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## Elucidating the microbial community associated with the protein preference of sludge-degrading worms

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### ABSTRACT

Sludge predation by aquatic worms results in an increased sludge reduction rate, which is mainly due to the specific 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 the *Tubifex tubifex* was investigated. The microbial diversity in the samples of the worm faeces (WF), predated activated sludge and protein-rich substrates were compared. The results indicated that predation on sludge resulted in a microbial change from Actinobacteria (44%) in the sludge, to Proteobacteria (64%) and Bacteroidetes (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 respired activated sludge samples. This similar microbial community could be due to microbial utilisation 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*.

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### KEYWORDS

Oligochaeta; tubifex; worm predation; microbial community; sludge reduction

### Highlights

- The microbial community in the sludge-based WF is related to the community in (predated) protein-rich substrates.
- The genera *Burkholderiales*, *Chryseobacterium*, *Flavobacterium* and *Massilia* seem to be associated with *T. tubifex*.
- Protein degradation is due to the synergistic activity of the aquatic worms and their intestinal microbial community.



### 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].

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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.

## 2. Materials and methods

### 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’.

### 2.2. Sludge characteristics

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

### 2.3. Reactor design

The worm incubation reactor comprised of two identical compartments, both containing 18 L 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  $20 \pm 1^\circ\text{C}$  and dissolved oxygen was above 5 mg/L. Reactor performance data can be found elsewhere [5].

### 2.4. DNA sample preparation and collection

Sludge samples were obtained from freshly obtained WAS and at 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, later they

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 tap water filtered over 0.45 µm. This container was passively aerated at room temperature (18–20°C). After 48 h, 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°C. Sludge-worms were externally washed and stored at –24°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 50% of protein, 11% of lipids and 24% carbohydrates. Fish food samples were acquired by dissolving 5 g of crushed Tetra Min fish food flakes in 0.5 L 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°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 predated fish food broth samples were frozen at –24°C. The worms were externally washed and stored at –24°C.

Table 1 summarises the samples analysed for microbial community composition.

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 bottle containing tap water. After 2 days the worms were transferred to 0.2 L bottle containing 0.11 g/L azocasein. After a 3-day incubation period, samples of the broth were stored at –24°C. The worms were externally washed and stored at –24°C.

## 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.

## 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

**Table 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 respired 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
Azocasein solution fed to sludge-worms		Azocasein	Azo

**Table 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
NO <sub>3</sub> <sup>-</sup> - N (mg N/L)	6.7 ± 0.14	13.9 ± 1.9	34.3 ± 0.14	
PO <sub>4</sub> <sup>3-</sup> - P (mg P/L)	0.45 ± 0.07	4.05 ± 0.1	9.1 ± 0.1	Previous study [5]
VS reduction (%)	-	9% ± 5	47% ± 15	
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 <sup>3+</sup> (mg/L)	0.02	0.03–0.05	0.11–0.15	

unweighted UniFrac metrics. The confidence cut-off was set as 0.5.

### 3. Results and discussion

#### 3.1. Sludge predation characteristics

The results of aerobic (worm) treatment of WAS are summarised in Table 2.

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.

#### 3.2. Overall microbial phylogenetic diversity

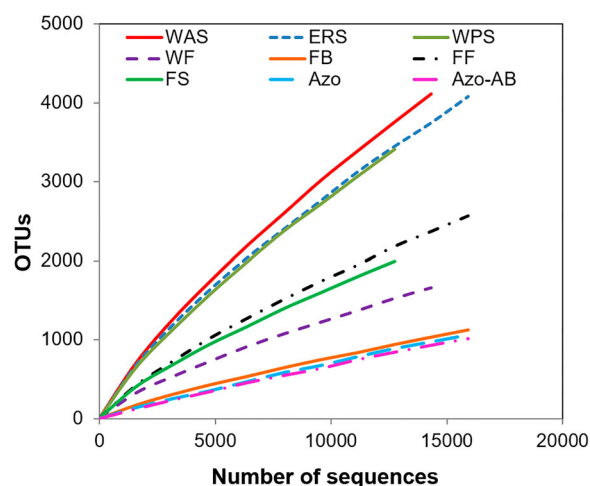
To investigate the changes in the microbial community in response to worm predation and the feeding of different substrates, 16S rRNA gene-based Illumina Miseq sequencing analysis was performed. In total, 194,043 high-quality reads were obtained for the nine samples. The RDP Classifier was used to assign OTUs to the different sequence tags. A total of 21,024 OTUs were identified based on the 97% identity cut-off.

