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Industrial sludge containing pharmaceutical residues and explosives alters inherent toxic properties when co-digested with oat and post-treated in reed beds

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Abstract

Background: Methane production as biofuels is a fast and strong growing technique for renewable energy. Substrates like waste (e.g. food, sludge from waste water treatment plants (WWTP), industrial wastes) can be used as a suitable resource for methane gas production, but in some cases, with elevated toxicity in the digestion residue. Former investigations have shown that co-digesting of contaminated waste such as sludge together with other substrates can produce a less toxic residue. In addition, wetlands and reed beds demonstrated good results in dewatering and detoxifying of sludge. The aim of the present study was to investigate if the toxicity may alter in industrial sludge co-digested with oat and post-treatment in reed beds. In this study, digestion of sludge from Bjorkborn industrial area in Karlskoga (reactor D6) and co-digestion of the same sludge mixed with oat (reactor D5) and post-treatment in reed beds were investigated in parallel. Methane production as well as changes in cytotoxicity (Microtox(R); ISO 11348–3), genotoxicity (Umu-C assay; ISO/13829) and AhR-mediated toxicity (7-ethoxyresorufin-O-deethylase (EROD) assay using RTW cells) were measured.

Results: The result showed good methane production of industrial sludge (D6) although the digested residue was more toxic than the ingoing material measured using $microtox_{30min}$ and Umu-C. Co-digestion of toxic industrial sludge and oat (D5) showed higher methane production and significantly less toxic sludge residue than reactor D6. Furthermore, dewatering and treatment in reed beds showed low and non-detectable toxicity in reed bed material and outgoing water as well as reduced nutrients.

Conclusions: Co-digestion of sludge and oat followed by dewatering and treatment of sludge residue in reed beds can be a sustainable waste management and energy production. We recommend that future studies should involve co-digestion of decontaminated waste mixed with different non-toxic material to find a substrate mixture that produce the highest biogas yield and lowest toxicity within the sludge residue.

Keywords: Biogas; Co-digestion; EROD; Industrial sludge; Microtox; Oat; Reed beds; Sustainable waste management; Toxicity; Umu-C

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Background

Methane production as biofuels is a fast and strong growing technique for renewable energy. According to Eurostat [1], total production of biogas in Europe was 100 million tonnes of oil equivalents (toe), corresponding to 9% of the total biofuel production in the European Union [1]. In Sweden, total renewable energy production was 9,993 ktonnes [1], corresponding to 1,363 GWh. Biogas from landfill, waste water treatment plants (WWTP), co-digestion plants and industrial plants accounted for 22%, 44%, 25% and 8%, respectively [2]. The aim of EU council [3] states that 20% of final energy consumption should be provided by renewable sources by 2020. Although the production of biofuels is growing, this goal is probably not realistic [4].

The part of 8% industrial waste (food waste, sludge from WWTP and industrial wastes) has potential to increase since some of these type of waste possess good methane gas potential comparable with common substrates for biogas production like grass, wheat and straw [5]. However, digested WWTP sludge is highly questioned for use as fertilizer in agriculture [6-9], and industrial waste can also possess inherent toxicity inhibiting digestion process and resulting in a digested residue needing post-treatment. Co-digesting of hardly degradable and toxic material together with some other substrate has also been demonstrated to be a realistic option [10-15].

The main alternatives for a digested residue with toxic or environmental hazardous properties are combustion, composting and/or use in less sensitive land applications such as covering of landfills. One alternative cost-efficient and low-technology demanding method, efficient in reducing nutrient content, carbons and toxicity, is the dewatering and treatment of sludge in constructed wetlands (CW) and reed beds [16-18]. More than 7,000 CW is operating in Europe and North America with increasing number in South America, Australia, New Zeeland as well as Africa and Asia [16-24]. The removal efficiency of nutrients and pollutants by CW and reed beds can be explained by the rhizosphere providing a large attachment area for both aerobic and anaerobic microorganisms [16,17,23,25-28] and as well as dewatering capacity by evapotranspiration and mechanical impact of shoots, roots and rhizome growth [17,22-24,29]. The capacity of planted beds in treating sludge from the same industrial area as in the present study, in comparison to filter beds without vegetation, has been demonstrated earlier [29,30]. Results showed that reed-planted beds were more efficient than unplanted at retaining toxicants, reducing the water-soluble toxicity [30], total organic carbon (TOC), biological oxygen demand (BOD) and chemical oxygen demand (COD) in the outgoing water [29,30].

Common reed has also demonstrated a high adaptive capacity to sewage sludge environment, and a doubling of shoot density compared to natural stands has been observed [20,24,29].

In this study, digestion of industrial sludge from Björkborn industrial area in Karlskoga containing nitroaromatic compounds, explosives and pharmaceutical residue and co-digestion of the same sludge mixed with oat was studied in parallel as well as post-treatment and dewatering of the digested sludge in reed beds. Earlier studies of the sludge used in this study industrial sludge from Björkborn industrial area in Karlskoga, showed good methane production potential during mesophilic conditions [31] but increased toxicity in the digested sludge [32-34]. Dewatering and treatment of this particular sludge demonstrated high dewatering capacity and reduced nutrient levels in outgoing water and sludge residue [29] as well as significantly reduced toxicity in outgoing water and bed material of reed beds measured with DR-CALUX, Umu-C assay and fish embryo toxicity test using *Danio rerio* [30].

