludge. From left to right: WP, ER5 and extended aerated (ER-30).*

3.3.6 Dewaterability and settleability

The change in SVI over the duration of the batch assay is shown in Figure 3-5.

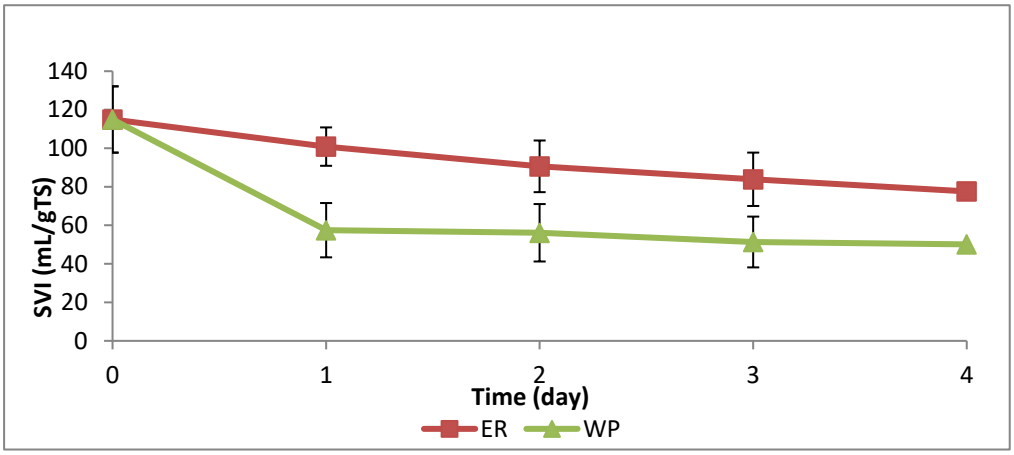

Figure 3-5: *Change in average SVI over the duration of the batch for endogenous respirated (ER) and worm predated (WP) sludges. Averaged values of 4 batches. Error bars represent standard deviations. Averaged VS reduction percentages were 40% ± 16 and 8% ± 3 for WP and ER respectively. The data point at day 4 was from 1 batch only.*

The velocity at which sludge settles, the sludge blanket volume was monitored over time (Figure 3-6).

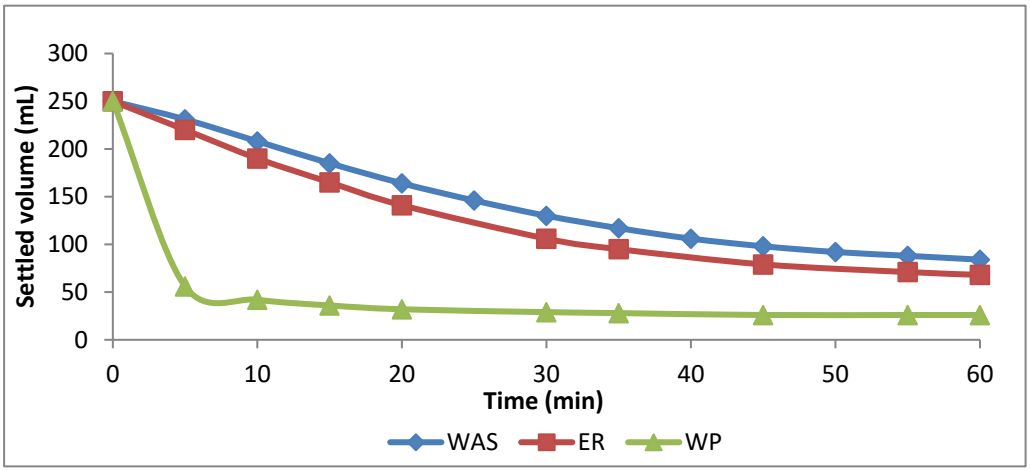

Figure 3-6: *Change in sludge blanket volume versus settling time for WAS, endogenous respirated (ER) and worm predated (WP) sludges. Results are from a 3-day batch experiment with VS reduction of 63% ± 3 and 13% ± 3 for WP and ER respectively using a worm/VS ratio of 15 g/g.*

It is clear that worm predation improved the settleability of the sludge (Figure 3-5). The SVI drops to roughly 50% of its starting value for WP, whereas the SVI for ER slowly decreased. Furthermore, WP settles almost completely in the first 5 minutes while WAS and ER needed 60 minutes to reach similar volumes (Figure 3-6). On the basis of the data from Figure 3-6, the zone settling velocity (ZSV) was calculated. The velocity increased from 0.248 m/h to 0.332 m/h and 2.29 m/h for WAS, ER and WP respectively which reflects the improved settling properties of WP sludge.

The improved settleability of worm predated sludge has been shown by other authors (Table 3.1) and is attributed to the increased density of worm faeces [15]. Additionally, it was observed that the worms accumulate sludge flocs around their bodies and over time these adhered flocs aggregate into larger particles and remain firmly attached to the worms and to other sludge aggregates as shown in Figure 3-7. A similar observation came from Inamori et al. [63], who found that bacterial floc size increased in the presence of the aquatic worm *Philodina erythrophthalma*.

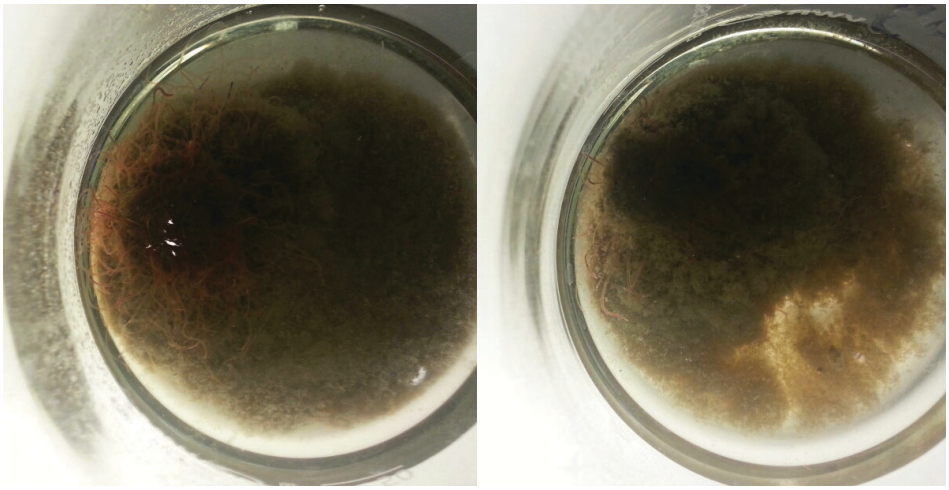

Figure 3-7: *Sludge adherence to T. tubifex. Left: Worms added to activated sludge. Right: Adhered sludge after 30 minutes of incubation.*

The change in SVI might also be linked to the significant change in EPS constituents after predation. Jin et al. [64] concluded that the improved settleability was in part correlated to a decrease in EPS concentration, which was also found it this study. More specifically, Chen et al. [65] found that the settleability in granular sludge improved, when loosely bound EPS was removed. Our current results strongly suggest that both the increased density of the faecal matter, the adherence of sludge flocs and the removal of proteins contribute to the decrease in SVI.

The dewaterability also changed in comparison with the feed WAS. The dewaterability of the different sludges were assessed with CST measurements and the results showed that the values for ER and WP slightly increased compared to the starting material: 5.3 ± 0.54, 6.9 ± 0.19 and 7.1 ± 0.31 (in seconds) for WAS, ER and WP, respectively. Unfortunately, the CST of ER-30 was not measured. However, CST deterioration was reported by Park et al. [66] who found that the CST increased from 50 to 517 seconds with an extended aeration time of 30 days for sludge stabilisation. The CST increased together with the amount of particles in the 2 µm range (Table 3.7). This suggests that the increase in CST is due to the increase in small particles generated by treatment of WAS, as was mentioned in the previous section. This notion is supported by Hall [67] who found that CST increased with the amount of small particles induced by sonication of activated sludge.

Overall, worm predated sludge exhibited better settling, due to the removal of EPS and faecal pellets, and a slightly worse dewaterability, due to the increase in small particles when compared to WAS and ER. (≤ 2μm) particles However when the aeration time is increased to 30 days, which results in a large fraction of ≤ 2μm particles, the settle ability and filterability both deteriorate

3.3.7 Biochemical characterisation of extended aerated and worm predated sludges

3.3.7.1 Soluble COD

In order to study the increased release of sCOD, the protein, carbohydrate and humic-like fractions in the various supernatants were measured. The averaged results of several batches are presented in Table 3.8.

Table 3.8: *Protein and carbohydrate fractions of dissolved COD in batch supernatants of waste activated (WAS), endogenous respirated (ER), worm predated (WP) and 30-day extended aerated (ER-30) sludges. Averaged result of 4 batch experiments, except the ER-30 and humic values which corresponds to 1 batch. Average worm/VS ratio was 13 g/g. The data, adapted from Park et al. [66] are the averaged values of 9 activated sludge samples, obtained from different WWTPs, that underwent aerobic treatment for 30 days.*

	Proteins (mg/L)	Carbohydrates (mg/L)	Humic-Like (mg/L)	VS reduction (%)
WAS	24.0 ± 8.6	4.8 ± 1.4	65.4 ± 8.0	-
ER	22.9 ± 3.3	11.4 ± 6.3	67.5 ± 4.4	9% ± 5.2
WP	24.9 ± 0.8	19.7 ± 4.1	75.6 ± 0.8	40% ± 13.6
ER-30	36.8 ± 2.1	39.6 ± 3.8	109 ± 2.5	29% ± 1.9
WAS Park et al. [66]	18.5 ± 21.1	7.92 ± 4.8	-	-
ER-30 Park et al. [66]	26.7 ± 11.1	38.2 ± 19.1	-	37% ± 11.2

The results show that the carbohydrate concentration, of the soluble COD in the supernatant, increased more for ER-30, followed by WP and ER when compared to WAS. A similar trend was observed for humic-like substances. The release of soluble carbohydrates and proteins, upon aerobic treatment of WAS, has been reported also by other authors [66,68]. In contrast to the increase in carbohydrates, the soluble protein concentrations remained relatively constant.

Protein concentrations for ER-30 increased more than the other samples. WP had higher VS reduction levels, yet less soluble protein compared to ER-30, which supports the preference for proteins by the worms.

Higgins et al. [69] showed that the removal of proteins from flocs, by addition of proteases, resulted in the release of carbohydrates and a decrease in particle size. Their results indicate that protein removal from the EPS (Table 3.5) by worm predation will also result in a release of carbohydrates. Although worm predation resulted in higher VS reduction compared to ER-30, only a limited carbohydrate release was observed in the WP batches compared to the ER-30 batches. This limited release might be due to carbohydrate consumption by the Table 3.5.

To gain a better understanding of the composition of the sCOD fraction, FEEMS analysis was performed (Figure 3-8).

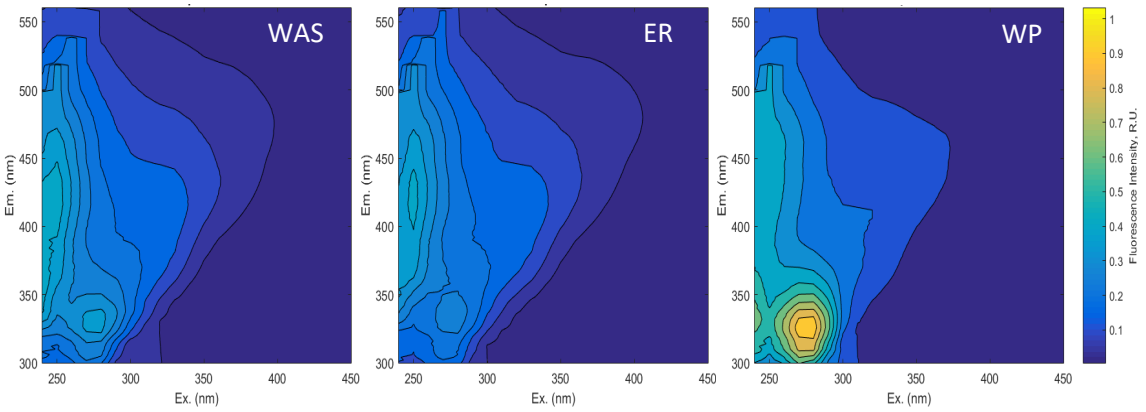

Figure 3-8: *FEEMS spectra of waste activated (WAS), endogenous respirated (ER) and worm predated (WP) sludges respectively. Measurements were done in triplicate. The plots are representative of the triplicates. VS degradation percentages of the samples were the following: ER 16%; WP 24%.*

Across all the three samples protein-like fluorophores believed to be from autochthonous sources [70] are present, along with less intense humic (Ex/Em 237-260/380-500nm range [43]) signatures. In the Ex/Em 270-280/320-350nm range, Tryptophan-protein-like substances (TPLS) increased in concentration after worm predation, compared to both WAS and ER samples. ER samples showed a lower concentration compared to WAS and WP.

Under worm predation, aromatic-protein-like substances (APLS) (Ex/Em 220-240/320-350nm range) showed an increase compared to WAS and ER samples.

WAS and ER samples had similar intensities. Additionally, the Tyrosine-like fluorophore (Ex/Em 225-237/309-221nm range [43]) appears. When dissolved organic matter is degraded, Tyrosine-like residue are exposed [70]. In contrast to the FEEMS results presented here, Tian et al. [23] reported a small decrease in TPLS and APLS after 25 days of operation, in a worm reactor that was part of a larger membrane bioreactor setup. Unfortunately, a control worm reactor (e.g. a worm reactor without worms) was not present so the influence of endogenous respiration and aeration on the release of aforementioned compounds could not be determined.

Although TPLS and APLS are referred to as proteins like regions, the soluble protein concentration did not increase as much as the soluble polysaccharides after worm predation. These differences in concentration could be attributed to the sensitivity of the fluorescent method compared to bulk protein and carbohydrate measurements. Alternatively, it is possible that the increase in TPLS and APLS is due to the increase in polysaccharide concentration and that these carbohydrates have a protein like component. This protein like component is partly in line with the proposal of Higgins et al. [69] that sludge flocs are predominantly hold together by lectin-like polymers, which are proteins with a carbohydrate binding domain. Another possibility is that these carbohydrates are glycoproteins or lipopolysaccharides as suggested by Park et al. [71] who found that the extracted EPS carbohydrates partly co-precipitated with protein, when exposed to $(NH_4)_2SO_4$.

The concentration of humic- and fulvic-like compounds slightly increased after predation. The fluorescence intensities of both WAS and ER remained similar. The observed increase might be attributed to a release of inert humic- and fulvic-like substances during VS reduction of humic/fulvic-bound substrates. Additionally, as previously discussed in section 3.3.3, the removal of protein-like components from the humic- and fulvic-like substances could have resulted in the release of these compounds into the supernatant. A small increase in humic and fulvic concentrations were not reported by Tian et al. [23]. Humic and fulvic substances are thought to inhibit hydrolysis rates by adsorption of enzymes [72]. Therefore, a decrease in the concentrations of these substances could have partly explained the increased hydrolysis rates during worm predation. In this case, humic and fulvic concentrations slightly increased however an inhibitory effect on conversion rates was not observed.

3.3.7.2 Total and dissolved cations

Multivalent cations are thought to be responsible for the formation and stability of sludge flocs. For this reason, total (sludge bound and dissolved cations) and dissolved cation concentrations were measured for the 3 studied sludges at the end of experiment; results are depicted in Figure 3-9A and B, respectively.

High concentrations of sodium may displace multivalent cations in an EPS matrix [73–75]; therefore, total and soluble sodium concentrations were measured as well. However, sodium concentrations were similar for all sludges and more or less at the same level as the bivalent cations. No impact of sodium at these concentrations is expected.

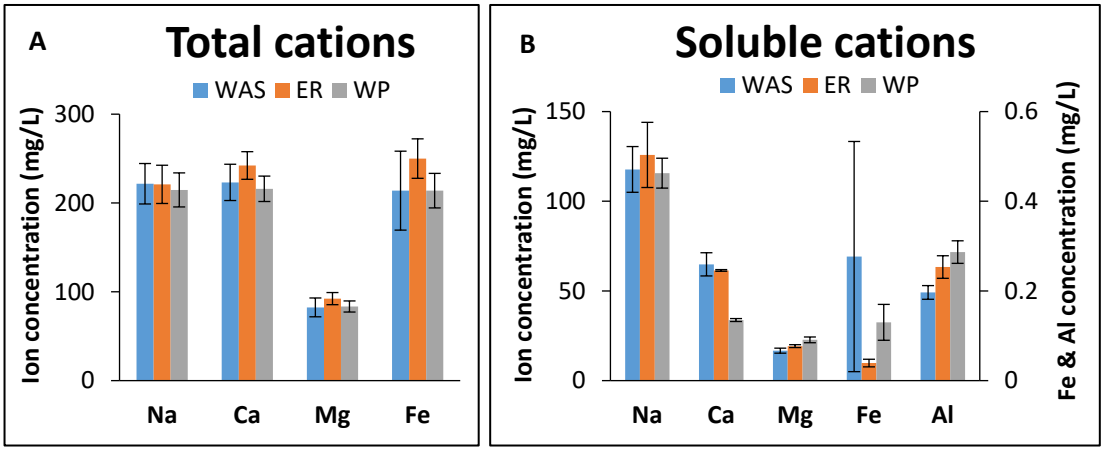

***Figure 3-9: A** Total and **B** dissolved cations for AS, ER and WP. Averaged results from 2 batches. Error bars represent the upper and lower value of the duplicate measurement. For Al^{3+} only the soluble fraction was measured. Average VS reduction was 10% and 35% for ER and WP respectively.*

Figure 3-9B shows a small distinct increase in soluble Mg^{2+} and Al^{3+} after treatment, with WP having the largest increase followed by ER. Total Mg^{2+} concentrations remained fairly constant (Figure 3-9A). The increasing soluble cation concentrations probably resulted from a release from the sludge flocs during VS destruction. Therefore, increased VS removal during worm predation coincided with an increased release of cations compared to the ER results. These observations are consistent with the observations made of the aforementioned author [64] who found values in the range of (WAS – ER-30) 18 – 38 mg Mg^{2+}/L and 64 – 103 mg Ca^{2+}/L. Furthermore, they concluded that the release of divalent cations was linked to aerobic VS destruction.

Interestingly the soluble Ca^{2+} concentration showed a large decrease after worm predation compared to WAS and ER, where a large increase was expected due to the concomitant Ca^{2+} increase with aerobic VS reduction [66]. The observed Ca^{2+} decrease was consistent over multiple batches. Several explanations for the Ca^{2+} decrease are viable. Most probably Ca^{2+} was taken up by the worms during the batch incubations. The haemoglobin in *T. tubifex* contains besides iron also calcium. The molar ratios of Fe:Ca were reported to be 160:70 [76].Additionally, precipitation of Ca^{2+} could have occurred with the increased release of phosphates during predation. Calcium phosphate precipitation was reported to be possible at slightly alkaline conditions and similar calcium concentrations [77,78]. However, a decrease in calcium was not observed in ER.

Alternatively, an increased amount of Ca^{2+} could have been bound to the released humic and fulvic substances that were liberated or made accessible by removal of VS through the action of the worms, as mentioned previously. Azman et al. [79] showed that calcium adsorbs to humic compounds and thereby mitigates the enzyme binding capacities of these humics. By this mitigation the hydrolysis rates were effectively increased during anaerobic digestion of cellulose [79]. It is not known whether such mechanism is of importance during worm predation. The calcium concentrations remain in sharp contrast to what other authors found with regard to the release of soluble Ca^{2+} [66,68]. A possible explanation for the relatively stable Ca^{2+} concentrations, when comparing WAS and ER, is the difference in batch duration which was 30 days in the aforementioned studies versus 4 days in our present study. Unfortunately, the metal content of the worms and ER-30 were not determined and hence, the reason for the lower soluble calcium concentration remains speculative.

Iron is associated with the protein fraction of EPS and iron is released during anaerobic storage of sludge [80,81]. Because of this iron-protein interaction and the removal of protein from the EPS, both total and soluble ferric iron were monitored (Figure 3-9 A and B). Regardless of the large uncertainty in the soluble WAS measurements, a clear difference between ER and WP is observed. For the total Fe^{3+} fraction an average of 36 mg /L iron was removed during worm predation. Concomitantly, the soluble Fe content in the WP supernatant was higher compared to the ER supernatant.

The results suggest that a part of the protein-bound Fe in the sludge was released during worm predation and another part absorbed by the worms as an iron source for their iron containing haemoglobin [76]. Unfortunately, the latter could not be verified because the metal content of the worms was not determined. Additionally, due to the possible occurrence of anoxic zones, being formed by clumps of worms, the microbial reduction of Fe^{3+} to Fe^{2+} might have had occurred. However, this is not very likely as this would result in ferrous precipitation with soluble phosphate [82,83]. These precipitates would be included in the total iron concentration, which would therefore not change. This is clearly not the case.

3.4 General Discussion

The objective of this study was to gain more insight into the mechanisms of hydrolysis and the general aspects of predation of activated sludge by aquatic worms. Results show that worm predation of activated sludge has a significant effect on the removal of volatile solids and dewaterability compared to the control without worms. The VS removal is accompanied by improved settling characteristics and an increased release rate of sCOD, inorganic nitrogen and phosphorus. The observed inorganic nitrogen release agreed with the drop in the EPS-protein fraction that *T. tubifex* specifically seems to target as the substrate.

