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High rate anaerobic treatment of LCFA-containing wastewater at low temperature

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Abstract

Fats, oil and grease (FOG) are a significant constituent in numerous wastewaters such as those in dairy industry. The hydrolysis of FOG result in the production of long chain fatty acids (LCFA) which destabilize the anaerobic treatment process due to their physico-chemical and microbial toxicity effects. Harnessing the high methanogenic potential of FOG necessitates effective treatment of high LCFA loads, wherein the feasibility of LCFA treatment at low temperatures has been not investigated up to now. The aim of this thesis was to study the feasibility of high-rate anaerobic treatment of LCFA-rich wastewaters at low ambient temperatures using dairy wastewater.

The screening of mesophilic inocula for treatment of mixed LCFA containing synthetic dairy wastewater (SDW) in batch studies showed that granular sludge inoculum achieved faster and higher methane yields (76-82% of theoretical yield) than the two municipal digestates (1-72%) at both 20 and 10°C. The LCFA degradation capacity in the granular sludge inoculum was attributed to the presence of β-oxidizing bacteria from the family *Syntrophaceae* (*Syntrophus* and uncultured taxa), the acetotrophic activity of *Methanosaeta* and the putative syntrophic acetate oxidizing bacteria (SAOB).

Continuous high-rate treatment of SDW was found to be feasible in expanded granular sludge bed (EGSB) reactors at 20°C (hydraulic retention time (HRT) 24 h, LCFA loading rate (OLR) 670 mgCOD-LCFA/L·d) with a soluble COD (sCOD) removal of 84–91% and methane yield of 44–51%. SDW feeding for longer than two months resulted in LCFA accumulation, which led to granular sludge flotation (36-57%) and disintegration (reduction in d_{50} of 24–33% and 75–84% in settled and washed-out granules, respectively). To counter the LCFA induced granular sludge disintegration and flotation, a novel reactor type, dynamic sludge chamber-fixed film (DSC-FF), was designed and achieved sCOD removal of 87-98% at HRTs from 12-72 h (LCFA loading rate 220-1333 mgCOD-LCFA/L·d) at 20°C. Moreover, even at the 12 h HRT, the unsaturated LCFAs (linoleate and oleate) were treated and only part of saturated LCFAs (stearate, palmitate) remained after treatment in the DSC-FF reactors. An increased methanogenic activity was established in the reactor sludges during reactor runs, which was evidenced by a higher acetotrophic activity in the granular sludge (from DSC), and a higher hydrogenotrophic activity in the biofilm (from FF) indicating development of distinct metabolic capabilities in the different reactor compartments.

High throughput 16S rRNA sequencing showed that the relative abundance of the acetoclastic methanogen, *Methanosaeta,* increased in EGSB reactors and in the active microbiomes of granules (from

DSC) and biofilm (from FF) when fed with increasing LCFA concentrations. This suggested acetoclastic methanogenesis as the predominant methanogenesis pathway for SDW and presumably, LCFA degradation at 20°C. Relative abundances of the taxa known to have β-oxidizing and methanogenic activity were high in the active microbiomes during SDW treatment in DSC-FF reactors at 20°C. The biofilm microbiome (from FF) had a prominent presence of the β-oxidizing bacteria *Syntrophus* and of the hydrogenotrophic methanogen *Methanospirillum* in comparison to the presence of the acetogenic bacteria, *Syntrophobacter, Desulfobulbus,* and *Geobacter,* and of the acetoclastic methanogen in the granular sludge microbiome, suggesting a role of these different taxa during LCFA degradation.

In summary, this work demonstrated successful inoculum selection at low temperatures (10 and 20°C), and high-rate anaerobic LCFA degradation at 20°C using novel reactor design (here, DSC-FF). The key bacterial and archaeal taxa involved in the anaerobic conversion of LCFA to methane at 20°C were also deduced.

Yhteenveto

Rasvat, öljyt ja rasvat (FOG) ovat merkittävä aineosa monissa jätevesissä, kuten elintarviketeollisuuden jätevesissä. Näiden hydrolyysi tuottaa pitkäketjuisia rasvahappoja (LCFA), jotka vaikuttavat anaerobisen jätevedenkäsittelyprosessin stabiilisuuteen fysikaalis-kemiallisten ja mikrobiologisten toksisuusvaikutustensa vuoksi. FOG:n korkean metaanintuottopotentiaalin hyödyntäminen edellyttää biokaasuprosessilta kykyä käsitellä suuria LCFA-kuormituksia, jota ei ole vielä tutkittu matalissa lämpötiloissa. Tämän opinnäytetyön tavoitteena oli tutkia suuria LCFA-pitoisuuksia omaavien elintarvikejätevesien anaerobista käsittelyä korkeakuormitteisissa reaktoreissa matalissa lämpötiloissa.

Synteettisen elintarviketeollisuuden jätevesien, jotka sisälsivät neljää eri LCFA:ta, anaerobista käsittelyä eri mesofiilisilla mikrobiyhteisöillä tutkittiin panoskokeissa. Granulalietteellä tuotettiin metaania nopeammin sekä saatiin suurempi metaanisaanto (76-82% teoreettisesta saannosta) kuin kahdella yhdyskuntamädätteellä (1-72%) 10°C:ssa ja 20°C:ssa. Granulalietteessä LCFA:iden anaerobista hajoamista edistivät β-hapettavat *Syntrophaceae*-heimon bakteerit (*Syntrophus* ja tuntemattomat lajit), *Metanosaeta*-suvun asetotrofiset metanogeenit, aktiivisuus ja oletetut syntrofiset asetaattia hapettavat bakteerit (SAOB).

Synteettistä elintarviketeollisuuden jätevettä käsiteltiin anaerobisesti jatkuvatoimisessa korkeakuormitteisessa EGSB-reaktorissa 20°C:ssa (viipymällä 24 h, LCFA-kuormituksella 670 mgCOD-LCFA/L·d). Kemiallisen hapenkulutuksen (COD) poisto oli 84-91% ja metaanisaanto 44-51%. Kahden kuukauden koeajojen aikana LCFA:ita kertyi reaktoriin, mikä johti granulalietteen nousemiseen reaktorin yläosaan (36-57%) ja hajoamiseen. LCFA:n aiheuttamaa granulalietteen hajoamista ja kellumista pyrittiin estämään suunnittelemalla uudenlainen reaktori, jossa oli dynaaminen lietereaktori yhdistettynä biofilmireaktoriin (DSC-FF). Tällä reaktorilla saavutettiin 20°C:ssa 87-98% liukoisen COD:n poisto viipymän ollessa välillä 12-72 h (LCFA-kuormituksella 220-1333 mgCOD-LCFA/L·d). Myös lyhimmällä 12 tunnin viipymällä tyydyttymättömät LCFA:aat (linoleaatti ja oleaatti) poistettiin syötetystä jätevedestä, kun taas osa tyydyttyneistä LCFA:ista (stearaatti ja palmitaatti) poistui reaktorista.

Asetotrofisen metanogeenin, *Methanosaeta*:n, suhteellinen runsaus (perustuen suurikapasiteettiseen 16S rRNA sekvensointiin) lisääntyi EGSB-reaktorissa sekä granulalietteessä (DSC) että biofilmissä (FF) LCFA-kuormituksen noustessa. Tämä osoittaa, että LCFA:n hajoamisen seurauksena metaania tuotettiin pääasiassa asetaatin kautta 20°C:ssa. Biofilmiin (FF) rikastui β-hapettavia bakteereja, *Syntrophus*-suvusta, sekä hydrogenotrofisia metanogeeneja, *Methanospirillum*-suvusta, kun taas granulalietteeseen rikastui

asetaattia hapettavia bakteereita suvuista *Syntrophobacte*r, *Desulfobulbus* ja *Geobacter*, sekä asetotrofisia metanogeeneja. Tämä osoittaa, että reaktorin eri osiin rikastuvilla mikrobiyhteisöillä oli erilaiset roolit LCFA:n anaerobisessa käsittelyssä.

Yhteenvetona voidaan todeta, että tämä väitöstyö osoitti mikrobiyhteisön valinnan tärkeyden matalissa lämpötiloissa (10 ja 20°C:ssa). Lisäksi LCFA:ta pystyttiin onnistuneesti käsittelemään anaerobisissa korkeakuormitteisissa reaktoreissa 20°C:ssa käyttämällä uudenlaista reaktorityyppiä (DSC-FF). Väitöstyössä selvitettiin myös pääasialliset bakteerit ja arkit, jotka ottavat aktiivisesti osaa LCFA:iden anaerobiseen käsittelyyn.

Sommario

Grassi, olio e unto (fats, oil and grease, FOG) sono componenti significativi in numerose tipologie di acque reflue come quelle del settore lattiero-caseario. L'idrolisi dei FOG provoca la produzione di acidi grassi a catena lunga (long-chain fatty acids, LCFA) che destabilizzano il processo di trattamento anaerobico a causa della loro tossicità fisico-chimica e microbiologica. Lo sfruttamento dell'alto potenziale metanogeno dei FOG richiede un trattamento efficace di elevati carichi di LCFA, di cui finora non è stata studiata la fattibilità a basse temperature. Lo scopo di questa tesi è stato quello di studiare la fattibilità del trattamento anaerobico di acque reflue ricche di LCFA a basse temperature utilizzando effluenti caseari.

Lo screening dell'inoculo mesofilo per il trattamento di LCFA misti in effluenti caseari sintetici (synthetic dairy wastewater, SDW) effettuato in condizioni batch ha mostrato come l'inoculo di fanghi granulari abbia prodotto una maggiore quantità di metano (76-82% della produzione teorica) rispetto ai due digestati municipali (1-72%) a 20 e 10 °C. La capacità degradativa degli LCFA da parte dell'inoculo di fanghi granulari è stata attribuita alla presenza di batteri β-ossidanti della famiglia *Syntrophaceae* (*Syntrophus* e taxa non coltivati), all'attività acetotrofica dei *Methanosaeta* e ai batteri putativi sintropici che ossidano l'acetato (SAOB).

