Lipolysis of Domestic Wastewater in Anaerobic Reactors Operating at Low Temperatures

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Poor breakdown of lipids is a major barrier to the anaerobic treatment of domestic wastewater at low temperatures. Lipids are degraded by extracellular lipases that are produced by, as yet, unknown bacteria. We wished to know if the poor degradation of lipids at low temperatures is due to a lack of lipase or to a lack of lipase activity. We therefore conducted an assay of the lipase activity in biomass adapted to the treatment of settled domestic wastewater at 4, 8 and 15 °C and compared this with the activity of the same biomass at 37 °C. Lipase activity decreased with temperature and was unmeasurable at 4 °C. When the lipase activity in the biomass was evaluated at 37 °C, the lipase activity was 21.7±14.0% greater in the biomass adapted to 4 °C than in the biomass adapted to 15 °C. We conclude that enzymes are produced at low temperatures but that the activity at these temperatures is low. Settled and unsettled wastewaters were used as controls and we were surprised to see lipolytic activity in the unsettled wastewaters at low temperatures. The activity was too high to be accounted for by the presence of biological detergents and we infer that is caused by bacteria naturally present in domestic wastewater. The presence of lipolytic activity at low temperatures was associated with the presence of members of the genera *Trichococcus* and *Devosia*, and the families *Caldilineaceae* and *Bacteroidaceae*.

1. Introduction

The use of anaerobic processes to treat domestic wastewater at low temperatures would be a revolutionary advance for the global water industry¹. Lipid hydrolysis (lipolysis) is arguably the most important single barrier to that goal. Lipids are a major fraction of the COD (up to 40%) of municipal wastewater². However, in studies of the anaerobic treatment of domestic wastewater at low temperatures lipids tend to accumulate whilst carbohydrates and proteins are typically removed^{3, 4, 5, 6}. However lipolysis is rarely studied in this context.

The hydrolysis and fermentation of lipids leads to the formation of VFA intermediates that are then used for methane production⁷. Lipases are of critical importance to this process as they are responsible for the hydrolysis of triacylglycerol and other lipids/fats. Limited lipolytic activity may lead to a reduced energy recovery, failure to meet the effluent discharge standards and to lipid accumulation in the reactor's mixed liquor⁸. Lipids can also form

agglomerates that settle in the bioreactor⁶, this can lead to biomass deterioration, washout^{9, 10} and treatment failure.

Most lipases consist of a catalytic triad formed by the amino acids serine-histidine-aspartate/glutamine¹¹ and a "lid" that controls access of the substrate based on hydrophobicity. Various mechanisms have been identified that allow these enzymes to function at low temperatures^{12, 13, 14, 15}. This supports the premise that lipid hydrolysis in anaerobic wastewater treatment at low temperature can be achieved in the presence of organisms that produce such enzymes.

The bacterial community participating in hydrolysis and fermentation of fats is complex and poorly understood^{16, 17}. We only have an indication of which phyla may play a role. *Bacteroidetes*, *Spirochaetes* and *Firmicutes* are the predominant phyla in anaerobic bioreactors treating fatty wastewater at mesophilic and thermophilic conditions^{18, 19}. These phyla were also observed in the microbiota that developed in mammalian, aquatic and serpent organisms' guts^{21, 21, 22, 23}. Far more detailed and sophisticated studies are required to confidently determine which species and genera produce which enzymes.

However, at this stage our first priority is fundamental information about the effect of temperature on the rates of lipolysis. In particular, if lipid accumulation can be attributed to the absence of

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enzymes, or merely the absence of enzyme activity. This study addressed that lacuna.

