

## Low-temperature limitation of bioreactor sludge in anaerobic treatment of domestic wastewater

Emma J. Bowen, Jan Dolfing, Russell J. Davenport, Fiona L. Read and Thomas P. Curtis

### ABSTRACT

Two strategies exist for seeding low-temperature anaerobic reactors: the use of specialist psychrophilic biomass or mesophilic bioreactor sludge acclimated to low temperature. We sought to determine the low-temperature limitation of anaerobic sludge from a bioreactor acclimated to UK temperatures ( $<15^{\circ}\text{C}$ ). Anaerobic incubation tests using low-strength real domestic wastewater (DWW) and various alternative soluble COD sources were conducted at 4, 8 and  $15^{\circ}\text{C}$ ; methanogenesis and acidogenesis were monitored separately. Production of methane and acetate was observed; decreasing temperature resulted in decreased yields and increased 'start-up' times. At  $4^{\circ}\text{C}$  methanogenesis not hydrolysis/acidogenesis was rate-limiting. The final methane yields at  $4^{\circ}\text{C}$  were less than 35% of the theoretical potential whilst at 8 and  $15^{\circ}\text{C}$  more than 75 and 100% of the theoretical yield was achieved respectively. We propose that the lower temperature limit for DWW treatment with anaerobic bioreactor sludge lies between 8 and  $4^{\circ}\text{C}$  and that  $8^{\circ}\text{C}$  is the threshold for reliable operation.

**Key words** | anaerobic treatment, domestic wastewater, mesophilic inoculum, psychrophilic

Emma J. Bowen (corresponding author)  
Jan Dolfing  
Russell J. Davenport  
Fiona L. Read  
Thomas P. Curtis  
Newcastle University,  
School of Civil Engineering of Geosciences,  
Newcastle upon Tyne,  
NE1 7RU,  
United Kingdom  
E-mail: [emma.bowen@newcastle.ac.uk](mailto:emma.bowen@newcastle.ac.uk)

### INTRODUCTION

In countries with a temperate climate such as the UK, aerobic activated sludge processes remain the principal technology for treating domestic wastewater (DWW) but these activated sludge processes do not recover any of the considerable amounts of energy contained in DWW and moreover, energy is consumed during treatment. Alternatively, anaerobic treatment processes have much lower energy demands, are able to recover energy from DWW as biogas and can be net energy producers (McKeown *et al.* 2012). In regions of temperate climate, anaerobic treatment is not widely applied for DWW treatment because the temperature of influent DWW is too low, typically below  $18^{\circ}\text{C}$  (Lettinga *et al.* 2001), to provide optimum conditions for the process microbiology. Low temperature is the 'Achilles' Heel' of anaerobic microbiology, impeding both the methanogenesis and hydrolysis reactions. Of course, anaerobic reactors can be heated to optimal temperatures for microbial growth and activity, thought to be in the mesophilic ( $25\text{--}37^{\circ}\text{C}$ ) or thermophilic ( $45\text{--}60^{\circ}\text{C}$ ) range but this requires energy. It is difficult to satisfy this energy demand from the process itself when treating high-volume dilute wastes

like DWW because the COD concentration is low and the biogas recovered is not sufficient, and so an energy demand is created which has economic and carbon-consumption costs. The need for carbon-sustainable DWW treatment which recovers resources from the waste has led to renewed interest in the application of anaerobic treatment at low temperatures in countries with temperate climates.

Anaerobic wastewater treatment becomes increasingly difficult as temperatures drop below  $20^{\circ}\text{C}$ . These difficulties can be attributed to changes in the physico-chemical nature of the wastewater and sludge and the slowing of biochemical reactions. Both have consequences for the microbiological processes in the different trophic levels of anaerobic digestion: hydrolysis, acid- and acetogenesis and methanogenesis. Early work on low-temperature reactors sought to resolve physico-chemical problems with interventions such as pre-settling of the DWW to reduce solids, mixing strategies to promote better mass-transfer and operation in two stages, to separate hydrolysis from acetogenesis and methanogenesis (van Lier *et al.* 1997; Lettinga *et al.* 1999, 2001). Anaerobic membrane bioreactors have been used successfully at

laboratory scale to uncouple hydraulic retention time from solids retention time so that slow growing anaerobes are better retained within the reactor (Smith *et al.* 2013).

Regarding the microbiology of these reactors, efforts to enhance the low-temperature microbial community have focussed almost exclusively on inoculating reactors with mesophilic sludge from existing reactors followed by a period of acclimation to increasingly lower temperatures (Sanz & Fernandez-Polanco 1990; Ndon & Dague 1997; Nachaiyasit & Stuckey 1997; van Lier *et al.* 1997; Rebac *et al.* 1999a, b; Lettinga *et al.* 1999; Langenhoff & Stuckey 2000; Connaughton *et al.* 2006; McKeown *et al.* 2009; Xing *et al.* 2009; Smith *et al.* 2013). The final temperature range of these acclimation experiments was generally 10–15 °C with some experiments reporting short operational phases of <26 days at very low temperatures 3–5 °C (Sanz & Fernandez-Polanco 1990; Lettinga *et al.* 1999). Pre-acidified or synthetic wastewater has usually been used in these experiments with relatively few examples using real DWW: van der Last & Lettinga (1992); Sanz & Fernandez-Polanco (1990); Smith *et al.* (2013).