Il trattamento in continuo di SDW è stato effettuato con elevate rese in reattori a letto granulare espanso (EGSB) a 20°C (tempo di ritenzione idraulica (HRT) 24 h, carico organico (OLR) 670 mgCOD-LCFA / L · d, 33% COD-LCFA) con una rimozione del COD dell'84–91% e una percentuale di metano nel gas prodotto del 44–51%. L'alimentazione di SDW per più di due mesi ha provocato un accumulo di LCFA, che ha portato alla flottazione (36-57%) ed alla disintegrazione dei fanghi granulari. Per contrastare ciò, è stato progettato un nuovo tipo di reattore, denominato dynamic sludge chamber-fixed film (DSC-FF), che ha ottenuto una rimozione di sCOD dell'87-98% con HRT da 12 a 72 h (carico organico 220-1333 mg COD-LCFA / L · d) a 20°C. Inoltre, persino ad un HRT di 12 ore, gli LCFA insaturi (linoleato e oleato) sono stati digeriti e solo una parte degli LCFA saturi (stearato, palmitato) è rimasta dopo il trattamento nei reattori DSC-FF. Una maggiore attività metanogenica nei fanghi è stata ottenuta durante l'esercizio del reattore; ciò è stato evidenziato da una maggiore attività acetotrofica nel fango granulare (DSC) e da una maggiore attività idrogenotrofica nel biofilm (FF), che indica lo sviluppo di capacità metaboliche distinte nei diversi compartimenti del reattore.

Il sequenziamento dell'rRNA 16S ad alto rendimento ha mostrato che l'abbondanza relativa del metanogenico acetoclastico, *Methanosaeta*, è aumentata nei reattori EGSB e nei microbiomi attivi di

granuli (da DSC) e biofilm (da FF) quando alimentati con concentrazioni di LCFA crescenti. Ciò ha suggerito la metanogenesi acetoclastica come via di metanogenesi predominante per SDW e presumibilmente, degradazione dell'LCFA a 20°C. Le abbondanze relative dei taxa noti per avere attività β-ossidante e metanogenica erano elevate nei microbiomi attivi durante il trattamento SDW nei reattori DSC-FF a 20°C. Il microbioma del biofilm (da FF) ha avuto una presenza preminente dei batteri β-ossidanti *Syntrophus* e del metanogenico idrogenotrofico, *Metanospirillum* in confronto alla presenza dei batteri acetogenici, *Syntrophobacter*, *Desulfobulbus* e *Geobacter* e methanogen del fango acetoclastico, suggerendo un ruolo di questi diversi taxa durante il degrado della LCFA.

In sintesi, questo lavoro ha dimostrato la riuscita selezione dell'inoculo a basse temperature (10 e 20°C) e una degradazione anaerobica LCFA ad alta velocità a 20°C utilizzando un nuovo design del reattore (qui, DSC-FF). Sono stati dedotti anche i principali taxa batterici e arcaici coinvolti nella conversione anaerobica di LCFA in metano a 20°C.

Résumé

Les gras, huiles et graisses (fats, oil and grease, FOG) constituent une fraction importante de nombreuses eaux usées, telles que celles de l'industrie laitière. L'hydrolyse des FOG entraîne la production d'acides gras à longues chaînes (long chain fatty acid, LCFA) qui déstabilisent le processus de traitement anaérobie en raison de leurs effets toxicologiques physico-chimiques et microbiens. L'exploitation du potentiel méthanogène élevé des FOG nécessite un traitement efficace des charges élevées en LCFA et la faisabilité d'un tel traitement à basse température n'a à ce jour pas été étudiée. L'objectif de cette thèse était ainsi d'étudier la faisabilité d'un traitement anaérobie à haute vitesse à basse température d'eaux usées riches en LCFA à l'aide d'une matrice d'eaux usées laitières.

Le criblage d'inoculas mésophiles dans le traitement d'eaux usées synthétiques reproduisants les caractéristiques d'eaux usées de l'industrie laitière (*synthetic dairy wastewater,* SDW) et chargées d'une variété d'LCFA au cours d'études par lots a montré que l'inoculum de boues granulaires obtenait des rendements en méthane plus rapides et supérieurs (76 à 82% du rendement théorique) que les deux digestâts municipaux (1 à 72%) à 20 et 10°C. La capacité de dégradation d'LCFA par l'inoculum provenant de boues granulaires a été attribuée aux présences de bactéries β-oxydantes de la famille *Syntrophaceae* (*Syntrophus* et taxons non cultivés), de l'activité acétotrophe de *Methanosaeta* et de bactéries oxydantes putatives d'acétate syntrophique (SAOB).

Un traitement continu à haute vitesse des SDW s'est avéré réalisable dans des réacteurs à lit de boue granulaire expansé (EGSB) à 20°C (temps de rétention hydraulique (HRT) 24 h, taux de charge en LCFA (OLR) 670 mgCOD-LCFA/L·d, 33% COD-LCFA) avec une élimination de la DCO soluble (soluble chemical oxygen demand, sCOD) de 84–91% et un rendement en méthane de 44–51%. Cependant, l'alimentation en SDW pendant plus de deux mois a entraîné une accumulation d'LCFA, ce qui a entraîné la flottaison des boues granulaires (36-57%) et leur désintégration. Pour contrer la flottaison et la désintégration des boues granulaires induites par les LCFA, un nouveau type de réacteur, constitué d'une chambre à rétention dynamique des boues et d'un film fixe (*dynamic sludge chamber – fixed film*, DSC-FF), a été conçu et a permis d'éliminer la sCOD de 87 à 98% avec HRT de 12 à 72 h et un taux de chargement de LCFA de 220-1333 mgCOD-LCFA/L·d à 20°C. De plus, même à 12 heures de HRT, les LCFA insaturés (linoléate et oléate) ont été traités et il ne subsistait après traitement qu'une partie des LCFA saturés (stéarate, palmitate) dans les réacteurs DSC-FF.

Une activité méthanogène accrue a été établie dans les boues du réacteur pendant les essais en réacteur; mise en évidence par une activité acétotrophe plus élevée dans les boues granulaires du DSC et par une activité plus élevée d'hydrogénotrophe dans le biofilm (FF), indiquant le développement de capacités métaboliques distinctes dans les différents compartiments du réacteur.

Le séquençage à haut débit du 16S rRNA a montré que l'abondance relative du méthanogène acétoclastique, *Methanosaeta*, augmentait dans les réacteurs EGSB et dans les microbiomes actifs des granules (de DSC) et du biofilm (de FF) lorsqu'ils sont nourris avec des concentrations croissantes d'LCFA. Ceci suggère que la méthanogenèse acétoclastique est la voie de méthanogenèse prédominante dans la dégradation des SDW et, vraisemblablement, des LCFA à 20°C. L'abondance relative des taxa réputés pour leur activités β-oxydante et méthanogène était élevée dans les microbiomes actifs au cours du traitement des SDW dans les réacteurs DSC-FF à 20°C. Ainsi, le microbiome du biofilm (de FF) présentait d'importantes concentrations de la bactérie β-oxydante *Syntrophus* et du méthanogène hydrogénotrophe *Methanospirillum*, par rapport à la présence des bactéries acétogènes, *Syntrophobacter*, *Desulfobulbus* et *Geobacter* et du méthanogène acétoclastique dans le microbiome des boues granulaires, suggérant que ces différents taxa ont un rôle spécifique lors de la dégradation des LCFA.

En résumé, ce travail a permis de démontrer la sélection réussie d''inocula à basses températures (10 et 20°C) et la dégradation anaérobie à haute vitesse des LCFA à 20°C en utilisant une conception novatrice de réacteur (ici, le DSC-FF). Les principaux taxa bactériens et archéens impliqués dans la conversion anaérobie des LCFA en méthane à 20°C en ont également été déduits.

Samenvatting

Vetten, oliën en vetachtige stoffen (fats, oil and grease, FOG) zijn een belangrijk bestanddeel van tal van afvalwaters, zoals van de zuivelindustrie. De hydrolyse van FOG resulteert in de productie van vetzuren met lange ketens (long chain fatty acids, LCFA) die het anaërobe behandelingsproces destabiliseren vanwege hun fysisch-chemische eigenschappen en microbiële toxiciteit. Het benutten van het hoge methanogene potentieel van FOG vereist een effectieve behandeling van hoge LCFA-belastingen, waarbij de haalbaarheid van de LCFA behandeling bij lage temperaturen tot nu toe niet is onderzocht. Het doel van dit proefschrift was het onderzoeken van de haalbaarheid van hoogwaardige anaërobe behandeling van LCFA rijk afvalwater bij lage omgevingstemperaturen met behulp van zuivelafvalwater.

Het screenen van mesofiele inocula voor de behandeling van gemengd LCFA met synthetisch zuivelafvalwater (synthetic dairy wastewater, SDW) in batch studies toonde aan dat korrelig slibinoculum snellere en hogere methaanopbrengsten (76-82% van de theoretische opbrengst) behaalde dan de twee digestaten van huishoudelijk afval (1-72%) bij zowel 20 als 10°C. De afbraakcapaciteit van LCFA in het korrelige slibinoculum werd toegeschreven aan de aanwezigheid van β-oxiderende bacteriën uit de familie *Syntrophaceae* (*Syntrophus* en niet-gekweekte taxa), de acetotrofe activiteit van *Methanosaeta* en vermeende syntrofische acetaat oxiderende bacteriën (syntrophic acetate oxidizing bacteria, SAOB).