2. Materials and Methods

2.1. Reactor set up

Reactors; seven quick fit 1-L glass batch reactors (Sigma Aldrich, UK) were assembled and incubated anaerobically at 4, 8 and 15 °C ($n = 2$, 2 and 3 for 4, 8 and 15 °C respectively). Mixing was only applied prior to sampling, at 140 rpm using a stainless-steel stirrer paddle to minimize likelihood of disturbance of the syntrophic communities^{24, 25, 26, 27, 28, 29}. The reactors were prepared based on Petropoulos *et al.*, 2017³⁰ and seeded volumetrically (250 ml) with an equal mixture of putatively cold-adapted sediment from Lake Geneva “N 46°23'04”, E 6°25'07” (average temperature -11 – 17 °C) and soils from Svalbard, in the high Arctic at various sampling points situated at “N78°, E11, 15,16°” (average temperature -16 – 6 °C). Reactors were operated at 4, 8 and 15°C for 1073 days to treat seven series of batches of wastewater prior to re-feeding for the 8th batch reported in the current study. The inoculum was re-fed with wastewater after the plateau phase in both methane and COD was reached. Re-feeding of the 8th batch followed the same strategy as for the previous batches (centrifugation (Cryofuge 5500i, Thermo Scientific, UK) at 4000rpm for 20 minutes at 10°C – emptying of the supernatant – seeding/feeding (seed:substrate 1:3 v/v, domestic wastewater)).

2.2. Substrate

Wastewater: wastewater was collected from the Tudhoe Mill (County Durham, UK) wastewater treatment plant (WWTP), which treats domestic wastewater. Prior to feeding the samples were stored at 4°C for 7-14 days. The first 5 batches were fed with raw wastewater of varying COD concentrations (600-1000 mg.L⁻¹) with a consistent composition of 45% lipids 35% carbohydrates and 20% proteins. For the subsequent batches primary settled influent was used (<300mgCOD.L⁻¹) to represent the wastewater stream that enters to the biological process, no pH adjustments were made. The composition was 60% carbohydrates and 38% lipids; proteins were less than 2%. The wastewater for the batch experiment reported here had a COD of 286±29 mg.L⁻¹ and sCOD of 99(±10) mg.L⁻¹ allowing an OLR of 0.29kgCOD.m⁻³.d⁻¹. The substrate's total suspended (TSS) and volatile (VSS) solids were gravimetrically estimated²⁷ from a 50 ml sample as 133(±14) mg.L⁻¹ and 83(±8) mg.L⁻¹ respectively. Anions (SO₄²⁻, NO₃⁻) after filtration (0.45µm) and analysis (Dionex, ICS-1000 Ion Chromatograph fitted with AS40 Automated Sampler) were found equal to 0.9 and 92 ppm for NO₃⁻ and SO₄²⁻ respectively. The reactors for this batch (batch 8) were monitored for 52 days after ensuring that a steady population and performance was achieved, analogous to the seven previous batches^{6, 26}. The feed wastewater was exposed to UV irradiation at a dose of 110 kJ.cm⁻²²⁶. The rationale behind sterilization was primarily to exclude microorganisms originating from the ‘mesophilic’ wastewater. The estimation of the lipid, protein and carbohydrates content of the biomass was carried out prior to re-feeding for the 8th batch (Day 1073). Regardless this prolonged incubation the 8 so far applied HRTs

highlight that the study aims in describing the limited hydrolysis phenomenon during the initial step of adaptation.

2.3. Chemical analysis

Proteins: The protein content of the mixed liquor and of the biomass (after lysis) was spectrophotometrically measured³¹, based on the interaction between protein and Coomassie Brilliant Blue G-250 (595nm) at unfiltered samples. A spectrophotometer from Merck (UK) was employed to measure the absorbance of both samples and standards with known BSA (Bovine Serum Albumin) protein concentration (Sigma Aldrich (UK)). The conversion to COD was carried out using (C₄H_{6.1}O_{1.2}N)_x as a point of reference³². The conversion of the proteins to cells was carried out based on the estimation of the proteins that are present in the mixed liquor and the assumption that 1 cell contains 2.35×10⁶ proteins (calculated based on the assumptions presented in Table 1; a similar value was proposed by Rittman and McCarty, 2001³³).

Lipids: The lipids content of the biomass was estimated gravimetrically using a methanol:chloroform extraction protocol³⁴ at a ratio 1:2. 1g of KCl was also added and mixed based on the optimized protocol suggested by Folch *et al.*, (1957)³⁵. 10ml of mixed liquor was used as sample volume for the particular study's lipid content. The conversion to COD was carried out stoichiometrically using octanol (C₈H₁₈O) as a point of reference. The sample size and the power of the test were estimated using the Minitab 17 statistical software (Minitab Inc., State College, PA, USA).