Furthermore, the results suggest that the release of soluble carbohydrates, cations and humic/fulvic substances is also due to the removal of protein. Concomitantly, sludge flocs disintegrate, resulting in smaller particles and thus increasing turbidity and CST. In contrast with the deteriorating dewaterability associated with ER-30, the settleability increased and the CST did not increase as much as ER-30, which can be attributed to the more compact worm faeces, the removal of VS and the aggregation of sludge particles through sludge – worm interactions. Recalcitrant flock biopolymers that can influence hydrolysis rates such as humic and fulvic substances, were not removed but slightly liberated. ALE, on the other hand, was partly consumed during worm treatment of WAS. Additional mechanisms related to sludge hydrolysis were not revealed by researching the biochemical and physical characteristics of worm predation.

Besides the aerobic removal of proteins and the concomitant release of soluble compounds, Park et al. [66] also showed that WAS conversion, using sequenced aerobic and anaerobic (or vice versa) conditions, reaches the same level of VS

reduction of the combined processes, reaching about 63%. Roughly 45 – 50% of the initial VS is removed in the first stage of either aerobic or anaerobic treatment after 30 days. This aerobic VS reduction is in the same order of magnitude as the averaged results presented in this study, which were about 47% ± 15 for worm predation and 30% for extended aeration (ER-30). Similar results were reported by Buys et al. [52], who showed that worm predated and endogenously respirated sludge both reached similar VS degradation levels of about 58% with a difference in incubation time of 46 days.

Surprisingly Tamis et al. [21] found 20 – 30% aerobic VS reduction by worm predation and an additional 40 – 55% VS reduction upon anaerobic storage of the worm predated sludges. A total of about 65% of the initial VS was removed during the aerobic and anaerobic treatment of WAS. Comparable results were reported by Hendrickx et al. [15] who showed that worm predation followed by anaerobic digestion of the worm faeces, resulted in a total of 50% VS reduction. In both examples the end point for the aerobic to anaerobic conversion reached similar values as the 63% reduction mentioned previously. The increased VS removal results in a lowering of the biological methane potential of worm predated sludges [84].

In conventional WWTPs where aerobic unit operations are predominantly followed up by anaerobic treatment for sludge digestion, 30 – 35 % of the initial aerobic VS is degraded during digestion. When the findings of Park et al. and Tamis et al. [21,66] and the results presented here are considered, it seems that the 45 – 50 % of the initial VS which remains undigested during anaerobic digestion, is digested by additional aerobic (worm) treatment. Furthermore, based on the similar VS reduction levels between WP and ER-30, it seems that the worms specifically target a fraction of the sludge that is predominantly biodegradable under aerobic conditions, yet at significantly higher degradation rates as compared to the endogenous decay of WAS.

The presented results call for further research concerning the aerobic and anaerobic biodegradability of predated sludges and a (re-) evaluation of implementing worm predation as a sludge reduction method. The latter could be of particular interest to WWTPs in which a large VS fraction of WAS seems to be left unaltered in current anaerobic digesters, resulting in large sludge disposal costs associated with the operation of these WWTPs. Note has to be taken of the

potential interference with liquid/solids separation in WWTPs due to the increase in small particles which are introduced by predation technologies. In addition to the bioconversion potential of applying worm predation to activated sludge, the biological cause of sludge reduction deserves further attention, especially to provide insight into the enzymatic activity responsible for the efficient reduction of polymers and possibly the reduction of microbial mass.

3.5 Conclusions

This research set out to gain more insight into the hydrolytic mechanisms and the general aspects of worm predation. It was found that worms specifically target the protein fraction of activated sludge. The removal of proteinaceous material from the activated sludge attributed to the increase in sCOD, inorganic nitrogen, the cations Mg^{2+}, Al^{3+} and Fe^{3+}, fulvic and humic substances as well as the disintegration of particles and partly the improved sludge dewater-ability. Additionally, *T. tubifex* seems to predominantly target the aerobic degradable fraction of activated sludge.

3.6 References

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3.7 Appendix

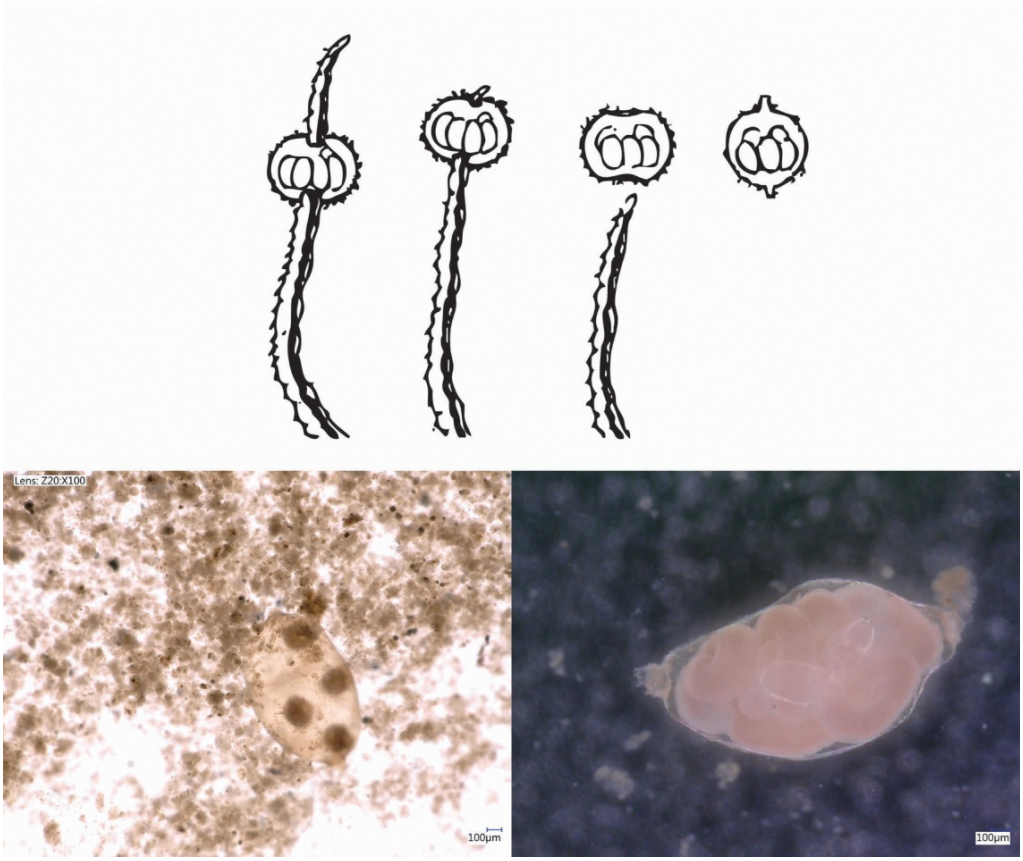


Figure 3-10: *T. tubifex cocoons. Note the extrusions at the top and bottom side of cocoon. Top: Cocoon formation and release in T. tubifex. Partly adapted from Hirao et al. [86]. Bottom-left: 100X magnification. Cocoon is visible. Bottom-right: Individual Tubifex worms visible in cocoon.*

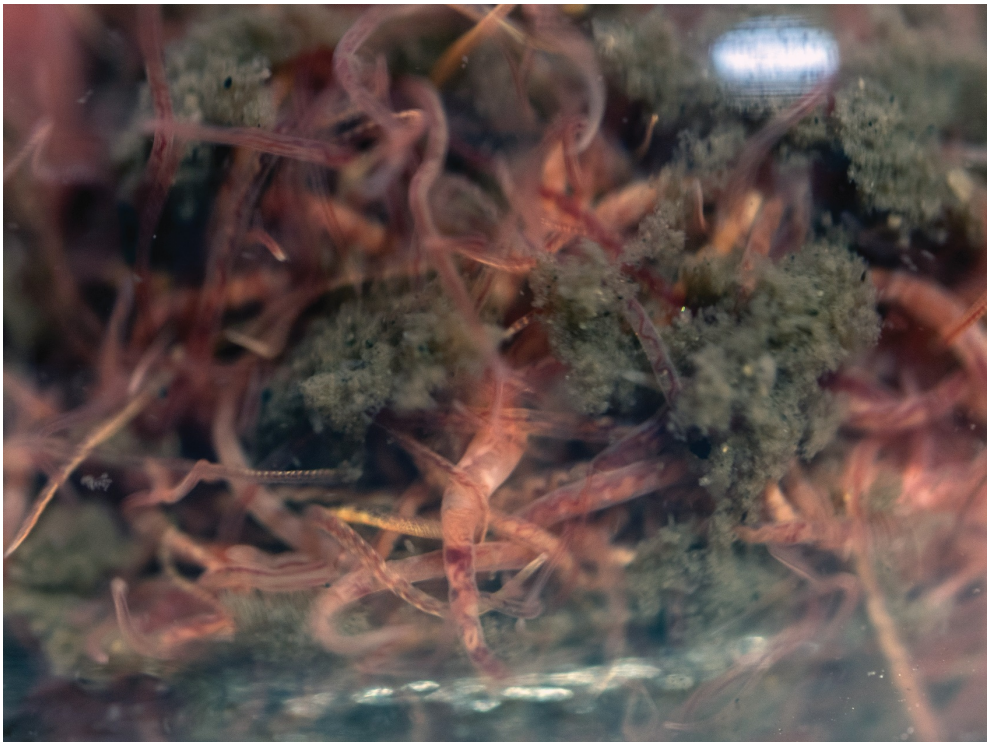
Table 3.9: *Worm biomass specific rates, corrected for endogenous respiration rates. Averaged results of 10 different 4-day batch tests except for N, P and sCOD release which were averaged from 6 batches. Errors are expressed as standard deviations. Wet weight of worms used in calculations.*

Average net specific conversion TS	mg TS/g worms d	9.79 ± 3.41
Average net specific conversion VS	mg VS/g worms d	7.14 ± 2.42
Average net specific conversion COD	mg COD/g worms d	10.06 ± 4.52
Average net specific conversion N	µg N/g worms d	7.21 ± 2.03
Average net specific release P	µg P/ g worms d	1.90 ± 1.28
Average net specific conversion sCOD	µg COD / g worms d	0.346 ± 0.20

4 The biodegradability of worm predated sludge: A sequential aerobic and anaerobic treatment approach

This chapter is based on:

Steef de Valk, Tales A. Tavares de Sousa, Ahmad F. Khadem, Jules B. van Lier and Merle K. de Kreuk (**2020**) *The biodegradability of aquatic worm predated waste activated sludge: a sequential aerobic and anaerobic treatment approach.* Bioresource Technology Reports, 12:100606



Abstract

The objective of this study was to investigate the effect of waste activated sludge (WAS) predation by the aquatic worm *Tubifex tubifex (*T*. tubifex*) on the overall biodegradability of WAS. The initial WAS biodegradability potential was determined in 80 days sequential batch-fed anaerobic and aerobic treatment combinations. These treatment combinations were used as a reference for comparison with the effect of 5-day predation and 40-day anaerobic treatment combinations. Predation and the subsequent anaerobic digestion of the predated solids shows superior solids removal and superior overall conversion rates compared to solely conventional anaerobic digestion. Strikingly, the predation and anaerobic treatment combinations reached the same chemical oxygen demand (COD) and volatile solids (VS) reduction as the reference processes, i.e. 58% and 49% for COD and VS, respectively. Our results show that predation and anaerobic treatment combinations increase solids removal rates, but do not alter the overall biodegradability potential of WAS.

4.1 Introduction

Waste activated sludge is a by-product from conventional sewage treatment. Due to stringent legislation [1], preventing the agricultural use of stabilised WAS in countries like the Netherlands, WAS treatment and final disposal largely contributes to the total sewage treatment costs [2]. To reduce the amount of WAS that requires further treatment and ultimately disposal, anaerobic digestion (AD) is widely applied. The average extent of WAS reduction in anaerobic digesters of conventional wastewater treatment plants that target biological nutrient removal reaches 30 – 35%, applying a solids retention time (SRT) of 25 – 30 days [3–5].

WAS biodegradability can be improved by applying pre- or in-line-sludge treatment methods prior to the anaerobic digestion process. In general, these additional treatments improve the solids reduction by an additional 5 to 35% [6]. Potentially, the biodegradability of WAS could reach 80 – 90%. However, due to presence of recalcitrant humic substances, which only make up 10 – 20% of the sludge organics [6] and other poorly biodegradable material, this value is never reached during the 25 – 30 days of treatment.

Interestingly, a positive effect on the biodegradability of WAS is observed in aerobic worm predated treatment. Aquatic worms, such as *T. Tubifex* have been found to naturally inhabit the aerobic zones of WWTPs. Sudden worm growth or worm blooms, have been associated with improved sludge settling characteristics and a lower WAS production. These beneficial characteristics resulted in a large research interest in WAS reduction using sludge worms [7]. In earlier studies, worm predation showed a similar WAS solids reduction compared to AD, with significant shorter residence times: worm predation resulted in 47% ± 15% solids reduction within 2 to 4 days of treatment [8,9]. Also, Tamis et al., (2011) suggested that worm predation as pre-treatment prior to anaerobic digestion enhances the overall WAS biodegradability compared to conventional AD in terms of solids removal and treatment time. Results showed that worm predation of WAS leads to 20 - 30% solids reduction, whereas this value increased to 65% after an anaerobic storage period of 60 to 100 days. In our previous work [8], we suggested that the aforementioned overall increased biodegradability was possibly due to presence of a sludge fraction that is only degradable under

aerobic conditions, such as the worm predation process and not under anaerobic conditions [11].

In order to properly research the contribution of predation on the biodegradability of WAS, a reference for the biodegradability potential of non-predated WAS is essential. To this end, the extent of WAS biodegradability will be estimated using the method of Park et al., (2006) and Novak et al., (2011) will be used. Their research showed that using sequential 30 day aerobic and 30 day anaerobic batch treatment, the first aerobic or anaerobic step, had the largest contribution to the overall solids removal, which was also the same regardless of the process order [11] and the overall VS removal reached 50 - 60% regardless of the process order.

In our present work, we investigated to which extent predation by the aquatic worm *T. tubifex* may contribute to the overall enhancement of the WAS biodegradability potential.

4.2 Materials and methods

4.2.1 Worms

T. tubifex worms were bought from a local wholesale (Aquadip b.v. The Netherlands). Details regarding the identification and handling can be found elsewhere [8].

4.2.2 Sludge characteristics

Activated sludge was sampled from waste water treatment plant (WWTP) Harnaschpolder (Den Hoorn, The Netherlands), which treats municipal sewage of 1.3 million people equivalents with an enhanced biological phosphorus removal (EBPR) system. Initial WAS concentrations were between 2.5 and 2.7 g/L.

4.2.3 Sludge treatment

4.2.3.1 Aerobic treatment

Extended aerobic treatment or endogenous respiration for the duration of 40 days (denoted as ER) was carried out in glass bottles filled with fresh or

anaerobically stabilised sludge. ER experiments were performed at room temperature (± 20 °C). Evaporated water was replenished with demineralised water. In case of foam formation, anti-foam (Antifoam A concentrate aqueous emulsion, A6582 – Sigma Aldrich) was used. The dissolved oxygen was maintained above 5 mg/L and was supplied with fine bubble aerators (aquarium stones). The sparging of air provided sufficient mixing.

4.2.3.2 Anaerobic/Anoxic treatment

WAS, worm predated sludges or ER sludges were incubated under anaerobic conditions for a period of 40 days. All incubations were performed in triplicate. 2L borosilicate glass bottles were filled with 2 L of sludge and inoculated with 125 µL /L of digestate (TS concentration of 21 g/L) to increase the microbial diversity in the incubations. Anaerobic conditions were created by sparging N_2 gas for 3 minutes. Bottles were incubated in a thermal shaker operated at 35 °C and 120 RPM.

Part of the 2L bottles were prepared to monitor biogas production and were coupled to an AMPTS II system (Bio-process Control, Sweden) for registering the methane production. The biogas was led through a hydroxide solution to remove the CO_2 from the produced biogas. The other part bottles were used to sample for sludge and liquid analysis during AD and were therefor not connected to the AMPTS. Biogas could freely escape by means of a connected fermentation lock. Nitrogen gas was used to replace the removed sample volume. Additionally, to generate sufficient anaerobically stabilised WAS for follow up experiments, an additional 20L of WAS was also incubated under anaerobic conditions.

The nitrate formed during the aerobic treatment stages could lead to anoxic conditions during the next degradation stage if not all nitrate is removed during the treatment process. Although, the subsequent anaerobic stage is referred to as AD, it will be indicated when nitrate was present. The removal of nitrate by adding an external carbon source was not considered as this could induce bacterial growth and thus alter the solids concentration and composition, which is different from the approach as proposed by Park et al., (2006).

4.2.3.3 Worm predation

Worm predation was performed in an airlift reactor that was composed of two identical compartments, both containing 18 L of WAS. The reactor was operated as a batch system. Predation and the associated control experiments lasted 5 days. Approximately 40 g/L wet weight worms were added to one compartment for worm predation (WP) of WAS and for the production of worm predated sludge (WPS). The other compartment did not contain worms and was used as a control to evaluate the endogenous respiration during 5 days of aeration (ER5). The dissolved oxygen was maintained above 5 mg/L. Detailed reactor operational data can be found elsewhere [7]. Evaporated water was replenished with demineralised water. The aquatic worms were separated from the predated sludges using a sieve with 200 µm mesh size and carefully rinsed with solids free filtrate to collect residual solids.

The worm predation of the stabilised sludges solids was carried out in the previously mentioned worm reactor. The experiment was performed in triplicate using a single initial WAS sample. The aerobically or anaerobically stabilised sludge was left to settle for 2 hours after which supernatant liquid was replaced with an equal volume tap water to minimise the concentration of ammonia [8] and potentially nitrate [13] which could be toxic for the aquatic worms. The initial nitrate concentrations did not exceed 7 mg N/L. The aerated experiments, that served as control for worm predation (i.e. without worms) were carried out in 3.5 L bottles with a working volume of 2.5 L, in triplicate. The dissolved oxygen was maintained above 5 mg/L and was supplied with fine bubble aeration, in which sparging of air provided sufficient mixing.

4.2.4 **Treatment process overview**

An overview of the different incubation experiments is given in Table 4.1. The aerobic endogenous respiration (ER) followed by anaerobic digestion (AD) will be denoted as ER-AD and vice versa as AD-ER. The AD and ER stages, in combination with worm predation (WP) will be denoted as ER-WP, WP-ER, AD-WP or WP-AD. The aerobic ER control experiments of worm predation of WAS will be denoted with the addition of the duration or SRT in days (i.e. ER5).

Table 4.1: *Abbreviations of the different experiments and duration of the process stages.*

Stage one	Duration (days)	Stage two	Duration (Days)	Abbreviation
Anaerobic digestion	40	Endogenous respiration	40	AD-ER
Endogenous respiration	40	Anaerobic digestion	40	ER-AD
Worm predation	5	Anaerobic digestion	40	WP-AD
Control	5	Anaerobic digestion	40	ER5-AD
Anaerobic digestion	40	Worm predation	5	AD-WP
Anaerobic digestion	40	Aerated Control	5	AD-ER5
Endogenous respiration	40	Worm predation	5	ER-WP
Endogenous respiration	40	Aerated Control	5	ER-ER5

4.2.5 Analytical methods

Total solids (TS), volatile solids (VS), total suspended solids (TSS) and volatile suspended solids (VSS) were measured in triplicate according to standard methods [14]. The sludge COD, nitrate and sulphate concentrations were measured in triplicate, using the photometric test kits LCK 014 and LCK 514, LCK 339 and LCK 153 respectively (Hach, Düsseldorf, Germany). Analytical methods were in accordance with the standard methods [14].

4.2.6 Reduction and rate calculations

The treatment processes consisted of two sequential incubation stages. The VS(S) and COD reductions in a particular incubation stage are expressed as fraction of the initial WAS sample. As such, the reduction percentages of the different stages in a sequential treatment process can be summed up to calculate the total overall conversion of that particular process.

$$Reduction\ as\ \%\ of\ initial\ WAS = \frac{[X]_{start\ of\ stage} - [X]_{end\ of\ stage}}{[X]_{initial\ WAS}}\ x\ 100\% \ .$$

(eq. 1)

with X = g COD/L or g VS(S)/L.

In order to determine the first order rate constants of the treatment stage, the integrated form of the first order rate equation was used.

$$\ln\left(\frac{X_t}{X_{t=0}}\right) = -k(t - t_0) + C \quad \text{(eq. 2)}$$

with X = the solids concentration, k the first order rate constant, t time in days and C the integration constant.

4.3 Results and discussion

4.3.1 The biodegradability of worm predated and waste activated sludge: determination of the extent in solids reduction

Firstly, in order to evaluate the solids removal potential of a defined treatment method, a simple percentual comparison of these methods against a control is insufficient. To put solids removal potential of a certain treatment method in the proper perspective, it is necessary to determine to what extent the solids potentially could be biodegraded in a given time frame. Therefore, the WAS biodegradability potential was determined and used as a reference point to assess the extent of WAS degradation through worm predation. The removal efficiencies of ER-AD and AD-ER were chosen, based on the long process time in both aerobic and anaerobic conditions to indicate the biodegradability potential of the WAS used in this study. The initial WAS biodegradability was used as a baseline for the other treatments.