Een behandeling van SDW met continue hoge snelheid bleek haalbaar te zijn in geëxpandeerde granulair slibbed (expanded granular sludge bed, EGSB) reactoren bij 20°C, hydraulische retentietijd (HRT) 24 uur, LCFA belasting 670 mgCZV-LCFA/L·d met een oplosbare CZV-verwijdering van 84-91% en methaanopbrengst van 44-51%. SDW-voeding langer dan twee maanden resulteerde in LCFA-accumulatie, wat leidde tot flotatie (36-57%) en desintegratie van het korrelslib. Om de LCFA-geïnduceerde korrelslib-desintegratie en flotatie tegen te gaan, werd een nieuw reactortype - het dynamische slibkamer-gefixeerde film (dynamic sludge chamber-fixed film, DSC-FF) – ontworpen, welke een oplosbare CZV-verwijdering van 87-98% behaalden bij HRT's van 12-72 uur (LCFA laadsnelheid 220-1333 mg CZV-LCFA/L·d) bij 20°C. Bovendien werden zelfs na 12 uur HRT de onverzadigde LCFA's (linoleaat en oleaat) behandeld en bleef slechts een deel van de verzadigde LCFA's (stearaat, palmitaat) achter na behandeling in de DSC-FF-reactoren. Een verhoogde methanogene activiteit van het reactorslib werd vastgesteld tijdens de reactor runs; bewezen door een hogere acetotrofe activiteit van het korrelige slib (van DSC), en een hogere hydrogenotrofe activiteit in de biofilm (van FF) die de ontwikkeling van verschillende metabole capaciteiten in de verschillende reactorcompartimenten aangeeft.

16S rRNA-sequentiebepaling met hoge doorvoer toonde aan dat de relatieve abundantie van het acetoclastische methanogeen, *Methanosaeta*, toenam in EGSB-reactoren en in de actieve microbiomen van korrels (van DSC) en biofilm (van FF) wanneer gevoed met toenemende LCFA-concentraties. Dit suggereerde acetoclastische methanogenese als de overheersende methanogenese-route voor SDW en vermoedelijk LCFA-afbraak bij 20°C. Relatieve hoeveelheden van de taxa waarvan bekend is dat ze β-oxiderende en methanogene activiteit hebben, waren hoog in de actieve microbiomen tijdens SDW-behandeling in DSC-FF-reactoren bij 20°C. Het biofilm microbioom (van FF) had een prominente aanwezigheid van de β-oxiderende bacteriën *Syntrophus* en de hydrogenotrofe methanogeen *Methanospirillum* in vergelijking met de aanwezigheid van de acetogene bacteriën, *Syntrophobacter*, *Desulfobulbus* en *Geobacter*, en acetoclastisch methanogen in het korrelslibmicrobioom, hetgeen een rol van deze verschillende taxa tijdens de LCFA-degradatie suggereert.

Samenvattend toonde dit werk succesvolle inoculum selectie aan bij lage temperaturen (10 en 20°C), en hoge anaërobe LCFA-afbraak bij 20°C met behulp van een nieuw reactorontwerp (hier DSC-FF). De belangrijkste bacteriële en archaeale taxa die betrokken zijn bij de anaërobe omzetting van LCFA in methaan bij 20°C werden ook vastgesteld.

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Contents

List of publications

I. Singh, S., Rinta-Kanto, J.M., Kettunen, R., Lens, P., Collins, G., Kokko, M., Rintala, J., 2019. Acetotrophic activity facilitates methanogenesis from LCFA at low temperatures: screening from mesophilic inocula. Archaea, 2019, 1–16.

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IV. Singh, S., Rinta-Kanto, J.M., Lens, P., Kokko, M., Rintala, J., O'Flaherty,V, Collins, G., 2020. Dynamics and assembly in active microbiome of granules and biofilms treating LCFA-rich wastewater in high-rate reactors. Submitted for publication.

Author's contribution

Paper I, Chapter 3:	Suniti Singh planned and performed the batch experiments, analyzed the parameters, and results, and wrote the manuscript. Jukka Rintala and Riitta Kettunen were involved in planning the experiment. Marika Kokko, Gavin Collins, Johanna Rinta-Kanto, and Piet Lens participated in the preparation and correction of the manuscript.
Paper II, Chapter 4:	Suniti Singh planned and performed the experiments, physical-chemical and bioinformatics analyses, and wrote the manuscript. Jukka Rintala and Riitta Kettunen were involved in planning the experiment. Henrik Tolvanen was involved in data analysis for particle size measurement. Marika Kokko, Gavin Collins, Johanna Rinta-Kanto, Piet Lens and Jukka Rintala participated in the preparation and correction of the manuscript.
Paper III, Chapter 5:	Suniti Singh and Vincent O'Flaherty were involved in the planning of experiments and design of reactors. Suniti Singh performed the experiments, and the related physico-chemical and data analysis, and wrote the manuscript. Conall Holohan performed the SMA batch experiments. Juan Castilla-Archilla helped in the set-up of reactors. Simon Mills helped in reactor operation. Vincent O'Flaherty, Jukka Rintala, Marika Kokko, Piet Lens, Conall Holohan and Gavin Collins participated in the preparation and correction of the manuscript.
Paper IV, Chapter 6:	Suniti Singh and Gavin Collins planned the experiments and analysis. Suniti Singh performed the experiments and the bioinformatics analysis, and, wrote the manuscript. Vincent O'Flaherty, Piet Lens, Johanna Rinta-Kanto, Marika Kokko and Jukka Rintala participated in the preparation and correction of the manuscript.

List of Symbols and Abbreviations

AD	Anaerobic digestion
AF	Anaerobic filter
COD	Chemical oxygen demand
CSTR	Completely stirred tank reactor
DGGE	Denaturing gradient gel electrophoresis
DSC	Dynamic sludge chamber
EGSB	Expanded granular sludge reactor
FOG	Fat, oil, and grease
FF	Fixed film
HRT	Hydraulic retention time
LCFA	Long chain fatty acids
MBR	Membrane bioreactor
mRNA	Messenger RNA
OTU	Operational taxonomic unit
OLR	Organic loading rate
PERMANOVA	Permutational multivariate analysis of variance
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
QIIME	Quantitative insights into microbial ecology
rRNA	Ribosomal RNA
sCOD	Soluble chemical oxygen demand
SRT	Sludge retention time
SAOB	Syntrophic acetate oxidising bacteria
SDW	Synthetic dairy wastewater
STP	Standard temperature and pressure
tCOD	Total chemical oxygen demand
TS	Total solids
UASB	Upflow anaerobic sludge blanket
VFA	Volatile fatty acids
VS	Volatile solids

1 GENERAL INTRODUCTION AND THESIS OUTLINE

1.1 Introduction

The United Nations 2030 Agenda for Sustainable Development mandated seventeen Sustainable Development Goals (SDGs), of which, four that belong to the focus areas of clean water, clean energy, sustainable communities, and climate action are inherently linked to the development of sustainable wastewater treatment and resource recovery systems. Utilization of water in domestic, agricultural or industrial sectors results in polluted wastewaters due to the introduction of biodegradable organics, nutrients, and inert; that would adversely impact the environment and human health if discharged as such (Crini and Lichtfouse, 2018). Wastewater treatment generally involves a combination of physical, chemical and biological processes. Several factors, for example, the wastewater and process characteristics, determine the selection of the effective treatment method. The physicochemical methods are typically used for pre- and primary treatment steps, whereas biological treatment is used typically as the secondary treatment step for the removal of organic matter. Within the biological treatment, the activated sludge processes implementing treatment at aerobic conditions are most widely used (Salsabil et al., 2010), but result in the generation of a considerable amount of excess sludge and high energy requirement for aeration. Anaerobic treatment is another treatment option, which converts organic compounds into biogas (methane and carbon dioxide) with the generation of a low amount of excess sludge; and eliminates the energy requirements expended on aeration. Currently, anaerobic treatment using high rate

reactors are widely used for warm industrial wastewaters and sewage treatment in moderate climates (Batstone and Jensen, 2011).

Several industrial wastewaters are emitted at low temperatures (≤20°C), including wastewaters from the bottling, malting, and brewing industries, soft drinks processes, as well as from the food, poultry, and dairy processing. Anaerobic high rate treatment of such cold wastewaters at the discharge temperatures would steer the treatment processes towards achievement of energy neutrality (Martin et al., 2011; Petropoulos et al., 2019).

The wastewaters produced from the food and dairy industries have a significant fat, oil, and grease (FOG) fraction, which should be harnessed for biogas production owing to the high methane production potential of lipids compared to that of carbohydrates and proteins (Alves et al., 2009). FOG hydrolysis results in the production of a mixture of long-chain fatty acids (LCFAs) (carbon length C12 - C18). Single LCFAs have frequently been used for investigating the anaerobic treatment of LCFA-rich wastewaters, despite the synergistic toxic effect exerted by LCFAs. LCFA accumulation may destabilize the anaerobic treatment process due to the physicochemical and microbial toxicity effects of LCFA and instigate operational limitations during the long-term anaerobic treatment of FOG or LCFA-rich wastewaters. Furthermore, LCFAs may inhibit the activity of different microbial groups in anaerobic consortia – hydrolytic bacteria, syntrophic bacteria, and methanogenic archaea (Davidsson et al., 2008; Hwu and Lettinga, 1997; Lalman and Bagley, 2001, 2000; Sun et al., 2013).

The physicochemical factors affecting the performance involve : (i) Mass-transfer limitations due to the formation of hydrophobic LCFA layer around the sludge aggregates due to LCFA sorption and entrapment, (ii) Sludge flotation due to the decrease in sludge density arising from LCFA accumulation on sludge aggregates, (iii) Sludge wash-out due to the disintegration of the granular sludge structure, (iv) Increased solubilization of the lipid bilayer (Desbois and Smith, 2010; Koster and Cramer, 1987) and the membrane proteins leading to direct cell toxicity and lysis (Hanaki et al., 1981; Rinzema et al., 1994), (v) Decreased cell permeability (Zhou et al., 2013), (vi) Disruption of cellular energy functions by disrupting the electron transport chain and uncoupling oxidative phosphorylation (Desbois and Smith, 2010), and, (vii) Enzyme activity inhibition (Zheng et al., 2005). These challenges associated with LCFA degradation are aggravated at low temperatures due to the alterations in the physicochemical characteristics, kinetics, thermodynamics, and hydrodynamics associated with reduced temperature, and the physiological adaptations needed by microbes at low temperatures.