Carbohydrates: Carbohydrates content was estimated by the anthrone method, a colorimetric method firstly reported by Hedje and Hofreiter (1962)³⁶ (1:4 sample:anthrone solution (dissolved in cold 95% H₂SO₄)). The concentration was estimated using a spectrophotometer from Merck (UK) at 630nm, standardizing a curve with known amounts of glucose. The conversion to COD was implemented stoichiometrically using glucose (C₆H₁₂O₆) as a point of reference.

VSS: The VSS content of the biomass was estimated gravimetrically as above, based on APHA, 2006³⁷.

Methane: CH₄ was monitored in the gas phase as % by volume, using gas chromatography. Samples of gas (100 µl) were removed from the headspace using a gas-tight syringe (SGE-Europe), and injected onto a Carlo Erba HRGC S160 GC fitted with an FID detector and HP-PLOTQ column (0.32 mm diameter, 30 m length and 20 µm film). The known headspace volume was used to convert the GC-measured gas concentrations into volume and COD_{CH₄} concentrations. In the calculation, the liquid dissolved gases were estimated using the Henry coefficients (Henry coef.CH₄: 433.6, 481.6, 574.8 at 4, 8 and 15 °C respectively³⁸. The Henry coefficients were used assuming equilibrium between the gas and the liquid phase. The production rates were expressed as COD mass that can be methanized per both gVSS in the mixed liquor and per cell estimated from the amount of proteins present in the mixed liquor (as described later).

VFA: Samples from the liquid phase were removed from reactors using sterile syringes, transferred to sterile 2 ml micro-centrifuge tubes and centrifuged (3 min at 13,000 × g) to obtain a supernatant for analysis. Volatile fatty acid concentrations in the supernatant were analysed by ion exchange based on a modified standard method as previously described³⁹. The detection limit of this instrument was 0.8 ppm.

Enzyme extraction: the lipolytic activity protocol was based on Gessesse et al., (2003)⁴⁰. 10ml of mixed liquor was taken from the reactors and were subjected to detergents (Triton X-100 and EDTA solution (optimised at the same study) before sonication (Labsonic, UK) to achieve cell lysis.

Enzyme assay: similarly, the assay protocol was based on Gessesse et al., (2003)⁴⁰ and Kim et al., (2012)⁴¹. *p*-Nitrophenyl palmitate (pNPP) (Sigma Aldrich, UK) was selected as substrate (*p*-NPP assay as described by Winkler and Stuckmann, (1979)⁴²). The main principle of the *p*-NPP assay is the estimation of the *p*-NP (*para*-nitrophenol) that is released following the 1:1 mole:mole reaction as a result of enzymatic hydrolysis of *p*-NPP^{43, 44, 45}.

20mM of stock was prepared in Isopropanol (Sigma Aldrich, UK). The solution was further diluted 1:20 in 20mM Trizma (Tris HCl, pH 8.0) Buffer (Sigma Aldrich, UK) containing 0.1% Gum Arabic and 0.4% Triton X-100 (Sigma Aldrich, UK).

Enzyme extract and substrate were set into a plastic cuvette (VWR, UK) at a 1:10 ratio. Samples were prepared in duplicates and incubated at the corresponding operational temperatures of 4, 8 and 15 °C. Additional samples were set at 37 °C. Unsterilized primary settled wastewater controls were also prepared to identify whether or not the activity originated from the cells in the seed or from the cells in the wastewater. An additional assay was carried out on raw wastewater samples. These WW controls were added to check our previous finding that wastewater originated cells work in synergy with the cells of the seed, contributing to wastewater hydrolysis. Controls with only *p*-nitrophenyl palmitate were also prepared and tested at all temperatures.

The cuvettes were set into a spectrophotometer (Merck, UK), and the absorbance was measured at 410nm. The readings were made every 20 minutes for the 1st hour and less frequently after that. The correlation was estimated by the curve formed by known concentration of *p*-nitrophenol standards. The standards were tested at all operational temperature for time: 0 and after 30 minutes to examine whether there is a change in their structure of the compound after a certain time. As no difference was observed, the curves for time 0 were used. The results were expressed in units (U) of lipase activity, which corresponds to the amount of enzyme that hydrolyses 1μmol of *para*-nitrophenyl (or produces 1 μmol of *para*-nitrophenol) per minute. The activity was expressed per ml of mixed liquor, per gVSS and per mg protein. The activity U was also expressed as COD, the conversion was stoichiometrically estimated. For the evaluation of the *k* coefficient of the lipolysis from the inoculum further normalization of its activity was carried out as per

the minimum concentration of proteins measured (for primary settled wastewater).