The aim of the present study was to investigate co-digestion of industrial sludge from Björkborn industrial area in Karlskoga and oat. We wanted to investigate if co-digestion of substrate, considered as waste, together with common crop is suitable for biogas production. Moreover, we wanted to check if oat could alter the methane yield and toxic properties of industrial sludge by measuring methane production as well as change of cytotoxicity, genotoxicity and dioxin-like activity. In addition, we also wanted to investigate if co-digestion produced a less toxic and post-treatment demanding residue when dewatered through reed beds.

Results and discussion

Biogas production

The results of the biogas measurements (Table 1) showed that the mixed reactor with sludge and oat (D5) produced methane gas at a level below the control reactor with oat (D4) but possessed a methane gas potentially higher than the sludge reactor (D6). However, gas production in reactor D6 (Table 1) was almost twice as high compared to methane production of the same sludge in a former study where a gas production of 2,000 ml/day at 37°C (60% methane) was achieved using the same organic loading rate (OLR) of 3 g VS/L reactor/day [31].

The measured toluene, benzene, ethylbenzene and xylen concentrations during routine controls (ALS; Table 2) exceeded the limits for land application and land use in Sweden [35]. These compounds are mainly degradation products of 2,4,6-trinitrotoluene (TNT), dinitrotoluens, nitrobenzoic acids and a range of other compounds used for the manufacturing of explosives, pharmaceutical and chemical intermediates [32-34].

Table 1 Biogas and methane yield

Reactor	Substrate	OLR (g VS/L/day)	CH4 (%)	Gas production (ml/day at 37°C)	Specific gas production (ml methane/g VS at 0°C)
D4	Oat	6	51	17,500	300
D5	Oat + sludge	3 + 3	53	14,000	270
D6	sludge	3	63	4,000	180

However, the OLR of 3 gVS/L used in the present study did not inhibited the digestion process and resulted in gas production comparable with ordinary municipal sewage sludge in Sweden with an average gas production of 160 to 350 m^3 CH_4/tonne VS [36,37]. OLR in D6 is half of OLR in D5, resulting in twice as high hydraulic retention time (HRT) which may give the microorganisms time for adaptation but can also explain the lower methane yield. The lower HRT in D5 (Table 2) may also explain lower toxicity. A previous study [38] found that decreasing HRTs results in higher feeding and outgoing flow rates and, consequently, rapid withdrawal of toxic intermediates and less accumulation of inhibiting intermediates [38]. Intermediates can originate from the degradation of aromatic amino acids [39].

Table 2 Content of organics and metals in a month sample of undigested sludge

Organic compounds		Metals	
Parameter	(mg/kg TS)	Parameter	(mg/kg TS)
TS (%)	20	As	<3
VS (% of TS)	68.4	Ba	79.8
AOX	84	Be	0.264
Benzene	0.76	Ca	25,600
Toluene	260	Cd	<0.1
Etylbenzene	0.27	Co	4.17
Xylene	13	Cr	74.3
Di-etylftalat	4.1	Cu	25.2
Di-n-butylftalat	0.19	Fe	63,000
Di-n-pentylftalat	32	Hg	<1
Di-(2etylhexyl)ftalat	3.1	Li	0.517
PAH (sum)	<4.3	Mn	85
4-Nonylphenol	<0.25	Mo	4.9
RDX	0.43	Na	2,050
HMX	0.45	Ni	4.08
TNT	0.82	P	24,600
		Pb	29.2
		S	17,300
		Sr	34.3
		V	21
		Zn	99.8

From Björkborn industrial area collected during routine sampling.

A decreased HRT can prevent the accumulation of toxic substances and inhibition of the digesting process [10,38], but as a consequence, the methane yield would be reduced [38]. Instead, co-digestion could be a promising alternative option. Olive mill waste (OMW) possesses a high energy potential (45 to 220 g of COD/L) but also a low pH, alkalinity and nitrogen content; additionally, a lipophilic fraction and phenolic compounds are present. These characteristics make this substrate toxic and complex to degrade during anaerobic conditions [14]. However, it is increasing the methane yield when co-digested with manure (Table 3). Earlier studies (Table 3) of co-digesting different substrates revealed promising biogas production and showed high methane yield.

Co-digesting of starch-rich and ammonia strong wastes obtained gas yields comparable with yields obtained in the present study when co-digesting sewage sludge and potato processing industrial waste [13]. Co-digesting of manure, slaughterhouse and agricultural waste revealed higher gas yield (Table 3) with higher diversity of substrate [13]. This is consistent with the study performed by Chan et al. [11] who tested co-digestion of sewage sludge and marine dredgings mixed with municipal refuse at 13 different ratios [11]. Additionally, results in this study are strengthened by other studies which also found that co-digesting enhanced biogas production compared to digestion of single material such as high-strength COD substrate [10,13,14].

The methane yield in this study is lower than in many other co-digestion studies (Table 3). The explanation can be the presence of hardly degradable and toxic nitro-aromatic compounds (Table 2). The present study of 4,000 ml biogas/day and 300 ml CH_4/g VS added (Table 1) are confirmed by earlier studies demonstrating digestion of nitroaromatic compounds where methane gas production of 2,300 ml/day was achieved using a nitro-benzene loading rate of 30 mg/L/day [40]. In another study, methane yields between 116 and 209 ml CH_4/gVS L^{-1} were obtained by adding p-nitrophenol [38].