High rate anaerobic FOG treatment has been investigated at mesophilic conditions (Cavaleiro et al., 2016; Dereli et al., 2015; Duarte et al., 2018; Jeganathan et al., 2006; Jensen et al., 2015; Kundu et al., 2013; Leal

et al., 2006; Passeggi et al., 2009; Ramos et al., 2014; Saatci et al., 2003; Silva et al., 2014) and thermophilic conditions (Hwu et al., 1997a, 1997b; Poh and Chong, 2014), while low-temperature anaerobic treatment has been studied using substrates with low FOG content (Bialek et al., 2012; Connaughton et al., 2006a; Esparza-Soto et al., 2013; McHugh et al., 2006; Sheldon and Erdogan, 2016). Thus, the possibility of high rate anaerobic treatment of LCFA-rich industrial wastewaters at low temperatures remains unknown.

In anaerobic high rate reactors, microbes are enriched from the inoculum during the operation wherein numerous factors, as operational temperature, substrate characteristics and loading among others may affect the active population and microbial interactions in the reactor (Braz et al., 2019; Lin et al., 2017). As the growth of anaerobic consortia is derived from the energy obtained from organics degradation, the populations of the different microbial groups may be used as an indicator of bioreactor performance (Carballa et al., 2015; Gonzalez-Fernandez et al., 2015). The active microbial populations in anaerobic reactors have been monitored by using both culture-dependent methods and culture-independent methods for linking the microbial community to interactions and functions (Vanwonterghem et al., 2014). The culture-dependent methods have been used for the enrichment and identification of FOG-and LCFA-degraders from anaerobic consortia (Grabowski et al., 2005b, 2005a; Hatamoto et al., 2007; Sousa et al., 2007). Multiple culture-independent methods such as denaturing gradient gel electrophoresis (DGGE), polymerase chain reaction (PCR), fluorescence *in situ* hybridization (FISH), restriction fragment length polymorphism (RFLP) and 16S RNA sequencing have been used for the profiling and identification of FOG- and LCFA-degraders (De Francisci et al., 2015; Grabowski et al., 2005b; Petropoulos et al., 2018, 2019; Treu et al., 2016; Ziels et al., 2017a), with a more recent transition towards the use of stable isotope probing (SIP) and advanced -omics platforms as metagenomics and transcriptomics to decipher the microbial interactions and functions (Hatamoto et al., 2007; Kougias et al., 2016; Treu et al., 2016; Ziels et al., 2017b). As FOG and LCFA degradation can be maximized by enriching and maintaining optimal concentrations of hydrolytic bacteria, synergistic (acidogenic and acetogenic) bacteria, and hydrogenotrophic and acetoclastic archaea; the identification, and enumeration of these FOG- and LCFA-degrading microbes would suggest new microbial functions, interactions and correlations to environmental parameters during low temperature LCFA degradation.

1.2 Objectives and scope of the study

The main objective of this thesis was to evaluate the feasibility of high rate anaerobic treatment of LCFA-rich feed at low ambient temperatures. The feasibility was evaluated in batch assays and continuous

anaerobic reactors using synthetic dairy wastewater (SDW) as a substrate. The specific objectives, which were studied using various experimental designs, were to evaluate:

1. The effects of inoculum source on methane production from LCFA-rich SDW in batch assays at low temperatures (Chapter 3),
2. The feasibility of using high-rate anaerobic reactors for treating LCFA-rich wastewater at low temperatures, and evaluating the effects of LCFA loading on sludge retention, granular sludge characteristics and reactor performance
    a) expanded granular sludge bed (EGSB) reactors (Chapter 4)
    b) dynamic sludge bed-fixed film (DSC-FF) reactors (Chapter 5)
3. The temporal microbial community dynamics and assembly in the sludge microbiomes in batch (Chapter 3) and continuous reactors treating LCFA-rich wastewater (Chapters 4 and 6).

1.3 Thesis Outline

This Ph.D. dissertation comprises of seven chapters, the main topics of which are shown in Fig. 1.1. The first chapter presents a general introduction regarding the background, problem statement, and research objectives. Chapter 2 provides the state of the art on the anaerobic treatment of LCFA at low temperatures by unifying the theoretical backgrounds from the low-temperature anaerobic treatment of industrial wastewaters, and, the anaerobic conversion of FOG and LCFA-rich wastewaters. In Chapter 3, the biochemical and microbiological effects of inoculum sources on the methane production from LCFA-rich SDW are evaluated in batch assays at low temperatures (10 and 20°C). Chapter 4 assesses the feasibility of EGSB reactors and the effects of OLR and LCFA concentration during continuous high-rate treatment of LCFA-rich wastewater at 20°C using biochemical, morphological, and temporal microbial community analysis. In Chapter 5, the feasibility of novel reactor design (DSC-FF) for continuous high-rate treatment of LCFA-rich wastewater is evaluated at 20°C by a gradual increase in OLR and LCFA loading loads with a decrease in HRT (72 to 12 h). Simultaneously, the use of a sludge mixture is evaluated to engineer microbial consortium suitable for SDW treatment. Chapter 6 evaluates the temporal microbial community dynamics and assembly in the active microbiomes of granular sludge, biofilm, and effluent; and the use of sludge mixture (as inoculum) on the microbial diversity during the high-rate treatment of LCFA-rich SDW in DSC-FF reactors at HRTs ranging from 72-12 h. Chapter 7 presents a comprehensive discussion of the results based on the experimental work in this thesis. This chapter presents an overview of the

practical applications of this research and provides recommendations and perspectives for future research.

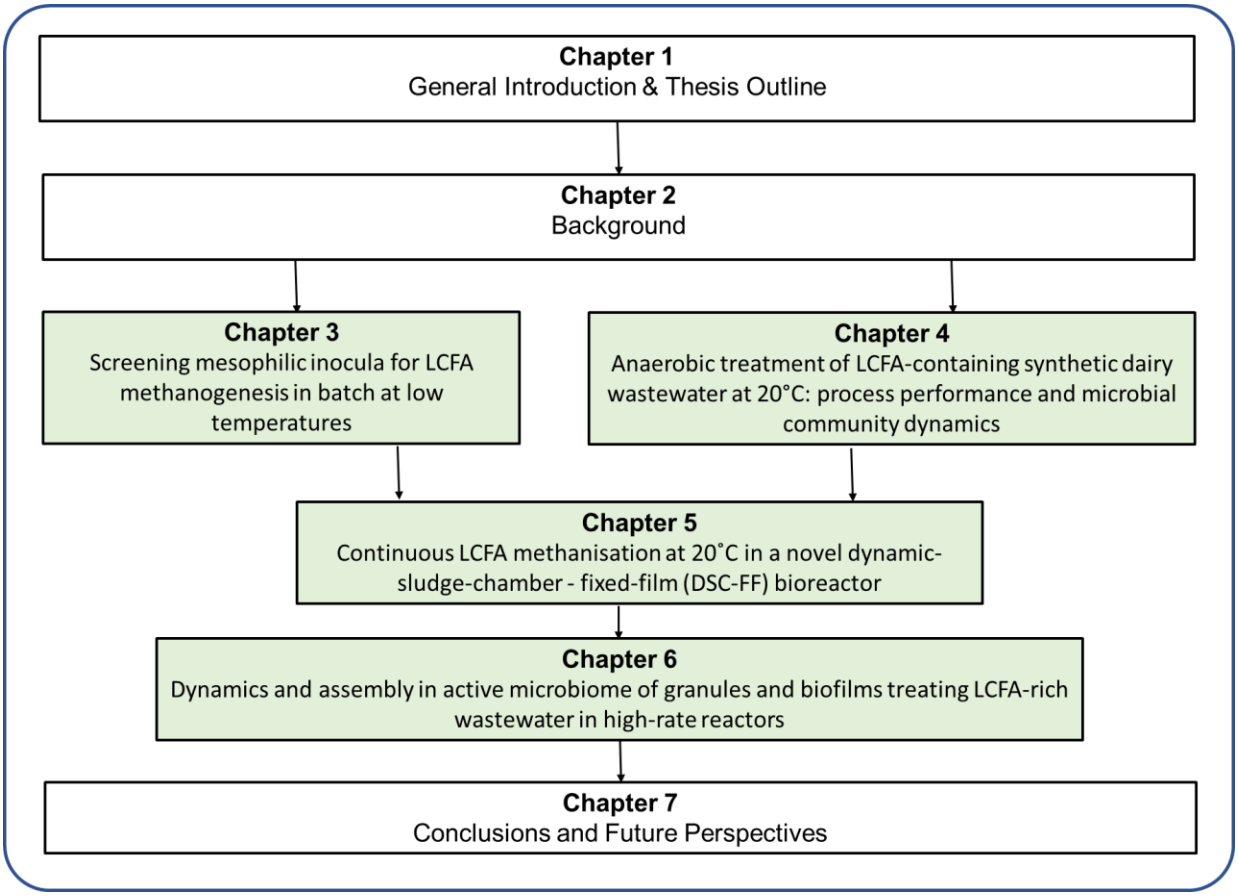

Fig. 1.1. Overview of the structure of this PhD thesis

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2 ANAEROBIC LOW TEMPERATURE TREATMENT OF LCFA-RICH WASTEWATER

2.1 LCFA-rich wastewaters and anaerobic degradation

2.1.1 LCFA-rich wastewaters

Numerous wastewaters are abundant in FOG content, notably those emitted from the oil mills, slaughterhouses, food processing, and dairy production units (Alves et al., 2009). Lipid catabolism produces LCFAs which have a variable chain length of carbon atoms in their aliphatic tail (C= 12- 18). The composition of various FOG-rich wastewaters is shown in Table 2.1, along with the LCFA concentrations shown in Table 2.2. The most commonly found LCFAs in wastewaters are - palmitate, stearate, oleate, and linoleate; wherein oleate is the most abundant (Alves et al., 2009). LCFAs inherently are carboxylic acids in saturated or unsaturated forms, with their degree of unsaturation dependent on the number of unsaturated (double or triple) bonds. The solubility of LCFAs in water decreases with an increase in the carbon chain length, but, increases with the degree of unsaturation (Bober and Garus, 2006; Yoke, 1958). Almost 90% of the total organic carbon (and thus, the methanogenic potential) of lipids is conserved within the LCFAs (Hanaki et al., 1981).