The microbiological and engineering advances described in these experiments exemplify our best efforts to date but the success of this strategy of acclimating mesophilic sludge to low temperatures is ultimately limited by the biological properties of the mesophilic biomass used. Importantly however, there is little consensus as to where those limits lie and no published evidence of this strategy being successfully implemented beyond pilot or laboratory scale (Lew *et al.* 2009). To consider this strategy against alternatives for start-up of low-temperature anaerobic bioreactors, it is important to determine the low temperature limits to which an acclimated mesophilic seed inoculum can be pushed before process failure, and to do so in the context of relevant temperature conditions. For example, it has been demonstrated that mesophilic sludge can be used in low-temperature anaerobic digestion (LTAD) at 15 °C, but this does not represent a low temperature for the UK and indeed, many other temperate countries. In north-east England (55°N), annual daily median wastewater temperatures are around 11.35 °C (95% C.I.s 10.94–11.23), with maximum temperatures below 15 °C for six months of the year (unpublished data from Northumbrian Water Ltd). Therefore, we are interested in the ability of the anaerobic reactor sludge to go from 15° to 4 °C rather than from 35° to 15 °C.

Experimental results from previous studies unfortunately have limited relevance to the challenge of treating DWW in a temperate climate because they (i) examine unrealistic

temperature ranges, (ii) use unrealistic wastes, and/or (iii) fail to distinguish which trophic level is the rate limiting process. To begin to address this we carried out a series of simple methanogenic incubation tests to explore the extent to which anaerobic membrane bioreactor sludge which had already been operated under UK ambient temperature conditions, could function at lower temperatures.

## MATERIALS AND METHODS

Methanogenic activity tests were carried at 4, 8 and 15 °C as a series of microcosm incubations containing the anaerobic sludge inoculum, various pure substrates: acetate; propionate; starch; hydrogen and carbon dioxide gas-mix; and low-strength soluble DWW. In these experiments, we used the soluble fraction of the DWW because the colloidal and suspended solid fractions are resistant to anaerobic degradation (Lew *et al.* 2009) and here we wanted to investigate the effects of low temperature in isolation. Substrate amendments were selected to act as precursors for different stages of the acidogenic and methanogenic pathways for comparison against real wastewater and both artificial substrates and real wastewater were added to the microcosms at the beginning of the incubation only. Propionate in particular was included as a substrate of interest because the degradation at low temperatures has previously been reported as problematic (Lettinga *et al.* 1999; Rebac *et al.* 1999b) and further, the biodegradation is carried out by a mixed microbial community of syntrophic bacteria and both hydrogenotrophic and aceticlastic methanogens. A parallel set of microcosms were prepared and supplemented with 2-bromoethanesulfonate (BES) to inhibit methanogenesis and facilitate the investigation of temperature and substrate on the hydrolytic/acidogenic component of anaerobic treatment.

General community diversity and quantitation analyses of microbial biomass sampled from microcosms and inocula were undertaken, but the resulting data did not add to the findings presented here, and so are not included.

### Microbial biomass

The sludge inoculum used to seed microcosms was collected from an anaerobic membrane bioreactor at the pilot facility of Cranfield University (Cranfield, UK). The reactor was not heated but experienced the ambient temperature conditions for the building in which it was housed and the influent DWW stream entering the pilot facility. The recorded reactor temperatures were in the range 10–25 °C (e.g. Garcia

*et al.* 2013). The sludge was transported in sterile plastic containers and stored at 4 °C until use. The volatile suspended solids (VSS) and chemical oxygen demand (COD) values of the sludge used to prepare microcosms were 4.4 g VSS l<sup>-1</sup> and 3.6 g COD l<sup>-1</sup>.

### Microcosm assembly

Two experiments were set up sequentially, the first with 'simple' substrates: acetate, propionate, H<sub>2</sub>/CO<sub>2</sub> and unamended controls (containing sludge and basal salts media only); the second experiment with 'complex' substrates: starch and DWW. A second series of acetate microcosms to provide reference between the first and second experiments to ensure that the microbial activity had not diminished between the first experiment being set up and the second being set up.

Batch microcosms were prepared in 125 ml Wheaton serum bottles, sealed with butyl rubber stoppers and aluminium crimps (Sigma Aldrich Ltd, Gillingham, UK). Microcosms were assembled by adding 20 ml of sludge inoculum, 30 ml of DWW or basal salts media + substrates to the Wheaton vial under a nitrogen atmosphere (BOC, Wallsend, UK). Unamended microcosms were included as controls, containing sludge and basal salts media only. Vials were stoppered and crimped and the headspace was given a final flush with N<sub>2</sub> gas.

