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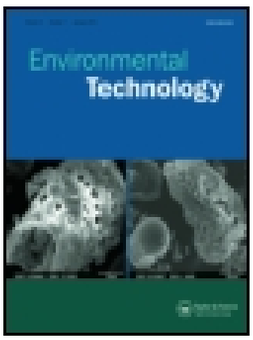

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Unravelling the protein preference of aquatic worms during waste activated sludge degradation

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ABSTRACT

Worm predation (WP) by *Tubifex tubifex* was investigated using waste activated sludge (WAS) as the substrate. In order to better understand the sludge degradation mechanisms during WP, the activity of five common hydrolytic enzymes was determined and compared among 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 the 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.

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Enzymatic activity; worm predation; sludge reduction; WAS; *Tubifex*

Highlights

- *Tubifex tubifex* and its intestinal biome release proteases.
- Protein hydrolysis is partly in synergy with the intestinal worm bacteria.
- Hydrolytic enzyme activities decline during aerobic sludge treatment.
- Enzymatic activity is predominantly associated with the solids fraction.

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 wastewater treatment plant (WWTP) [2]. To reduce these costs, sludge minimisation 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 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 macrofauna [7].

Predation by macrofauna, for example with aquatic worms, has gained increased attention in the past decades. For instance, both Tamis et al. [8] and Lou et al. [9] researched full-scale worm reactors for sludge reduction. Both studies showed a higher degree of sludge reduction and thus showed a 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].

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Although the effects of worm predation (WP) on sludge reduction are well researched, it is not yet clear why a higher degree of sludge reduction occurs with WP when compared to using extended aeration and AD. 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 have 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 are 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 important roles in the hydrolysis of WAS [18]. Knowledge concerning the hydrolytic activity of these enzymes in relation to WP 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 WP process, this paper presents a comparative analysis of the relevant hydrolytic enzymatic activities between the initial feed activated sludge, that is, WAS, the sludge after 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 (ABs) to suppress the bacterial activity.

2. Material and methods

2.1. Laboratory-scale worm reactor

Tubifex 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 WWTP Harnaschpolder (Den Hoorn, The Netherlands), which treats the domestic wastewater of 1.3 million people equivalents and comprises a biological nutrients removal plant. The laboratory-scale reactor consisted of two identical 18 L compartments: one contained about 700 g of worms for generating the worm predated sludge (WPS), and the other served as a control for ER, producing endogenous respiration 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. [8].

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°C ± 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].

2.2. Analytical procedures

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

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, PA, USA), were incubated with different substrates (Table 1) in an Innova 40 thermal shaker (New Brunswick Scientific Co., Inc., Enfield, CT, USA) at 25°C ± 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*g*, 90 s, at room temperature), and the obtained supernatant was filtered using 0.45 µm membrane filters.

Table 1. Substrates for enzymatic activity assay.

Enzymatic activity	Substrate	Medium	Reaction concentration	Wavelength
Lipase	4-Nitrophenyl palmitate	[a]	1 mM	410 nm
Protease	Azocasein	Tris HCl 20 mM 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

[a]Stock solution of 80 mM of 4-nitrophenyl palmitate in isopropanol. For 50 mL substrate solution, 23.5 ml of Tris HCl 20 mM pH 7, 23.3 mL DMSO, 1 mL Trition ×100 and 2.5 ml of nitrophenyl palmitate stock were mixed in this order.

The filtrate was mixed with NaOH solution to an end concentration of 1 M. Subsequently, absorbance was measured using a Genesys 10S UV–VIS spectrophotometer (Thermo Fisher Scientific, Inc. Waltham, MA, 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.

2.4. Azocasein conversion and AB 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 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 h, which was sufficient to ensure that the worms ingested the maximum amount of azocasein. Gillis et al. [20] showed that *tubifex* needs approximately 24 h to purge their intestines and that the defaecation rate is linear in time. The incubation took place in different combinations of azocasein and AB solutions, as shown in the incubation section of Table 2. After 20 h, the incubation solutions were discarded, and fresh solutions were added. The 20-h 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 supplementary information section (Figure S1).

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 defaecation section of Table 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 dissolved in a 0.2 g/L streptomycin-tap water solution.

One millilitre 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.

Table 2. Overview of the different samples with their corresponding incubation and defaecation solutions.

Sample	Incubation phase	Defaecation 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

Note: Streptomycin was used as antibiotic (AB).

2.5. Flow cell cytometry

In order to assess the contribution of bacteria towards azocasein hydrolysis, the number of total and intact cells was measured using flow cytometer (FCM) according to Prest et al. [21]. Total cells were stained using SYBR® Green I, and intact cells with SYBR® Green propidium iodide. The samples were measured using a BD Accuri C6® FCM (BD Accuri cytometers, Belgium). When necessary, dilutions were made using filtered (0.22 µm Millex-GP) Evian® bottled water.