Table 2.1. Composition of FOG-rich wastewaters

Wastewater source	pH	COD (g/L)	Solids (g/L)	FOG (g/L)	References
Cheese production	5.5-7.7 (6.7#)	0.79-6.55 (2.93#)	1.1-6.4 (2.75#) (TS)	0.1-0.6 (0.29 #)	Gutiérrez, Encina and Fdz-Polanco, 1991
Ice cream production	6.6-7.3 (6.9#)	3.77-6.10 (4.94#)	0.6-.4 (1.1#) (TSS)	0.8#	Hawkes, Donnelly and Anderson, 1995
Cheese production	5.5-9.5 (7.3#)	1.0-7.5 (4.4#)	0.5-2.5 (1.1#)	0.2-1.8 (0.7#)	Monroy H. *et al.*, 1995
Whey production	4.3-8.7	5.4-77.3	3.9-58.9 (TS)	0.4-5.7	Kalyuzhnyi, Martinez and Martinez, 1997
Cheese production	5.2#	5.34#	4.21#	na	Strydom, Britz and Mostert, 1997
Milk processing	6.9#	4.65#	2.75#	na	Strydom, Britz and Mostert, 1997
Butter production	5.8#	1.91#	1.72#	na	Strydom, Britz and Mostert, 1997
Milk and cream bottling plant	8-11	2-6	0.4-1.0 (TSS)	0.3-0.5	Ince, 1998
Dairy wastewater	na	56.6-140.2(18#)	1.7-12.6 (7.2#) (TSS)	0.1-10.6 (4.8 #)	Arbeli et al., 2006
Cheese production	3.7-4.3, (4.0#)	11-29.5 (20.3#)	1.4-9.4 (5#)	0.5-3.3 (1.9#)	Vlyssides *et al.*, 2012

- mean, na – not available, TS - total solids, TSS - total suspended solids

Table 2.2. Composition of LCFAs in FOG-rich raw materials and wastewaters (shown as % of total LCFA)

	Saturated LCFA				Unsaturated LCFA				
Substrate	**Laurate (C12:0)**	**Myristate (C14:0)**	**Palmitate (C16:0)**	**Stearate (C18:0)**	**Palmitoleate (C16:1)**	**Oleate (C18:0)**	**Linoleate (C18:2)**	**Others**	**Reference**
Palm oil	nd	1.4	42.9	4.8	0.7	39	10	nd	Alves *et al.*, 2009
Olive oil	nd	nd	14.3	2.4	1.4	71.4	5.5	nd	Alves *et al.*, 2009
Soybean oil	nd	1	11	4.8	nd	21.9	49	nd	Alves *et al.*, 2009
Cotton seed oil	nd	1.4	25.7	2.9	1	15.2	51.9	nd	Alves *et al.*, 2009
Cocoa butter	nd	nd	26.7	32.9	0.5	33.8	4.3	nd	Alves *et al.*, 2009
Chicken fat	nd	1.4	21	4.3	6.7	42.4	20	nd	Alves *et al.*, 2009
Beef tallow	1	2.6	28.1	20	3.8	37.6	2.9	nd	Alves *et al.*, 2009
Slaughterhouse wastewater	nd	nd	35	15	nd	50	0	0	Hwu *et al.*, 1998
Domestic sewage	nd	2.2	16.4	8.1	0.9	30.5	29.2	nd	Quéméneur and Marty, 1994
Whole milk	nd	nd	21	6	nd	39	13	21	Komatsu, Hanaki and Matsuo, 1991)
Whole milk	7	6	21	6	2	39	13	na	Hanaki et al 1981
Dairy wastewater	nd	nd	27	7	nd	37	13	nd	Kim, Han and Shin, 2007

nd – not detected, na – not available

2.1.2 Principles of anaerobic degradation

Anaerobic degradation is a microbially-mediated process that involves the degradation of different organic fractions, i.e., carbohydrates, proteins, and lipids, resulting in the production of biogas. In natural and engineered environments, the carbon flow proceeds sequentially through the different groups in an anaerobic consortium, involving the hydrolytic, acidogenic, and acetogenic bacteria, and the methanogenic archaea.

During hydrolysis, complex macromolecules (carbohydrates, proteins, and fats) are hydrolysed to simpler solubilized forms by the action of exocellular enzymes produced by hydrolytic bacteria. This is followed

by acidogenesis, wherein solubilized monomers such as monosaccharides, amino acids, and LCFAs are degraded by acidogenic bacteria into volatile fatty acids (VFAs), alcohols, lactate, carbon dioxide, and hydrogen; mediated at low concentrations of formate and low partial pressures of hydrogen (Fig. 2.1). Subsequently, acetate is produced from the VFAs by acetogenic bacteria during acetogenesis. The terminal step during anaerobic digestion is methanogenesis, wherein, methanogenic archaea convert acetate or hydrogen and carbon dioxide to methane (Fig. 2.1). Major pathways for methanogenesis are:

i. Acetoclastic methanogenesis: The methyl end of acetate gains an electron from the carboxyl end, resulting in one molecule each of methane and carbon dioxide. It is the prevailing pathway and contributes to about 72% of the methane production, mediated by the action of acetoclastic archaea.
ii. Reductive methanogenesis: Hydrogen molecule donates an electron to carbon dioxide, resulting in the production of one methane and two water molecules. It contributes to about 28% of the methane production, mediated by the action of hydrogenotrophic archaea.
iii. Reductive methanogenesis coupled to syntrophic acetate oxidation: The methyl and the carboxyl ends of the acetate are oxidized to carbon dioxide along with the production of hydrogen during syntrophic acetate oxidation. The hydrogen is further converted to methane by hydrogenotrophic methanogenesis.

2.1.3 Anaerobic degradation of LCFAs

Hydrolysis of lipids is carried out by exocellular lipases resulting in the production of glycerol and LCFA (Fig. 2.1). Lipid hydrolysis is slower and more challenging than the hydrolysis of carbohydrates, such as lactose (Pavlostathis and Giraldo-Gomez, 1991; Perle et al., 1995; Vidal et al., 2000); due to the low bioavailability of lipids (Petruy and Lettinga, 1997) and the need for a sludge retention time (SRT) long enough for the growth of fatty-acid oxidizing bacteria (Miron et al., 2000). However, during lipid catabolism, LCFA degradation often is the rate-limiting step (Hanaki et al., 1981; Novak and Carlson, 1970; Pavlostathis and Giraldo-Gomez, 1991). LCFA inhibition on acetoclastic methanogens was modelled based on the inhibitory concentration, and the LCFA adsorbed onto sludge with a non-competitive inhibition model and a cell-functionality (inhibition) model (Zonta et al., 2013). These two proposed models suggested a high sensitivity of acetoclastic methanogens to LCFA than the acidogenic bacteria (Zonta et al., 2013). Ma *et al.* (2015) also found methanogens as the most inhibited by LCFAs based on the kinetics of inhibition factors of the individual AD microbial groups. Under the LCFA inhibition conditions, methanogenesis was the rate-limiting step, whereas lipid hydrolysis was the fastest AD step which was

strongly affected by the inoculum to substrate ratio. In contrast, under the conditions without LCFA inhibition, the lipid hydrolysis was the rate-limiting step (Ma et al., 2015).

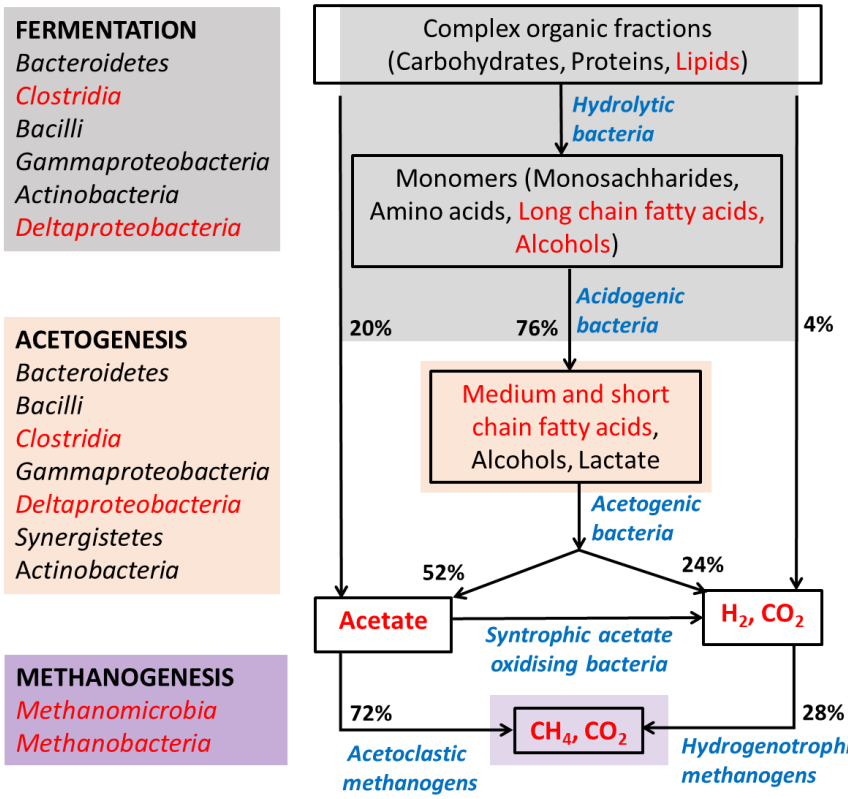

Fig. 2.1. Interactions between microbial groups and carbon flow during anaerobic degradation of lipids (Amani et al., 2010; Cai et al., 2016). The microbial classes involved in substrate degradation in fermentation, acetogenesis, and methanogenesis are presented on the left. The microbial classes and metabolic intermediates known to be involved in the degradation of FOG-rich substrates are highlighted in red.