A low methane yield can also be explained by low carbon/nitrogen (C/N) ratio. The C/N ratio of 1.7 (Table 4) makes the industrial sludge unfavourable for digestion since a ratio of 16 is required to balance the anaerobic degradation between accumulation of volatile fatty acid (VFA) during digestion with high C/N ratio or accumulation of ammonia with low C/N ratio [10,13]. C/N ratio in oat mixed with sludge was higher (3.6) but still

Table 3 Examples of previously performed studies of co-digestion

Substrate	(Mixed ratios *v/v*)	HRT (days)	OLR (g VS/L/day)	Methane yield (ml CH_4/g VS/day)	References
OMW + cattle manure	75% + 25%	13	3,4	700 to 1,000	Angelidaki and Ahring [10]
OMW + pig manure	69% + 31%	6	2.9 + 2.6	2,700	Sampaio et a.l [14]
Sewage sludge + potato waste	44% + 56%	20	2.7	600	Murto et al. [13]
Industrial waste + pig manure	17% + 83%	30	2.6	800	Murto et al. [13]
Industrial waste + pig manure + slaughterhouse waste	17% + 71% + 12%	28	3.1	900	Murto et al. [13]
Industrial waste + pig manure + slaughterhouse waste	17% + 66% + 12% + 5%	36	2.6	1,000	Murto et al. [13]
Sewage sludge + marine dredgings + municipal refuse	20 + 5% + 75%	36 (batch)		900 to 1,200	Chan et al. [11]
OMW + piggery effluent	83% + 17%	6 to 7	3.5	1,300	Marques [12]
Oat + sludge	50% + 50%	28	6	500	Present study

unfavourable according to [13]. On the other hand, if the activity of methanogenic bacteria is low, less of the proteins will be degraded to free ammonia ions, inhibiting the digestion process [5].

Post-treatment in reed beds

Reduction of nutrients and carbon throughout the three months of digestion and dewatering in reed beds is shown in Table 4. The results show a high reduction of ammonia for both reed bed lines (Table 4) despite the short treatment time. This result is consistent with the findings of an earlier study of dewatering the sludge from Björkborn industrial area [29]. The authors found that reed beds were able to reduce COD and TOC to more than 90%, BOD and total nitrogen (N_{tot}) to more than 80% and total phosphorous (P_{tot}) to over 85% during the growth period (April to October). During the resting period (November to March), reduction of COD, BOD, N_{tot} and P_{tot} decreased to 66%, 28%, 35% and 55%, respectively [29].

Table 4 Nutrient and organic change in the sludge and sludge + oat reactor

(mg/L)	N_{tot}	TOC	P_{tot}	NH_4	C/N
Ingoing sludge D5	103	369	4.2	46.7	3.6
Digested D5	110	215	1.5	89.0	2.0
Sludge residue D5	28	275	1.4	0.4	9.9
Bed material D5	49	8	1.3	17.2	0.2
Outgoing water D5	Nm	118	1.5	2.7	0.2
Red (%)		68	65.0	94.2	*−0.2*
Ingoing sludge D6	811	1,340	10.7	800	1.7
Digested D6	777	1,290	13.4	746	1.7
Sludge residue D6	439	350	3,4	0,31	0,8
Bed material D6	123	373	32.3	24.3	3
Outgoing water D6	347	151	10.1	164	0.4
Red (%)	57	89	5.6	79.5	1.6

Before and after digestion and dewatering through reed beds after 3 months. Reduction (%) of nutrients and organics is calculated between ingoing sludge and outgoing water from reed beds. Italic number means increase. nm, not measured.

Additionally, several other studies have shown a high post-treatment capacity of reed beds. BOD removal efficiency of 63% to 79% independent from the season or age of the system was reported [41]. Moreover, other studies demonstrated removal efficiency of nutrients, nitrogen, phosphorus, BOD and total suspended solids (TSS) using constructed wetlands [21,42].

Toxicity tests

Microtox

The result of the present study showed an increased toxicity and an accumulation potential of non-reduced nitroaromatic compounds of the industrial sludge in reactor D6 during digestion. This is consistent with earlier studies investigating the same sludge where an increased acute toxicity and decreased cell vitality were measured after exposure to extracts of digested sludge using the microtox [31] and neutral red assay [32], respectively. Reed beds containing industrial sludge (D6) showed a decreased toxicity in sludge residual and bed material, suggesting that some of the compounds causing toxicity were transformed to water-soluble compounds and rapidly transported through the reed beds, ending up in the outgoing water. A large portion of those compounds, which are not trapped in the bed material or flushed out with the outgoing water, were probably degraded in the reed beds as shown in earlier studies of dewatering sludge from Björborn industrial area [30].

