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The biodegradability of aquatic worm predated waste activated sludge: A sequential aerobic and anaerobic treatment approach

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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.

1. Introduction

Waste activated sludge is a by-product from conventional sewage treatment. Due to stringent legislation (Commission Of European Communities, 1998), 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 (Metcalf et al., 2004). 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 (Bhattacharya et al., 1996; Hiraoka et al., 1985; Ruffino et al., 2015).

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% (Gonzalez et al., 2018). 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 (Gonzalez et al., 2018) 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 (Ratsak and Verkuijden, 2006). In earlier studies, worm predation showed a similar WAS solids reduction compared to AD, with significant shorter residence times: worm predation resulted in $47\% \pm 15\%$ solids reduction within 2 to 4 days of treatment (de Valk et al., 2017b; Hendrickx et al., 2010). 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 (de Valk et al., 2017b), we suggested that the aforementioned overall increased biodegradability was possibly due to presence of a sludge

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fraction that is only degradable under aerobic conditions, such as the worm predation process and not under anaerobic conditions (Park et al., 2006).

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 (Park et al., 2006) 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.

2. Materials and methods

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 (de Valk et al., 2017b).

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.

2.3. Sludge treatment

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.

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. 2 L borosilicate glass bottles were filled with 2 L of sludge and inoculated with 125 μ L/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 min. Bottles were incubated in a thermal shaker operated at 35 °C and 120 RPM.

Part of the 2 L bottles were prepared to monitor biogas production and where 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 of the bottles where 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 20 L 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).

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 (de Valk et al., 2017b). Evaporated water was replenished with demineralised water. The aquatic worms were separated from the predated sludges using a sieve with 200 μ m 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 h after which supernatant liquid was replaced with an equal volume tap water to minimise the concentration of ammonia (Hendrickx et al., 2010) and potentially nitrate (Gomez Isaza et al., 2020) 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.

2.4. Treatment process overview

An overview of the different incubation experiments is given in Table 1. The aerobic endogenous respiration (ER) followed by anaerobic digestion 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 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

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 (A.P.H.A., 2012). 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 (A.P.H.A., 2012).

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.

$$\text{Reduction as \% of initial WAS} = \frac{[X]_{\text{start of stage}} - [X]_{\text{end of stage}}}{[X]_{\text{initial WAS}}} \times 100\% \quad (1)$$

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.

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

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

3. Results and discussion

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 percentage 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 2.

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 (Novak et al., 2003; Park et al., 2006) 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 Harnaspolder 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 (Park et al., 2006; Ramdani et al., 2010), 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 $47\% \pm 15$ with conversions in the ER5 control of $9\% \pm 5$ (de Valk et al., 2017b). Furthermore, in previous research we found that the ER VS removal of 30 days (ER30) reached similar values as WP (de Valk et al., 2017b). 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 ($36\% \pm 6$). 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

Table 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 where 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

solids (Bhattacharya et al., 1996; Hiraoka et al., 1985; Ruffino et al., 2015), 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 (Gonzalez et al., 2018), ii) the tightly bound extracellular polymeric substances (EPS) fraction that is hard to degrade (Ye et al., 2014) and iii) the available process time as well as iv) mixing conditions (Leonzio, 2019) that are both not optimized to reach the maximum biodegradability in full scale reactors.

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 Fig. 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 rate constants over the first 26 days of the digestion processes in Fig. 1 are listed in Table 3.

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

Table 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 Fig. 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	
		VSS (d^{-1})	COD (d^{-1})	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

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 stabilised. 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 $\text{N-NO}_3^-/\text{L}$, which was only by 50% converted at the end of the second stage (final concentration 55 ± 21 mg $\text{N-NO}_3^-/\text{L}$). 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.

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

Table 4

COD/VS ratios of the 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

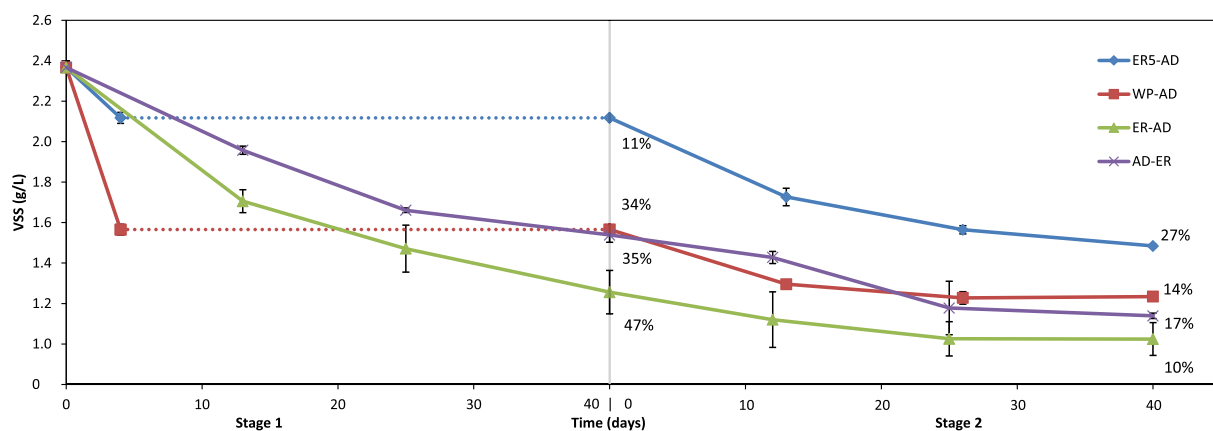


Fig. 1. The change in VSS concentration during the treatment stages. Results are from a single initial WAS sample. The dashed lines are only a visual aid as to have WP and ER5 in the same treatment stage as ER and AD and better reflect the VSS removal rate. The percentual change in VSS reduction of the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