For the DWW microcosms, primary settled DWW with a COD of 280 mg l<sup>-1</sup> was collected from Howden municipal wastewater treatment plant, Newcastle upon Tyne, UK and filtered using a 0.45 µm pore filter to yield a filtrate containing only the soluble-COD fraction. Filtered DWW (final COD 146 mg l<sup>-1</sup>) was sparged with nitrogen gas to reduce the dissolved oxygen concentration just prior to use in the microcosms.

The liquid phase for the 'substrate treatment' microcosms consisted of basal salts medium supplemented with trace elements, sodium bicarbonate, oxygen scavenger and redox indicator (Widdel & Bak 1992). Substrates: acetate (300 mg l<sup>-1</sup>), propionate (370 mg l<sup>-1</sup>), and starch (600 mg l<sup>-1</sup>) were added to the liquid phase to achieve a COD in the range (300–500 mg l<sup>-1</sup>). For the hydrogen/carbon dioxide microcosms, the gaseous substrate was supplied by flushing the headspace of the sealed microcosm with the gas mix (80:20% by vol., Scientific and Technical Gases, Newcastle-under-Lyme, UK) once the microcosm had been assembled. For the methanogenesis-inhibited microcosms, BES was added to the basal salts media at a concentration of 10 mM. All solutions for the liquid phase of the microcosms were

de-gassed by sparging with nitrogen gas, and sterilised by autoclaving or filtering through a 0.2 µm filter as appropriate.

Sealed microcosms were inverted and incubated in the dark at the three incubation temperatures: 4, 8, and 15 °C. Temperature was logged inside the incubation chambers using a dedicated temperature monitoring microcosm which was equipped with a thermocouple connected to an EasyLog USB data logger (Lascar Electronics Ltd, Salisbury, UK).

### Sampling and chemical analysis

For the purposes of these incubation tests, only methane and volatile fatty acids (VFAs) were monitored as the products of anaerobic activity in the methanogenic- and methanogenesis-inhibited microcosms, respectively. Liquid and gas phases of the microcosms were sampled concomitantly. Microcosms were removed from the temperature controlled incubators and sampled under ambient conditions but efforts were made to minimise temperature increase during this procedure by cooling with ice blocks: the temperature recorded by the loggers did not increase above 20 °C and returned to incubation temperature within 2h from when the microcosms were removed from the incubators.

Methane was monitored in the gas phase as % by volume, using gas chromatography. Samples of gas (100 µl) were removed from the microcosm headspace using a gas-tight syringe (SGE-Europe Ltd, Milton Keynes, UK), and injected directly onto a Carlo Erba HRGC S160 GC fitted with a flame ionisation detector (FID) and HP-PLOTQ column (0.32 mm diameter, 30 m length and 20 µm film; Agilent, Wokingham, UK). Hydrogen was used as the carrier gas at a flow rate of 250 ml/min and the oven temperature was 35 °C. Methane was not measured in the dissolved phase in these experiments because the system under closed batch conditions is at equilibrium and therefore, methane could be estimated using Henry coefficients at the appropriate temperatures.

Samples of the liquid phase were removed from the microcosms using sterile syringes, transferred to sterile 2 ml micro-centrifuge tubes and centrifuged (3 min at 13,000 rpm) to remove biomass; the supernatant was collected for analysis of VFA content. VFAs were analysed by ion exchange chromatography based on a modified method of Manning & Bewsher (1997). Aqueous samples were syringe-filtered through 0.45 µm filters, acidified 1:1v/v with oxysulfonic acid and sonicated in a sonic bath for 30 minutes to remove carbonate from the samples as carbon dioxide. The resulting samples were analysed on a DIONEX ICS-1000

equipped with an Ionpac ICE-AS1, 4 × 250 mm column using a 1.0 mM heptafluorobutyric acid eluent solution. The volume of the injection loop was 10 µl and flow rate was 0.16 ml/min. The cation regenerant solution used for the AMMS-ICE II Suppressor was 5 mM tetrabutylammonium hydroxide.

### Data analysis

Raw headspace methane and VFA data were converted from units of concentration to µmol values per microcosm bottle and mean data from triplicate microcosm treatments was subsequently used to calculate specific rates of acetogenesis and methanogenesis. The maximum rate was estimated from the maximum linear gradient between µmol and 'experiment day' and the overall rate for the first 175 days was also calculated as this best represented the period in which the substrate amendments were consumed. Methane production was based on headspace data since the amount dissolved in the gas phase was <1%, as estimated using Henry coefficients, according to Dolfig & Janssen (1994) and Amend & Shock (2007).

## RESULTS AND DISCUSSION

### Acidogenic activity in BES microcosms

Hydrolytic and acidogenic activity was assessed from VFA accumulation where methanogenesis was inhibited using BES. Acetate was the main VFA formed with trace levels of butyric and valeric acid appearing after day 140 in some treatments. Acetate increased in all BES microcosms including the unamended microcosms and those fed with acetate as a substrate (Figures 1 and 2), suggesting that the sludge inoculum provided an additional carbon substrate for anaerobic activity either from COD contained therein or from the decay of some of the sludge biomass.