A contrary result was observed by testing a mixture of sludge and oat from D5. Ingoing sludge of D5 was less toxic compared to ingoing sludge of D6 (Figure 1). The

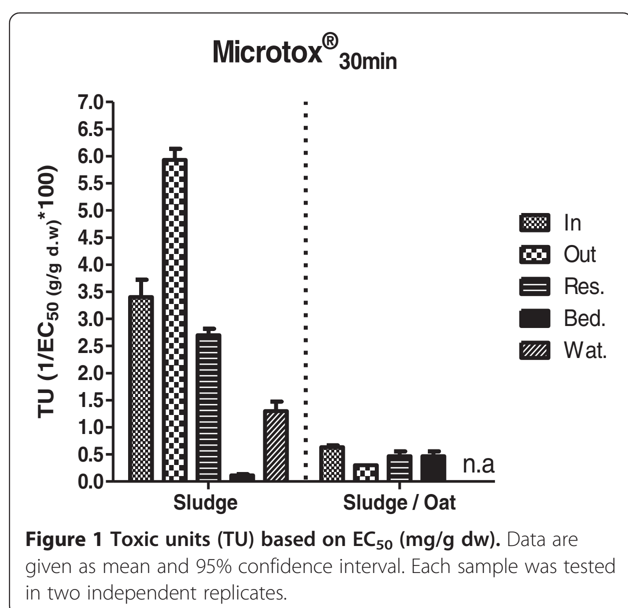

Figure 1 Toxic units (TU) based on EC_{50} (mg/g dw). Data are given as mean and 95% confidence interval. Each sample was tested in two independent replicates.

differences between the reactors (Figure 1) are larger than the dilution effect of oat by a factor of 2 (Table 1). Additionally, sludge from D5 showed decreased toxicity after digestion in opposite to digested D6 sludge demonstrating increased toxicity. The comparison of both reactors after digestion demonstrates a larger difference in toxicity that cannot be explained by dilution effect alone. Acute toxicity could not be detected in outgoing water from reed beds of D5. Sludge residue and bed material trapped toxic compounds, demonstrated by slightly increased TU values in D5 (Figure 1, Table 5).

Umu-C

In the Umu-C assay (ISO 13829), a genotoxic effect is significant if the induction factor is above 1.5 compared to the negative control. Figure 2 shows the used concentrations of the different samples reaching an induction factor of 1.5. Genotoxicity above 1.5 was detected in the undigested and digested D6 sludge with LID values of 82.5 and 41.25 μl/ml, respectively (Figure 2, Table 5). This result clearly demonstrates that the toxicity increased after digestion with only half the concentration needed to be genotoxic compared to undigested sludge. Outgoing water from reed beds treating D6 sludge showed higher LID values indicating that genotoxic compounds may have been adsorbed in bed material or degraded.

The mixed material with sludge and oat from reactor D5 showed unchanged genotoxic activity before and after digestion but decreased LID values in bed material which points at adsorption of genotoxic compounds in the bed material (Figure 2).

The genotoxic properties of this industrial sludge have been demonstrated before. A previous study [32] showed significant genotoxic potential in the digested sludge compared to undigested, tested in the comet assay with RTL-W1-cells. A former study of large scale anaerobic treatment of sludge from Björkborn industrial area demonstrated an increased genotoxic activity during treatment from induction factor 1.5 to induction factor 2.8 in the Umu-C assay [34]. This is higher as the genotoxic potential of D6 in the present study, although the pattern is the same, an increasing induction factor during anaerobic treatment (Figure 2, Table 5).

Different publications describe the genotoxic properties of nitro-aromatic compounds such as TNT, nitrobenzoic acids, nitrobenzenes and degradation products [43-46] and increasing toxicity with increasing number of nitro-groups [34,47]. Additionally, literature has also shown higher toxicity with nitro-substituted aromatics compared with their corresponding amines [38,48-50]. This may explain the increased genotoxicity, although weak, by the presence of unreduced nitro-aromatics within the sludge used in this study. 7-Ethoxyresorufin-O-deethylase (EROD) EROD activity could only be detected in three samples (Figure 3, Table 6). The EC_5 was comparably low in D5 Res (36.8 mg/ml), D6 Res (20.4 mg/ml) and in D6 Bed (68.5 mg/ml). All other samples did not induced EROD activity.

After 3 months of loading, detectable levels of EROD inducers could only be found in sludge residue on top of the reed beds from both D5 and D6 and in the bed material from D6. However, the levels are very low, and it was impossible to calculate Bio-TEQ values. Mesophilic digestion can increase EROD activity compared to undigested material. In a former study by [33], the same sludge obtained from the same manufacturing area as in this study, demonstrated three to six times higher levels of EROD activity in the digested sludge than in the incoming flux [33]. Additionally, earlier studies of methanogenic digestion of household waste showed that acid anaerobic conditions [51,52].

The identity of these EROD inducers was neither clarified in the former study by [33] nor in this present study. Additionally, it has been shown that a variety of different compounds apart from the well-known dioxins and polychlorinated biphenyls (PCBs) can induce EROD activity. For instance, conversion of proteins like tryptophan to indole acetic acid (IAA) and transformed compounds like indole-3-carbinole (I3C) and indolo-3.2-β-carbazole (ICZ) demonstrated 1×10^2 and 1×10^5 times higher AhR binding affinity than the parent compound, respectively [53,54]. Additionally, several phytochemicals including caffeic acid, chlorogenic acid, diosmin, ferulic acid and resveratrol showed both inhibition and induction of EROD [55].

Conclusions

Digestion of sludge from Björkborn industrial area (D6) resulted in a methane production of 180 ml CH_4/g VS,

Table 5 Summary table of the result of ecotoxicity test

Sample	EROD		Umu-C induction > 1.5	Microtox TU
	EC_5	EC_{10}	LID (μl/ml)	(mg/g dw)
D5 undigested	n.a		165	0.6
D5 digested	n.a		165	0.3
D5 sludge residue	36.8	100.8	165	0.5
D5 bed material	n.a		165	0.11
D5 outgoing water	n.a		n.m	n.a
D6 undigested	n.a		82.5	3.3
D6 digested	n.a		41.25	5.9
D6 Sludge residue	20.4	37.8	n.m	2.7
D6 Bed material	68.5		n.m	0.04
D6 Outgoing water	n.a		82.5	1.3

n.a, not available; n.m, not measured.

comparable with methane production of WWTP sludge. However, the digested residue was more toxic than the ingoing material measured using $\text{microtox}_{30\text{min}}$ and Umu-C.