The LCFA degradation proceeds sequentially, with an initial LCFA adsorption to the cell surface, followed by the activation of saturated and unsaturated LCFAs for their transport into the cytosol in prokaryotes. Within the cytosol, the degree of unsaturation affects LCFA degradation. In the case of mono-unsaturated LCFAs (e.g., oleate C18:1), isomerase converts the configuration of mono-unsaturated LCFA (cis) to mono-unsaturated LCFA (trans). In the case of poly-unsaturated LCFAs (e.g., linoleate C18:2), isomerase converts the configuration of di-unsaturated LCFA (cis) to di-unsaturated LCFA (trans) which is further converted to a saturated trans-configuration by the action of reductase (Fig. 2.2) (Sousa et al., 2009). The mono-unsaturated LCFAs in trans configurations are converted to their saturated forms and transported intracellularly for further degradation through β-oxidation (Fig. 2.2). Recently, another pathway has been suggested for the saturation of unsaturated LCFAs (Cavaleiro et al., 2016), that is independent of hydrogen partial pressure and suggested the involvement of non-syntrophic microbes. During each cycle of β-oxidation, the LCFAs are shortened by 2 carbons in chain length, producing one fatty acid molecule with

smaller chain length and one acetate molecule (Fig. 2.2). The conversion of LCFA to lower molecular weight C_{n-2} fatty acid proceeds through cyclic β-oxidation up until the production of an equivalent number of acetate or propionate molecules from the LCFA is achieved (Fig. 2.2).

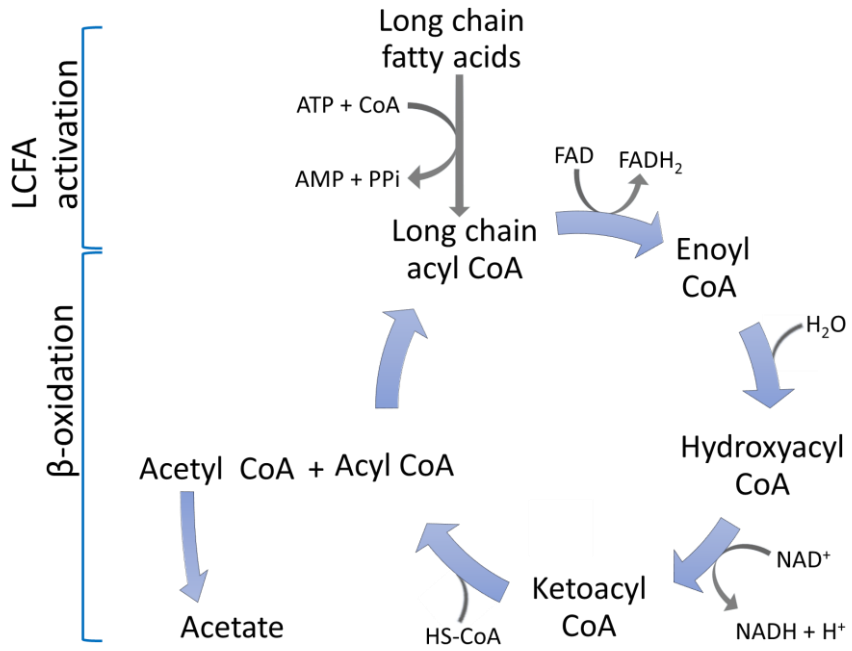

Fig. 2.2. LCFA catabolism via cyclic β-oxidation in *Escherichia coli* (even-numbered LCFA) (Sousa et al., 2009).

The hydrolytic bacteria usually belong to the classes *Bacteroidetes, Clostridia, Bacilli, Gammaproteobacteria*, and, *Actinobacteria* (Amani et al., 2010; Cai et al., 2016). To date, only seven species are known to degrade LCFA (carbon atoms > 12) from the classes *Clostridia* (family *Syntrophomonadaceae*) (Hatamoto et al., 2007; Sousa et al., 2007; Wu et al., 2007) or *Deltaproteobacteria* (family *Syntrophaceae*) (Jackson et al., 1999); and only four species (*Syntrophomonas sapovorans, Syntrophomonas curvata, Syntrophomonas zehnderi, and Thermosyntropha lipolytica*) from the class *Clostridia* are currently known to degrade the unsaturated LCFAs, e.g., oleate (C18:1) and linoleate (C18:2) (Sousa et al., 2009).

2.2 Anaerobic wastewater treatment at low temperatures

2.2.1 Anaerobic high rate reactors and low temperatures

High treatment capacity of wastewaters is needed in anaerobic reactors to accomplish treatment in smaller reactor volumes and treatment space and improve cost benefits. This can be achieved by the use of high-rate reactors, referring to systems wherein the SRT is uncoupled from the HRT (van Lier et al., 2015); consisting of anaerobic consortia in suspension, biofilm or self-aggregated granular form. A higher

biomass concentration can be maintained in anaerobic reactors by using different retention principles, viz., (i) settling characteristics of the granules in granular sludge reactors, (ii) settling characteristics of the sludge (suspended/flocculent) in sequencing batch reactors, (iii) microbial biofilm formation on support matrix in biofilm reactors, (iv) membrane-based physical separation of liquid from solids in membrane bioreactors, and (v) retention through flotation in certain specialized reactor configurations (anaerobic flotation reactor, inverted anaerobic sludge blanket). Few of the widely applied anaerobic high-rate reactor configurations are shown below along with an example of a conventional digester, anaerobic contact process (ACP) (Fig. 2.3).

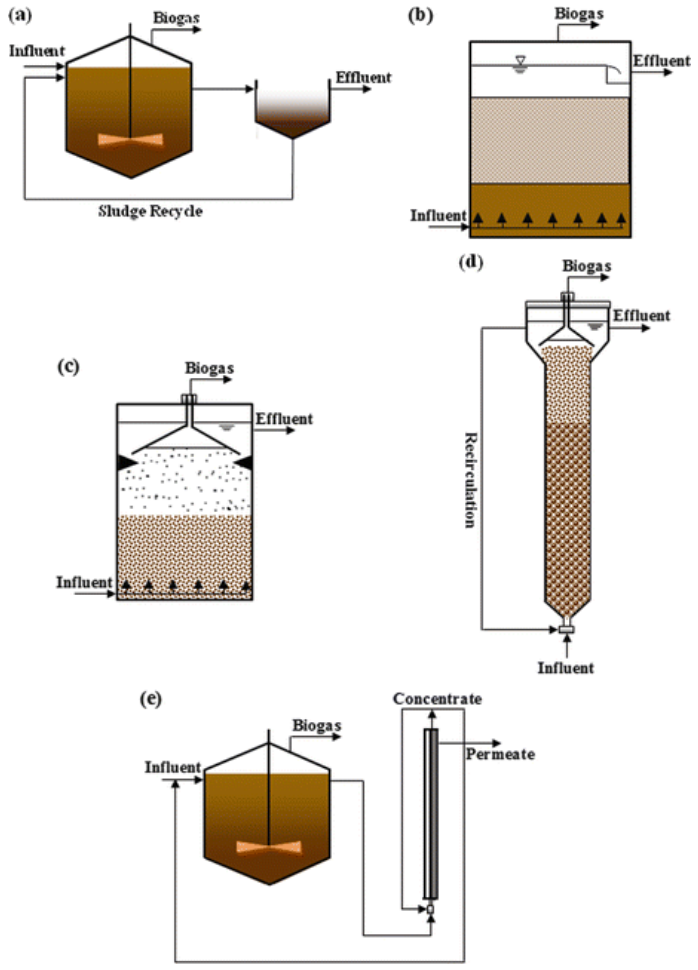

Fig. 2.3. Examples of high rate reactors: (b) Anaerobic filter (AF), (c) Upflow anaerobic sludge blanket (UASB) reactor, (d) Expanded granular sludge bed (EGSB) reactor, (e) Membrane coupled completely stirred tank reactor (CSTR), along with conventional clarifier-coupled digester – anaerobic contact process (ACP). Figure is based on (van Lier et al., 2015).

The advantages of granular sludge reactors involve the capability to withstand the shock loads and to function across different temperature ranges (Couras et al., 2015; Karadag et al., 2015; Lettinga et al., 2001; Mao et al., 2015; van Lier et al., 2015). The applicability of a reactor design is subject to the

composition and strength of wastewater. The treatment of complex wastewater, such as dairy wastewater, has been achieved at full-scale in various reactor types, i.e., granular sludge reactors, membrane bioreactors, anaerobic filter reactors, flotation reactors, fluidized bed reactors, and sequential batch reactors (Frijters et al., 2014; Lier et al., 2015; Omil et al., 2003; Passeggi et al., 2012).

Eshtiaghi *et al.*, (2013) established the need for engineering the hydrodynamic parameters for optimal bioreactor performance, based on the influence of operational parameters, such as, temperature. For example, liquid viscosity and gaseous solubility increase with a decrease in temperature. The increased liquid viscosity induces a lowered mixing of the solid-, liquid-, and gas-phase components, and incurs additional costs due to higher energy needed for mixing. The increased gaseous concentrations in the liquid phase may affect other parameters, for example, increased carbon dioxide dissolution reduces the liquid pH at low temperature. Furthermore, a drop in the temperature decreases the diffusion of soluble compounds and liquid-solid separation, thereby decreasing the mass transfer (substrate and product transfer) and particle settling, respectively. Apart from physicochemical implications, the kinetic and thermodynamic parameters are also impacted by a decrease in temperature (section 2.2.2).