3. Results and discussion

3.1. WP 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 3). Organic (VS and chemical oxygen demand (COD)) removal rates during WP were about 5-fold higher compared to the control without worms, that is, endogenous respiration. Additionally, an increased release of P - PO_4^{-3}, N - NH_4^+—NO_3^- and soluble COD (sCOD) was observed, as described elsewhere [13].

Table 3. Average worm concentrations and removal and release rates for WP and ER after 4 days of sludge treatment.

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 - 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	2.1 ± 1.4	3.6 ± 1.1
Soluble COD release rate	mg sCOD/d	0.06 ± 0.06	0.30 ± 0.2

Note: 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. [13].

Table 4. Enzymatic activities of the mixed liquor of WAS, WPS and ERS.

	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

Note: 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 four times in order to achieve a linear substrate conversion. Average VS reduction was 24% ± 5% and 10% ± 4% for WP and ER, respectively.

3.2. Enzymatic activities in treated sludge

3.2.1. Mixed liquor sludge

The enzymatic activities of five common hydrolytic enzymes were determined before and after aerobic (worm) treatment of WAS. The results, presented in Table 4, show that, in general, the enzymatic activities in the mixed liquor decreased after treatment of the activated sludge. The graphs of the enzymatic activity assays can be found in the supplementary information section (Figure S2).

The decrease in the 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% to 90% within the first 5 days of aerobic digestion of the activated sludge, was reported by Novak et al. [22], Yu et al. [23] and Lou et al. [9]. 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].

The literature values of studied hydrolytic enzymes in the mixed liquor of different wastewater sources show a large range in activity (Table 5). When comparing the results presented in Table 4 to the literature values in Table 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. [24] 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 different sludges.

Table 5. Literature values of enzymatic activity in WAS recalculated to the same unit (μmol/g VS(S) min) when necessary.

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

Note: Values marked with [a] were estimated from a graph. AS: activated sludge; bioP: biological phosphorus removal; p.e.: population equivalents.

There are several potential explanations for the general decrease in enzyme activities upon aerobic (worm) treatment of WAS: Firstly, Frølund et al. [27] and Cadoret et al. [25] found that enzymatic activity is predominantly bound to sludge solids, more specifically the EPS matrix. These studies indicate that VS reduction, upon aerobic (worm) treatment, is related to the degradation of solids-bound enzymes. Secondly, Foladori et al. [28] 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 notable. Furthermore, bacterial cells are known to contain high concentrations of proteins, that is, 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 bacteria that produces enzymes, and/or the actual enzymatic proteins. This degradation of enzymes and/or their producers could explain the larger reduction in activities observed after WP 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 6).

Results show that per % point VS removal, a higher reduction in α-glucosidase, β-glucosidase and alkaline phosphatase enzyme activity was observed after WP 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

Table 6. Reduction in enzymatic activity expressed as per cent change compared to the baseline enzymatic activity values of WAS.

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

Note: Net change in enzyme activity/net change in VS [%/%]. Errors are expressed as standard deviations.

the WPS compared to ERS. These calculations suggest that a part of the protease activity was conserved or maintained during WP.

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, due to either worm activity or the previously mentioned decay during aerobic treatment, could promote the growth of other proteolytic bacteria on the released bacterial proteins. Changes in the microbial community due to the presence of aquatic worms have been reported by others [31].

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 7). 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. [27] and Cadoret et al. [25], amongst others, stating that enzymatic activity is predominantly bound to the solids fraction in the sludge. The reduced in the protease activity in the sludge mixed liquor during WP (Table 6) was not reflected as an increased activity in the supernatant (Table 7). This implies that the increase in protease activity remained associated with the solids fraction.

Furthermore, Cadoret et al. [25] 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 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 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.

3.3. The effect of ABs on the conversion of azocasein in the worm gut

Bacterial association and interaction with the intestines of aquatic worms are well described in the literature [15–17]. Given their homology to higher organisms, it is highly 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 AB streptomycin to suppress intestinal bacterial activity, a distinction can be made between the proteolytic activity of the worm and its intestinal bacteria. The (AB-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 defaecation gives an

Table 7. Enzymatic activity of the supernatants of WAS, WPS and ERS.

	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

Note: 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.