Secondly, a control without worms was used (ER5-AD) to be able to validate the results of solids removal due to WP-AD in comparison to the maximum biodegradability potential, or baseline experiment. An overview of the averaged solids removal in terms of COD and VS for the different treatment processes is presented in Table 4.2.

Table 4.2: *VS and COD reduction of WAS after aerobic, anaerobic or predation treatment. Results are presented as averages of replicates, namely, AD-ER was replicated two times, WP, ER5 and ER-AD were replicated four times. After the average values, the standard deviations are shown. In case of AD-ER the variation between duplicates is presented.*

Stage 1	Stage 2	VS reduction stage 1	VS reduction stage 2	Total VS reduction	COD reduction stage 1	COD reduction stage 2	Total COD reduction
WP	AD	21% ± 6	26% ± 7	47% ± 5	37% ± 6	17% ± 9	57% ± 1
ER5	AD	4% ± 3	34% ± 4	37% ± 5	19% ± 7	28% ± 4	44% ± 5
ER	AD	35% ± 3	12% ± 3	46% ± 2	52% ± 1	7% ± 2	59% ± 3
AD	ER	36% ± 6	7% ± 3	43% ± 3	40% ± 5	16% ± 4	59% ± 3

The results clearly show that the first aerobic or anaerobic digestion stage showed the largest contribution to the total VS and COD removal, which is in agreement with other research [10,15] and can be explained by the sequenced degradation of readily biodegradable sludge parts followed by the more complex parts. Furthermore, the reference treatments AD-ER and ER-AD, showed that the order of the process conditions had no significant influence on the total amount of VS removed, even though the second phase in ER-AD remained anoxic.

Although the biodegradability extent in both treatments are similar, they differ from the 63% VS removal for both process sequences reported by Park et al., (2006). It is likely that this high reduction in the experiment of Park et al., (2006) was due to the relatively limited stabilised WAS. Park et al.,(2006) used WAS from a WWTP that was operated at an SRT of 7 days, while the WWTP Harnaschpolder that was used in our experiment, was operated at an SRT of 16 days. Furthermore, difference in biodegradability is highly dependent on influent composition and other process conditions [10,16], which also might have differed between two WWTPs.

Regarding the overall COD removal, Martinez-Garcia et al., (2016) showed during a 120 day batch digestion experiments with lab grown sludge, that sole anaerobic, aerobic or hypoxic conditions resulted in 57 – 70% COD removal. Although the incubation time differed considerably, the COD removal in the first treatment stages of ER and AD, are in the same order of magnitude as was reported by Martinez-Garcia et al., (2016).

Predation of activated sludge, in the first stage of the WP-AD treatment resulted, as expected in a higher average VS and COD reduction compared to 5 days of endogenous respiration (ER5) which served as a control for WP. Additionally, the conversions in the second stage of ER5-AD and WP-AD were higher than the conversions in the second stage of ER-AD due to lower solids removal in the first stage of ER5 and WP. Compared to previous research, the VS removal in WP as first stage is distinctly lower than the potential VS removal range that aquatic worms showed before, which was 47% ± 15 with conversions in the ER5 control of 9% ± 5 [7]. Furthermore, in previous research we found that the ER VS removal of 30 days (ER30) reached similar values as WP [7]. The ER presented here was performed over 40 days, which is 10 days longer than in earlier studies. Very likely, this increased the VS removal and increased the difference with WP. But more importantly, the here presented results show that the previously reported similarity between ER30 and WP was apparently coincidental and is likely due to the fact that the determination of ER30 and WP was not performed using the same initial WAS sample.

In relation to the overall solids removal, the reduction in WP-AD after 45 days is comparable to the removal in the reference AD-ER and ER-AD processes after 80 days of treatment. Based on these results it is clear that worm predation and anaerobic treatment combinations significantly improve sludge process time but do not alter the overall biodegradability potential of the sludge compared to the reference process. Interestingly, the WP-AD solids reduction was considerably higher than when only AD is applied (first stage AD-ER) during 40 days (36% ± 6). Based on this difference, Tamis et al., (2011) hypothesized that the biodegradability of the sludge was increased due to worm predation.

The similarity in the COD and solids removal percentages, between the AD-ER combinations and WP-AD suggest that in conventional activated sludge systems with AD, which removes about 30 – 35% of the solids [3–5], about 20 to 25% of the biodegradable material remains untreated. Worm predation technology can remove the remaining biodegradable COD in a time-efficient manner. Limitations to the extent of sludge biodegradability can be attributed to various factors: i) the presence of recalcitrant humic substances that may account for 10 – 20% of the sludge organics [6], ii) the tightly bound extracellular polymeric substances (EPS) fraction that is hard to degrade [18] and iii) the available process time as well as

iv) mixing conditions [19] that are both not optimized to reach the maximum biodegradability in full scale reactors.

4.3.2 Process performance

To gain more insight into the sequential degradation processes, the VSS removal of the different processes using a single initial WAS sample is shown in Figure 4-1. At the end of the treatment, the total amount of removed solids were in the same range but differed slightly after the second stage, except for ER5-AD which served as a control for WP-AD. The observed trend indicated that the reduction was not yet complete after the 40 days of AD in the ER5-AD sequence. In contrast, the WP-AD and ER-AD reached full conversion already after 26 days AD and did not show further conversion during the last 14 days. Interestingly, although the redox states differed between the ER-AD process presented here (anoxic with final N-NO_3^- concentrations of 55 ± 21 mg/L) and the ER-AD process of Park et al., (2006) (anaerobic), this seemingly did not influence the overall solids removal which was comparable to that of AD-ER.

The rates constants over the first 26 days of the digestion processes in Figure 4-1 are listed in Table 4.3.

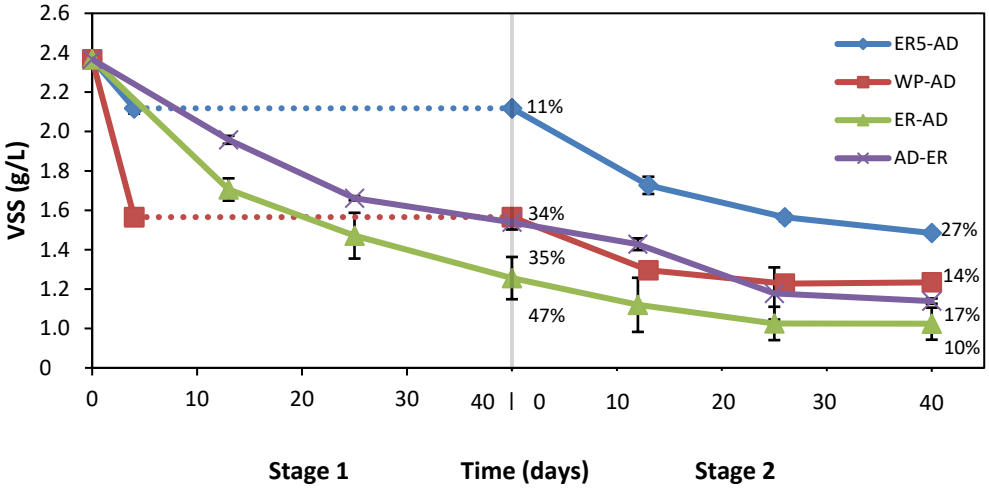


Figure 4-1: *The change in VSS concentration during the treatment stages. Results are from a single initial WAS sample. The dashed lines are only a visual aid as to have WP and ER5 in the same treatment stage as ER and AD and better reflect the VSS removal rate. The percentual change in VSS reduction of the stages is displayed. Standard deviations are shown.*

Table 4.3: *First order rate constants for the different treatments. Rate constants were calculated based on the VSS and COD degradation of the experiments shown in Figure 4-1. The first 26 days of both treatment stages were used. Standard deviations are shown. The average R^2 values were 0.96 ± 0.04.*

Stage 1	Stage 2	Stage 1	Stage 2	Stage 1	Stage 2
		VSS (d^{-1})		COD (d^{-1})	
ER5	AD	0.028 ± 0.005	0.007 ± 0.001	0.035 ± 0.008	0.009 ± 0.001
WP	AD	0.103 ± 0.005	0.009 ± 0.001	0.126 ± 0.006	0.008 ± 0.002
ER	AD	0. 016 ± 0.002	0.009 ± 0.002	0.018 ± 0.002	0.007 ± 0.001
AD	ER	0.012 ± 0.001	0.007 ± 0.001	0.012 ± 0.001	0.009 ± 0.001

In general, the higher rate constants in the first stages compared to the second stage indicate that easily biodegradable material was primarily removed at a higher conversion rate and leaving the more recalcitrant material for the second stage. It could also indicate that a relevant hydrolytic microbial community was initially lacking during the second stage, which resulted in lower rate constants. The rate constants in the second stages were in the same order of magnitude. The results further show, that the COD and VSS rate constants during WP were an order of magnitude higher compared to the first stage of the other treatment processes. The rate constants of the first aerobic and anaerobic process stages are in the same order of magnitude as the results found by Martinez-Garcia et al., (2016) who used 120 days of batch digestion, revealing constants (d^{-1}) of 0.024 ± 0.002 and 0.021 ± 0.002 for the aerobic and anaerobic conditions respectively.

The cumulative productions of biogas, consisting of CH_4 and N_2 in the different experiments were minimal, 0.2 to 1.1 NmL per day during the last 5 to 10 days of treatment. This implies that the differently treated sludge in AD-ER, ER5-AD and WP-AD was apparently fully stabilized. As expected, due to the 40 days of aeration in the first stage, the ER-AD treatment scheme remained anoxic in the second stage due to the formation of about 101 ± 10 mg $N\text{-}NO_3^-$/L, which was only by 50% converted at the end of the second stage (final concentration 55 ± 21 mg $N\text{-}NO_3^-$/L). A detailed analysis of the COD balances of the incubations is discussed in the supplemental information section.

A comparison of the sludge COD/VS ratios is presented in Table 4.4.

Table 4.4: *COD/VS ratios of one initial WAS sample and after the different treatment processes. Standard deviations are shown.*

Stage 1	Stage 2	Stage 1	Stage 2
Initial WAS	-	1.64 ± 0.01	-
ER5	AD	1.45 ± 0.08	1.46 ± 0.03
WP	AD	1.25 ± 0.06	1.40 ± 0.01
ER	AD	1.17 ± 0.03	1.24 ± 0.10
AD	ER	1.53 ± 0.05	1.22 ± 0.02

Results show that the COD/VS ratio in all treatments of stage 1 decreased compared to the initial WAS COD/VS ratios. After stage 1, the lowest COD/VS ratios were found for ER and WP were full aerobic conditions led to the highest degree of carbon oxidation. The COD/VS ratios after the anaerobic or anoxic incubations remained more or less the same or showed a slight increase. The latter might be attributed to VS solubilisation under anaerobic/anoxic conditions. A similar pattern was observed for other experiments (Supplemental information section). WP and ER showed a similar low COD/VS ratio after the first stage despite the 35 days difference in incubation time. Possibly, the oxidation of bacterial lysis products and EPS during prolonged aeration in ER is similar in magnitude as in the WP treatment. It should be noted that worms have the ability to consume and oxidize bacteria [20–23] and EPS [7].

4.3.3 Predation of the aerobic and anaerobic treated sludge fractions

Instead of using WP as pre-treatment before AD, as was shown in the previous section, it can also be used as post treatment. The solids of aerobically and anaerobically stabilised sludges were used as substrates for *T. tubifex* predation, in order to investigate if the aquatic worms can release and degrade additional COD or VS after more conventional aerobic or anaerobic sludge stabilisation. After this additional worm treatment, the processed sludges were anaerobically digested again, to investigate if the worms increased the overall conversion efficiency. The VS and COD reductions are shown in Table 4.5.

Table 4.5: *VS and COD reductions after predation of aerobically and anaerobically stabilised WAS solids and the subsequent anaerobic digestion of the predated solids. The removals of the reference process, AD-ER and ER-AD were around 46% VS and 57% COD. The initial sludge is from a different batch than those presented in section 4.3.1. Standard deviations are shown. n.a. = not applicable due to the death and decay of worms in this experiment.*

Stage 1	Stage 2	VS reduction stage 1	VS reduction stage 2	Subtotal VS reduction	Extra AD	Total VS reduction
AD	WP	29% ± 1%	21% ± 1%	51% ± 2%	8% ± 0.3%	58% ± 2%
AD	ER5	29% ± 1%	0.2% ± 0.01%	29% ± 1%	13% ± 1%	43% ± 2%
ER	WP	36% ± 1%	-8% ± 0.4%	n.a.	n.a.	n.a.
ER	ER5	36% ± 1%	2% ± 1%	39% ± 2%	10% ± 1%	49% ± 3%

Stage 1	Stage 2	COD reduction stage 1	COD reduction stage 2	Subtotal COD reduction	Extra AD	Total COD reduction
AD	WP	25% ± 1%	31% ± 1%	57% ± 1%	4% ± 0.1%	60% ± 1%
AD	ER5	25% ± 1%	3% ± 0.1%	28% ± 1%	13% ± 1%	42% ± 2%
ER	WP	53% ± 2%	-12% ± 0.4%	n.a.	n.a.	n.a.
ER	ER5	53% ± 2%	2% ± 1%	55% ± 1%	6% ± 0.3%	61% ± 2%

The degradation during the second stage of both control (AD-ER5 and ER-ER5) experiments, showed little extra reduction, since the sludge used in the experiment were already largely stabilised (Figure 4-1). The addition of the extra AD stages, (Table 4.5) for the control experiments resulted, for the ER-ER5-AD process in a similar overall solids removal as to the ER-AD and AD-ER reference processes (Table 4.2). The AD-ER5-AD process, which is essentially 80 days AD,

showed a 13% extra removal which was probably due to the influence of the 5 days extra aeration and the weakening and lysis of anaerobic bacteria and rapid growth of aerobic bacteria which could be degraded in the next treatment step. Nonetheless, it seems that due to the lack of a prolonged aeration stage, the reduction levels of the reference process where not reached.

The extent of sludge biodegradability, in terms of COD removal in the worm predation processes AD-WP (57% ± 1%, Table 4.5) and WP-AD (57% ± 1%, Table 4.2) were the same. The VS removal was in the same order of magnitude (51%±2% and 47%±5%, respectively). Additionally, the solids and COD removals in the WP stages were similar. These observations indicate that the process order for anaerobic digestion and predation combinations is not relevant.

Hendrickx et al., (2010) also tested the WP-AD and AD-WP combinations and found a 10% higher solids removal for the WP-AD track in comparison to AD-WP. The total VSS reduction (re-calculated from the reported mass balance) was as high as 76% for the WP-AD track. Possible reason for the higher total VS reduction compared to this study, could be related to their reactor design that separates all worm faeces from the aerated sludge compartment. The subsequent AD process is then solely performed with worm faeces, instead of the AD of worm predated sludge, which will be a combination of sludge particles that crossed the worm track and sludge particles that were aerated for 5 days. Furthermore, Hendrickx et al., (2010) used a more traditional biological methane potential test, where the COD ratio between inoculum and substrate is >2. Whereas the anaerobic incubation presented here only used a minimal amount of inoculum/ seed sludge.

The additional AD stage in the AD-WP-AD process showed a small increase in VS and COD removal compared to the WP-AD process. It seems that the predation process improved the sludge anaerobic biodegradability compared to the control AD-ER5-AD. It is likely that the improved biodegradability is due to the activity of the worms. Possibly, the release of worm associated intestinal bacteria could also play a role. As was mentioned previously, due to soluble COD limiting conditions during aerobic treatment, sludge growth is limited [24,25], however the intestinal bacterial community of the aquatic worms do grow [26] and are released along with the worm faeces [27]. Possibly, these released bacteria could assist in the degradation of sludge.

In contrast to predation of anaerobically stabilised sludge (AD-WP), the predation of aerobic stabilised sludge (ER-WP) resulted in worm death and solubilisation of worm biomass. These results are in contrast with Elissen, (2007) who performed similar sequential treatment experiments, e.g. the AD-WP and ER-WP processes with the aquatic worm *L. variegatus* in which a negative effect or even worm death was not observed if the pre-treatment time did not exceed 20 days. In that study, TSS removal increased (estimated from graph) from about 55 to 65% in case of ER-WP and from 19 to 29% in the AD-WP process. Although different process conditions were used, aquatic worms apparently can further degrade stabilised WAS solids as is also shown in work the presented here. Unfortunately, the reference processes, AD-ER and ER-AD were not determined.

The exposure to the different redox conditions in the sequential treatment stages might have resulted in soluble COD- and/or soluble BOD- limited conditions. Under these conditions, sludge reduction can be attributed to EPS reduction, microbial decay and mineralisation [24]. Decaying or lysed bacteria might have been used as substrate for maintenance metabolism [25,29,30] or, alternatively, as substrate for bacterial growth. Net microbial growth is especially prevalent at the beginning of a process stage when biodegradable organic matter is still abundant. Ultimately, all these processes are together responsible for the lower observable sludge yield for processes utilising alternating anaerobic and aerobic conditions [24,25,31–33].

In practical sludge cycling applications, the WAS is recirculated between an external anaerobic or anoxic substrate deficient tank and the main aerobic, substrate rich process. This sludge cycling process, with some process modification is termed the oxic-settling-anaerobic (OSA) process [34].
The order of the anaerobic and aerobic process conditions in OSA-like processes is important from an energy recovery perspective as solids removal and rate constants are highest in first stages. OSA-like processes can be improved by the addition of a worm predation stage after the first anaerobic stage (e.g. AD-WP). This would result in improved overall solids removal rates while maintaining the possibility to maximize energy recovery trough methane production. Although promising, due to the increased ammonia concentrations after an anaerobic conversion process, ammonia toxicity has to be taken into account [8]. The benefits of the addition or incorporation of a predatory stage to alternating process conditions for improved solids removal was also indicated by Jung et al.,

(2006). Furthermore, predation has a positive effect on sludge settling and compacting [7,8] which can further improve the efficiency of OSA-like processes that employ a settling stage.

4.3.4 Worm death during aerobic predation

Because worm death was observed in different occasions when aerobic and anaerobic conditioned sludges were fed, it was decided to separate the solids by sedimentation and only feed the settled fraction to the worms. In this way, the high NH_4^+ concentration from the anaerobic digestate was avoided and could not lead to toxicity [7]. Additionally, possible inhibiting compounds originating from the aerobic sludge were also not fed in this way. A toxic effect was observed in conditioned sludge predation experiments with the aquatic sludge worm *L. variegatus.* Elissen et al. [39] showed that worm growth on aerobic conditioned sludge was possible. However, the aerated conditioning period of the sludge could not exceed 48 days, because after this period unexplained worm death occurred. Despite this precaution and an aeration of only 30 days in this study, worm death could not be avoided during the predation of the aerobic conditioned sludge solids.

A possible cause of worm death could be related to the food source of the aquatic worms. As was mentioned previously, both bacteria [27–30] and EPS [6] are considered an important food source for *T. tubifex*. The low COD/VS ratio of the ER sludges are indicative of the previously mentioned substrate limited conditions which are dominated by lysis and cryptic growth. More specifically, on a microbial level Foladori et al. [18] showed by using flow cell cytometry on a 12 day SRT ASSR system, that bacterial decay and lysis of activated sludge predominantly occurs in extended aerobic conditions whereas in anaerobic conditions the bacterial count remained stable. The extended aerobic treatment could reduce the sludges' nutritional value (e.g. bacteria which also coincides with the decrease in COD/VS ratios for ER sludges (Table 4.4) in aerobically stabilised sludge indicate that there is hardly any biodegradable carbon present in this sludge that could serve as substrate for the aquatic worms. In contrast, anaerobically stabilised sludge has a higher bacterial count and higher measured COD/VS ratio and is thus more suitable for worm predation.

The impact of ingesting solids with a low biodegradability by Tubificidae is to our knowledge not investigated. Ingestion studies, where micro plastics were used as nutrient poor solids, showed a decrease in energy reserves in marine worms [39] and decreased growth rates in terrestrial worms [40]. Possibly, *T. tubifex* suffered from similar effects when the aerobically stabilised sludge solids were ingested, resulting in worm decay. The presented results strongly suggest that the worm-preferred sludge fraction should contain an abundancy of bacteria and EPS. It has been documented that aquatic worms could prefer gram negative bacteria over gram positive [41]. Interestingly, predation on anoxic or anaerobic WAS, which contains an abundance of gram negative bacteria [42], resulted in higher worm biomass growth rates and yields, as opposed to the worm biomass growth rates and yields on aerobically stabilised sludge grown bacteria and EPS [8].

4.3.5 Aerobic and anaerobic degradable sludge fractions

In our previous research [7] we suggested that *T. tubifex* predominantly feeds on an aerobically degradable fraction. It was reasoned that the similar solids reduction of WP and ER, matched the difference in solids removal between WP-AD and AD as reported by Tamis et al., (2011), which should be indicative of a presence of a distinct 'aerobic degradable fraction' that was responsible for the improved biodegradability of predated sludge. However, the presented results show that the aforementioned matching degradation patterns were only coincidental and that the extent of the sludge biodegradability is not influenced by the predation process. Additionally, it is clear that sequential aerobic (predation) and anaerobic processes are essential to reach the biodegradability potential.