The lipase assay was carried out on the 52nd day of the 8th batch, at the very early stage of the plateau phase in methane production to ensure that activity would be at the highest possible level.

2.4. Community structure analysis

DNA extraction: Biomass samples were obtained from the pellets produced during the preparation of samples for VFA analysis, as described. Total genomic DNA was extracted from biomass pellets collected during the VFA sampling using a FastDNA[®] SPIN for soil kit (Q-BIOgene, Cambridge, UK) according to the manufacturer's instructions.

Amplification: A 411-base-pair (bp) section of the 16s rRNA gene spanning the V4-V5 region (*Escherichia coli* positions 515 to 926) was selected for amplification following the protocol from McCann et al., (2016)⁴⁶.

Following PCR, samples were purified with PCR Purification Kit (Qiagen, UK) according to the manufacturer's instructions and eluted in 50 μl EB buffer (10mM Tris Cl, pH 8.5) (Qiagen, UK).

Library quantification; The concentration of DNA in purified PCR samples was then quantified using Quant-iT[™] PicoGreen[®] dsDNA Reagent (Invitrogen, UK) using a Nanodrop 3300 Fluorospectrometer (Thermo Scientific, Wilmington, USA).

Sequencing and bioinformatics; Sequencing was carried out by NERC Biomolecular Analysis Facility in the University of Liverpool (Liverpool, UK) using the Roche 454 sequencing GS FLX Titanium Series. The 16s rRNA gene data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 pipeline⁴⁷. Briefly, the sequences were binned into OTUs using a 97% identity threshold, and the most abundant sequence was selected as a representative sequence for each OTU. The microbial taxonomy was assigned to OTUs against a subset of the GreenGene database (13.8 version). UPARSE was used to align OTU, trim quality and remove chimera OTU⁴⁸; <https://www.drive5.com/uparse/>). We obtained 603359 sequences in total, and 6118 to 41454 sequences per sample. The sequencing data were randomly pruned to 6100 per sample for downstream analysis⁴⁹. The differences in the bacterial communities between reactors and wastewater were statistically verified in the STAMP[®] platform; heatmaps show data matrices where colouring gives an overview of the numeric differences at genus level. The detailed significance between different genera was checked using White's non-parametric *t*-test.

3. Results

Accumulation phenomena: After 1023 days and eight batches of feed, the lipids, carbohydrates and proteins in the mixed liquor were quantified and expressed in units of COD (Fig. 1.a). Lipids

accumulated in the bioreactor to a higher degree than proteins and carbohydrates at all temperatures (4, 8 and 15 °C).

Proteins and cell content: In principle, the amount of bacterial biomass in the reactor can be measured by both the protein and the VSS concentrations in the mixed liquor (Table 1). However, the presence of plant material in the inoculum could have interfered with the latter biomass measure. We therefore only used the protein content approach to estimate the cell abundance (Table 1). Both proteins and VSS appeared to highest in the 8 °C reactors but the differences between reactors were not statistically distinguishable ($P > 0.05$).

Reactor efficiency: After an acclimation period ≈ 400 days the reactors showed satisfactory COD removal efficiencies irrespective of the wastewater type (raw or primary settled) (effluent quality and corresponding retention times after acclimation in Table 2). The COD:CH₄ conversion ranged between 40–60% at 4 °C whilst at 15 °C it ranged from 50–90%^{6,26} of the theoretically expected value. Methane was produced at all temperatures on all wastewater batches (Fig. 1b); importantly, considerable methane production rates were achieved at low temperature (4 °C). The methane in the aqueous phase did not exceed the 1% of the measured in the headspace volume. The putative cell specific methanogenic activity was estimated as 20.1 ± 3.2 , 8.0 ± 1.6 and 23.7 ± 5.7 fgCOD.cell_{protein}⁻¹.day⁻¹ for 4, 8 and 15 °C respectively.