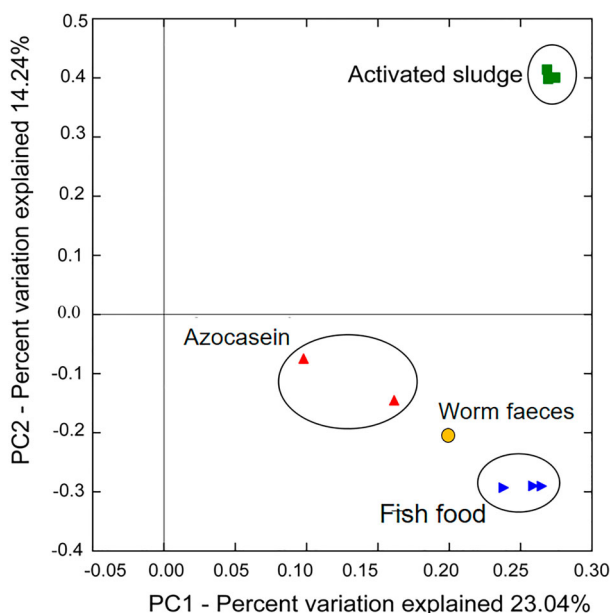
**Table 3.** The distribution of the identified OTUs and  $\alpha$ -diversity indices across the samples.

Samples	OTUs	No. of reads	Faith's PD	Chao1	Shannon
WAS	4111	15,614	159	14,313	8.62
ERS	4084	24,212	161	16,093	8.72
WPS	3410	13,021	136	12,795	7.89
WF	1656	15,107	46	6019	5.81
FB	1123	16,231	44	5954	2.40
FF	2571	27,393	89	10,189	6.56
FS	1995	13,958	74	7423	6.73
Azo	1059	29,363	44	11,997	1.73
Azo-AB	1015	39,144	20	9046	1.63

The distribution of the identified OTUs across the samples and the calculated  $\alpha$ -diversity indices (Table 3) shows that the (treated) activated sludge samples (WAS, ERS and WPS) were characterised by a high microbial diversity compared with 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 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 1.** Rarefaction curves of the OTU obtained from 16S rRNA gene analysis of the microbial community in (aerobically treated) activated sludge, WF, fish food and azocasein samples.



**Figure 2.** Principal coordinate analysis plots (PCoA) of sample fractions determined using the unweighted Unifrac distance metric.

In order to further assess the relationships between the different samples, the principal coordinate analysis (PCoA) was performed (Figure 2). 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 Azo-AB sample, which contained AB. This separated lineage is subdivided into three lineages that separate the fish food and azocasein samples and the WF.