Co-digestion of toxic industrial sludge and oat (D5) showed higher methane production (270 ml CH_4/g VS) despite the fact that just half of the HRT was used. Moreover, the digested residue was significantly less toxic than the sludge residue of D6. The differences in toxicity (i.e. Microtox) cannot be explained by dilution effects (OLR and HRT) as discussed in the 'Results and discussion' section and in this section. This clearly demonstrates the benefits of co-digestion of industrial sludge together with oat. Furthermore, dewatering and treatment in reed beds showed low and non-detectable toxicity in reed bed material and outgoing water as well as reduced ammonium (NH_4^+), N_{tot} and TOC. Moreover, toxicity of the dewatered D5 sludge on top of the reed beds were significantly lower than corresponding D6 for all three bioanalytical tests used in this study.

A less-contaminated waste stream demands less energy and monitoring during treatment. Therefore, digestion of sludge resulting in a less toxic residue, with a shorter and less complex post-treatment is the most cost-efficient option. We have demonstrated that co-digestion of industrial sludge with oat fulfilled that requirement. Additionally, dewatering and treatment of sludge in reed beds can be recommended as a post-treatment method of digested sludge.

Future studies should involve co-digestion of this industrial sludge or other waste mixed with different straw and grass in different proportions in order to find a substrate mixture that produces the highest biogas yield and lowest toxicity within the sludge residue. Using waste as a substrate in a sustainable way can also increase the possibilities to reach the aim of the EU council [3],

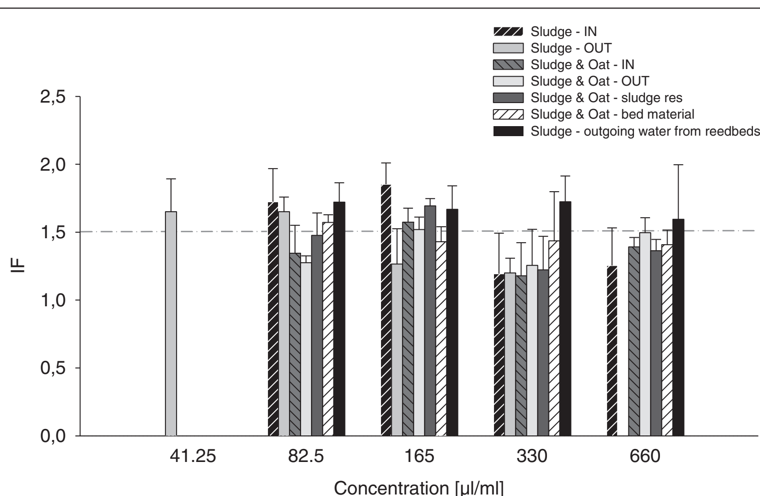

Figure 2 Mean and std dev of concentrations that caused induction factor above 1.5 compared to control. *n* = 3.

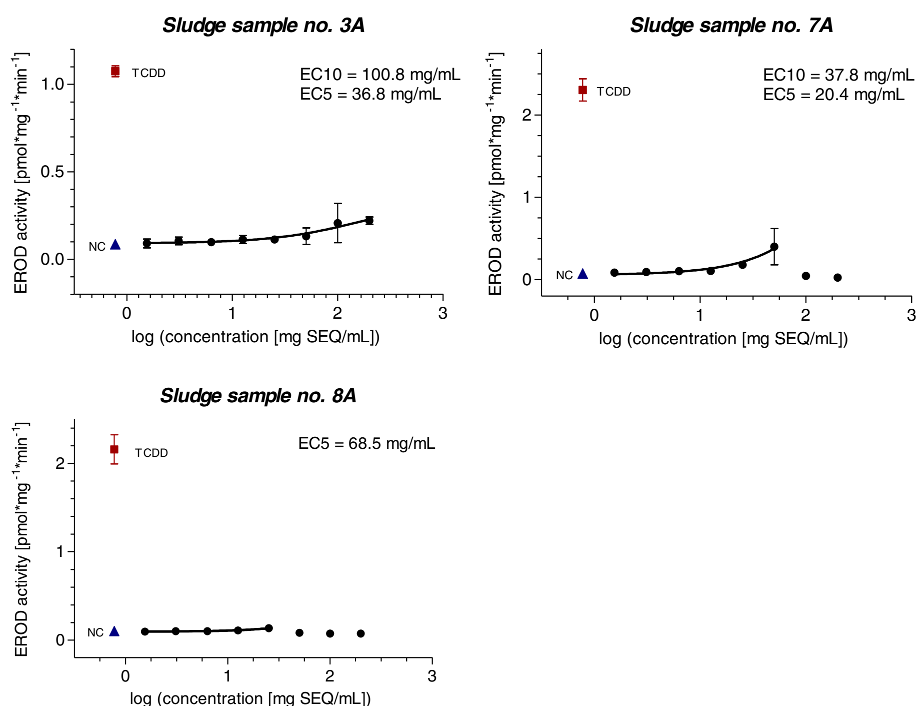

Figure 3 EROD activity detected in three samples. EC5 and EC10 in sludge residue of reactor D5 (no. 3a), in sludge residue of reactor D6 (no. 7a) and bed material in reed beds treating sludge from reactor D6 (no. 8a).

stating that 20% of final energy consumption should be provided by renewable sources by the year 2020.