In a broader context, our present research revealed an important pitfall when comparing different solids reduction process. For evaluating the biodegradability potential of a certain treatment process, it is insufficient to only compare the percental changes in an assay with a control. Under all circumstances, a reference point for the biodegradability potential of the initial sludge is required. By doing so, a treatment process can be more accurately evaluated and the performance compared to other processes.

4.4 Conclusions

The objective of this study was to investigate the effect of sludge predation, on the biodegradability of WAS by using a sequential anaerobic and aerobic treatment method. The following conclusions where made:

- The WAS biodegradability extent was not affected by the predation and AD process combinations.
- *T. Tubifex* improved sludge conversion rates and may thus reduce retention times in consecutive processes.
- The natural breakdown of sludge in consecutive anaerobic and aerobic conditions reached the same limit in biodegradability irrespective of the process order.
- The first stages in consecutive sludge treatment processes has the largest contribution towards the sludge biodegradability extent.

4.5 References

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4.6 Appendix

Table 4.6: *COD/VS ratios of one initial WAS sample and after the different treatment processes. Standard deviations are shown.*

Stage 1	Stage 2	Stage 1	Stage 2
Initial WAS		1.69 ± 0.06	-
ER5	AD	1.41 ± 0.02	1.54 ± 0.01
WP	AD	1.34 ± 0.04	1.51 ± 0.05
ER	AD	1.22 ± 0.01	1.23 ± 0.02
AD	ER	1.63 ± 0.03	1.37 ± 0.05

Table 4.7: *COD balance of the anaerobic and/or anoxic incubation stages of the different sequential treatment processes. Total volumes of produced gas, the theoretical volume of produced nitrogen gas and the COD values are shown. The theoretical nitrogen gas production was calculated based on the amount of removed nitrate. This value was used to calculate the methane production from the total produced gas volumes, which is composed of CH4 and N2. COD requirement for denitrification and sulphate reduction are based on stoichiometry. The overall COD balance is presented in the last column, which ideally should give a normalised value of 1. Results derived from a single initial WAS sample; digestion experiments, and thus gas production measurements, were performed in triplicate. Standard deviations are shown.*

	Total produced gas (CH4/N2) (NmL)	**Theoretically produced N_2 (NmL)**	**Total COD added (g)**	**Total COD removed (g)**	**COD for denitrification (mg)**	**COD for sulphate reduction (mg)**	**COD in CH_4 (mg)**	**COD balance (-)**
ER5-AD	354 ± 1	54 ± 1.6	6.38 ± 0.15	1.98 ± 0.19	192 ± 6	124 ± 18	848 ± 5	0.6 ± 0.1
WP-AD	217 ± 9	83 ± 0.6	4.42 ± 0.13	0.88 ± 0.05	296 ± 4	113 ± 6	378 ± 24	0.9 ± 0.1
ER-AD	94 ± 3	85 ± 50	3.56 ± 0.29	0.67 ± 0.09	266 ± 143	17 ± 29	114 ± 62	0.5 ± 1.2
AD-ER	516 ± 24	4 ± 0.1	7.33 ± 0.05	2.28 ± 0.08	13 ± 1	85 ± 1	1447 ± 68	0.7 ± 0.1

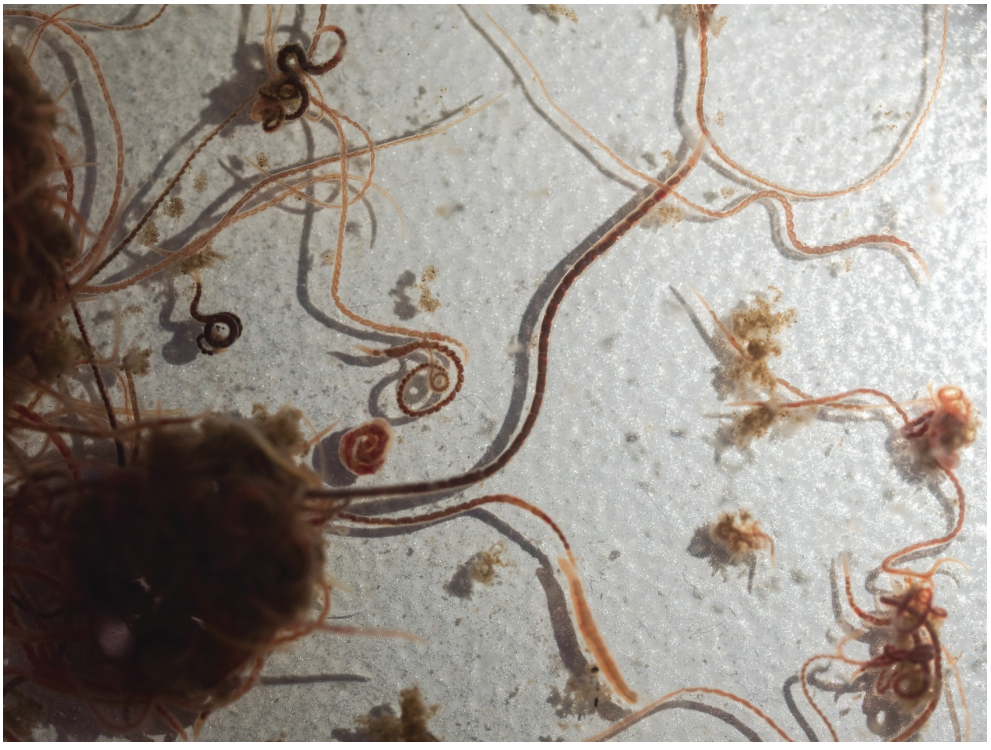
Theoretically, (at NTP with molar gas volume of 22.7 L) the denitrification of about 46 ± 25 mg N-NO_3^- in the ER-AD process could result in 75 ± 40 NmL N_2 gas which is close to gas production of 94 ± 2.7 NmL, when not taking N assimilation in account (Table 4.7). Additionally, the theoretical specific N_2 gas production based on the stoichiometry of denitrification, using biomass as carbon and energy source ($C_5H_7O_2N + 4NO_3^- \rightarrow 2N_2 + 5CO_2 + NH_3 + 4OH^-$) is 283.72 NmL N_2 per gram removed COD. This value is in the same order of magnitude as the 143 ± 23 NmL per gram removed COD obtained for ER-AD.

However, the large difference in nitrate concentrations between triplicates and the small deviation in the produced gas of the triplicates, are indicative of N assimilation and possibly the occurrence of methane production from anaerobic zones. With the exception for the WP-AD incubation, the COD balances over the anaerobic/anoxic processes could not be closed. Possibly the introduced errors are from the theoretical approach and the limitations of the experimental procedure that was focused on sludge solids removal rather than maximizing biogas production.

5 UNRAVELLING THE PROTEIN PREFERENCE OF AQUATIC WORMS

This chapter is based on:

Steef de Valk, Ahmad F. Khadem, Jules B. van Lier and Merle K. de Kreuk (**2018**) *Unravelling the protein preference of aquatic worms during waste activated sludge degradation*. Environmental Technology, 39:2, 182-189



Abstract

Worm predation by *T. tubifex* was investigated using waste activated sludge (WAS) as the substrate. In order to better understand the sludge degradation mechanisms during worm predation, the activity of 5 common hydrolytic enzymes were determined and compared between the initial feed activated sludge, endogenous respirated sludge and worm predated sludge. The results showed that the enzymatic activity decreased upon aerobic (worm) treatment of WAS and that this activity was predominantly associated with the removed solids fraction of the sludge. Interestingly, the protease activity showed a smaller decrease in activity when worms were present. Flow cell cytometry revealed the release of intestinal bacteria from the worms, which are presumed to be largely responsible for the observed protease activity. Additionally, experiments in which *T. tubifex* were treated with antibiotics showed that the worms are responsible for a maximum of 73% of the observed proteolytic activity. The remaining 27% is attributed to the intestinal bacteria that exhibit a synergistic relationship with *T. tubifex* towards protein hydrolysis.

5.1 Introduction

Waste activated sludge (WAS) is produced in large quantities as a side product of conventional activated sludge-based wastewater treatment processes. WAS is considered a waste stream that needs to be properly discarded [1]. The processing cost of the WAS can amount to up to 50% of the total operational costs of a waste water treatment plant (WWTP) [2]. To reduce these costs, sludge minimization techniques are widely researched and applied, with anaerobic digestion (AD) of WAS being the most prevalent.

Current approaches to increase the efficiency of AD are based on increasing the extent of hydrolysis and concomitant hydrolysis rates during sludge treatment since hydrolysis of sludge particles is considered to be the rate limiting step in sludge digestion [3]. Biochemical and physicochemical techniques such as enzyme dosing, ozonation, sonication and thermal treatment aim to improve the solubilisation of the WAS, thereby increasing hydrolysis rates [4]. Other methods focus more on minimising the production of WAS. These sludge reduction methods are based on either cell lyses and cryptic growth mechanics [5], as applied in the cannibal process [6] or on predation by macro fauna [7].

Predation by macro fauna, for example with aquatic worms, has gained increased attention in the past decades. For instance both Tamis et al. (2011) and Lou et al. (2011) researched full scale worm reactors for sludge reduction. Both studies showed a higher degree of sludge reduction and thus showed great potential for full scale application. Similar results were found in several different lab-scale reactor setups, independent of the aquatic worm species used [8,10–13].

Although the effects of worm predation on sludge reduction are well researched, it is not yet clear why a higher degree of sludge reduction occurs with worm predation when compared to extended aeration and anaerobic digestion. In this regard, the apparent preference of the aquatic worms to degrade the protein-like fraction of the extracellular polymeric substances (EPS) within sludge flocs, is an important finding [14] as it suggests protease activity.

Thus far, the nature and origin of these proteases has remained unclear. They could be produced by the worms themselves, or alternatively, by the bacteria inhabiting the intestines of aquatic worms [15–17]. Additionally, there is evidence

that aquatic worms can degrade entire bacteria that are consumed [15–17]. Further insights into this phenomenon is imperative to optimise the application potentials of enzymatic pre-hydrolysis of WAS.

In addition to proteases, other hydrolytic enzymes, such as glycosidases, phosphatases and lipases, play an important role in the hydrolysis of WAS [18]. Knowledge concerning the hydrolytic activity of these enzymes in relation to worm predation will likely provide the required fundamental insights to develop a feasible sludge minimisation technique based on enhanced enzymatic pre-hydrolysis.

In order to further elucidate the enzymatic activities that are essential to the worm predation process, this paper presents a comparative analysis of the relevant hydrolytic enzymatic activities between the initial feed activated sludge, i.e. WAS, the sludge after worm predation (WP), and the sludge after endogenous respiration (ER). Additionally, to distinguish between the enzymatic activity of the aquatic worms and their intestinal bacteria, a selected group of the aquatic worms were treated with antibiotics to suppress bacterial activity.

5.2 Material and Methods

5.2.1 Lab-scale worm reactor

T. tubifex was purchased from a local wholesale supplier (Aquadip B.V., The Netherlands). The aquatic worms were used in batch experiments in a lab-scale reactor. WAS was used as the substrate and was obtained from the domestic waste water treatment plant Harnaschpolder (Den Hoorn, The Netherlands), which treats the domestic waste water of 1.3 million people equivalents and is comprised of a biological nutrient removal (BNR) plant. The lab-scale reactor consisted of two identical 18L compartments: one contained about 700 grams of worms for generating the worm predated sludge (WPS), and the other served as a control for endogenous respiration, producing ER sludge (ERS). The design of the reactor is a modified lab-scale version of the full-scale worm reactor that was used by Tamis et al. (2011).

Both compartments were aerated and mixed using an airlift system. The average dissolved oxygen (DO) concentration was ≥ 5 mg/L, and the temperature was

maintained at 20 ± 1°C. The pH, left unaltered, was 7.5 ± 0.2. The duration of one batch cycle was 4 days. Distilled water was used to compensate for evaporation losses. Details regarding the taxonomy and handling of the worms and the performance of the worm reactor can be found in a previous study [13].

5.2.2 Analytical procedures

Total solids (TS) and volatile solids (VS) were measured in triplicate in accordance to standard methods [19].

5.2.3 Enzymatic activities

WAS, ER and WP mixed liquor samples and their corresponding supernatants, which were obtained by filtration of the mixed liquor sludge using 0.45 µm polyethersulfone membrane filters (VWR International LCC, Radnor, Pennsylvania, USA), were incubated with different substrates (Table 5.1) in an Innova 40 thermal shaker (New Brunswick Scientific Co., Inc., Enfield, Connecticut USA) at 25 ± 1°C at 100 rpm. The pH of the sludge samples was adjusted to 7. Samples were taken at regular intervals, and the enzymatic reaction was immediately stopped by the addition of trichloroacetic acid (TCA) dissolved in demineralised water, 15% w/w (reaction concentration). The samples were stored at -8 °C until further analysis.

After thawing, the samples were centrifuged (16,000 x g, 90s, at room temperature), and the obtained supernatant was filtered using 0.45 µm membrane filters. The filtrate was mixed with NaOH solution to an end concentration of 1M. Subsequently, the absorbance was measured using a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA) with demineralised water as a blank. The absorbance values were plotted, and the slope was determined using linear regression. A calibration curve was made, using nitrophenyl solution as a standard. All chemicals and enzymatic substrates were purchased from Sigma-Aldrich.

Table 5.1: *Substrates for enzymatic activity assay.*
**Stock solution of 80mM of 4-Nitrophenyl palmitate in isopropanol. For 50 mL substrate solution, 23.5ml of Tris HCl 20mM pH 7, 23.3 mL DMSO, 1 mL Trition X100 and 2.5 ml of Nitrophenyl palmitate stock, were mixed in this order.*

Enzymatic activity	**Substrate**	**Medium**	**Reaction concentration**	**Wavelength**
Lipase	4-Nitrophenyl palmitate	*	1 mM	410 nm
Protease	Azocasein	Tris HCl 20mM pH 8	0.2% w/w	440 nm
α-glucosidase	P-nitrophenyl-α-D-glucopyranoside	Demi water	1 mM	400 nm
β-glucosidase	P-nitrophenyl-β-D-glucopyranoside	Demi water	1 mM	400 nm
Phosphatase	P-nitrophenyl-phosphate	Demi water	1 mM	400 nm

5.2.4 Azocasein conversion and antibiotic treatment

In order to differentiate between the protease activity of *T. tubifex* and the intestinal bacteria, the release of ingested azocasein from the worm gut was monitored. For this purpose, worms were incubated with several combinations of the antibiotics (AB) Streptomycin sulphate salt and azocasein, and the release of the azo-dye from the worm gut was recorded.

The incubation period was set to 40 hours, which was sufficient to ensure that the worms ingested the maximum amount of azocasein. Gillis et al. (2004) showed that tubifex needs approximately 24 hours to purge their intestines and that the defecation rate is linear in time. The incubation took place in different combinations of azocasein and antibiotic solutions, as shown in the incubation section of Table 5.2. After 20 hours, the incubation solutions were discarded, and fresh solutions were added. The 20-hour duration period was selected based on experimental results that found azocasein hydrolysis to be negligible within this time frame. Azocasein hydrolysis may occur as a result of the growth of worm-associated bacteria. Details regarding this particular experiment can be found in the appendix (Figure 5-3).

At the end of the incubation period, the worms were thoroughly rinsed in flowing tap water to remove residual azocasein. Subsequently, the worms were transferred to 250 mL Erlenmeyer flasks containing 75 mL of the solutions listed in

the defecation section of Table 5.2. In all incubation steps, adequate passive aeration was ensured by setting the height of the 75 mL solution, including the worms, to approximately 2 cm. The following solutions were used: 0.5% (w/w) azocasein dissolved in tap water or in 0.2 g/L Streptomycin in tap water.

Table 5.2: *Overview of the different samples with their corresponding incubation and defecation solutions. Streptomycin was used as antibiotic (AB).*

Sample	**Incubation phase**	**Defecation phase**
Control	Water	Water
Control	AB	Water
Control	AB	AB
Release	Azocasein in water	Water
Release	Azocasein in AB	Water
Release	Azocasein in AB	AB

1 mL samples were periodically taken to follow the release of the azo-dye. The samples were mixed with 0.25 mL 45% (w/w) TCA to stop any enzymatic conversion and to precipitate non-hydrolysed azocasein. Next, the samples were frozen at -24 °C for later analysis. After thawing, the samples were filtered over 0.45 µm membrane filters, and 1 mL of filtrate was mixed with 0.25 mL 4M NaOH solution. Subsequently, the absorbance was measured at 440 nm using the aforementioned photo-spectrometer with demineralised water as a blank. The experiment was performed in triplicate. All chemicals were obtained from Sigma-Aldrich.

5.2.5 Flow cell cytometry

In order to assess the contribution of bacteria towards azocasein hydrolysis, the number of total and intact cells were measured using flow cytometer (FCM) according to Prest et al. (2013). Total cells were stained using SYBR® Green I, and intact cells with SYBR® Green Propidium Iodide. Samples were measured using a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium). When necessary, dilutions were made using filtered (0.22 µm Millex-GP) Evian® bottled water.

5.3 Results and discussion

5.3.1 Worm predation nutrient release and sludge reduction rates

The presence of *T. tubifex* had a significant impact on the extent and rate of excess sludge hydrolysis (Table 5.3). Organic (VS and chemical oxygen demand (COD)) removal rates during worm predation were about 5-fold higher compared to the control without worms, i.e. endogenous respiration. Additionally, an increased release of $P\text{-}PO_4^{-3}$, $N\text{-}NH_4^{+}\text{-}NO_3^{-}$ and soluble COD was observed as described elsewhere [13].

Table 5.3: *Average worm concentrations and removal and release rates for worm predation (WP) and endogenous respiration (ER) after 4 days of sludge treatment. Table shows averaged results with standard deviations of 10 different 4-day batch tests except for N, P and sCOD release, which were averaged from 6 batches. Table adapted from de Valk et al. (2016).*

Parameter	**Units**	**ER**	**WP**
Ratio Worms / VS	g Worms /g VS	-	14.1 ± 1.4
Worm concentration	g Worms/L	-	40.2 ± 5.9
TS removal rate	g TS/d	1.4 ± 0.94	8.2 ± 2.0
VS removal rate	g VS/d	1.3 ± 0.73	6.2 ± 1.5
COD removal rate	g COD/d	1.7 ± 1.8	7.2 ± 4.4
$N\text{-}NH_4^{+}\text{-}NO_3^{-}$ release rate	mg N/d	5.7 ± 3.1	10.5 ± 0.9
$P\text{-}PO_4^{-3}$ release rate	mg P/d	2.1 ± 1.4	3.6 ± 1.1
Soluble COD release rate	mg sCOD/d	0.06 ± 0.06	0.30 ± 0.2

5.3.2 Enzymatic activities in treated sludge

5.3.2.1 Mixed liquor sludge

The enzymatic activities of 5 common hydrolytic enzymes were determined before and after aerobic (worm) treatment of WAS. The results, presented in Table 5.4 show that in general, the enzymatic activities in the mixed liquor decreased after treatment of activated sludge. The graphs of the enzymatic activity assays can be found in the appendix Figure 5-4.

Table 5.4: *Enzymatic activities of the mixed liquor of waste activated sludge (WAS), worm predated sludge (WPS) and endogenous respirated sludge (ERS). Enzymatic activities are expressed as µmol substrate·gVS^{-1}min^{-1}, except for the protease activity, which was expressed as the increase in colour intensity of azo-dye: Absorbance·gVS^{-1}·min^{-1}. The sludges in the lipase assay were diluted 4 times in order to achieve linear substrate conversion. Average VS reduction was 24% ± 5 and 10% ± 4 for WP and ER respectively.*

	Protease	**α-glucosidase**	**β-glucosidase**	**Lipase**	**Alkaline Phosphatase**
WAS	0.017 ± 0.001	0.41 ± 0.006	0.34 ± 0.008	0.32 ± 0.011	1.17 ± 0.026
WPS	0.012 ± 0.001	0.20 ± 0.016	0.14 ± 0.005	0.11 ± 0.008	0.40 ± 0.005
ERS	0.014 ± 0.0004	0.33 ± 0.009	0.30 ± 0.016	0.20 ± 0.013	0.89 ± 0.013

The decrease in enzymatic activity was more prevalent for WPS than for ERS, for which enzymatic activities were closer to the activities of the original WAS. The averaged activities decreased by 53% ± 14 and 17% ± 5 for WPS and ERS respectively, compared to WAS. A decrease in hydrolytic enzyme activity, ranging from 50 - 90% within the first 5 days of aerobic digestion of activated sludge, was reported by Novak et al. (2003), Yu et al. (2008) and Lou et al. (2011). These literature values are similar to the activity decrease in WPS rather than ERS. The apparent low activity reduction in ERS is likely due to a difference in sludge composition between the studies [24].

Literature values of studied hydrolytic enzymes in the mixed liquor of different wastewater sources show a large range in activity (Table 5.5). When comparing results presented in Table 5.4 to the literature values in Table 5.5, it becomes clear that they are in the lower activity range. In regard to the differences in enzymatic activity between the different studies and the results presented here, Nybroe et al. (1992) noted that in general, hydrolytic enzyme activities are related to the composition of the influent and the process conditions of the activated sludge process and may differ significantly between the different sludges.