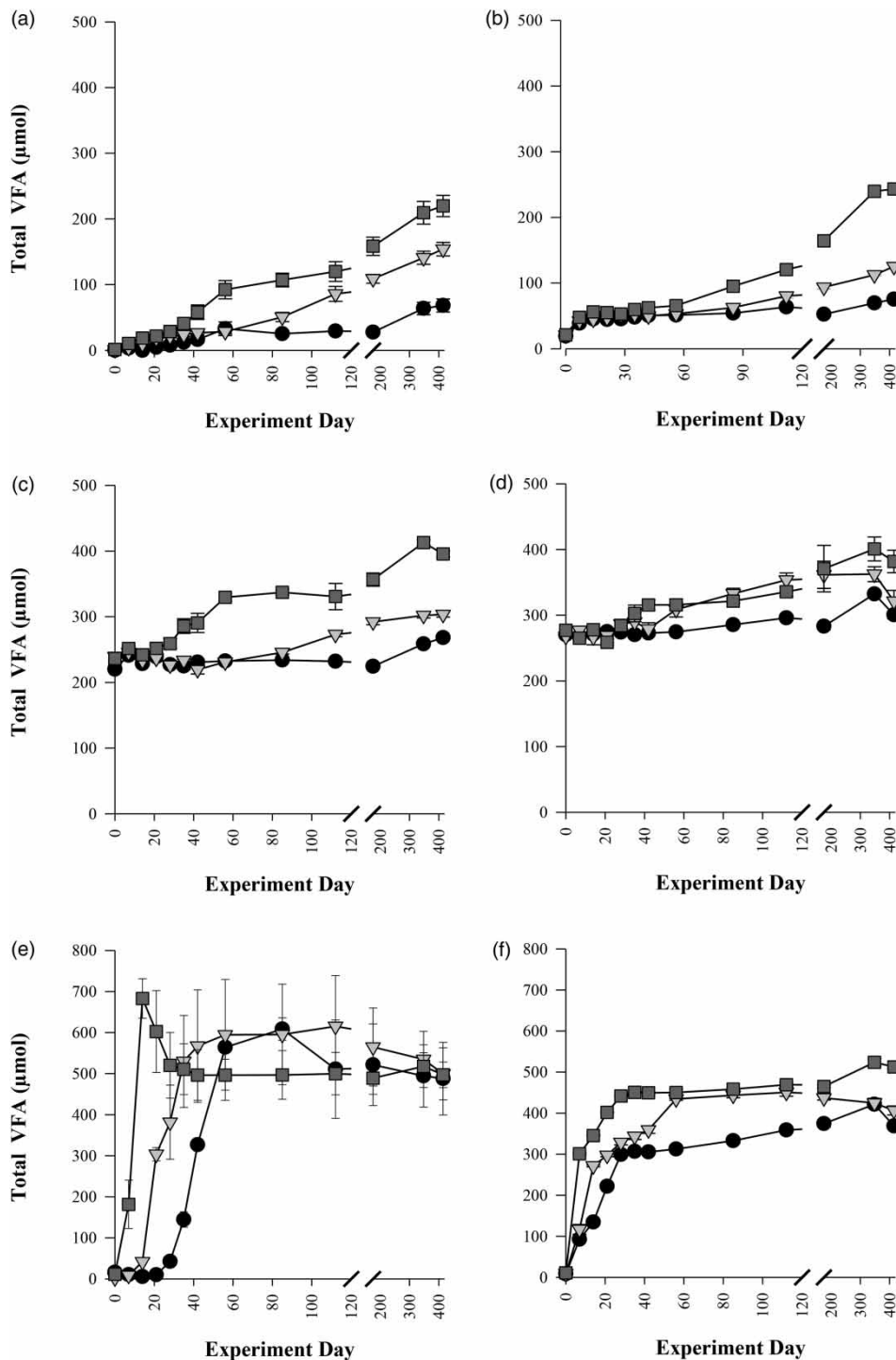
In microcosms incubated with DWW-BES, the acetate approximately doubled during the first 7 days in all temperatures. There was a subsequent 60 day lag before any further appreciable increase in VFA yield and generally VFA dynamics in these microcosms were similar to the unamended microcosms (Figure 1(a)). This similarity was attributed to the low COD substrate load of the DWW which highlights the difficulties experienced when working with this type of wastewater. In spite of the low yields of acetate however, maximum rates of acidogenesis were higher for the DWW than the unamended microcosms and surprisingly, the rate for DWW at 4 °C was comparable

to that at 15 °C at 2.80 and 3.81 µmol VFA d<sup>-1</sup> respectively (shown in Table 1).

The VFA profiles for starch-BES was characterised by a sharp increase in acetate during the first 28 days, when maximum rates of acidogenesis were observed (Figure 1(f), Table 1), suggesting that the starch was rapidly hydrolysed and acidified. A gradual increase in acetate concentration was observed after day 56, indicative of digestion of the sludge inoculum at a lower rate; butyric and valeric acids were detected in the microcosms during this phase. The rate of acidogenesis over the whole incubation period was comparable across all three temperatures, showing clearly that the acclimated mesophilic sludge inoculum was capable of degrading the starch to produce methanogenic substrates at 4 °C, and that a similar yield could be reached given an appropriate residence time within anaerobic reactors.