Methods

Substrates and inoculums used in the study

Industrial sludge used as feeding material to the bioreactors was collected from the wastewater treatment plant of Björkborn industrial area, (Karlskoga, Sweden). Approximately 25 kg of dehydrated sludge was collected and carefully mixed and aliquots were stored in 1 L polyethylene bottles at −20°C. Total solid content (TS) of the sludge mounted 15.8, and volatile solid (VS) was 65.8% of TS. The oat was received from Söderslätts Spannmålsgrupp and milled to a grain size of 1 mm prior storage at room temperature in 1 L polyethylene cans during the experimental period. TS of the mounted 96%, and the VS of TS was 97.3%. Inoculum for the laboratory digesters consisted of digested sewage sludge from Reningsverket Nykvarn (Linköping, Sweden) and cow manure from Swedish dairy farm (Hags gård, Rimforsa, Sweden).

Digestion of sludge and oat in bioreactors

In the study, two digesters and one control were operated at 37°C with 20 days of HRT for the co-digestion of industrial sludge, milled oat (D5) and industrial sludge (D6), in parallel with a control reactor fed with milled oat (D4). The control reactor was operated according to the same protocol as the experiment reactors. Each digester contained an active liquid volume of 4 L and was equipped with a tube for feeding substrate/withdrawal of reactor material, a gas outlet and a central placed

Table 6 Samples, extraction solvents and analysis used in this study

Sampling point	***Sample***	***Analysis***			
		TOC, NH_4^+, Tot N, Tot P	***Microtox***	***Umu-C assay***	***EROD assay***
Ingoing sludge	IN	Water phase	Water phase	Water phase	Toluene (Soxhlet)
Digested sludge	Out	Water extract	Water extract	Water extract	Toluene (Soxhlet)
Sludge residual	Res	Water phase	Water phase	Water phase	Toluene (Soxhlet)
Bed material	Bed	Water extract	Water extract	Water extract	Toluene (Soxhlet)
Outgoing water	Wat	Native water	Native water	Native water	Toluene

The material used in the analysis is in bold in the first column. The first row in bold and italics describes the analysis used in the study, and the cells from the second row and column describes the extracts used.

impeller (Ø:70 mm) for mixing. Mixing was performed in 15 min intervals four times a day and for about 10 min in connection to feeding by use of a servomotor (MAC050-A1; All motion technology, New York, NY, USA) at 500 rpm.

Digester D5 was inoculated with 2.5 L digested sewage sludge and 500 g of cow manure followed by the addition of 1 L deionized water. The following day, 200 g of digester liquid was withdrawn followed by the feeding of 200 g of a substrate blend consisting of the industrial sludge (2.0 g VS/L/day), oat 0.25 (g VS/L/day) and deionized water. The same feeding procedure was done for 28 days. To start reactor D6, digested sludge (200 g per day) from reactor D5 was collected for the last 10 days and transferred to a digester. At an active volume of 2 L in D6, 1 L digester liquid was transferred from D5 to D6.

Digester D5 was fed with the same substrate blend until an active volume of 4 L was resumed. The loading rate was allowed to increase with 0.5 kg VS/L/day every 5 days until 3 g VS/L/day was reached first with the industrial sludge and then 3 g VS/L/day with oat. Digester D5 was then fed with this substrate blend for 60 days when the experiment was terminated. Digester D6 was fed with the industrial sludge (2 g VS/L) and deionized water until an active volume of 4 L was resumed. The loading rate was then allowed to increase with 0.5 kg VS/L/day every 5 days until 3 g VS/L/day was reached. This loading rate was kept for the remaining experimental period of 60 days.

Gas production was recorded on daily basis. The methane content of the produced gas was measured once a week. The produced gas was collected in a balloon during 24 h, and the gas composition was determined using a portable gas detector (Gas data, GFM series, Whitley, Coventry, UK). Analyzed gases, besides CH_4, were CO_2, O_2 and H_2S. Samples were also taken from the reactor liquid; concentrations of individual VFAs (acetic, propionic, butyric, isobutyric, capronic, isocapronic, valeric and isovaleric acid) were determined twice a week by GC-FID [56], pH at least twice a week and TS/VS once a week following the protocols from Swedish Standard SS-EN 12176 and SS 028113, respectively.

Post-treatment in reed beds

Two lines of reed beds treating sludge from D5 and D6 were constructed. Three beds with 32 cm of sand and gravel, from top to bottom 10 cm of sand, 9 cm of coarse sand, 7 cm of gravel and 6 cm of small stones (Figure 4) with vertical flow, were constructed indoors with a volume of 25 L and a upper surface area of approximately 700 cm^2 (Figure 1). The beds were planted with common reed (*Phragmites australis*) and kept indoors under controlled conditions with 300 mmol photons/m^2 s^{-1} and loaded with sludge (diluted to 1.2% dry weight, 1.0 L/day); retention time was 2 h. The loading was continued within 3 months.