Lipase activity of the seed: The lipase activity at all operating temperatures (and at 37 °C) is shown in Table 3. As expected, lipase activity decreased (and lipid accumulation increased) as the temperature decreased (Fig. 2; Fig. 3). However, when measured under the same conditions (37 °C) (Table 3) the lipases from the 4 °C reactor were found to be equally or, plausibly, more active ($P < 0.1$) than those formed from the biomass acclimated at 8 and 15 °C respectively. The effect of temperature on enzyme activity appeared to vary from reactor to reactor (Fig. 4). To facilitate comparisons with other studies the activity was also expressed: per gram VSS (U.gVSS⁻¹ or $\mu\text{mol pNPP.gVSS}^{-1}.\text{day}^{-1}$) and specific activity (fgramCOD.cell⁻¹.day⁻¹) at 4, 8, 15 and 37 °C (Table 3).

Effect of temperature on the properties of the substrate: Temperature not only affected the enzyme kinetics but also the physical properties of the lipids used in the assay. The optical density (OD) of the lipids (p-nitrophenyl palmitate) was affected by the temperatures at which the assays were undertaken. The OD values after 20 minutes' incubation were 1.04, 0.64, 0.61 and 0.27 at 4, 8, 15 and 37 °C respectively.

Lipolysis and methanogenesis: Lipolysis was not detectable at 4 °C and therefore may be regarded as the rate limiting step at this temperature. The concomitant increase in lipid accumulation was observed (Fig. 3).

Lipase activity of the substrate: Lipolysis activity in primary settled wastewater was negligible (~ 0.00) at 4, 8 and 15 °C but evident at 37 °C (Table 3). By contrast the raw non-settled wastewater showed

considerable lipolytic activity ranging from 0.45 to 0.68 U.ml⁻¹. The raw WW activity was hardly affected by temperature (Fig. 4) and was found to be similar or higher than to the activities observed for the mixed liquor solids in the reactors (Table 2).

Bacterial community structure: The bacterial sequences were dominated by ten phyla (SI. Figure I) with *Proteobacteria* the predominant phylum at all operational temperatures. *Firmicutes* and *Chloroflexi* were the second most abundant groups, with the former more abundant at lower temperatures versus the latter at higher temperatures. The phyla that were detected in the raw wastewater sample (SI. Figure I) were similar to those identified in the reactors, except for the absence of the *Chlorobi*, the presence of the *Spirochaetes* and the predominance of *Bacteroidetes*.

At a family/genus level the picture remained unchanged for the inoculated reactors (Similarity $< 90\%$; $P: 0.05$), the differences between the cells originating from wastewater and those from the inoculum were more distinct though (Figure 5; similarity $< 20\%$; $P < 0.05$). The genera *WCHB1-05*, *SHD-231* and unclassified *Bacteroidaceae*, *Caldilineaceae*, *Clostridiales* were the most abundant groupings in wastewater, and almost absent ($P < 0.05$) the reactors at all temperatures (abundance $> 15\%$). This, combined with the presence of *Devosia*, *Fusibacter*, *Thichococcus*, *Acetobacterium* and *Phenylobacterium* could possibly explain the origins of the lipolytic activity observed in the wastewater.

4. Discussion

4.1. The limit of wastewater lipolysis

It is well known that lipids accumulate at low temperatures in anaerobic wastewater treatment systems^{4, 6, 8, 50, 51}. However this is the first study to quantify the effect of temperature on the rate on the lipolysis of domestic wastewater. This is a valuable first step towards tackling an important barrier to the use of anaerobic methods to treat wastewater at low temperatures.

Our work indicates that this accumulation of lipids is due to a lack of lipolytic activity in the biomass and not a lack of an indigenous capacity to produce lipase. The close association between lipase activity and both methane production and lipid accumulation at differing temperatures is clear evidence that enzyme activity is contributing to both phenomena. This does not preclude the possibility that some other temperature related mechanism is also affecting lipid accumulation (e.g. changes in the lipid structure based on the measured O.D. of the p-nitrophenyl palmitate). But there is no other obvious additional mechanism.

4.2. The lipolysis activity from the seed

At first glance, the lipase activity in our biomass appears at temperatures $> 4^\circ\text{C}$. The rates are high when compared with activities in biomass from conventional digesters and activated sludge: 60 and 65⁴¹ and 300⁴⁰ U/g VSS at 37 °C respectively (versus 401, 506 and 793 U/gVSS at 37 °C in this study). Even at 8 and 15 °C

our adapted bacteria had comparable activity (35 and 68 U/gVSS) to mesophilic and thermophilic biomass at warm temperatures. Higher rates could have been achieved if the system was operated in a high rate feeding regime in the presence of higher cell abundances.