The H<sub>2</sub>/CO<sub>2</sub>-BES microcosms also showed rapid acetate accumulation, indicative of homoacetogenic activity (Figure 1(e)). Decreased incubation temperature increased the time lag for VFA accumulation and reduced the maximum rate of acidogenesis (Table 1): at 8 °C the rate was 52% of that at 15 °C; and at 4 °C, 38% of that at 15 °C. However overall yields of acetate for the total incubation period were close to the theoretical yield (625 µmol) from homoacetogenesis; at 4 and 8 °C, ~600 µmol were produced. The yield in the microcosms at 15 °C exceeded this theoretical value presumably because of some acetate production from the organic matter in the inoculum. These results highlight the strong activity of the homoacetogens at low temperatures (when H<sub>2</sub> is not limiting), as previously described for permanently cold anoxic soil environments (Kotsyurbenko *et al.* 2007). Although in this case the high H<sub>2</sub>/CO<sub>2</sub> creates an artificial substrate condition not likely to be observed in DWW, these results are interesting because they suggest that similarly to microbial communities present in soil environments, the bio-reactor community harbours a homoacetogenic population which is active at very low temperatures.

In the propionate-BES microcosms (Figure 2(a), (c) and (e)), propionate decreased during the incubation period and acetate increased, indicating that acetogenesis had occurred in the absence of methanogenesis. This was an unexpected result since propionate oxidation is thermodynamically unfavourable in the absence of a syntrophic partner organism (de Bok *et al.* 2005). In anaerobic wastewater treatment this syntrophic partner is generally a methanogen. We speculate that in our experiments, the oxidation of propionate may have been coupled to homoacetogenic activity but further experiments are necessary to explore this. Decreasing incubation temperature resulted in increased