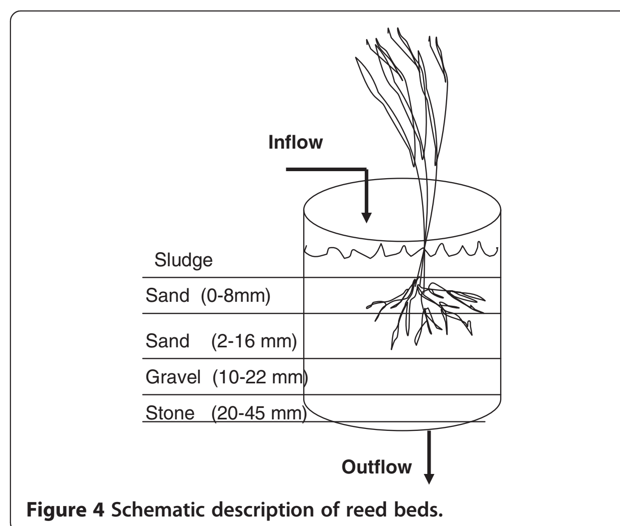

Figure 4 Schematic description of reed beds.

Sampling, preparation, extraction and cleanup

All tests in this study were performed on the undigested sludge, digested sludge and outgoing water (Figure 5) of the last month sample after 90 days of digesting. Outgoing water was collected daily and pooled into a monthly sample and stored at −20°C prior to analysis. Sludge residue on top of the reed beds and bed material (Figure 5) was collected at the end of the study.

Approximately 500 g of the bed material and sludge residual was collected from the upper part of the beds consisting of sand. Only the upper layer was collected

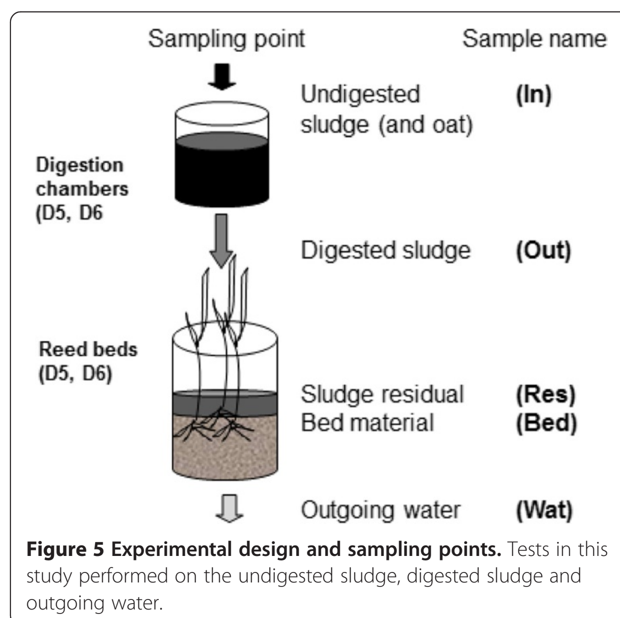

Figure 5 Experimental design and sampling points. Tests in this study performed on the undigested sludge, digested sludge and outgoing water.

for the experiments based on the assumption that most of the compounds will be trapped in the upper part of the beds due to the large specific area of this bed material compared to the coarse material below the upper part. In brief (Figure 5), ingoing sludge (In), digested sludge (Out), sludge residue (Res), bed material (Bed) and outgoing water (Wat) were extracted with water and toluene (Table 6) according to a former study in order to estimate both the bioavailable toxicity and the total toxic potential, respectively [30].

For toxicity testing of sludge and bed material, a Soxhlet extraction was conducted (24 h, 3 cycles/h) by using toluene (Riedel-de Haën, >99.8% (GC)), Envisolv according to [57]. The toluene extracts for the EROD assay were cleaned up using a multi-layer silica column in order to isolate the persistent lipophilic compounds according to the protocol shown in [58] and [59]. The silica column consisted from the bottom of 1 cm^3 copper powder to precipitate the remaining sulphate and 5.3 cm^3 KOH, 0.88 cm^3 neutral silica, 5.3 cm^3 40% H_2SO_4, 2.65 cm^3 20% H_2SO_4, 1.76 cm^3 neutral silica and 1.76 cm^3 $NaSO_4$ (monohydrate). The remaining fraction contained persistent dioxin-like compounds and included, e.g. PCDDs/PCDFs and PCBs and were eluted with *n*-hexane. The solvents were evaporated under a nitrogen stream, and the sample was transferred to DMSO (Sigma assay (GC) minimum 99.5%, Sigma-Aldrich, St. Louis, MO, USA) for the subsequent EROD assay.

The ingoing sludge, bed material and sludge residue were also prepared for analysis of toxicity, TOC, N_{tot}, NH_4^+ and P_{tot} by shaking with 1:5 proportion of deionized water for 24 h followed by centrifugation at 5,700 × *g*. The water-phase supernatant was used for testing. The outgoing water from the beds was collected, and 100 ml from each time point was pooled to a monthly sample and stored at −20°C until analysis. The water was centrifuged and tested undiluted.

Analysis of organics and metals were performed of the ingoing sludge of reactor D6 (Table 2) at the commercial laboratory, ALS Scandinavia AB, Luleå, Sweden (ALS).