Table 5.5: *Literature values of enzymatic activity in waste activated sludge recalculated to the same unit (µmol/g VS(S) min) when necessary. Values marked with * were estimated from a graph. Abbreviations: AS – Activated Sludge, bioP – biological phosphorus removal, P.E. – people equivalent.*

Enzyme	**Source**	**Activity** (µmol/g VS(S) min)	**Reference**
Protease (Abs/min/g VS)	AS – average loaded, 300.000p.e.	5.54 – 8.00	[25]
	AS – Anaerobic-anoxic-oxic process	18.1 – 27	[26], [23]*
α-glucosidase	AS – average loaded	0.95 – 2.52	[25]
	AS – pilotscale alternating aerobic/ anoxic	0.67	[24]
	AS – bioP – 100.000p.e.	0.04 – 0.08	[27]*
	AS – Anaerobic-anoxic-oxic process	2.5 – 40.9	[26], [23]*
β-glucosidase	AS – bioP – 100.000p.e.	0.15	[27]*
	AS – biological nutrient removal – Anoxic-oxic process	0 – 4.1 [mUnit/gTS]	[22]*
Lipase	AS – bioP – 100.000p.e.	0.04 – 0.08	[27]*
Alkaline Phosphatase	AS – Anaerobic-anoxic-oxic process	11	[23]*

There are several potential explanations for the general decrease in enzyme activities upon aerobic (worm) treatment of WAS: Firstly, Frølund et al. (1995) and Cadoret et al. (2002) found that enzymatic activity is predominately bound to sludge solids, e.g. the EPS matrix. These studies indicate that volatile solids reduction, upon aerobic (worm) treatment, is related to the degradation of solids-bound enzymes. Secondly, Foladori et al. (2015) used flow cell cytometry to show that bacterial decay is a crucial factor in VS reduction during aerobic treatment of WAS. These findings indicate that the decay of bacteria, which in fact are enzyme producers, will lead to a reduction in enzymatic activity.

In this respect, the evidence that *T. tubifex* degrades entire bacteria [15–17] is noteable. Furthermore, bacterial cells are known to contain high concentrations of proteins, i.e. about 60% on a dry weight basis [29]. The latter coincides with the observation that sludge degrading worms preferentially hydrolyse and consume the protein-like fraction in sludge [13,30].

The preferred protein-like fraction that will be degraded by the worms may very well include the enzyme producers e.g. bacteria and/or the actual enzymatic proteins. This degradation of enzymes and/or their producers could explain the larger reduction in activities observed after worm predation compared to the endogenous respired sludge. In order to gain more insight into the relation between enzymatic activity and VS reduction, their percentile ratio was analysed (Table 5.6).

Table 5.6: *Reduction in enzymatic activity expressed as percent change compared to the base enzymatic activity values of waste activated sludge. Net change in enzyme activity / net change in VS [%/%]. Errors are expressed as standard deviations.*

Enzyme	**ERS**	**WPS**
α-glucosidase	1.42 ± 0.23	2.19 ± 0.15
β-glucosidase	0.95 ± 0.30	2.61 ± 0.17
Protease	3.17 ± 1.32	0.95 ± 0.19
Lipase	3.67 ± 1.04	3.25 ± 0.54
Alkaline Phosphatase	1.78 ± 0.27	2.85 ± 0.18

Results show that per % point VS removal, a higher reduction in α-glucosidase, β-glucosidase, and Alkaline Phosphatase enzyme activity was observed after worm predation compared to the control. However, lipase activity showed similar ratios between ER and WP, and for the protease activity, the ratio was a factor of 3 lower for the WPS compared to ERS. These calculations suggest that a part of the protease activity was conserved or maintained during worm predation.

The observed 'conservation' of protease activity could be the result of several processes. Firstly, proteases could be released by either the worms or by the intestinal bacteria. Secondly, bacterial decay, either due to worm activity or the previously mentioned decay during aerobic treatment, could promote growth of other proteolytic bacteria on the released bacterial proteins. Changes in the microbial community due to the presence of aquatic worm have been reported by others [31].

5.3.2.2 Enzyme activities in the supernatant

The enzymatic activities in the sludge supernatants were determined in order to distinguish the solids-bound enzyme activity from the enzymes in solution (Table 5.7).

Table 5.7: *Enzymatic activity of the supernatants of waste activated sludge (WAS), worm predated sludge (WP) and endogenous respirated sludge (ER). Enzymatic activities are expressed as µmol substrate·gVS^{-1}·min^{-1} except protease activity, which was defined as the increase in colour intensity of liberated azo-dye: Absorbance·gVS^{-1}·min^{-1}. Average values and standard deviations were calculated from triplicates.*

	Protease	α-glucosidase	β-glucosidase	Lipase	Alkaline Phosphatase
WAS	0.0002 ± 0.0006	-0.025 ± 0.045	0.007 ± 0.007	0.0049 ± 0.0126	-0.0034 ± 0.0257
WPS	0.0003 ± 0.0007	0.008 ± 0.017	0.018 ± 0.035	0.0135 ± 0.0065	0.109 ± 0.052
ERS	0.0001 ± 0.0001	0.022 ± 0.045	0.012 ± 0.035	0.0050 ± 0.0072	0.04 ± 0.004

Taking the standard deviations of the measurements and the low enzyme activities in the supernatant into account, it can be concluded that the averaged enzyme activities in the sub 0.45 µm or soluble fraction remained stable within the error margins after aerobic (worm) treatment.

In comparison with the mixed liquor, the enzyme activities in the supernatant were significantly lower. This low activity is in agreement with the observations of Frølund et al. (1995) and Cadoret et al. (2002) amongst others, stating that enzymatic activity is predominantly bound to the solids fraction in sludge. The reduced reduction in the protease activity in the sludge mixed liquor during worm predation (Table 5.6) was not reflected as an increased activity in the supernatant (Table 5.7). This implies that the increase in protease activity remained associated with the solids fraction.

Furthermore, Cadoret et al. (2002) found an increased enzymatic activity after using cation exchange resin (CER) or sonication to disperse the sludge flocs and disrupt the EPS matrix. Due to these dispersions, the fraction of particles with a diameter less than 4 µm increased to 99%. This particle size reduction could have resulted in the release of some enzymes into the supernatant that had been loosely bound to the EPS matrix.

Similar to CER and sonication, increased numbers of particles smaller than 2 µm upon aerobic (worm) treatment of activated sludge have been observed as discussed elsewhere [13]. Nevertheless, considering the standard deviation of the enzyme activities in the supernatant, no significant differences in supernatant enzyme activity were found (Table 5.7). However, the enzymatic activities in the supernatant followed a similar trend with the increase in small particles, namely WPS>ERS>WAS, except for the α-glucosidase activity, where WPS was lower in activity compared to ERS. Although not statistically warranted, this suggests that due to the reduction in particle sizes, some bound enzymes are released from the sludge flocs, resulting in a small increase in enzymatic activity in the supernatant.

5.3.3 The effect of antibiotics on the conversion of azocasein in the worm gut.

Bacterial association and interaction with the intestines of aquatic worms is well described in the literature [15–17]. Given their homology to higher organisms, it is very plausible that gut associated bacteria in the worms play a similar hydrolytic role. The hydrolytic role of associated microorganisms has been demonstrated in the midgut of earthworms [32], the hindgut of termites [33], the rumen of cows [34] and the human gut [35]. With respect to the removal of protein-like components from sludge, we postulate that proteolytic bacteria in the worm gut play an important role.

By treating *T. tubifex* with the antibiotic Streptomycin to suppress intestinal bacterial activity, a distinction can be made between the proteolytic activity of the worm and its intestinal bacteria. The (antibiotic treated) worms were fed azocasein, which is a protein substrate. When azocasein is ingested and subsequently hydrolysed, the azo-dye will be released. The release of the azo-dye from the worm through defecation gives an indication of the hydrolytic activity inside the intestines of the worms. Furthermore, to quantify the decrease in bacteria excreted after incubation with antibiotics, the defecated intestinal bacteria were counted using flow cell cytometry (Figure 5-1).

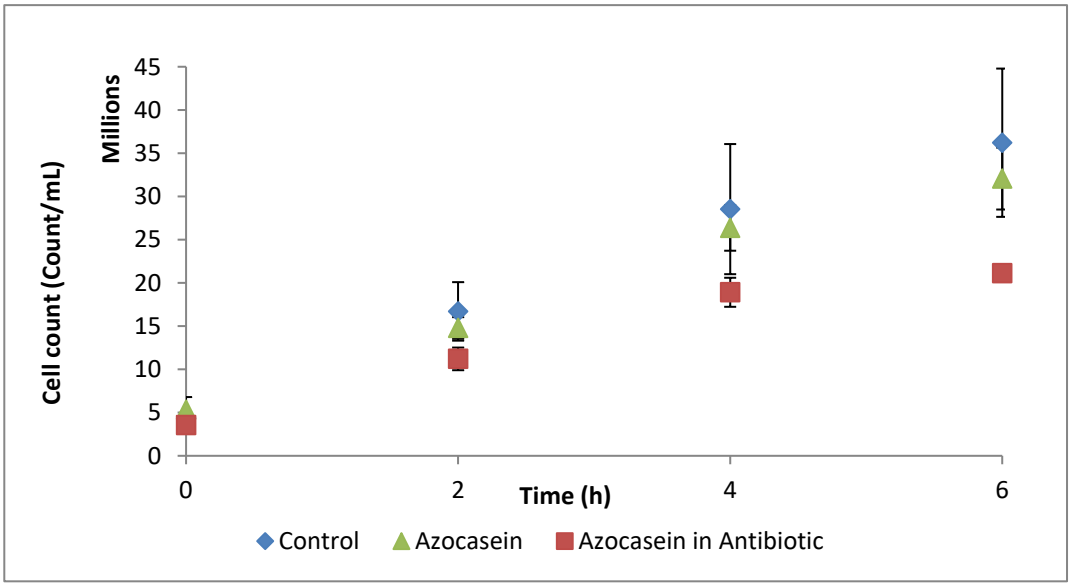


Figure 12: *The release of intestinal bacterial cells from the worm gut by defecation. Worms were incubated for 40 hours with different combinations of azocasein and antibiotics (AB). Upon transfer to 0.45 µm filtered water, the release of intestinal bacteria was monitored using flow cell cytometry. Average values and standard deviations were calculated from triplicates.*

Based on the differences between the azocasein incubations with and without antibiotics, the presence of antibiotics shows a clear effect on the number of bacteria that are released from the worm gut in time; after 4 hours, no additional organisms were released from the gut, and the overall release was lower in the antibiotic incubated worms. Bacterial release between the control and azocasein samples was similar. It should be noted that there was no difference in motility (e.g. tail waving and crawling) between worms incubated in water or in the substrate mixtures. This indicates that the worms were not physically affected by the incubations.

Exponential bacterial growth was absent during the experiment, which indicates that the increase in cell counts was predominantly due to accumulation of defecated bacterial cells. The accumulation of bacterial cells of the azocasein-incubated worms and the control is almost linear in time, which corresponds with the results of Gillis et al. (2004), who showed that the defecation rate of *T. tubifex* is linear based on the weight decrease due to defecation.

Interestingly, the released intestinal bacteria showed proteolytic activity (appendix Figure 5-3), suggesting that the released intestinal bacteria, which are part of the solids fraction of the sludge (Figure 5-1), produced the additional proteolytic activity (Table 5.6). However, protease activity was not determined separately for the worm faeces, and thus worm-based proteases cannot be ruled out.

The released azo-dye, which is liberated upon hydrolysis of the protein moiety in casein, is presented in Figure 5-2.

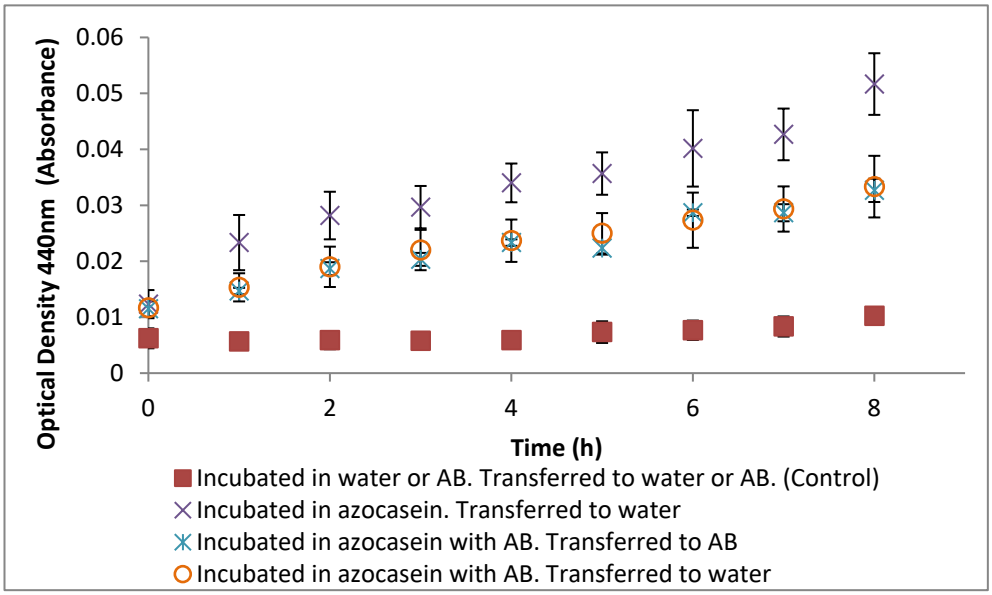


Figure 5-2: *Averaged azo-dye excretion from the worm gut by defecation. Worms were incubated for 40 hours with different combinations of azocasein and/or antibiotics (AB). Upon transfer to a clear medium, the release of the hydrolysation product, azo-dye, was measured at 440nm using a spectrophotometer. Control measurements were grouped together. Average values and standard deviations were calculated from triplicates.*

The difference between azocasein incubation with or without antibiotics clearly show that antibiotics affect the release rate of the azo-dye. Furthermore, the release of azo-dye is almost linear, which matches with the findings of the previously mentioned linear defecation observed by Gillis et al. (2004). The control samples show that no interfering substances were released during the experiment. The slopes of the release curves were calculated using linear regression. Due to the initial increase in optical density at time 0 and 1, these data points were not taken into account for the calculations.

Table 5.8 shows that the presence of antibiotics had a negative influence on the release rate of azo-dye.

Table 5.8: *Averaged azo-dye release rates. Slopes were calculated using linear regression on the triplicate measurements and subsequently averaged. The first two data points were not taken into account to reduce the bias caused by the initial increase in optical density. The averaged R^2 values of the individual replicates were 95.1% ± 3.3. The slopes of the control samples were grouped and averaged. Average values and standard deviations were calculated from triplicates.*

	Incubated in azocasein, transferred to water	**Incubated in azocasein and AB, transferred to water**	**Incubated with azocasein and AB, transferred to AB**	**Averaged controls**
Change in optical density per hour	$3.1x10^{-3} \pm 1.6x10^{-4}$	$2.3x10^{-3} \pm 3.6x10^{-5}$	$2.2x10^{-3} \pm 3.6x10^{-4}$	$4.9x10^{-4} \pm 1.9x10^{-4}$

A difference in azo-dye release rates of about 27% was observed between antibiotic treated and non- treated worms. This outcome suggests that the hydrolytic activity within the worm gut was negatively

influenced upon antibiotics treatment and that intestinal proteolytic bacteria within the worm gut had a significant influence on the conversion of azocasein. Additionally, these results indicate a synergistic relationship between the worms and their intestinal bacteria towards protein hydrolysis. Despite the fact that the antibiotic treated worms were not completely sterile (Figure 5-1), a maximum of about 73% of the azo-dye release rate can be attributed to the proteolytic activity of the worms. Altogether, the results show that the hydrolysis of the preferred protein-like fraction in sludge can be mainly attributed to the proteolytic activity of *T. tubifex*.

5.4 Conclusion

The activities of 5 common hydrolytic enzymes were predominantly associated with the solids fraction of waste activated sludge. Upon aerobic (worm) treatment of activated sludge, the enzymatic activities declined. Interestingly, the decline in protease activity during worm predation, in relation with the amount of solids removed, was lower compared to the ratio found for endogenously respirated sludge. This difference in the decline of protease activity in the sludge mixed

liquor and the apparent stable enzyme activity in the supernatant suggest that this difference is due to the synthesis of protease that remained associated with the solids fraction. The synthesis of protease could partially be due to the release of intestinal proteolytic bacteria. Experimental results using antibiotics in a selection of the incubations showed that *T. tubifex* is responsible for a maximum of about 73% of the protein hydrolysis rate. The remainder is due to intestinal bacteria working in synergy with *T. tubifex.*

To summarise:

- Enzymatic activity is predominantly bound to the solids fraction of waste activated sludge.
- Enzymatic activity decreases during aerobic treatment of activated sludge.
- The presence of worms decreased the reduction in proteolytic activity upon aerobic digestion in comparison to the control that was aerated without worms.
- Protein conversion is mainly due to *T. tubifex* and partly in synergy with intestinal bacteria.

5.5 References

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5.6 Appendix

5.6.1 Azo casein conversion and antibiotic treatment

5.6.1.1 Material and methods

In order to make a distinction between the protease activity of *T. tubifex* and the intestinal bacteria, the worms were treated with antibiotics to suppress bacterial growth and subsequently incubated with azocasein. The experimental setup consisted of actively aerated bottles, with a working volume of 200 mL containing 9 g worms. The intestines of the worms were purged in running tap water for 48h prior to the experiment. Compressed air was filtered (0.22 µm Millex-GP) before being sparged into the bottles.

Antibiotic treatment consisted of daily dosing the antibiotics (AB) Streptomycin and/or Tetracycline, both obtained from Sigma-Aldrich. From a stock solution of 6 g/L antibiotic, a 1 mL dose was given daily for 3 constitutive days. After antibiotic treatment, the worms were rinsed with sterile tap water. In some cases, AB was present during the experiment. In these cases, 3 mL of the stock AB solution was added. Worms that were not treated with antibiotics were incubated in tap water for the same duration. After the incubation period, the worms were transferred to bottles containing 0.5% (w/w) azocasein solution in autoclaved tap water. Bottles containing only azocasein solution served as a control.

The conversion of azocasein was determined using the method described in the enzymatic activity section. Materials and the non-chlorinated tap water were autoclaved before being used in these experiments.

5.6.1.2 Result, Discussion and conclusions

The graphs in Figure 5-3 indicates that azocasein conversion and thus azo-dye release started around 21 hours.

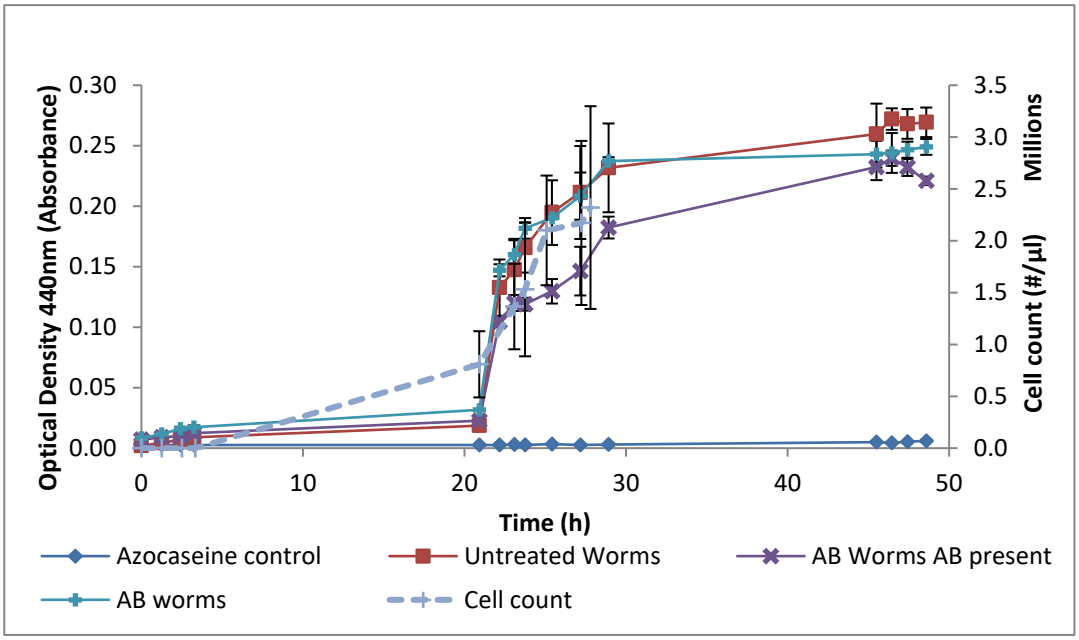

Figure 5-3: *Azocasein conversion, bacterial counts and the influence of antibiotics on casein conversion. Errors are expressed as standard deviations.*

This conversion took place at the same time for all the samples. Interestingly the number of living bacteria increased more or less exponentially, exactly when the highest casein conversion rate was observed. Conversion by the worms themselves is not considered because the worms where thoroughly rinsed before being used in the experiments and it takes approximately 20- 24h before the first ingested azocasein is defecated [1].

Furthermore, antibiotics primarily affect growing bacterial cells and thus the effect of the antibiotic treatment without azocasein substrate present is minimal. The presence of antibiotic combined with azocasein clearly shows that conversion is inhibited. This further supports that the azocasein conversion was mainly due to

bacteria. These proteolytic bacteria were not present in the azocasein control as no conversion took place. For this reason, it highly likely that the bacteria originated from the aquatic worms.