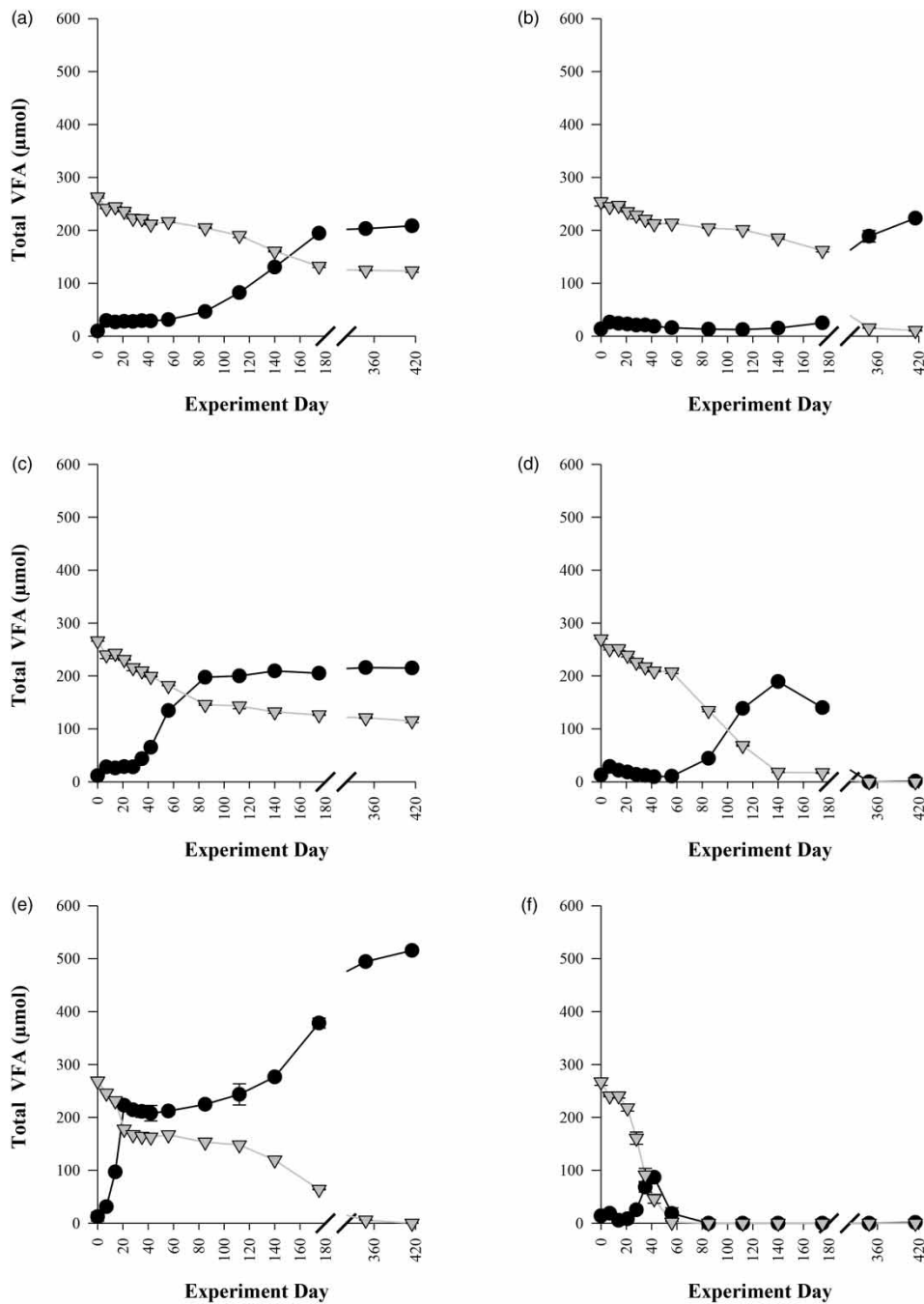


**Figure 1** | Cumulative total VFA in BES-microcosms at 15, 8 and 4 °C (square, triangle and circle respectively). (a) Unamended; (b) DWW; (c) acetate I; (d) acetate II; (e) H<sub>2</sub>/CO<sub>2</sub>; (f) starch. Values are mean data from triplicate microcosms; error bars represent standard error of the mean. Propionate data are presented as a separate set of graphs in [Figure 2](#).

lag time for propionate oxidation activity ([Figure 2\(b\), \(d\)](#) and [\(f\)](#)) and decreased reaction rates, as shown in [Table 1](#). In all three temperature regimes the initial phase of propionate oxidation reached a plateau, after which all of the

propionate was converted to acetate at 15 °C but no further activity was observed in the lower two temperatures. It was not clear why the lower temperature regimes became inhibited in this manner.





**Figure 2** | Acetate (circles) and propionate (triangles) dynamics in propionate-amended microcosms at 15, 8, and 4 °C with and without BES (methanogenic): (a) 4 °C + BES; (b) 4 °C methanogenic; (c) 8 °C + BES; (d) 8 °C methanogenic; (e) 15 °C + BES; (f) 15 °C methanogenic. Values are mean data from triplicate microcosms; error bars represent standard error of the mean.

### Methanogenic activity

In the methanogenic microcosms, methane accumulation was observed in all microcosms (Figure 3). Methanogenic activity decreased in the order 15 > 8 > 4 °C and decreasing

temperature resulted in increased lag times for the onset of methanogenesis.

In the microcosms amended with acetate, starch and H<sub>2</sub>/CO<sub>2</sub>, methanogenesis progressed similarly; starch and H<sub>2</sub>/CO<sub>2</sub> were initially converted to acetate which

**Table 1** | Maximum and mean (day 0–175) rates of acidogenesis<sup>a</sup> and methanogenesis

Substrate	Temp. (°C)	Max. acidogenesis rate (μmol VFA d <sup>-1</sup> )	Mean acidogenesis rate (d0–175) (μmol VFA d <sup>-1</sup> )	Max. methanogenesis rate (μmol CH <sub>4</sub> d <sup>-1</sup> )	Mean methanogenesis rate (d0–175) (μmol CH <sub>4</sub> d <sup>-1</sup> )
Un-amended ± BES	4	0.59	0.16	0.19	0.07
	8	0.49	0.62	0.88	0.69
	15	2.46	0.90	6.58	1.19
DWW ± BES	4	2.80	0.19	0.23	0.13
	8	3.04	0.42	3.54	0.65
	15	3.81	0.82	3.84	1.27
Acetate I ± BES	4	–	–	–	–
	8	–	–	–	–
	15	–	–	–	–
Acetate II ± BES	4	–	–	–	–
	8	–	–	–	–
	15	–	–	–	–
H <sub>2</sub> /CO <sub>2</sub> ± BES	4	18.63	1.53	0.43	0.42
	8	23.24	2.05	6.37	1.93
	15	47.99	2.05	7.53	3.38
Starch ± BES	4	10.38	0.61	0.49	0.41
	8	18.72	2.44	5.65	2.14
	15	41.42	2.59	5.97	3.12
Propionate ± BES	4	0.84	0.31	1.14	0.62
	8	2.39	0.32	6.73	2.59
	15	7.30	0.95	12.77	3.68

Rates based on data from triplicate microcosms, VFA data for acetate microcosms not included as acetate were added as a substrate.