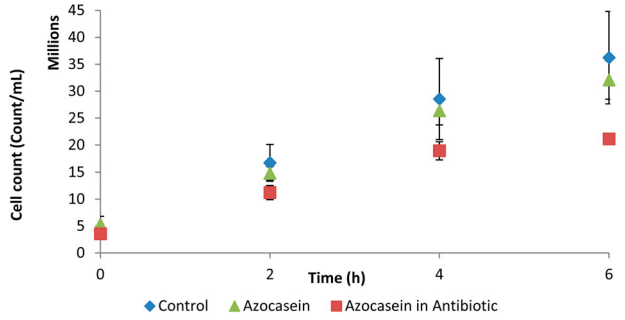

Figure 1. The release of intestinal bacterial cells from the worm gut by defaecation. Worms were incubated for 40 h with different combinations of azocasein and antibiotics (ABs). Upon transfer to 0.45 µm filtered water, the release of intestinal bacteria was monitored using flow cell cytometry. Average values and standard deviations were calculated from triplicates.

indication of the hydrolytic activity inside the intestines of the worms. Furthermore, to quantify the decrease in bacteria excreted after incubation with ABs, the defaecated intestinal bacteria were counted using flow cell cytometry (Figure 1).

Based on the differences between the azocasein incubations with and without ABs, the presence of ABs shows a clear effect on the number of bacteria that are released from the worm gut in time; after 4 h, no additional organisms were released from the gut, and the overall release was lower in the AB-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 the accumulation of defaecated 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. [20], who showed that the defaecation rate of *T. tubifex* is linear based on the weight decrease due to defaecation.

Interestingly, the released intestinal bacteria showed proteolytic activity (Figure S1, supplemental information section), suggesting that the released intestinal bacteria, which are part of the solids fraction of the sludge (Figure 1), produced the additional proteolytic activity (Table 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 2.

The difference between azocasein incubations with and without ABs clearly shows that ABs 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 defaecation observed by Gillis et al. [20]. 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 8 shows that the presence of ABs had a negative influence on the release rate of azo-dye.

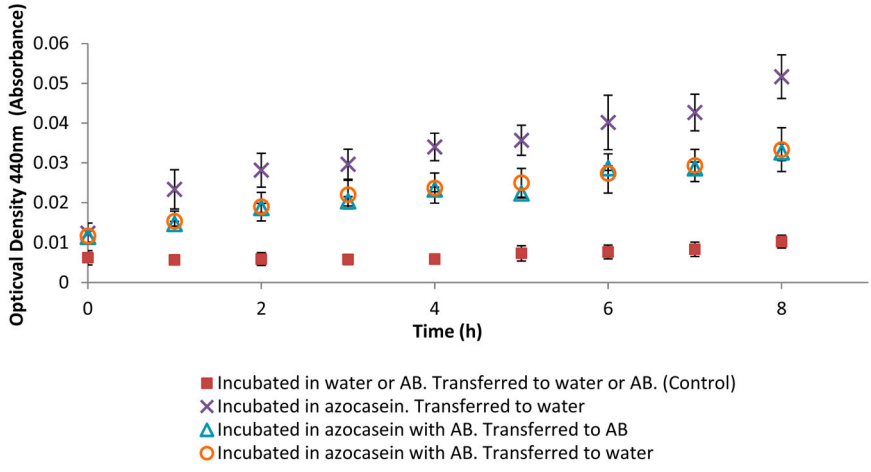

Figure 2. Averaged azo-dye excretion from the worm gut by defaecation. Worms were incubated for 40 h with different combinations of azocasein and/or antibiotics (ABs). Upon transfer to a clear medium, the release of the hydrolysation product, azo-dye, was measured at 440 nm using a spectrophotometer.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

Table 8. Averaged azo-dye release rates.

	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	$3.1 \times 10^{-3} \pm 1.6 \times 10^{-4}$	$2.3 \times 10^{-3} \pm 3.6 \times 10^{-5}$	$2.2 \times 10^{-3} \pm 3.6 \times 10^{-4}$	$4.9 \times 10^{-4} \pm 1.9 \times 10^{-4}$

Note: 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.

A difference in azo-dye release rates of about 27% was observed between AB-treated and non-treated worms. This outcome suggests that the hydrolytic activity within the worm gut was negatively influenced upon ABs 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 AB-treated worms were not completely sterile (Figure 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*.

The protein hydrolysis that is attributed to *T. tubifex* provides opportunities to further investigate the worm-based enzymes that are responsible for the removal of the protein fraction of sludge. This study confirms that proteases play an important role in worm-based hydrolysis of WAS. This knowledge coupled with the alleged hydrolysis of bacterial cells by the aquatic worms can be used to improve existing enzymatic pre-hydrolysis techniques of WAS, as described by various studies [36–39].

4. Conclusion

The activities of five common hydrolytic enzymes were predominantly associated with the solids fraction of WAS. Upon aerobic (worm) treatment of the activated sludge, the enzymatic activities declined. Interestingly, the decline in protease activity during WP, in relation to 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 ABs 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*.

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