Nevertheless, the drop in hydrolysis rates with temperature is the main barrier to the anaerobic treatment of domestic wastewater in temperate climates. Even in our supposedly “adequately acclimated to cold” biomass, taken from the arctic and adapted to wastewater treatment, the loss of lipolysis is disproportionately responsible for the loss of hydrolysis at lower temperatures. The difference between the cell specific rates of lipid hydrolysis and the overall rates of cell specific hydrolysis (as estimated by Petropoulos et al., 2017³⁰) drop at temperatures <15 °C and stop at 4 °C.

However, this is not due to a lack of lipase *per se*, but rather due to a lack of lipase activity: The same protein extracts that were inactive at 4 °C displayed considerable activity when tested at 37 °C. What is less clear is why the activity at low temperature was so poor for our adapted biomass. We noted a considerable increase in the optical density in the lipid containing enzyme assays at low temperatures. This leads us to suspect that the increased rigidity of lipids at low temperatures⁵² is important and could prevent the enzymes from accessing their targets in the solidifying substrate.

If lipid removal, and thus lipase activity, is key to a successful anaerobic treatment of domestic wastewaters at low temperatures the nature of the lipids in the feed is potentially critical. We do not yet know if the fats used in artificial wastewaters (for example soya oil and milk powder) will faithfully represent real wastewaters. Disintegration-related parameters are also potentially important, but are rarely taken into consideration⁵³. However, we speculate that the putative greater diversity of lipids in real wastewaters may require a greater diversity of enzymes for successful treatment. By the same token, an improved picture of lipolytic activity would be garnered using a realistic mixture of many lipid substrates in enzyme assays. Numerous studies^{12, 21, 40, 41, 54} have previously relied on p-nitrophenyl palmitate as a proxy for the most difficult to degrade lipid (saturated fatty acid, melting point: 63 °C, solubility: 0.000145 mg.L⁻¹ at 25 °C).

4.3. The lipolysis activity from the wastewater

The discovery of a background level of lipolytic activity in unsettled wastewater was an unexpected benefit of including real wastewaters in this study. Although the activity levels are low, they could contribute to the treatment of wastewaters at low temperatures. The origin of this activity is unknown and curious in two respects: (i) the activity is almost unaffected by changes in temperature and (ii) is largely removed by primary sedimentation. The second point implies that the activity is associated with larger solids and is supported by the difference of the protein content between the WW at the two steps. The reduction of enzyme activity in settled wastewater has been previously observed⁵⁵, but the capacity of these enzymes was not previously defined.

An intriguing hypothesis is that some of the lipase activity is derived from enzymes in detergents. Lipase activity in a domestic detergent was also found to be relatively insensitive to temperature⁵⁶ and this could be a signature of detergent related lipases in wastewater. However, the same study found there to be 122 U of lipase activity per gram of washing powder; this is only enough to provide 0.0011 U of lipase activity per ml (assuming 5Kg of detergent per person per year and 150 liters of water per person per day⁵⁷). This is too low to account for the lipase activity seen in our raw wastewater. This implies that some of the raw-WW originated cells could express enzymes that have evolved to function at low temperatures^{12, 13, 14, 15}.

4.4. Community structure and metabolism of lipids

It therefore seems more likely that this lipase activity is associated with the ecology of the sewer. It is plausible that bacteria present in the wastewater and the sewer system produce the low temperature lipolytic activity. Two phyla, *Bacteroidetes* and *Spirochaetes* that have been reported in reactors treating fatty wastewaters^{18, 23, 58} were indeed present in the wastewater and notably absent from the reactors. Our sequencing data suggest that *Trichococcus* and unclassified *Caldilineaceae* and *Bacteroidaceae* are likely to be the link with the high lipolysis observed in wastewater^{59, 60, 61}. We suspect that *WCHB1-05* is also important in this respect as this genus is often observed in reactors poor in available organic material^{62, 63}, a situation very likely in our reactors where part of the organic material was unavailable (lipids) at low temperatures. The genus *WCHB1-05* is also associated with bioremediation of hydrocarbons⁶⁴, this increases the likelihood of its potential to degrade other oils/fats as well⁶⁵. All these genera were found to be present in only low numbers in the inoculated reactors ($P < 0.05$) in which the families of *Bacteroidales*, *Planococcaceae* and *Conexibacteriales* were the predominant ones; the two first were absent from the wastewater.