Toxicity tests

Microtox

Toxicity to *Vibrio fischeri* of water extracted samples was assessed according to the Microtox® ISO 11348–3 test protocol (2007) by using Microtox Omni™ Software (Azur Environmentals, Newark, DE, USA). The samples and two controls consisting of deionized water were adjusted to a salinity of 2 ppt. Light inhibition in the sample compared to the control was measured after 30 min of incubation. Samples were diluted by a 1:2 series, and each dilution step was prepared in duplicates. The sample concentrations tested were 80%, 50%, 33.33%, 25%, 16.67%, 12.50%, 8.33% and 6.25%. EC_{50} values (30 min) were determined from concentration-response curves. Toxic units TU (*g*/*g* dw) was calculated using the formula: $TU = 1/(EC_{50} \times 100)$.

Umu-C

Genotoxicity of water extracts from the sludge and bed material as well as outgoing water from the beds was detected using the Umu-C test with *Salmonella typhimurium* TA1535/pSK1002 according to the standard protocol ISO 13829 (2000). The bacteria were cultured in tryptone/glucose medium in 96-well plates (Labdesign, TCT, Lake Charles, LA, USA). All concentrations were tested in triplicates. As positive and negative control, 50 μg 4-nitroquinoline-1-oxide (4-NQO)/L and pure medium was used, respectively. Induction of genotoxicity, expressed as β-galactosidase activity was measured as the absorbance at 420 nm after 2 h of exposure followed by 2 h post-incubation. Growth was measured as the absorbance at 600 nm. Absorbance was measured with a microplate reader (Expert 96, MikroWin 2000, Asys/Hitech, Eugendorf, Austria). The result was calculated as an induction ratio related to growth in Equation 1.

$$\text{Induction ratio} = \left(1/\text{Growth}_{\text{Abs600 nm}} \times \left(\text{Samples}_{\text{Abs 420 nm}}/\text{Control}_{\text{Abs420 nm}}\right)\right) \quad (1)$$

The test was considered valid if the growth factor at a wavelength of 600 nm of exposed bacteria versus negative control was not below 0.5 and the induction ratio measured at 405 nm of the positive control was at least twice compared to the negative control. The samples were considered genotoxic if the induction factor exceeded 1.5 (exposed bacteria versus negative control) measured at 405 nm.

EROD assay

Induction of 7-ethoxyresorufin-O-deethylase was measured in the CYP 1A expressing permanent fish cell line RTL-W1 (rainbow trout liver, *Oncorhynchus mykiss*). Cells were obtained from Dr. Niels C. Bols (University of Waterloo, Canada) [60] and maintained at 20°C in 75 cm^2 plastic culture flasks (TPP, Trasadingen, Switzerland) without additional gassing in Leibovitz medium (L15) supplemented with 9% foetal bovine serum (Th. Geyer, Renningen, FRG), 1% penicillin/streptomycin (Sigma-Aldrich). Induction of EROD was measured in confluent cell monolayers in 96-well microtiter plates (TPP) with 3 to 4×10^5 cells/ml according to a previously published method [61,62]. Before exposure to the sludge extracts, cells were seeded in 96-well microtiter plates at a density of 3 to 4×10^5 cells/ml and allowed to grow at 20°C to confluency for 72 h. Subsequently, the medium was removed and the cells were exposed for 72 h with the sludge extracts water dilutions in L15 medium, negative control (L15 medium) and positive

control using 100 to 3.125 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, Sigma-Aldrich, Deisenhofen, FRG). After exposure, all plates were shock-frozen and stored at −80°C for at least 1 h until EROD measurement.

For measurement of the EROD activity, the plates were thawed for 10 min, the protein standard solution (10 to 1.25 mg/ml bovine serum albumin (Sigma-Aldrich) in phosphate buffer (0.1 M Na_2HPO_4-solution; Malinckrodt Baker, Deventer, Netherlands) adjusted to pH 7.8 with 0.1 M NaH_2PO_4-solution (Merck, Darmstadt, FRG) was added in triplicates and 100 to 3.125 nM resorufin standard (Sigma-Aldrich) in phosphate buffer was added in duplicates. The 7-ethoxyresorufin solution (100 μl, 1.2 μM, Sigma-Aldrich) was added to each well except the wells containing either the protein standard or the resorufin standard. The plates were incubated for 10 min. NADPH (50 μl, 0.09 mM, Sigma-Aldrich) was added to all wells and the plates were incubated 10 min at room temperature. The deethylation reaction was stopped by adding 100 μl of 0.54 mM fluorescamine (in acetonitrile) to each well. The production of resorufin was measured in a fluorescence plate reader (TECANinfiniteM200, Tecan Austria GmbH, Grödig, Austria; excitation 544 nm, emission 590 nm) after 15 min. The EROD activity was expressed as picomole resorufin produced per milligramme protein per minute (pmol/(mg protein/min)). Protein was determined fluorometrically using the fluorescamine method (excitation 355 nm, emission 465 nm) [57,63]. Concentration-response curves and EC_5, EC_{10} and EC_{25} values were calculated using non-linear regression analyses of GraphPad Prism 5.0 (GraphPad, San Diego, USA).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
LG designed and conceived of the study, collected and prepared the samples, carried out the microtox, participated in the EROD assay and the Umu-C assay and drafted the manuscript. SH carried out the EROD assay. JE carried out the digestion part, and VR performed the Umu-C assay. HH participated in its design and coordination and helped to draft the manuscript. SK participated in the design of the study, supervised the study in Aachen and performed the statistical analysis. All authors read and approved the final manuscript.

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