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5.6.2 Enzymatic activity measurements

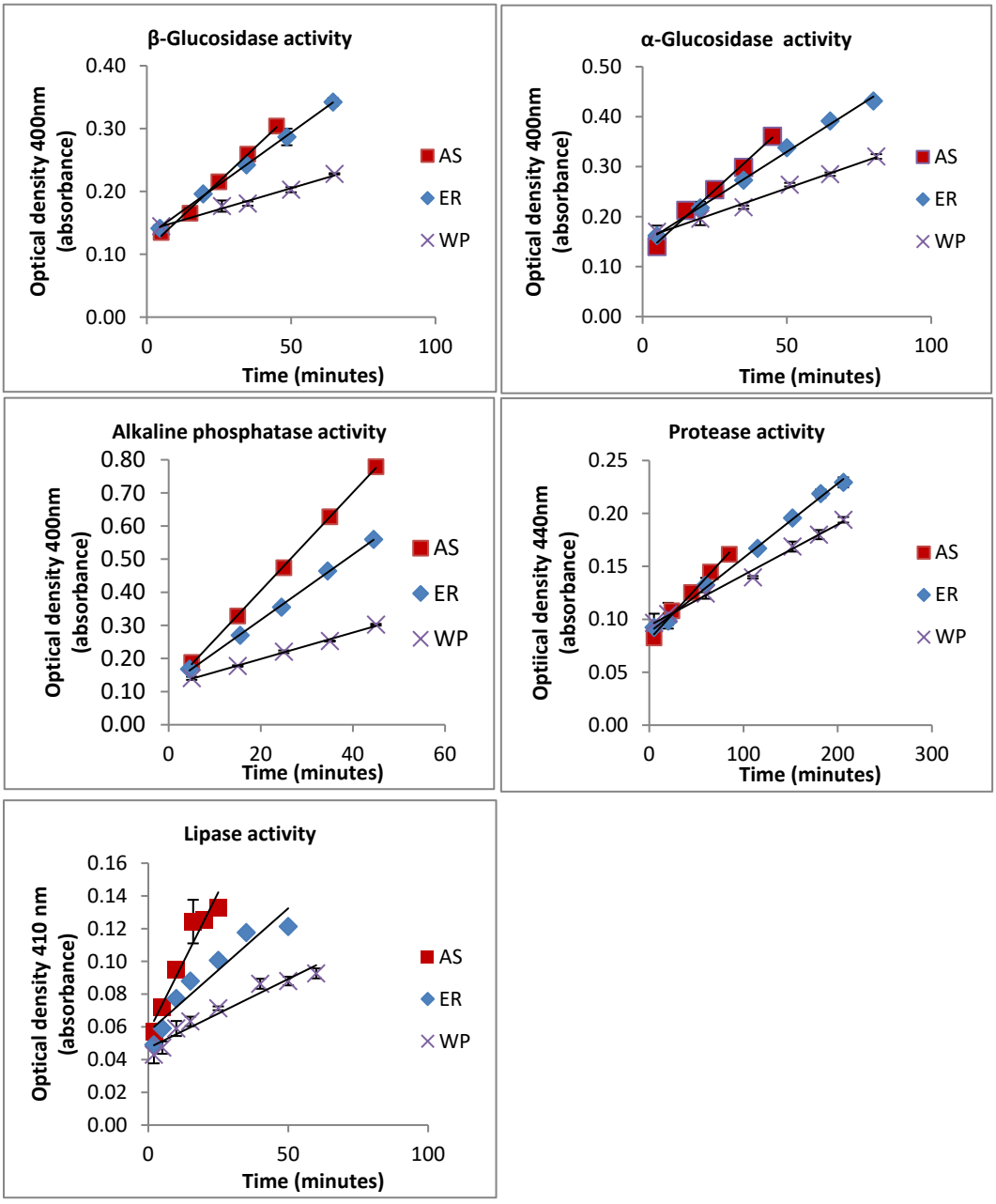

Figure 5-4: *Enzymatic activity in the mixed liquor of Activated sludge (AS), Endogenous respirated (ER) and worm predated (WP) sludge. The sludge in the Lipase assay was diluted 4 times prior to use. Results presented here are the absorbance data. Average values and standard deviations were calculated from triplicates.*

6 ELUCIDATING THE MICROBIAL COMMUNITY OF SLUDGE-DEGRADING WORMS

This chapter is based on:

Steef de Valk & Cuijie Feng, Ahmad F. Khadem, Jules B. van Lier and Merle K. de Kreuk (**2019**) *Elucidating the microbial community associated with the protein preference of sludge-degrading worms.* Environmental Technology, 40:2, 192-201

Abstract

Sludge predation by aquatic worms results in an increased sludge reduction rate, which is mainly due to the removal of a protein fraction from the sludge. As microorganisms play an essential role in sludge hydrolysis, a better understanding of the microbial community involved in the worm predation process will provide more insight into the relations between the aquatic worms, their associated microbiome and the efficient sludge reduction. In this study, the microbial community associated with predation by *T. tubifex* was investigated. The microbial diversity in samples of the worm faeces (WF), predated activated sludge and protein-rich substrates were compared. The results indicated that predation on activated sludge resulted in a microbial change from Actinobacteria (44%) in the sludge, to Proteobacteria (64%) and Bacteriodites (36%) in the WF. Interestingly, the faecal microbial community was more related to the community in (predated) protein-rich substrates than to the community in predated or endogenously respirated activated sludge samples. This similar microbial community could be due to microbial utilization of protein hydrolysis products. Alternatively, conditions in the worm gut could facilitate a protein hydrolysing community which assists in protein hydrolysis. The genera *Burkholderiales*, *Chryseobacterium* and *Flavobacterium* were found to be associated with predation by *T. tubifex*.

6.1 Introduction

The processing of waste activated sludge (WAS), which is a produced as a by-product in waste water treatment plants (WWTP), is mandatory in the European Union [1]. The processing of the waste sludge can amount to 50% of the operational costs of a WWTP [2,3]. Due to the increasing number of WWTPs and thus increasing production of WAS [4] and the associated disposal costs, sludge reduction technologies have been researched extensively. One of these proposed methods is sludge reduction through predation by aquatic oligochaete worms.

Aquatic worms such as *Tubifex tubifex* [5], *Lumbriculus variegatus* [6,7] and *Aulophorus furcatus* [8] have shown to be highly efficient in reducing sludge solids. Between 20% and 50% of the volatile solids (VS) can be removed through worm predation [5,7–9] in a matter of days. Similar reduction values can be found for aerobic and anaerobic digestion, however in a time frame of 30 days [5].

The solids removal during worm predation is mainly due to the reduction of the protein fraction in the sludge [5,10] and is accompanied by the release of degradation products, such as phosphate and inorganic nitrogen, but also soluble chemical oxygen demand (COD), which partly consists of polysaccharides and a limited fraction of proteins [5,6,11–13]. The protein reduction can be attributed to the synergistic activity of the oligochaeta and their intestinal bacterial community [14].

Besides the aforementioned synergistic activity, several authors have suggested that *T. tubifex* and other aquatic worms selectively consume bacteria as a food source [15–17]. In this perspective, the removal of proteins from the extracellular polymeric substances (EPS) matrix, during sludge predation could be due to the consumption of bacteria residing in the EPS. The removal of bacteria from ingested particles, which can be referred to as 'microbial stripping' [18], results in changes in microbial community of the natural sediments the worms inhabit [19]; or in case of sludge reduction, changes in the microbial community of the sludge reduction system the worms inhabit [20].

Ample evidence for changes in the microbial community of the worm gut and habitat was also found for terrestrial oligochaete [21–24]. These microbial changes are probably not only due to the worms' removal or consumption of bacteria and the excretion of degradation products, but also due to the type of

substrate the worms consume [25]. In turn, these environmental changes could result in optimised growth conditions for specific bacterial species associated with the worms.

The importance of intestinal bacteria on the hydrolysis of organic matter is well described for other organisms such as cows, humans and termites [26–28]. These studies (amongst others) reveal that the key concept in the interactions between host and intestinal bacteria is mutualism. This interaction is marked by mutual support either by providing hydrolysed substrates for the host organism or a steady supply of substrate and favourable growth conditions for the intestinal organisms.

Additionally, the types of ingested substrate and the chemical conditions (e.g. pH, redox potential, etc.) within the intestines of oligochaete earthworms can influence the structure of the intestinal microbiome [23] or even increase the scope of hydrolysable substrates such as plastic degradation by meal worms [29]. Although limited evidence is available for aquatic worms, it is highly likely that the interaction between the aquatic worms and bacteria is similar to terrestrial worms as they share a large similarity in biology.

In this perspective, it is important to gain a better understanding of the microbes associated with the aquatic worms as they play an important role in the hydrolysis of organic matter [14]. A better understanding of the intestinal microbial population could provide more insight in to the effective and rapid sludge reduction due to predation by aquatic worms. Thus far, there is still a scarcity of information regarding the microbiology of aquatic worms and the relation between the type of substrate and its influence on the worm-associated microbial community.

In this study, the molecular methodology Illumina Miseq sequencing was applied on the predation process of the aquatic worm *T. tubifex* to determine the diversity within the intestinal microbiota and to investigate the influence of different protein-rich substrates on the microbial community structure.

6.2 Materials and Methods

6.2.1 Worms

T. tubifex worms were bought from a wholesale supplier (Aquadip b.v. the Netherlands). Details regarding the identification and handling of the worms can be found elsewhere [5]. The general composition of *T. tubifex* consists as a % of dry matter mostly of protein (60%), lipids (11 – 33%) and carbohydrate (16%)[30,31]. Worms that have been preconditioned to activated sludge were designated as 'sludge-worms'. Worm that arrived from the wholesale supplier were designated as 'fresh worms'.

6.2.2 Sludge characteristics

Fresh activated sludge was obtained from the WWTP Harnaschpolder (Den Hoorn, the Netherlands). This WWTP treats municipal wastewater of 1.3 million people equivalents and is comprised of a biological nutrient removal plant. Sludge solids consisted, as a percentage of dry matter mostly protein (50%) followed by carbohydrates (20%) and lipids (3%) (Personal communication, H. Guo).

6.2.3 Reactor design

The worm incubation reactor comprised of two identical compartments, both containing 18L of WAS and operated under the same conditions. One compartment contained about 700 g of worms for generating the worm predated sludge (WPS), and the other served as a control for endogenous respiration (ER), producing ER sludge (ERS). The duration of the batch incubations was 4 days. The temperature was 20 ± 1^{o}C and dissolved oxygen was above 5 mg/L. Reactor performance data can be found elsewhere [5].

6.2.4 DNA sample preparation and collection

Sludge samples were obtained from freshly obtained WAS and from the end of the 4-day batch incubation period. In order to collect worm faeces (WF), worms were fed fresh activated sludge for 2 days. After the incubation period the worms were extensively rinsed with tap water. Only worms without sludge attached to their skin were selected individually by pipetting and were transferred into a

plastic container with 100 mL 0.45 µm filtered tap water. The container was passively aerated at room temperature (18 – 20^{o}C). After 48h the worms were removed and the broth containing the faecal matter was concentrated using an Eppendorf mini centrifuge (14,000 rpm, 5 min, RT). Samples were stored at -24^{o}C. The sludge-worms were washed externally and stored at -24^{o}C.

Tetra Min fish food flakes were used as a protein-rich substrate. The composition as adapted from [32] consists as percentage of dry matter of 50% of protein, 11% of lipids and 24% of carbohydrates. Fish food samples were acquired by dissolving 5g of crushed Tetra Min fish food flakes in 0.5L of aerated tap water at room temperature and incubating for a period of 20 days. Subsequently, samples of this broth were collected and frozen at -24^{o}C.

Part of the fish food broth was fed to 10 g freshly acquired worms that did not have prior contact with waste water nor sludge. To this end, the worms were incubated in bottles with a working volume of 0.2 L in tap water. Bottles were actively aerated. Bottles were fed every 2 – 4 days with settled solids from the fish food broth bottle. After 15 days of incubation with worms, the samples of the predated fish food broth were frozen at -24^{o}C. The worms were washed externally and stored at -24^{o}C.

Azocasein was also used as a protein-rich substrate. The azocasein samples were obtained in the following manner: 10 g of worms were incubated in tap water with or without a mixture of the antibiotics (AB), namely, tetracycline (3 g/L) and streptomycin (3 g/L), for 2 consecutive days in an aerated 0.2 L (working volume) bottle. After 2 days the worms were transferred to a bottle containing 0.2 L; 0.11 g/L azocasein and incubated for 3-days. After the incubation period, samples of the broth were stored at -24^{o}C. The worms were washed externally and stored at -24^{o}C. Table 6.1 summarises the samples analysed for microbial community composition.

Table 6.1*: Summary of the samples for microbial community analysis. Incubations were all carried out in aerated conditions.*

Source	**Description**	**Name**	**Abbreviation**
Waste activated sludge	Fresh waste-activated sludge	Waste-activated sludge	WAS
	WAS aerated for four days	Endogenous respirated sludge (control)	ERS
	WAS fed to worms in batch for four days	Worm-predated sludge	WPS
	WAS fed to worms, faeces collected separately.	Worm faeces	WF
Fish food	Fish food incubated for 20 days	Fish food Broth (control)	FB
	Fish food broth fed to fresh worms	Fish food fresh worms	FF
	Fish food broth fed to sludge-worms	Fish food sludge-worms	FS
Azocasein	Azocasein solution fed to sludge-worms with AB present	Azocasein with AB	Azo-AB
	Azocasein solution fed to sludge-worms	Azocasein	Azo

6.2.5 Total DNA extraction and Illumina Miseq sequencing

DNA extraction was performed using the MoBio Ultra Clean Microbial DNA isolation kit (MoBio Laboratories, Inc., CA, U.S.A.). DNA isolation was confirmed by agarose gel electrophoresis. The amplification and sequencing of the bacterial 16S rRNA gene were performed by Research and Testing Laboratory (Lubbock, Texas, USA) with the following primers: U28F (5'-GAG TTT GAT CNT GGC TCA G -3') and U388R (5'- TGCTGCCTCCCGTAGGAGT-3') [33] used with a high coverage over 90% for each domain. All Illumina Miseq sequencing was performed at the Research and Testing Laboratory (Lubbock, TX, U.S.A.). In this study, the archaeal community was not investigated due to low PCR amplification which implied a low archaeal presence in the samples. Unfortunately, not enough microbial DNA could be isolated from the worm biomass. Therefore, a comparison with the actual intestinal bacteria was not made.

6.2.6 Data analysis

After completing Illumina Miseq sequencing, all failed sequence reads, low quality sequence ends and chimaeras were removed using a custom analysis pipeline

based on USEARCH [34]. The downstream analysis was performed by combining different programmes from the Quantitative insights into microbial ecology (QIIME) pipeline, version 1.6.0 [35].

The 16S rRNA gene sequences were classified into operational taxonomic units (OTUs) by a 0.03 difference (97% similarity) and were assigned to a taxonomy by using the Ribosomal database project (RDP) as described by Wang et al. [36].The OTU numbers were counted for each sample as the species richness.

Additionally, the rarefaction curves, the diversity indices including the richness estimators Chao1 and Shannon (H'), phylogenetic diversity index (Faith's PD) and principal component analysis (PCoA) were calculated using QIIME v1.9.0 (http://www.qiime.org) [37]. PCoA was plotted using weighted and unweighted UniFrac metrics. The confidence cut-off was set as 0.5.

6.3 Results and Discussion

6.3.1 Sludge predation characteristics

The results of aerobic (worm) treatment of WAS are summarised in Table 6.2.

Table 6.2: *Summary of the characteristics of waste-activated sludge (WAS), endogenous respiration sludge (ERS) and worm-predated sludge (WPS). Protein and carbohydrate measurements were performed with BSA and glucose-D as standards.*

Parameter	**WAS**	**ERS**	**WPS**	**Study**
Solids concentration (g VS/L)	2.8 ± 0.05	2.4 ± 0.03	1.7 ± 0.04	This study
N-NO_3^- (mg N/L)	6.7 ± 0.14	13.9 ± 1.9	34.3 ± 0.14	
P-PO_4^{3-} (mg P/L)	0.45 ± 0.07	4.05 ± 0.1	9.1 ± 0.1	
VS reduction (%)	-	9% ± 5	47% ± 15	Previous study [5]
SVI (mL/g VS)	115 ± 17	84 ± 14	51 ± 13	
EPS protein-like content (mg/g VS)	17.6 ± 2.4	17.6 ± 2.3	6.7 ± 1.6	
EPS carbohydrate-like content (mg/ g VS)	17.0 ± 3.0	17.8 ± 2.9	12.9 ± 2.5	
Soluble carbohydrates-like substances (mg /L)	4.8 ± 1.4	11.4 ± 6.3	19.7 ± 4.1	
Soluble protein-like substances (mg/L)	24.0 ± 8.6	22.9 ± 3.3	24.9 ± 0.8	
Soluble Fe^{3+} (mg/L)	0.02	0.03 – 0.05	0.11 – 0.15	

Aerobic treatment of WAS, during four days resulted in a VS reduction of 39% ± 2 for worm predation and 14% ± 2 for ER. Additionally, inorganic nitrogen and phosphorous were released, while the pH remained stable at 7.3 ± 0.2. These results are in line with several other studies [7,8,38]. In contrast to ER, worm predation was accompanied by a relevant reduction in protein-like fractions in the EPS and a lower reduction of carbohydrate-like EPS.

6.3.2 Overall microbial phylogenetic diversity

To investigate the changes in the microbial community in response to worm predation and the feeding of different substrates, 16S rRNA gene-based Illumina Miseq sequencing analysis was performed (Table 6.3).

Table 6.3: *The distribution of the identified OTUs and α-diversity indices across the samples.*

Samples	**OTUs**	**No. of reads**	**Faith's PD**	**Chao1**	**Shannon**
WAS	4111	15614	159	14313	8.62
ERS	4084	24212	161	16093	8.72
WPS	3410	13021	136	12795	7.89
WF	1656	15107	46	6019	5.81
FB	1123	16231	44	5954	2.40
FF	2571	27393	89	10189	6.56
FS	1995	13958	74	7423	6.73
Azo	1059	29363	44	11997	1.73
Azo-AB	1015	39144	20	9046	1.63

In total, 194,043 high-quality reads were obtained for the nine samples. The RDP Classifier was used to assign OTUs to the different sequence tags. A total of 21,024 OTUs were identified based on the 97% identity cut-off. The distribution of the identified OTUs across the samples and the calculated α-diversity indices shows that the (treated) activated sludge samples (WAS, ERS and WPS) were characterised by a high microbial diversity compared to the other samples. Additionally, the sludge samples were comparable to the microbial diversity of activated sludge systems of other WWTPs [39]. Furthermore, the predation of WAS resulted in a lower microbial diversity in WPS while the ERS remained similar

to the WAS. The decrease in diversity after predation is more profound when the WF are compared to the sludge samples. In contrast with sludge predation, predation of fish food resulted in an increase in diversity compared to the un-predated substrate and contained a more diverse microbial community compared to the WF.

The relation between OTUs and the number of sequences (Figure 6-1) shows that the non-sludge samples have a lower microbial diversity compared with the sludge samples. This can be ascribed to the differences in substrate composition.

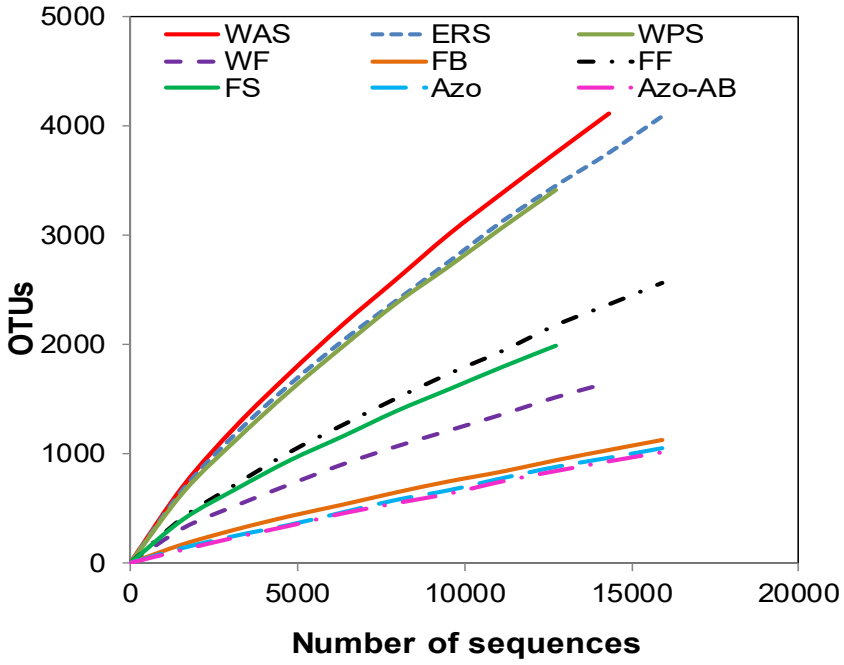

Figure 6-1: *Rarefaction curves of the OTU obtained from 16S rRNA gene analysis of the microbial community in (aerobicly treated) activated sludge, worm faeces, fish food and azocasein samples.*

In order to further assess the relationships between the different samples, the principal coordinate analysis (PCoA) was performed (Figure 6-2).