The presence of a cold-adapted/psychrophilic genus *Devosia* in the wastewater^{99, 67} could be significant^{68, 69}. Significant enzymatic (but not lipolytic) activity has been observed in sewer biofilms⁷⁰; this phenomenon should be revisited as it could help us develop low temperature anaerobic treatments.

A definitive answer to the source of the enzymes for hydrolysis in general and lipolysis in particular would benefit from a coupled metagenomics and metaproteomics study. Linking lipolysis with specific species, especially in raw WW could be the next step to overcome decelerated lipolysis at low temperature (e.g. via DNA-SIP). Unfortunately, we were unable to visualise proteins on an SDS gel using established of protein extraction methods. Metaproteomics, is a rapidly evolving field and we expect the challenge will succumb to a more sophisticated approach^{71, 72}.

5. Conclusions

This is the first study of the effect of temperature on the rates of lipolysis in domestic wastewaters. Lipid hydrolysis, and specifically reduced lipolysis activity, but not the absence of lipase is the main factor limiting the removal of COD in the anaerobic treatment of domestic wastewater at low temperature. There is an unknown, and unexpected, source of lipolytic activity in unsettled wastewaters, that appeared to be almost unaffected by temperature. We do not know which bacteria produce the lipases but we suspect *Firmicutes*, *Spirochaetes* and *Bacteroidetes* to be the key phyla, and specifically the genus *Trichococcus* and the families *Caldilineaceae* and *Bacteroidaceae*. However, a study of the metaproteomics and metagenomics of the biomass is needed to determine which organisms produce which enzymes. For the time being, we recommend that anaerobic reactors treating domestic wastewater at low temperatures are fed with unsettled wastewater.

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Conflicts of interest

There are no conflicts to declare.

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Fig. 1 – a) Lipids, carbohydrates and proteins in the mixed liquor of reactors treating domestic wastewater at 4, 8, and 15 °C; b) Daily methane formation rate per gram of mixed liquor VSS; error bars account for statistical error after nested analysis of variance, $n = 2, 2$ and 3 for $4, 8$ and 15 °C respectively.

Fig. 2 – Normalized lipase activity for the seeded reactors from ‘4’, ‘8’, ‘15’ incubated at the corresponding temperatures and at ‘37’ $^{\circ}\text{C}$ for all samples; normalization ($U.\text{ml}^{-1}$) to the minimum protein content detected in the study (ps WW: $0.2 \text{ mg}.\text{ml}^{-1}$) for comparison of activities with different protein abundance - 4 $^{\circ}\text{C}$ was set as twice reduced from the minimum detected ($0.005 \text{ U}.\text{ml}^{-1}$); error bars account for statistical error after nested analysis of variance, $n = 2, 2, 3$ and 2 for, 4, 8, 15 and 37 $^{\circ}\text{C}$ respectively.

Fig. 3 - Relationship between methane formation rate (grey markers), lipid accumulation (white markers) and lipolysis rate at 4, 8 and 15 °C (rhombus, triangle, square in dark grey respectively); error bars account for statistical uncertainty (95%) after nested analysis of variance, $n = 2, 2$ and 3 for 4, 8 and 15 °C respectively.

Fig. 4 - K Arrhenius plot for all enzyme activities extracted from the reactors at all temperatures, and for raw wastewater, incubated at 8, 15 and 37°C. All activities were normalized based on the minimum protein content (WW: 0.2 mg.ml⁻¹); sample at 4°C was excluded (0.0 U); trendline equations from top to bottom correspond to 4, 15 and 8°C, far right for raw WW.

Fig. 5 – 16S bacterial heatmap; colour intensity gives an overview of the numeric differences at genus level; from left to right (dark grey to light grey; samples from WW $n = 1$, Reactor 15°C $n = 3$, Reactors 8°C $n = 2$; Reactors 4°C, $n = 2$)

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Table 1 - Solids, proteins and bacterial abundance in the mixed liquor and the raw and primary settled (ps) wastewaters.