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 (McMurtry, 1983; Tsuchiya and Kurihara, 1979; Wavre and Brinkhurst, 1971; Whitley and Seng, 1976) and EPS (de Valk et al., 2017b).

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 5.

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 (Fig. 1). The addition of the extra AD stages, (Table 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 process (Table 2). The AD-ER5-AD, 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 were not reached.

The extent of sludge biodegradability, in terms of COD removal in the worm predation processes AD-WP (57% ± 1%, Table 5) and WP-AD (57% ± 1%, Table 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

Table 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 3.2. 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%

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 (Foladori et al., 2015; Wei et al., 2003), however the intestinal bacterial community of the aquatic worms do grow (de Valk et al., 2017a) and are released along with the worm faeces (de Valk et al., 2017c). 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 the work 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 (Wei et al., 2003). Decaying or lysed bacteria might have been used as substrate for maintenance metabolism (Canales et al., 1994; Foladori et al., 2015; Hamer, 1985) 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 (Ferrentino et al., 2016; Foladori et al., 2015; Semblante et al., 2014; Urbain et al., 1993; Wei et al., 2003).

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 (Chudoba et al., 1992). 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 through methane production. Although promising, due to the increased ammonia concentrations after an anaerobic conversion process, ammonia toxicity has to be taken into account (Hendrickx et al., 2010). 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 (de Valk et al., 2017b; Hendrickx et al., 2010) which can further improve the efficiency of OSA-like processes that employ a settling stage.

3.4. Worm death during aerobic predation

Because worm death was observed in different occasions when aerobic and anaerobic stabilised sludges was fed, it was decided to separate the solids by sedimentation and only feed the settled fraction to the worms. For the anaerobic sludge, the high NH_4^+ concentration could lead to elevated NH_3 concentrations which is toxic for aquatic worms. Toxicity LC_{50} values for aquatic worms are reported to be between 0.29 and 1.20 mg-N- NH_3/L (Hickey and Vickers, 1994; Schubaur-Berigan et al., 1995; Whiteman et al., 1996). The anaerobic sludge contained 163 ± 1 mg/L N- NH_4^+ , while the pH increased to 8.5 during the aeration phase. Since the pK_a of NH_3 is 9.25, NH_3 concentrations could go up to 28 mg N- NH_3/L . In case of aerobically stabilised sludge, an unknown toxic effect was observed during predation experiments with the aquatic worm *L. variegatus*. Elissen (2007) showed that worm growth on aerobically treated sludge was possible if the aerated conditioning period of the sludge did not exceed 48 days because otherwise unexplained worm death would occur. Despite the precaution of only using the solids fraction and an aeration process of 30 days, worm death could not be avoided during the predation of the aerobically stabilised sludge solids.

A possible cause of worm death might be related to the food source of the aquatic worms. As was mentioned previously, both bacteria (McMurtry, 1983; Tsuchiya and Kurihara, 1979; Wavre and Brinkhurst, 1971; Whitley and Seng, 1976) and EPS (de Valk et al., 2017b) are considered an important food source for *T. tubifex*. The low COD/VS ratio of the ER sludges are indicative of the previously mentioned soluble BOD limited conditions in the sludge, which are dominated by lysis and EPS reduction. More specifically, on a microbial level Foladori et al. (2015) showed, using flow cell cytometry on the sludge of an 12 day SRT OSA-like process, that bacterial decay and lysis of activated sludge predominantly occurs in extended aerobic conditions, whereas in anaerobic conditions the bacterial count remained stable. The low bacterial count, as shown by Foladori et al. (2015) and the low COD/VS ratio (Table 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 (Wright et al., 2013) and decreased growth rates in terrestrial worms (Huerta Lwanga et al., 2016). 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 abundance of bacteria and EPS. It has been documented that aquatic worms could prefer gram negative bacteria over gram positive (Ratsak and Verkuiljen, 2006). Interestingly, predation on anoxic or anaerobic WAS, which contains an abundance of gram negative bacteria (Van Lier et al., 2008), 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 (Hendrickx et al., 2010).

3.5. Aerobic and anaerobic degradable sludge fractions

In our previous research (de Valk et al., 2017b) 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. 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 were 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 toward the sludge biodegradability extent.

CRedit authorship contribution statement

Steeff de Valk: Conceptualization, Methodology, Investigation, Writing - original draft. **Tales A. Tavares de Sousa:** Investigation. **Ahmad F. Khadem:** Methodology. **Jules B. van Lier:** Supervision, Writing - review & editing. **Merle K. de Kreuk:** Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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