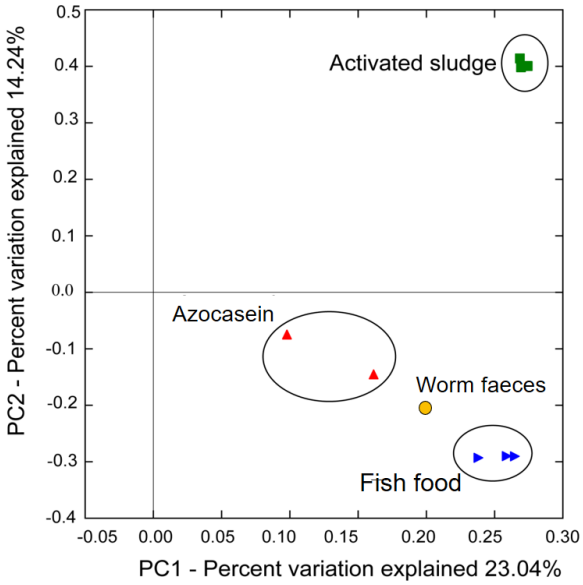

Figure 6-2: *Principal Coordinate Analysis plots (PCoA) of sample fractions determined using the unweighted Unifrac distance metric.*

The PCoA shows that the (treated) sludge samples (WAS, ERS and WPS) are distinctly different from the WF and the protein-rich substrates fish food and azocasein. The fish food samples (FS, FF and FB), azocasein (Azo) and WF formed a separate lineage due to the similarity in their microbial communities, except for the antibiotic containing Azo-AB sample. This separated lineage is subdivided into three lineages that separates the fish food and azocasein samples and the WF.

Interestingly, the microbial diversity in the WF is more related to the diversity of the two protein substrates than to the (treated) sludge samples. To be more specific, the worm faeces shared a similar microbial community with Azo. This similarity in the bacterial community could be related to the metabolism of the worms, which primarily converts the protein fraction of the sludge, which is also shown by the lower protein content in the EPS (Table 6.2). Additionally, De Valk et al. [14] showed, by suppressing bacterial activity in *T. tubifex* using antibiotics, that bacteria play an important role in the hydrolysis of protein.

6.3.3 Phyla level similarities between T. tubifex predated substrates

In order to explore the taxonomic diversity of the microbial communities in the different samples, the RDP identifier was used to assign sequence tags to the different taxonomic levels, ranging from phylum to genus (Figure 6-3).

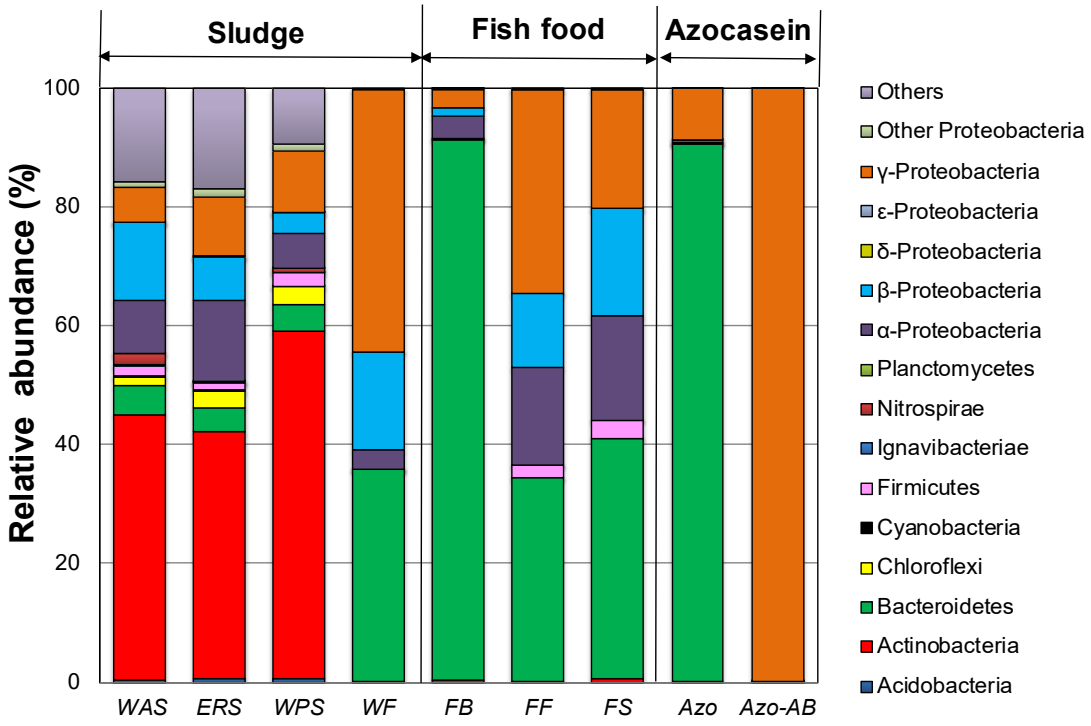

Figure 6-3: *The microbial community of the nine samples on the phyla level.*

A total of 11 abundant phyla were detected across the different samples. In accordance with the α-diversity indices (Table 6.3), the microbial composition of the (treated) sludge samples was similar and larger in diversity compared to the diversity in the WF, fish food and azocasein samples. The diversity between the fish food samples was similar. Predation of the fish food samples resulted in the appearance of Firmicutes (2 – 3%) and a change in abundancy from Bacteroidetes (90%) to Proteobacteria (64%).

The passage of sludge through the gut of *T. tubifex* resulted in a reduction from 11 to 4 abundant phyla in the WF: the dominant phylum of Actinobacteria (44%) in

WAS was replaced by Bacteroidetes (36%) and Proteobacteria (64%), consisting of γ-Proteobacteria (44%), β-Proteobacteria (15%) and α- Proteobacteria (5%) in the WF. This change in diversity can be attributed to an environmental difference between the worm gut and the sludge, which thus resulted in a different microbial composition. However, this diversity change might possibly also result from bacterial degradation by the worms.

Similar to the WF, Bacteroidetes and (α-, β-, and γ-) Proteobacteria were also present in all the protein samples. This suggests that protein degradation during worm gut passage leads to a similar microbial composition as the resulting microbial composition after worm gut passage of WAS, a protein-rich substrate.

6.3.4 Genus-level differences between T. tubifex predated substrates

In order to gain more insight into the microbial composition after gut passage, heat maps were constructed that compares the worm faeces with the sludge samples (Figure 6-4) and with the protein samples (Figure 6-5).

		WAS	ERS	WPS	WF
Actinobacteria	*Tetrasphaera*	1.08	3.64	1.24	0.00
	Candidatus Microthrix	40.14	32.42	50.67	0.03
Bacteroidetes	*Cytophaga*	0.06	0.06	0.03	0.01
	Chryseobacterium	0.05	0.00	0.00	30.36
	Flavobacterium	0.12	0.06	0.08	2.20
Chloroflexi	*Caldilinea*	1.09	2.10	2.68	0.00
Firmicutes	*Bacilli*	0.67	0.29	0.32	0.00
	Clostridium	0.91	0.84	1.69	0.04
Nitrospirae	*Nitrospira*	1.99	0.14	0.81	0.00
α-proteobacteria	*Brevundimonas*	0.00	0.01	0.01	2.94
	Fulvimarina	0.31	0.67	0.12	0.00
	Bradyrhizobium	0.56	1.08	0.71	0.00
	Pedomicrobium	0.95	1.91	0.99	0.00
	Rhodobacter	2.25	1.91	0.76	0.00
	Sphingopyxis	0.11	0.13	0.09	0.01
	Comamonadaceae	1.09	0.22	0.20	1.56
β-Proteobacteria	*Massilia*	0.00	0.00	0.00	14.56
	Burkholderiales	3.32	2.25	1.12	0.33
	Dechloromonas	5.32	1.72	0.24	0.07
	Zoogloea	0.51	0.05	0.00	0.00
	Rhodocyclales	0.57	0.39	0.16	0.00
γ-Proteobacteria	*Aeromonas*	0.01	0.00	0.00	2.65
	Shewanella	0.00	0.00	0.00	0.63
	Chromatiales	1.87	2.70	4.67	0.00
	Acinetobacter	0.02	0.03	0.03	38.39
	Pseudomonas	0.19	0.22	0.12	1.94
	Candidatus Competibacter	1.19	1.88	1.95	0.00
	Dokdonella	0.82	2.21	0.95	0.00

Figure 6-4: *Heatmap displaying the microbial diversity on phyla and genus levels of the aerobically (worm) treated sludges. A comparison between 28 selected genera. The selection was based on a relative abundance larger than 1% at the genus level.*

		WF	FF	FB	FS	Azo	Azo-AB
Bacteroidetes	*Chryseobacterium*	30.4	0.1	0.0	0.0	90.6	0.0
	Flavobacterium	2.2	4.7	90.1	10.9	0.0	0.0
	Flavobacteriia other	3.0	28.1	0.7	25.6	0.0	0.0
Firmicutes	*Clostridium*	0.0	2.1	0.1	2.9	0.0	0.0
α-proteobacteria	*Brevundimonas*	2.9	0.3	0.1	0.6	0.3	0.0
	Bosea	0.0	3.1	0.4	1.6	0.0	0.0
	Rhizobium	0.0	3.1	1.0	5.2	0.0	0.0
	Azospirillum	0.0	1.4	0.7	2.7	0.0	0.0
β-Proteobacteria	*Comamonadaceae*	1.6	10.9	1.2	16.8	0.0	0.0
	Massilia	14.6	0.0	0.0	0.0	0.0	0.0
	Burkholderiales	0.3	0.4	0.0	0.7	0.1	0.0
γ-Proteobacteria	*Aeromonas*	2.7	0.1	0.1	0.0	0.0	0.0
	Citrobacter	0.4	4.1	1.2	3.0	0.0	0.0
	Enterobacter	0.0	1.3	0.3	1.1	0.0	0.0
	Acinetobacter	38.4	0.0	0.0	0.3	0.1	0.0
	Pseudomonas	1.9	0.3	0.1	0.4	8.4	0.0
	Lysobacter	0.0	25.5	0.3	9.4	0.0	0.0
	Pseudoxanthomonas	0.0	2.3	0.7	5.3	0.0	0.0
	Stenotrophomonas	0.0	0.0	0.0	0.2	0.0	100.0

***Figure 6-5**: Heatmap comparing the microbial diversity, on phyla and genus levels, of the protein rich substrates, azocasein and fish food against the sludge-based worm faeces. Nineteen genera were selected for comparison based on the presence in the faecal samples and the abundance in the protein-rich samples.*

Notable changes in abundancy were observed for the Actinobacteria – *Candidatus Microthrix* that declined in abundancy from 40% in WAS to 0% in the worm faeces. *Candidatus Microthrix* is known for their filamentous colony formation and relation to sludge bulking [40,41]. The absence of this genus could play a role in the improved sludge settling characteristics of the worm faeces [6]. However, this is in contrast with the higher abundance in the WPS that contradicts with the improved settleability associated with the WPS in terms of SVI (Table 6.2).

Additionally, as previously mentioned, the four phyla that increased in abundance after gut passage of WAS (Figure 6-4) were the Bacteroidetes – *Chryseobacterium* (30%), α- Proteobacteria – *Brevundimonas (3%)*, β-Proteobacteria – *Massilia (15%)* and γ-Proteobacteria – *Acinetobacter (38%)*. Although the phyla of Proteobacteria and Bacteroidetes in the worm faeces are similar to those of the (predated) protein substrates (Figure 6-3), considerable differences at genus level are found (Figure 6-5).

The main differences for the Bacteriodetes phylum are within *Chryseobacterium* (30%) in the WF that is 'replaced' by *Flavobacterium* and *(other) Flavobacteriia* in the fish food samples. More specific, within the fish food samples, predation of the fish food broth (FB) resulted in a population shift from *Flavobacterium* (90%) to the 'other' *Flavobacteriia (*25 – 28%) in the predated fish food samples (FS and FF).

Within the Proteobacteria phylum, genus-level differences were mainly with the γ-Proteobacteria phylum. The dominant *Acinetobacter* genus in the worm faeces (40%) was 'replaced' by *Lysobacter* in the predated fish food samples (FF (25%) and FS (9%)). The β-Proteobacteria *Massilia* was present in the WF (15%) while low in abundance (< 1%) in the (predated) protein samples. Additionally, *Comamonadaceae*, showed an abundancy increase from ≤ 1.6% in WF and FB to 11% and 17% in FF and FS respectively. The changes for the α-Proteobacteria where not as pronounced as within the other phyla. A diverse distribution of *Bosea*, *Brevundimonas*, *Rhizobium* and *Azospirillum* was found within the (predated) protein-rich samples and worm faeces.

The relation between the substrate and specific microbial environments within the worm gut has been investigated in earth worms. Thakuria et al. [25] found that differences in substrate composition can result in microbial shifts of the gut wall-associated bacteria. However, the strongest determinant in the selection process of the gut wall-associated bacteria is the ecological group (anecic or endogeic) followed by the habitat the host occupies and lastly the species of earthworm [25]. Only the habitat constraint, which is related to types of substrate present, is relevant for the worms used in this research.

Therefore, the differences in the microbial presence between the sludge-based WF and protein-rich substrates are obviously due to the different substrate compositions. Furthermore, the differences between predated and un-predated samples are most likely due to the specific growth environment within the worm gut. Additionally, no distinct differences were found between sludge and 'fresh' *Tubifex* worms that adapted to different habitats.

6.3.5 **Microorganisms associated with T. tubifex.**

Based on the presence of the different genera (Figure 6-4 and Figure 6-5) in the WF and (predated) protein-rich substrates, several genera that seemed to be associated with *T. tubifex*, or increase in abundance after predation of certain substrates, are listed in Table 6.4.

***Table 6.4**: Genera associated with T. tubifex. Based on the presence or abundancy differences between worm faeces and (predated) protein rich substrates.*

Genus	**Sample presence**	**Indications**	**References**
Acinetobacter	Sludge-worm predated samples.	*Acinetobacter* originated from sludge and remained associated with the sludge-worms.	This work
Burkholderiales	Sludge-and fresh-worm predated samples.	The presence in FF could indicate that a close association with *T. tubifex* exists that is not related to contact with sludge.	This work
Chryseobacterium	Worm faeces and sludge-worm predated azocasein.	Strong indications that a favourable niche was established.	This work
Flavobacterium (others)	Worm faeces and predated fish food.	Strong indications that a favourable niche was established.	In natural sediments [15,16,42], Submerged membrane reactor combined with worm predation [20]
Lysobacter	(Predated) fish food broth.	Indication that *Lysobacter* proliferated when fish food broth was predated.	This work
Comamonadaceae	Sludge, Worm faeces and (predated) fish food broth.	Fish food predation resulted in an increase in abundancy.	This work
Massilia	Worm faeces (14%) and predated fish food (≤ 0.1%).	Proliferation only after gut passage of sludge	This work
Aeromonas*, *Pseudomonas	Present in all samples	None	In natural sediments [15,16,42]
Clostridium and Pseudomonas	Present in all samples except *Clostridium* in Azo	None	Submerged membrane reactor combined with worm predation [20]

6.4 General discussion

Results of the conducted research increased insights into the microbial communities associated with sludge-reducing worms and led to a better understanding of the degradation of proteinaceous substrates in aquatic worms.

Present results confirm that aquatic worms prefer the protein fraction of the consumed substrates or the proteins of substrate-associated bacteria. Irrespective of the protein source, the released products from protein hydrolysis, such as amino acids, can be directly taken up by aquatic worms, such as *T. tubifex* and *L. hoffmeisteri* [42]. In this respect, the gut of *T. tubifex* can be considered as a stimulating environment for a protein degrading bacterial community. Alternatively, the worm gut excretes enzymes to degrade protein-sources and that these degradation products stimulate the proliferation of certain genera (Table 6.3).

Either way, the consequence of predation is a change in the microbial community towards a biome related to the degradation of proteins, which could contain the previously mentioned *Burkholderiales*, *Chryseobacterium* and *Flavobacterium* genera. These worm associated genera, that live in a synergistic relationship [14], could assist the worms with additional proteolytic functionality or play an important role in protein degradation within the worm gut. For these reasons, these associated classes deserve further attention in future research.

Additionally, due to the possibility of lytic activity by the worms, the appearance of *Lysobacter* is interesting as this genus is known for its anti-microbial effects [43]. This anti-microbial function could be of high importance for the worms in the degradation of bacteria. Additional research into the lytic activity in the worm predation system is considered of importance for developing enzymatically assisted hydrolysis of sludge.

6.5 Conclusions

Microbial community analysis revealed that the worm faeces produced through predation of WAS share more similarities in microbial structure with predated protein rich substrates as compared to the sludge itself. Additionally, these similarities coincide with the protein preference of *T. tubifex*. These microbial changes could therefore be related to gut-specific processes such as the release of

protein hydrolysing enzymes of which the degradation products support a protein-degrading community. Alternatively, the worm gut could provide a favourable environment for protein hydrolysers. In general, other microbial changes could be induced by the activity of the tubifex worms by microbial grazing, optimal conditions in the worm intestines and the excretion of degradation products. Some genera, within this shifted microbiome, such as *Burkholderiales*, *Chryseobacterium* and *Flavobacterium* are associated with predation by *T. tubifex* and are likely to be related to protein degradation.

To summarise:

- The genera *Burkholderiales, Chryseobacterium, Flavobacterium* and *Massilia* seem to be associated with predation by *T. tubifex*.
- The microbial change towards a microbiome related to protein degradation could be due to
    - The facilitation of a protein-degrading microbial community by the worm gut.
    - The use of protein-related hydrolysis products by bacteria due to worm-based protease enzymes released in the worm gut.
- In general, other microbial changes could be induced by the activity of the tubifex worms by microbial grazing, optimal conditions in the worm intestines and the excretion of degradation products.

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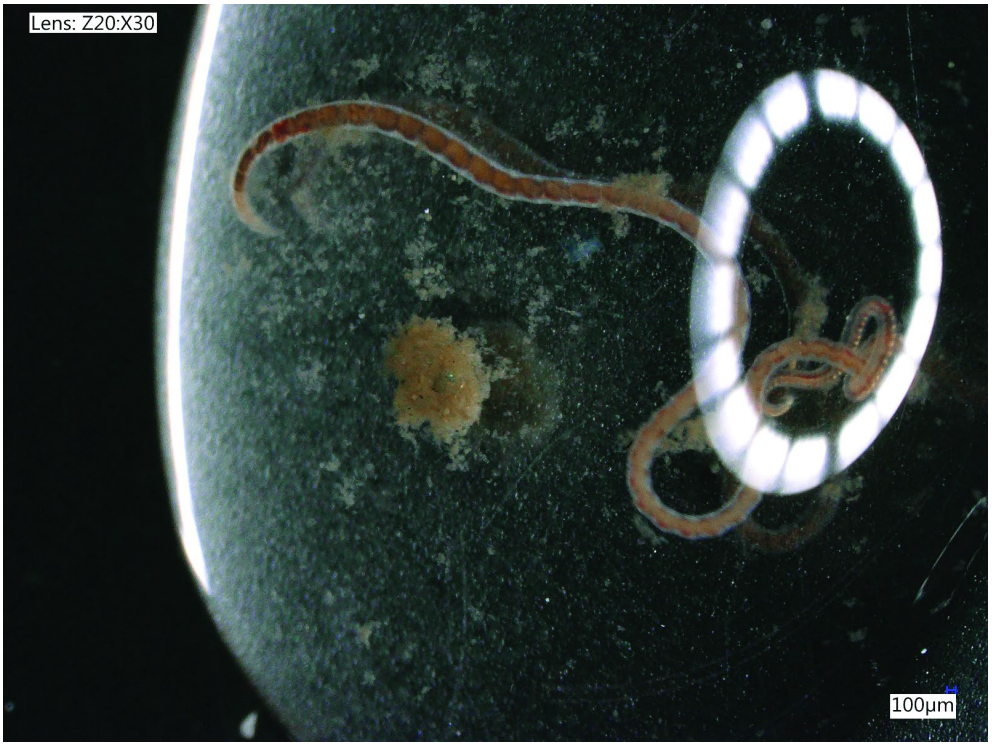
7 OVERALL CONCLUSION, RECOMMENDATIONS AND OUTLOOK



7.1 General summary

The work presented in this thesis focused on ways to improve the extent and rate of WAS hydrolysis by using the sludge degrading aquatic worm *T. tubifex* as a 'model biochemical reactor' and as the starting point for the research. Published work had revealed that predation by these aquatic worms resulted in high sludge degradation efficiencies. However, so far, the bioconversion process inside the aquatic worm were approached as a black box system. The research presented in this thesis was focused on unravelling the worm-based enzymatic processes for improving WAS hydrolysis.

In order to relate physical and biochemical changes in WAS to worm predation, a comparative analysis between the effects of endogenous WAS respiration under extended aeration conditions and worm predation of WAS was made (chapter 3). The results showed that the improved WAS conversion is related to the efficient removal of protein-like and to a smaller extent polysaccharide-like substances from the sludge matrix. Additionally, recalcitrant flock biopolymers such as humic and fulvic substances, were not removed but slightly liberated.