	VSS* (g.L ⁻¹)	mg protein ** .ml ⁻¹	N protein *** .ml ⁻¹	Σ cells **** .ml ⁻¹
4°C	5.48 (±1.18)	0.35 (±0.15)	7.0×10 ¹⁵	3.0×10 ⁹
8°C	7.09 (±1.05)	1.03 (±0.11)	2.1×10 ¹⁶	8.7×10 ⁹
15°C	5.78 (±1.11)	0.68 (±0.16)	1.4×10 ¹⁶	5.8×10 ⁹
ps WW	0.08 (±0.01)	0.20 (±0.02)	4.1×10 ¹⁵	1.7×10 ⁹
raw WW	0.51 (±0.20)	0.54 (±0.20)	1.1×10 ¹⁶	4.6×10 ⁹

*VSS content of the reactors mixed liquor; **protein content of the reactor samples after cell lysis; values in parenthesis stand for standard error, n = 2, 2, 2, 3 for WW, 4, 8 and 15 °C respectively. Assumptions made: ***one protein molecule weighs approximately 2×10⁻¹⁶ grams; Avogadro number: 6×10²³ Da.g⁻¹; average protein length l_{aa}: 300 aa.protein⁻¹ (E. coli and other bacteria); 1Da = 100 aa; 1 cell contains 2.36×10⁶ proteins (All assumptions based on Rittman and McCarty, 2001³³).

Table 2 – Average influent and effluent COD quality achieved after acclimation of the reactors to the corresponding temperatures (acclimation with raw WW after Batch 3 (day $\approx$ 400); with primary settled wastewater, n = 2, 2, 4 at 4, 8 and 15°C respectively); time stands for the time required to achieve the reported effluent quality.

	primary settled WW (mg.L ⁻¹)	time (d)	raw WW (mg.L ⁻¹)	time (d)
COD in	189 ($\pm$ 15.0)		763.3 ($\pm$ 23.6)	
COD effluent. at 4°C	116.3 ($\pm$ 8.5)	15-22	112.3 ($\pm$ 1.5)	37-54
COD effluent. at 8°C	100.0 ($\pm$ 2.0)	<9	124.3 ($\pm$ 7.0)	37-54
COD effluent at 15°C	81.0 ($\pm$ 20.1)	<9	75.3 ($\pm$ 10.9)	37-54

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Table 3 - Lipolysis activity per mixed liquor volume, per gram of protein extracted, per gram VSS of mixed liquor and per cell per day at all operation and incubation temperatures (4, 8, 15, 37°C, ps WW (wastewater), raw WW)

Temperature (°C)*	4	8	15	4-37	4-37	15-37	ps WW 37	raw WW 4	raw WW 8	raw WW 15	raw WW 37
U.ml _{mixed liquor} ⁻¹	0.0	0.25 (±0.04)	0.41 (±0.10)	2.79 (±0.59)	3.66 (±0.88)	4.78 (±1.05)	0.01 (±0.20)	0.45 (±0.00)	0.66 (±0.11)	0.60 (±0.12)	0.68 (±0.04)
U.mg _{protein} ⁻¹	0.0	0.26 (±0.05)	0.68 (±0.22)	11.46 (±0.68)	3.89 (±0.40)	8.96 (±1.72)	0.05 (±0.10)	0.84 (±0.07)	1.23 (±0.22)	1.11 (±0.21)	1.25 (±0.00)
U.gVSS ⁻¹	0.0	34.5 (±5.60)	67.5 (±16.0)	401.2 (±85.2)	506.3 (±122.1)	793.5 (±175.5)	124.3 (±238.0)	882.4 (±4.3)	1294.1 (±220.6)	1176.5 (±230.8)	1333.3 (±73.0)
fgCOD.cell ⁻¹ .day ⁻¹	0.0	115.7 (±17.8)	286.0 (±68.)	3821.4 (±812.0)	1698.7 (±407.0)	3361.1 (±742.2)	24.0 (±53.6)	397.8 (±1.9)	583.5 (±99.5)	530.4 (±104.2)	601.2 (±32.9)

*4, 8, 15 correspond to the operational and incubation temperatures; 37°C, 4-37, 8-37, 15-37 correspond to the operational and incubation temperature respectively e.g. 4-37 signifies operational temperature 4°C, incubation for the essay 37 °C, ps WW stands for primary settled wastewater. Error bars account for statistical error after nested analysis of variance, n = 2, 2, 4 at 4, 8 and 15°C respectively.