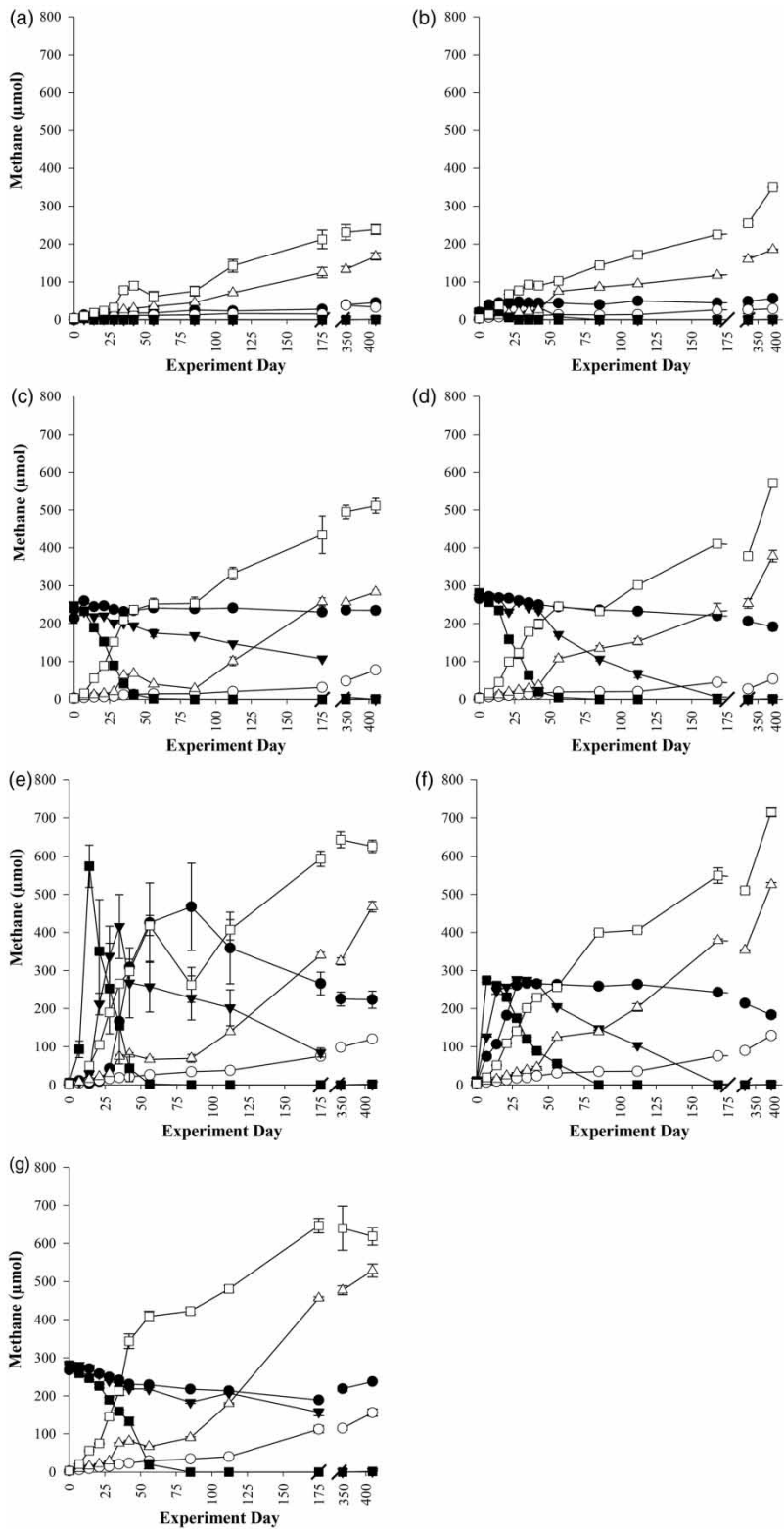
<sup>a</sup>Acidogenesis based on increase in total VFA in BES-amended microcosms, except propionate-amended microcosms, where acidogenesis was based on specific acetate formation data.

accumulated and was thus available as a precursor for acetoclastic methanogenesis (Figure 3(c)–(f)). At 15 °C methane production commenced immediately with concomitant acetate accumulation, followed by a phase of acetate consumption and methane production in stoichiometric ratios (1:1). At 8 °C, there was a lag prior to the onset of methanogenic activity, after which, the overall pattern of acetate consumption and methane production was essentially repeated with a longer transient phase of acetate presence in the microcosms. The maximum rates of methanogenesis at 8 °C were between 84 and 94% of the maximum rates at 15 °C (Table 1). At 4 °C, little methanogenesis was observed and acetate, either supplied directly or formed from acidogenesis, remained in the microcosms at the end of the incubation. Rates of methanogenesis at this temperature were between 3 and 8% of those at 15 °C. The persistence of acetate in the microcosms at 4 °C, which was not consumed with prolonged incubation periods of >420 days, indicates that acetoclastic methanogens in the source community were particularly affected at temperatures between 8 and 4 °C.

In the DWW microcosms acidogenic activity was evident at all temperatures in the first 7 days; acetate

accumulated but was consumed after day 21 and 56 at 15 and 8 °C respectively. At 4 °C, this acetate remained in the microcosm liquid phase for the duration of the incubation period (Figure 3(b)). The maximum rates of methanogenesis at the two higher temperatures were comparable at 3.84 and 3.54 μmol CH<sub>4</sub> d<sup>-1</sup>, relative to 0.23 μmol CH<sub>4</sub> d<sup>-1</sup> at 4 °C. Similarly to the other substrates therefore, methanogenic activity is reduced at 4 °C.