2.2.2 Anaerobic microbiology at low temperature

Psychrophiles have evolved features, genotypes, and phenotypes to acclimate to low temperatures. These involve (i) the regulation of membrane fluidity, (ii) production of extracellular polymeric substances that function as cryoprotectants or buffers, and (iii) expression of cold shock protein domains and functional enzymes at low temperatures (D'Amico et al., 2006). Moreover, the enzymatic machinery of the psychrophiles is adaptable to the mesophilic temperature ranges, resulting in increased metabolic activity with an increase in temperatures up to the optimum temperature (Schulz et al., 1997). In contrast, the metabolic activity in mesophilic consortia decreases with a decrease in temperature. Yet, mesophilic inocula have been used for the low temperature (4 to 25°C) anaerobic treatment of a variety of industrial wastewaters (Table 2.3), based on the ease of availability of anaerobic sludges from mesophilic conditions whereas the use of psychrophilic inoculum sourced from operational reactors is rare due to the lack of psychrophilic AD applications.

Table 2.3. Origin of inocula used in continuous anaerobic treatment of industrial wastewaters at low temperatures (5-25°C).

Inoculum characteristics				Treatment conditions			References
Inoculum origin	**Temperature**	**Sludge structure**	**Source environment**	**Wastewater type**	**Reactor configuration**	**Temperature (°C)**	
Anaerobic sludge treating brewery wastewater	Mesophilic	Granular	Engineered system (full-scale UASB reactor)	Soft drink industry wastewater	EGSB/UF-MBR	13.7-24.2	Sheldon and Erdogan, 2016
Anaerobic sludge	Mesophilic	Granular	Engineered system (full-scale reactor)	Brewery wastewater	EGSB-AF	15.37	Connaughton, Collins and O'Flaherty, 2006
Anaerobic sludge treating alcohol wastewater	Mesophilic	Granular	Engineered system (IC reactor)	Synthetic whey-based wastewater	Anaerobic hybrid reactors	20, 18, 16, 14, 12	McHugh, Collins and O'Flaherty, 2006
Anaerobic sludge treating citric acid production wastewater	Mesophilic	Granular	Engineered system (a full-scale IC reactor)	Ethanol:acetone:propanol:methanol = 1:1:1:1 (COD ratio)	EGSB-AF	15, 37	Enright *et al.*, 2009
Anaerobic sludge treating industrial alcohol production wastewater	Mesophilic	Granular	Engineered system (a full-scale IC reactor)				
Sludge mixture of cattle manure, waste activated sludge and a variety of non-granular anaerobic sludges (1:2:2)	Mesophilic origin, stored at 4°C for 2 years	Suspended	Engineered and natural				
Anaerobic sludge treating industrial alcohol production wastewater	Mesophilic	Granular	Engineered system (full-scale IC reactor)	Synthetic dairy wastewater	IFB, EGSB	37, 25, 15	Bialek *et al.*, 2012
Sludge treating soft drink production wastewater	Mesophilic	Granular	Engineered system (full-scale treatment plant)	Molasses wastewater	FBR	5-18	Zhang *et al.*, 2012
Anaerobic sludge treating leachate	Mesophilic	Suspended	Engineered system (lab-scale reactors)	Solid waste leachate	MBR	37, 10	Trzcinski and Stuckey, 2010

Anaerobic sludge	Mesophilic	Granular	Engineered system (UASB)	Coffee bean processing wastewater	Upflow FBR (support material - blast furnace cinders, polyurethane foam and crushed stone)	6.4-32.9 (average 18)	Fia *et al.*, 2012
Anaerobic sludge treating alcohol wastewater	Mesophilic	Granular	Engineered system (full-scale IC reactor)	Organic wastewater (ethanol:acetone:propanol:methanol 1:1:1:1)	EGSB-AF	4-15	McKeown *et al.*, 2009
Anaerobic sludge treating municipal wastewater	Mesophilic	Suspended	Engineered system (anaerobic hybrid pilot-scale reactor)	Fruit juice factory wastewater	Two-stage UASR followed by AS system.	25	El-Kamah *et al.*, 2010
Anaerobic sludge treating cereal wastewater	Acclimated to 17°C for 300 d	Granular	Engineered system (pilot-scale UASB)	Chocolate-processing industry wastewater	UASB	18	Esparza-Soto *et al.*, 2013
Anaerobic sludge	Acclimated to 20°C	Granular	Engineered system	VFA-based wastewater	EGSB	5-15	Syutsubo et al., 2008
Waterfowl lake sediment	Natural site (ambient air temperature of 0°C)	Suspended	Natural source	Synthetic brewery wastewater (diluted beer)	Upflow reactor with polyurethane foam	15	Xing et al., 2010
Mixture of lake sediment and arctic soil (1:1)	Natural site (ambient air temperatures of -16 – 17°C)	Suspended	Natural sources	Real settled municipal wastewater	UASB, MBR	15	Petropoulos et al., 2019

UASB - Upflow anaerobic sludge blanket, EGSB - Expanded granular sludge bed, UF - Ultrafiltration, MBR – Membrane bioreactor, AF – Anaerobic filter, IC – Internal circulation, CSTR – Completely stirred tank reactor, FBR – Fixed-bed reactor, IFB - Inverted fluidized bed, UASR - Upflow anaerobic sponge reactor, AS – Activated sludge.

Laboratory studies have been performed using psychrophilic and cold-adapted inocula from diverse environments. Xing et al., (2010) compared inocula from different cold natural locations (lake sediment, pond silt, and wetlands) to mesophilic granular sludge and found that the highest specific methane production rates from glucose in batch assays at 15°C was achieved by a psychrophilic inoculum (waterfowl lake sediment) (Xing et al., 2010). Syutsubo et al., (2008) used granular sludge adapted to 20°C to treat VFA-based wastewater in EGSB by gradually decreasing the temperature from 15 to 5°C, and, monitored the activity of retained sludge at 10, 15, 20, 35 and 45°C over a 400-d duration. The authors confirmed an increased microbial activity (acetoclastic, hydrogenotrophic and propionate-oxidizing/degrading) at different low-temperatures (10, 15 and 20°C) compared to their activities in the inoculum, demonstrating the effectiveness of microbial adaptation to low temperature conditions (Syutsubo et al., 2008). However, Bowen et al., (2014) used mesophilic suspended sludge adapted to ambient temperatures (10 - 25°C) as cold-adapted inoculum for the oxidation of VFA, acetate, and H_2/CO_2 at 4, 8 and 15°C. It was found that methanogenesis was absent at temperatures below 8°C, and accordingly, the acclimation of mesophilic sludge to lower temperatures was concluded as an unsuitable approach for psychrophilic methanogenesis at temperatures ≤8°C (Bowen et al., 2014). Thus, the enrichment of specific taxa during reactor operation is subject to the initial composition of the inoculum, substrate composition, and operational temperature. Due to the lack of consensus on the general applicability of the inoculation approaches for psychrophilic anaerobic digestion, investigations are necessitated for evaluating inoculum performance for LCFA degradation at low operational temperatures.

For successful implementation of anaerobic treatment at low temperature, the balanced growth of different microbial groups is essential. Microbes can be classified based on their temperature ranges and optima, i.e. hyperthermophiles (65-110°C, T_{opt} - 90-100°C), thermophiles (40-75°C, T_{opt} - 60-65°C), mesophiles (10-47°C, T_{opt} – 33-37°C), psychrophiles (-7-18°C, T_{opt} -10-14°C) or psychrotrophs (0-30°C, T_{opt} - 18-22°C), and may have a rapid decline in their growth rates to either side of the optimum growth temperature (Pommerville, 2014). Organisms belonging to domain *Archaea* have a wider temperature tolerance and optimum growth range than those in domain *Bacteria* or *Eukarya* (Pommerville, 2014).

As a general rule, the temperature dependence of the maximum substrate utilization and microbial activity (Pavlostathis and Giraldo-Gomez, 1991) or enzyme activity (Bergamo et al., 2009; D'Amico et al., 2006; Petropoulos et al., 2018) is based on the Arrhenius relationship (Equation 1).

$$k = A\, e^{-Ea/RT}$$

where, k is microbial growth rate/process rate/enzyme activity rate, Ea the activation energy (J/mol), R the gas constant (8.314 J/mol·K), and T the absolute temperature (K).

A decrease in operational temperature generally leads to a decrease in maximum substrate degradation kinetics and specific growth rates, but may also increase the net biomass yield of the methanogens and acidogens (Nachaiyasit and Stuckey, 1997). Although different microbial stages have been proclaimed to be rate-limiting in low temperature anaerobic treatment, there is a lack of consensus with studies reporting hydrolysis or acidogenesis as the rate-limiting step (Kotsyurbenko et al., 1993; Petropoulos et al., 2018). For example, the hydrolysis of particulate matter has been suggested to be low at temperatures below 15°C (Lettinga et al., 2001), and lipid hydrolysis has been shown as the rate-limiting step in real lipid-rich wastewater around 4°C (Petropoulos et al., 2018). On the other hand, acidogenesis from different organic substrates has been shown as the rate-limiting step in a study evaluating hydrolysis and acidogenesis at temperatures from 6 to 28°C (Kotsyurbenko et al., 1993).