The aforementioned polysaccharide-like substances possibly consisted of the alginate like exopolymer (ALE) fraction that was partly consumed during worm treatment of WAS. The removal of these fractions was related to the disintegration of sludge flocs and a resulting release of floc bound fulvic and humic substances as well as the cations Mg^{2+}, Al^{3+} and Fe^{3+} from the sludge matrix. These released compounds have a known structural function in the EPS of sludge flocs and are, therefore, most likely to be tightly associated with the removed protein-like fraction. From these results it indirectly becomes clear that protein-like substances play an important role in floc stability.

Because the removal of the protein-like fraction had such pronounced effects on WAS reduction, the effects of worm predation on the biodegradability of WAS, and indirectly, enzymatic sludge treatment in the worm intestines was further explored. By applying sequential 40 days aerobic and 40 days anaerobic (or anoxic) conditions, the full biodegradability potential of the initial feed WAS was determined and compared to the biodegradability extent of anaerobic digestion and worm predation treatment combinations (chapter 4). The results showed that the first treatment step always removed the majority of the organics and that the

feed WAS could not be further degraded than about 50% in VS or about 60% in COD. Furthermore, worm predation and anaerobic treatment combinations reached the full biodegradability potential of the initial feed sludge in almost half the process time than the reference aerobic and anaerobic sequences. It was further found that worm death occurred when 40 days aerobically treated sludge was fed to worms. Worm death is possibly related to the prolonged substrate limited conditions to which the sludge was exposed to. Very likely, these conditions promote bacterial decay. As mentioned above, bacteria and EPS are part of the diet of sludge degrading worms.

In general, hydrolytic enzymes are responsible for the efficient conversion of organic polymeric molecules. Investigation of several hydrolytic enzymes in the worm predation process revealed an improved protease activity which corroborated with the removed protein-like fraction (chapter 5). The protease activity was predominately associated with the predated solids fraction. These findings suggested that the activity improvement could be related to attachment of intestinal proteolytic bacteria and/or worm-based enzymes to the sludge matrix. It was further found that *T. tubifex* is up to 73% responsible for protein hydrolysis while the remainder is due to synergy with intestinal bacteria.

Bacteria play an important role in the worm predation process and hydrolysis in general. By exploring the synergistic relation between bacteria and sludge worms, more insight has been gained in the microbial community associated with the predation process (chapter 6). It was found that the microbiome in worm faeces, produced through WAS predation are more related to the community in predated protein rich substrates than to the WAS-community in the feed sludge. This similarity in the microbial community could be due to microbial utilization of protein hydrolysis products produced by the worms. Alternatively, conditions in the worm gut could have facilitated a protein hydrolysing community, which assisted in protein hydrolysis. Additionally, the genera *Burkholderiales*, *Chryseobacterium*, *Flavobacterium* and *Massilia* were found to be associated with the predation process of *T. tubifex* and are likely to be related to protein degradation.

7.2 Recommendations

Although research and apparent interest in worm predation technology as means to reduce WAS had its peak almost 10 years ago (Figure 7-1), the potential of worm predation for WAS reduction remains unaffected.

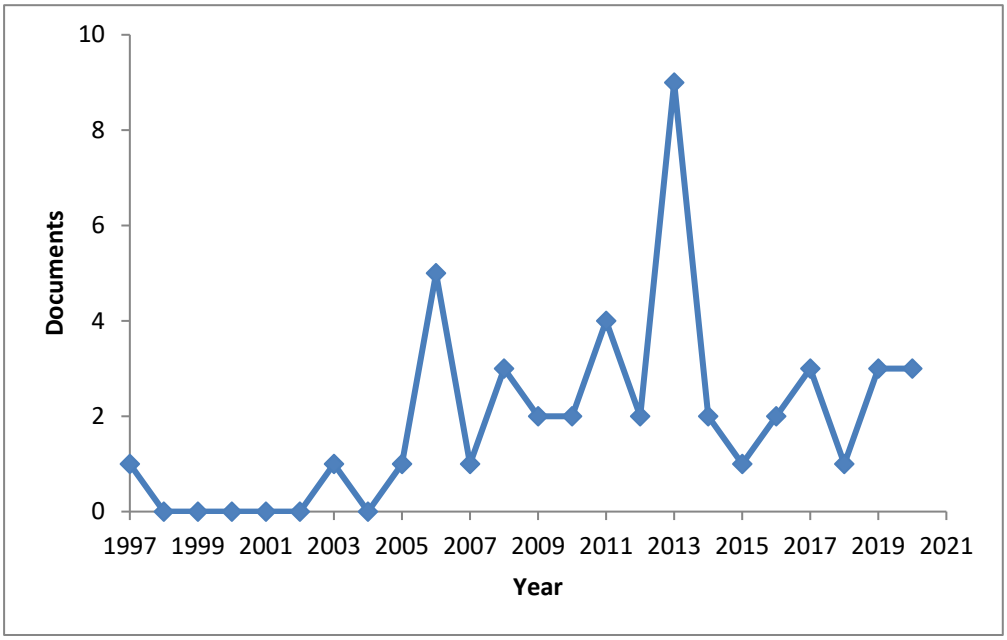


Figure 7-1: *Worm predation related articles per year. Data retrieved from www.scopus.com with search string: worms; sludge; predation.*

After years of research, the author is convinced that worm predation technologies belong to the most time efficient biological treatment methods available for the reduction of WAS. Although this knowledge was already available a decade ago and successful efforts have been made to maintain worm biomass in worm reactors, predation technology is still not used in practice. Possibly, the integration of a worm bioreactor in a conventional wastewater treatment plant is more cumbersome than microbiology-based treatment units. However, despite this diminished interest, the aquatic worms can still teach us a lot about efficient WAS solids degradation.

7.2.1 Solids removal

The biodegradability of WAS solids is heavily dependent on the duration, type of the digestion process and possible pre-treatment [1]. As discussed in this work, worm predation showed high solids removal rates. The high solids removal rate of predation technologies is especially useful in combination with anaerobic treatment of WAS. The biodegradability potential of this combination reached a level of about 50%. Nowadays anaerobic digestion of WAS reaches 30 – 35%, while applying a solids retention time (SRT) of about 25 – 30 days [2–4]. As such, potentially about 20% less sludge solids could be disposed of, when using worm predation in combination with anaerobic digestion technology for secondary sludge (chapter 3). It must be noted that the WP-AD combination is preferred over the AD-WP combination, because additional sludge separation and dilution steps can be avoided due to the high ammonia concentration in anaerobic sludges after AD, which can be toxic for aquatic worms[5].

About 10 – 20% of WAS solids consist of poorly biodegradable substances such as humic matter [1]. If this poorly degradable fraction is added to the assessed solids biodegradability potential of 50% (chapter 4), then, theoretically, a fraction of 30 – 40% of the solids remains unaffected. Apparently, the remaining solids in this 30 – 40% fraction, which include bacteria and their structural EPS [6,7], are more recalcitrant. Very likely, removal of this fraction requires more lengthy biological treatment or additional chemical or physical pre-treatment techniques in order to be further biodegraded. The advantage of first removing the relatively easy biodegradable WAS components is that less solids must be disposed of or have to undergo additional processing in order to further improve the biodegradability extent.

Obviously, the clear benefits of WAS solids removal trough predation followed by AD has to be put into perspective of the current activated sludge process with concomitant sludge stabilisation using AD. The economic feasibility of this process combination can be determined by a cost benefit analysis. In order to do so, several important cost estimations, relevant to the worm process must be considered: i) aeration and heating of the worm predation process, ii) the extra nitrogen and phosphorus load due to nutrient release during worm predation, iii) the effects of less solids and more worm biomass on biogas production and iv) less sludge solids to dispose of and lastly v) the changes in coagulant needs.

Although, a proper cost benefit analysis could further support predation, it is out of the scope of this thesis.

7.2.2 Enzymatically enhanced hydrolysis

The main aim of the EnzyFOR project was to gain more insight into enzymatically enhanced hydrolysis of organic (waste) streams. The first steps into elucidating the worm process showed that for efficient WAS hydrolysis, targeting protein-like and ALE sludge fractions are important. Additionally, it was recently reported that glycosylated proteins were found in EPS from aerobic granular sludge [8]. These findings corroborate the observed removal of ALE and protein-like substances (chapter 3). Insight into the type of protease (e.g., cysteine-, metallo-, serine-proteases etc.) using specific protease inhibitors on cell free enzyme extracts [9–12] could reveal the specific type of protease active in the worm process. Such knowledge would be of interest in order to investigate to what extent aquatic worms can degrade the ALE fraction in aerobic granular sludges, as these granules contain a larger ALE fraction, with possibly different composition, compared to conventional activated sludge flocs [13]. The results could reveal which enzyme class is active in both sludge types. Obviously from a resource recovery perspective it is not recommended to degrade the ALE fraction in granules considering the current investments made to extract and process ALE from aerobic granular sludge as novel biopolymer [14]. However, this is not the case for flocs as the ALE concentration in this sludge mass is relatively low but still significant for attaining the floc stability.

Besides protein-like substances and ALE, also the bacterial fraction in activated sludge is important, which can be as high as 23% of the total sludge mass [15]. There are strong indications that aquatic worms consume the bacteria present in WAS [16–22]. In hindsight, indications of bacterial degradation are presented in chapter 3 using fluorescence excitation emission matrix spectroscopy (FEEMS). The increased aromatic-protein and tryptophan-protein like substances due to predation are also released when a bacterial culture is lysed using lysozyme [23]. This lytic aspect of aquatic worms should be further investigated to gain more insight into effective methods for cell lysis which is one of the limiting steps preceding hydrolysis [24]. To this end, specific bacterial feeding trials with common sludge bacteria could be performed in combination with flow cell cytometry to distinguish between living and dead cells.

The bacteria selection should include both gram-positive and gram-negative sludge bacteria, as Ratsak et al. (2006)[25] discussed that aquatic worms have a possible selectivity for gram-negative bacteria. Possibly, an adaptation of the feeding method devised by Laarhoven et al. (2016)[26] could be used for such a study.

In enzymatically assisted sludge hydrolysis, proteases and lysozymes are already applied in enzyme cocktails, which often also include amylases and cellulases [23,27–29]. Because there is no consensus on what the optimal enzyme mix should consist of, or what type of enzymes should be included, confusing and sometimes conflicting results are obtained [28]. A structured approach towards optimal enzyme mixtures tailored to the substrate is needed. More insight into the dominant components of EPS could narrow the scope of suitable hydrolytic enzymes. Additionally, a statistical approach could minimise the search for optimum enzyme dosing procedures which is highly recommended. Various parameters such as contact times, sludge concentrations, enzyme types and concentrations are important and will influence changes in (soluble) COD, VSS and TSS, biogas formation and sludge processing (appendix Table 7.2). The design of experiments and more specifically, optimal design statistics are therefore recommended [30].

A preliminary economic evaluation of enzyme pre-treatment of WAS, made by the EnzyFOR project partner TAUW compared the operational costs (including depreciation) of enzymatically enhanced AD to conventional AD (Table 7.1). The base case (1) represented a conventional AD process, which was augmented with enzyme pre-treatment and used solids reduction and settleability improvements similar to the results from the worm predation process (chapter 4). Additionally, the effect of varying several important cost parameters on the operational costs where evaluated: (2) A lower degree of solids reduction, (3) a doubling of the amounts of dosed enzymes, (4) no improvement in sludge dewatering or an increase in PE use, and lastly, (5) a one third drop in the sludge disposal costs. (Table 7.1), sludge dewatering and disposal costs, energy and heat balances and in-company information regarding construction and operational costs. As not all information of the EnzyFOR partner TAUW can be shared due to market sensitive information, only the relative changes in operational costs are shown.

Table 7.1: *Decrease in yearly operational costs (in %) for enzymatically assisted AD relative to conventional AD of WAS, for different scenarios. The base case (1) represents a conventional AD process augmented with enzyme pre-treatment and used the solids reduction and settleability improvements of the worm predation process, as reported in this thesis as input. The effects of varying selected cost parameters on the overall operational costs are shown in scenarios 2 to 5. The enzyme costs used in the calculation where (due to confidentiality) set at an expected and realistic price.*

Scenario	**Decrease in yearly operational cost relative to the costs of a conventional AD process.**
1) Base case: Conventional AD process augmented with enzymatic treatment	-23%
2) Lower sludge dry weight reduction 5% instead of 7%	-20%
3) Double enzyme dosage 0.1 -> 0.2 g/kg dry weight	-23%
4) No improved dewatering or additional PE usage	-15%
5) Lower sludge disposal costs 75 -> 50 €/m^3	-25%

The evaluation showed a positive economic feasibility of enzyme assisted hydrolysis of WAS for all assessed cases, even for the worst-case scenarios 3 and 4, in which costs related to coagulant application and level of dewatering are the major costs components. Interestingly, scenario 5 indicates that the sludge disposal costs, which are inherently linked to dewatering and coagulant costs have a major impact on the feasibility of enzymatic pre-treatment. In this respect, removing WAS solids up to the biodegradability potential (chapter 3), prior to AD could potentially further improve the economic balance for enzymatically assisted AD as possibly more recalcitrant solids can be removed.

Furthermore, in addition to an economic evaluation and the development of an optimum enzyme dosing strategy, the successful implementation of enzyme assisted hydrolysis of WAS requires a proper research methodology to efficiently evaluate and compare the different strategies. The required methodology should include: i) references to the biodegradability potential of the sludge before and after enzyme treatment, ii) incorporation of the appropriate control experiments using inactivated enzymes, iii) the COD quantity linked to enzyme addition, and iv) a uniform way of reporting data for proper results evaluation. Fortunately, the indicative results (Table 7.1) show that there is an economic incentive to indeed provide funding for more research into enzymatically assisted hydrolysis of WAS.

7.2.3 Methodology

For determining the efficiency of WAS treatment technologies, it is of paramount importance that a framework is provided to determine how a certain solids reduction or pre-treatment process performs in relation to other treatment schemes. The review of Gonzalez et al. (2018) on sludge pre-treatments [1] clearly shows the need for a general approach to determine treatment efficiencies, as literature values cannot be compared easily. The research into the biodegradability potential and other aspects of worm predation, relied heavily on control experiments and reference points obtained with the initial sludge samples as well as control experiments without worms. By doing so, degradation rates and removal percentages of combined biological processes, such as worm predation and endogenous respiration or sequential processes, could be properly evaluated and compared.

7.3 The future of aquatic worms

In the past two decades several research groups, of mainly Dutch and Chinese origin, were actively researching aquatic sludge worms and predation of WAS (Figure 7-2).

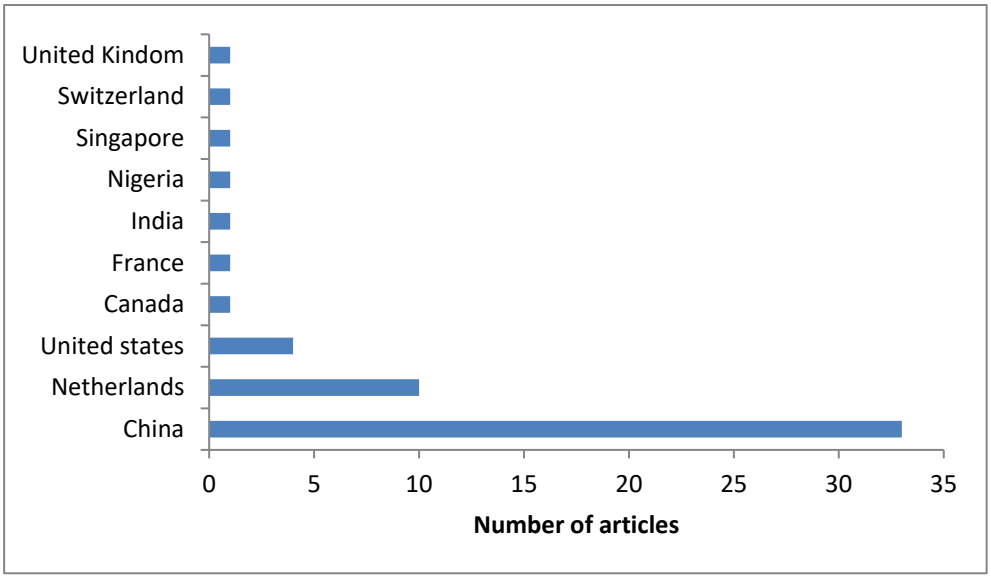


Figure 7-2: *Sludge predation related publications per country. A total of 54 articles have been published since 1997. Data retrieved from www.scopus.com with search string: worms; sludge; predation.*

Despite the numerous publications in the field, the translation of lab-scale worm predation technology to pilot scale was only reported by Tamis et al. in 2011 [31]. At the moment of writing, several research groups in China are still actively researching WAS treatment with aquatic worms. Some research is aimed for membrane fouling mitigation in MBR systems [32] using aquatic worms. Other research is investigating the effects of increased sCOD release during worm predation and the (positive) effect of this release on denitrification [33].

In the research field of aquatic toxicology, the application of aquatic worms is very relevant, using the worms for water quality assessments as biomarkers [34–38]. Recently, worm characteristics such as bioturbation and oxygenation effects of sediments [39] and the positive effects of aquatic worms on denitrification [33] and denitrifying phosphate removal [40] are researched to successfully improve the nutrient removal capacity of constructed wetlands [41,42]. Alternatively, worm technology can be used as a means to improve the valorisation of defined organic waste streams. For example, aquatic worms convert the organic material into worm biomass which is rich in protein and lipids and could therefor serve as feedstock [26,43,44].

Although various research groups have shown the added value of aquatic worms and the research during the past decades solved most of the hurdles that limited implementation, the actual reason for the diminishing interest remains elusive. Possibly, working with higher life forms in engineered treatment systems at sewage treatment plants is regarded as a constraint, as worms are less predictable than bacteria. Moreover, worms are worms and therefore inherently have a ‘yuck’ factor. Such a ‘yuck’ factor is known to hamper implementation of novel ideas such as the use of treated sewage [45] and using insects as a protein source [46].

To be frank, there are plenty of opportunities to show case the benefits of aquatic worms, but as long as worms are found ‘yucky’, implementation of worm-based technology will remain low. Fortunately, the worms are unaware of this factor and will always remain as they are: “The humble creature, who knows nothing of the benefits she confers upon mankind […]” as expressed by Charles Darwin (1881) [47].

7.4 References

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7.5 Appendix

Table 7.2: Literature overview of enzymatic pre-treatment.

Scale	Substrate	Enzyme	Activity U/g	Contact or measurement time (h)	Dose g /gDS or w/w	VSS reduction	CH_4 production (L/g)	Polymer dose kg/ tDS	Dewatering sludge concentration	Notes	Ref.
Batch Lab	WAS	α-amylase	6000	8	0.06 w/w	15% - 33%	-	-	-		[1]
Batch Lab	WAS	α-amylase	6000	4	0.06 w/w	40%	-	-	-		[2]
Batch Lab	WAS	Protease	5000	4	0.06 w/w	55%	-	-	-		[2]
Batch Lab	WAS	Mixed	-	4	0.06 w/w	25% - 58%	-	-	-		[2]
AnMBR Lab	Synthetic	Mixed	-	-	3,6mL /g	22%	from 0.27 to 0,34	-	-	HRT 8.5h, SRT 50d	[3]
Batch Lab	Cellulosic WAS	Cellulase (T.reesei)	8300	24	0.096	13%	-	-	-		[4]
Full scale	WAS (primary + secondary)	Glycosidic		-	2,5 kg /tonne DS	-	10-20% increase	-	from 27% to 31% solids	SRT 24d, Sludge loading 45 m^3/d	[5]
Batch lab	Pulp paper mill sludge	Lysozyme	40	-	0,015 g/gDS	-	-	from 11% to 6% (v/v)	from 5.6 to 8.9% DS		[6]
Batch lab	AD	Amylase-Protease	-	24	0.47 U/mg VSS	-	from 224 to 264 mL biogas/gVS	-	-	SRT 30d	[7]
Pilot/lab	AD	commercial mix	unknown	16	1 mg/gDS	-	-	15 mg/gDS	from 18% to 20%		[8]
Batch Lab	Lipase	1.771	dairy wase		12	-	from 133 to 226 - 276 ml biogas /gTS		-	SRT 15d	[9]

7.6 References

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Steef de Valk, Ahmad F. Khadem, Christine M. Foreman, Jules B. van Lier and Merle K. de Kreuk (**2017**) *Physical and biochemical changes in sludge upon Tubifex tubifex predation*. Environmental Technology, 38:12, 1524-1538

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Steef de Valk, Tales A. Tavares de Sousa, Ahmad F. Khadem, Jules B. van Lier and Merle K. de Kreuk (**2020**) *The biodegradability of aquatic worm predated waste activated sludge: A sequential aerobic and anaerobic treatment approach.* Bioresource Technology Reports, 12:100606

CURRICULUM VITAE

Steef de Valk, born on the 23rd of May 1983 in Amsterdam, the Netherlands, completed a Bachelor and Master degree in Life Science & Technology at the Delft University of Technology between 2002 and 2011. After cultivating an environmental engineering and waste to resource mind-set at the faculty of Environmental Biotechnology, he endeavoured into the world of wastewater treatment as a PhD candidate at the Sanitary Engineering section at the TU Delft faculty of Civil Engineering and Geosciences. The subject of sludge degrading aquatic worms has provided Steef with the specific details of the worm predation process and a broader insight in wastewater treatment. Steef is currently enjoying a post-doctoral position at the same faculty and is working as a project manager in several international water treatment and reclamation collaborations.