In propionate microcosms, propionate degradation and methane production commenced within the first 7 days (Figure 3(g)). Maximum rates of methanogenesis were significantly affected by temperature, more so than for any other substrate (Table 1). However, these microcosms generally showed the best ‘performance’ of all substrates examined, in terms of methane production. This contrasts with the previous findings of Lettinga *et al.* (1999) and Rebac *et al.* (1999b) who describe methanogenic activity on propionate-based synthetic wastewater as ‘especially sensitive to low temperature’ and ‘the rate limiting step’, respectively. A transient accumulation of acetate was observed in propionate treatments at 15 and 8 °C incubations (Figure 2(a) and (c)) where the rate of acidogenesis



**Figure 3** | (a)–(d) Total VFA (closed symbols) and methane (open symbols) in methanogenic microcosms at 15 (square), 8 (triangle) and 4 °C (circles): (a) unamended; (b) DWW; (c) acetate I; (d) acetate II. Values are mean data from triplicate microcosms; error bars represent standard error of the mean. (e)–(g) Total VFA (closed symbols) and methane (open symbols) in methanogenic microcosms at 15 (square), 8 (triangle) and 4 °C (circles): (e) H<sub>2</sub>/CO<sub>2</sub>; (f) starch; (g) propionate. Values are mean data from triplicate microcosms; error bars represent standard error of the mean.



was periodically greater than the rate of methanogenesis, possibly due to a higher growth rate of the propionate oxidisers relative to their syntrophic methanogen partners or due to an overload of acetate from syntrophic propionate oxidation to acetate coupled to homoacetogenesis, as proposed for the BES-propionate microcosms. At 4 °C methane production reached a plateau after 175 days, however propionate oxidation to acetate continued (Figures 2(a) and 3(g)). The acetate produced remained for the duration of the incubation and was not consumed in methanogenesis.

### The limitations of bioreactor sludge at low temperatures

Our experiments assess the ability of anaerobic bioreactor sludge acclimated to operational temperatures below 15 °C to be challenged with temperatures as low as 4 °C. At 15 °C anaerobic activity was observed immediately and over the incubation period generally >80% of the COD was reduced to methane in agreement with previous data for soluble COD (Ndon & Dague 1997; Nachaiyasit & Stuckey 1997; van Lier *et al.* 1997; Rebac *et al.* 1999a, b). At 8 °C anaerobic activity was established following a lag period after which the dynamics of acidogenesis and methanogenesis were similar to those at 15 °C. Once anaerobic reactions had commenced, maximal rates were not markedly different from those at 15 °C suggesting that after an initial temperature shock in going from 15 to 8 °C, the microbial community recovered.

At 4 °C methanogenesis was inhibited although both acetoclastic and hydrogenotrophic precursors were available in the microcosms. Conversely, acidogenic reactions occurred at all temperatures indicating that the methanogens rather than the acidogenic organisms were more sensitive to the decrease in temperature. Homoacetogenic organisms were apparently able to compete for hydrogen and carbon dioxide at 8 and 4 °C, further limiting the potential for methane to be formed by the hydrogenotrophic methanogenic pathway and increasing the amount of acetate present. We propose that there is a threshold of functional limitation between 4 and 8 °C for bioreactor sludge acclimated to psychrophilic temperatures, where the original source community was taken from a bioreactor operated at mesophilic temperatures. Molecular microbial investigation using denaturing gel-electrophoresis and quantitative polymerase chain reaction (qPCR) confirmed that methanogens were present in the microcosms in the order of  $10^8$  cells ml<sup>-1</sup> throughout the incubation (data not presented here), and thus, we

conclude that the inhibition of methanogenesis was due to inhibition of activity, rather than the absence of a methanogen population.

Our results contrast with the few studies which have reported methanogenesis at temperatures below 8 °C using acclimated mesophilic sludge: Sanz & Fernandez-Polanco 1990; Lettinga *et al.* 1999; McKeown *et al.* 2009. It could be that we have by chance only captured methanogens that function poorly at 4 °C in the biomass interned in our microcosms but in using an inoculating sludge that was well-adapted to DWW and low temperatures (15 °C), we would anticipate some degree of enrichment towards these conditions already. Moreover, modest samples of biomass will capture most of the diversity of low diversity groups such as methanogens (Curtis & Sloan 2006). At the minimum our results show that methanogenesis may fail at 4 °C in bioreactors inoculated using acclimated sludge. Collectively, our findings and previous studies suggest that LTAD of DWW in the 0–15 °C range in reactors seeded with acclimated bioreactor sludge is variable at best, and unreliable or risky at worst.

### CONCLUSIONS

We have shown clearly that sludge sourced from a bioreactor acclimated to treating DWW at 15 °C could not function below 8 °C. Methanogenesis was inhibited even with simplified COD (comparable to pre-acidified/pre-settled waste). We conclude that this strategy of adapting mesophilic bioreactor sludge to psychrophilic temperatures cannot reliably inoculate reactors for anaerobic treatment of DWW, where influent waste and ambient temperatures will drop below 8 °C.

In the future, we would hope for a more scientific and quantitative approach to reactor seeding in which we rationally evaluate the probability of a desirable organism being present in the seed and the time required for that organism to play a role in the treatment, in this case for example, a psychrophile. This ambition could be realised as we gain deeper understanding of the distribution of rare and abundant taxa, better and sequencing protocols and improved methods for predicting microbial dynamics and adaptation. Novel seeding strategies involving specialist psychrophilic enrichments or cold-adapted biomass, together with an understanding of the microbial ecology of LTAD, may enable us to move towards design of fully ambient plants for energy-sustainable wastewater treatment.

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