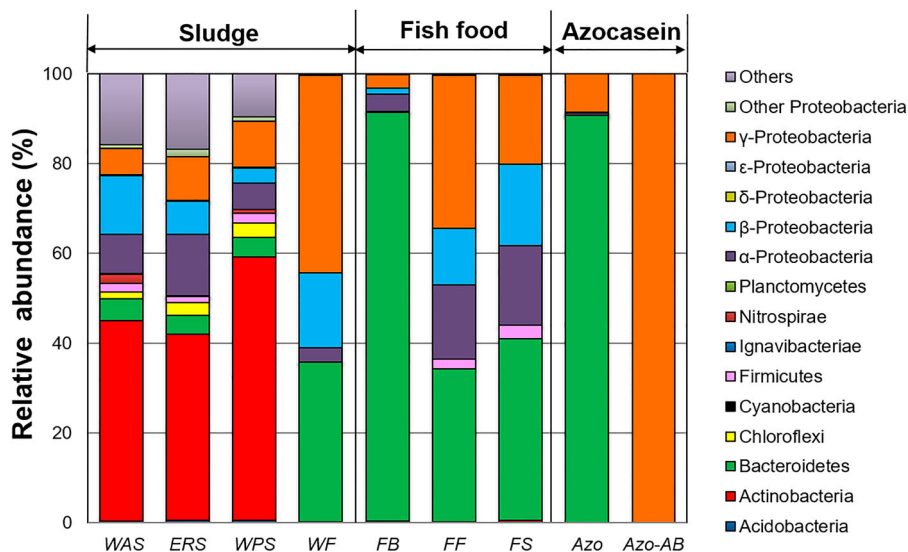
Interestingly, the microbial diversity in the WF is more related to the diversity of the two protein substrates than to the (treated) sludge samples. To be more specific, the WF shared a similar microbial community with Azo. This similarity in the bacterial community could be related to the metabolism 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 2). Additionally, De Valk et al. [14] showed, by suppressing bacterial activity with AB in *T. tubifex*, that bacteria play an important role in the hydrolysis of protein.

### 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 3).

A total of 11 abundant phyla were detected across the different samples. In accordance with the  $\alpha$ -diversity indices (Table 2), the microbial composition of the (treated) sludge samples was similar and larger in diversity compared with 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 abundance 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



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

Proteobacteria (64%), consisting of  $\gamma$ -Proteobacteria (44%),  $\beta$ -Proteobacteria (15%) and  $\alpha$ -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 ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -) 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 compared with the resulting microbial composition after worm gut passage of WAS, a protein-rich substrate.

### 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 WF with the sludge samples (Figure 4) and with the protein samples (Figure 5).

Notable changes in abundance were observed for the Actinobacteria – *Candidatus Microthrix* that declined in abundance from 40% in WAS to 0% in the WF. *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 WF [6]. However, this is in contrast with the higher abundance in the WPS that contradict with the improved settleability associated with WPS in terms of SVI (Table 2).

Additionally, as previously mentioned, the four phyla that increased in abundance after the gut passage of WAS (Figure 4) were the Bacteroidetes – *Chryseobacterium* (30%),  $\alpha$ -Proteobacteria – *Brevundimonas* (3%),  $\beta$ -Proteobacteria – *Massilia* (15%) and  $\gamma$ -Proteobacteria – *Acinetobacter* (38%). Although the phyla of Proteobacteria and Bacteroidetes in the WF are similar to those of the (predated) protein substrates

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

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

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

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

(Figure 3), considerable differences at the genus level are found (Figure 5).

The main differences for the Bacteroidetes 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  $\gamma$ -Proteobacteria phylum. The dominant *Acinetobacter* genus in the WF (40%) was 'replaced' by *Lysobacter* in the predated fish food samples (FF (25%) and FS (9%)). The  $\beta$ -Proteobacteria *Massilia* was present in the WF (15%) while low in abundance (<1%) in the (predated) protein samples. Additionally, *Comamonadaceae*, showed an abundance increase from  $\leq 1.6\%$  in WF and FB to 11% and 17% in FF and FS, respectively. The changes for the  $\alpha$ -Proteobacteria were 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 WF.

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. 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.

### 3.5. Microorganisms associated with *T. tubifex*

Based on the presence of the different genera (Figures 4 and 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 4.

## 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



**Table 4.** Genera associated with *T. tubifex*. Based on the presence or abundance differences between WF and (predated) protein-rich substrates.

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

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 4).

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.

## 5. Conclusions

Microbial community analysis revealed that the WF produced through sludge predation 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.

- 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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