Recently, the microbial communities in psychrophilic inoculum treating lipid-rich municipal wastewater at 15°C were evaluated using DGGE (Petropoulos et al., 2017), qPCR, and 16S rRNA sequencing (Petropoulos et al., 2019, 2018); and an important role of the bacterial classes *Carnobacteriaceae* (genus *Trichococcus*), *Caldilineae* (family *Caldilineaceae*), *Bacteroidia* (family *Bacteriodaceae*), *Betaproteobacteria* (family *Comamonadaceae)* and of the archaeal classes *Methanomicrobia* (genera *Methanosaeta*, *Methanosarcina*) and *Methanobacteria* (genus *Methanospirillum*) was deduced.

2.3 Anaerobic treatment of LCFA rich wastewaters at low temperature

Dairy effluents are an example of complex wastewaters, and rich in carbohydrates, proteins, and FOG fractions wherein lactose and casein are the chief carbohydrate and protein constituents (Demirel et al., 2005). While evaluating the influence of the relative concentrations of carbohydrates, fats, and proteins on anaerobic biodegradability of dairy wastewaters, a reduced hydrolysis rate was found in FOG-rich wastewater than in the carbohydrate or protein-rich wastewaters (Vidal et al., 2000).

The continuous treatment of LCFA-rich wastewaters has been evaluated in granular sludge reactors such as UASB and EGSB (Table 2.4). Hwu, Lier and Lettinga (1998) used EGSB to investigate the effect of reactor hydrodynamics on the anaerobic treatment of LCFA mixture (oleate: palmitate = 82:18) at 30 and 55°C. They suggested the operation of granular sludge reactors as UASB at lower upflow velocities (1 m/h) than as EGSB at higher upflow velocities (4-7 m/h) for achieving higher methane conversion (70% vs 39-50%) (Hwu et al., 1998a). The sludge flotation started at LCFA loading rates of 0.09 gCOD/gVSS·d, and flotation of the entire granular sludge bed was observed within 5 d (Hwu et al., 1998b). Saatci, Arslan and Konar (2003) treated FOG and LCFA-rich sunflower oil factory wastewater and achieved more than 70% removal of the lipids and fatty acids with a methane conversion of 46-99% (OLRs 1.6-7.8 gCOD/L·d) in UASB (Saatci

et al., 2003). Pereira et al., (2005) used EGSB reactors to treat oleate and palmitate and achieved a low methane conversion (20-25%) up to a period of 75 d despite a high COD removal (80-93%), as a significant portion of LCFAs was accumulated in the reactors due to entrapment, precipitation and adsorption of LCFAs (Pereira et al., 2005). Leal et al., (2006) also observed accumulation of fats while treating FOG supplemented dairy wastewater in UASB at FOG concentrations of 600 and 1000 mg/L (Leal et al., 2006). Such FOG accumulation on sludge was found to lead to sludge flotation and washout which increased the FOG loading, eventually resulting in the failure of anaerobic treatment process (Jeganathan et al., 2006). Consequently, the critical accumulated FOG loading was identified as 1.04 gFOG/gVSS for the treatment of oily wastewater (from food industry) in UASB at 35°C (Jeganathan et al., 2006). Recently, the treatment of real municipal wastewater containing (70% lipids) at 15°C was compared in UASB and anMBR reactors over 375 d at short HRTs (7.7-84 h) and found the accumulation of unhydrolyzed COD that were majorly constituted of lipids. The lipid accumulation in anMBR reactors was about three-folds higher (4.5 mgCOD/L) than in the UASB reactors (1.4 mgCOD/L) (Petropoulos et al., 2019), and indicated the constraints of using anMBR for the treatment of FOG-rich wastewater at low temperatures.

Table 2.4. Feed and reactor characteristics for high-rate anaerobic treatment of FOG and LCFA-rich wastewaters.

Feed	COD (mg/L)	FOG content (mg/L)	Reactor type	HRT (h)	Temperature (°C)	References
Synthetic dairy wastewater with oil and grease content	1800-3700	200, 600, 1000	UASB	19	35	Leal *et al.*, 2006
Dairy industrial wastewater	2520	495	Modified UASB with internal fat separator and digester	17	32-38	Passeggi, López and Borzacconi, 2009
Long chain fatty acid mixture (oleate:palmitate = 82:12)	100-1000	100-1000#,*	EGSB	0.6-6	30, 55	Hwu, Lier and Lettinga, 1998
Sunflower oil factory wastewater	5600-15500	540-7640, 237-1294#	UASB	48-67	37	Saatci, Arslan and Konar, 2003
Rendering wastewater	3000-26000	1200-14600	UASB	30-120	35± 3	Jeganathan, Nakhla and Bassi, 2006
Settled municipal wastewater	100-800	70¤	UASB, MBR	7.7-84	15	Petropoulos et al., 2019

#LCFA content, * concentration in mgCOD/L, ¤ - percentage of lipids

Although the operational challenges associated with sludge flotation, granular disintegration, and sludge washout have been encountered in granular sludge reactors; the anaerobic treatment of FOG and LCFA-rich wastewaters is feasible at FOG concentrations contingent to the 'critical accumulated FOG loading'. The use of modified reactor designs may be beneficial in sludge retention and enhance the FOG and LCFA degradation capacity. For example, Passeggi, López and Borzacconi (2009), used a modified UASB with an internal fat separator to extract floating FOG content and coupled it to a digester for degradation of the

extracted fatty material. The sludge retention in this modified reactor design was achieved by using a lamella settler during the treatment of industrial dairy wastewater (Passeggi et al., 2009).

The challenges in the anaerobic treatment of FOG-rich wastewaters are compounded at low temperatures due to the temperature effects on process parameters as described in section 2.2.1. The treatment of dairy wastewater at low ambient temperatures has been performed in various granular sludge reactors. Dague et al., (1998) treated non-fat dry milk in ASBR at HRT of 6-24 h at 5-25°C, with highest methane yield of 280 mL-CH_4/g-COD at 25°C; and found the methane yields and COD removal efficiencies to decrease with temperature. In comparison, when a pilot-scale static granular bed reactor (SGBR) was used to treat dairy processing wastewater at low temperatures (10-25°C) at an HRT of 9-48 h, a COD removal of 94% and methane yield of 200 mL-CH_4/g-COD was achieved at the average operational temperature (19°C) (Park et al., 2012). Even lower temperatures (10°C) were used by Bialek, Cysneiros and O'Flaherty (2013), and the treatment of synthetic dairy wastewater was found to be feasible at 12-48 h HRT (OLR 0.5-2 gCOD/L·d) in EGSB reactor with a COD removal of 85% (Bialek et al., 2013).

Numerous biofilm reactors also have been used for treating dairy wastewaters. Treatment of dairy effluents was investigated in anaerobic fixed bed reactor (AFBR) at sub-mesophilic temperatures of 22-26°C, and, achieved COD removal of 67% and methane yield of 110 mL-CH_4/gCOD at an HRT of 3 d (OLR 8 gCOD/L·d) during semi-continuous operation (Nikolaeva et al., 2013). Wang et al., (2009) treated milk permeate wastewater having acidic pH (3-6.5) at 20-25°C at long HRTs (>9 d) (OLR 0.5-6.5 g-COD/L.d) in MBBR, and, achieved a COD removal of 56-92% and high methane yield (267-302 mL-CH_4/g-COD) (Wang et al., 2009). Ramasamy and Abbasi (2000) treated synthetic dairy wastewater at room temperatures in a CSTR consisting of nylon-mesh biofilm support also at long HRTs (10-15 d), and, achieved COD removals of 46-59% (Ramasamy and Abbasi, 2000). Treatment of dairy wastewater in an anaerobic filter (AF) was reported to achieve high COD removal (75-95%) at 4 d HRT at 21 to 12.5°C, however, the COD removal decreased (40-70%) with the decrease in HRT to 1d. The methane production, as well as the COD removal, declined with temperature (21 to 12.5°C) at all the HRTs (Viraraghavan and Kikkeri, 1990). Bialek, Cysneiros and O'Flaherty, (2014) used IFB reactor for treating synthetic dairy wastewater at lower temperature (10°C), and, reported lower COD removals (33-69%), presumably due to the shorter HRTs (12-48 h), but achieved higher methane yields (107-241 mL-CH_4/gCOD) than reported by Viraraghavan and Kikkeri, (1990). Furthermore, synthetic dairy wastewater was treated at 25°C (HRT of 10-11 d) in MBR, and, a COD removal % of 37-56% and methane yield of 175 mL-CH_4/gCOD_{in} was reported (Al-Malack and Aldana, 2015).

Table 2.5. Feed characteristics and reactor performances for anaerobic treatment of dairy wastewater at low ambient temperatures.

Substrate	Reactor	Temp (°C)	LCFA loading rate (g-COD/L.d)	OLR (g-COD/L.d)	HRT (d)	COD removal (%)	Methane yield at STP (mL/g-COD.d)	References
Non-fat dry milk	ASBR	5-25	a	0.6-2.4	0.25- 1	60-98	95-290	Dague, Banik and Ellis, 1998
Synthetic dairy parlour wastewater	Two phased-UASB septic tank	10-20	NR	0.1-0.24	3.5+ 1.5	33-62	NR	Luostarinen and Rintala, 2005
Dairy processing wastewater	SGBR	10-25	NR	0.6-9.7	0.38- 4	>90	90-340	Park *et al.*, 2012
Synthetic dairy wastewater	EGSB	10	a	0.5-2	0.5-4	84-90	151-806	Bialek, Cysneiros and O'Flaherty, 2013
Dairy washing wastewater	AFBR	22-26	NR	4.4-24	1 -5.5	28-82	70-180	Nikolaeva et al., 2013
Milk permeate wastewater	MBBR	20-25	NR	0.5-6.5	9	56-92	267-302	Wang et al., 2009
Synthetic dairy wastewater	CSTR with biofilm support	25	a	0.015-0.022[b]	10-15	46-59	NR	Ramasamy and Abbasi, 2000
Dairy wastewater	AF	12.5, 21	NR	0.63-4.03	1-6	45-85	30-260	Viraraghavan and Kikkeri, 1990
Synthetic skimmed dairy wastewater	IFB	10	a	0.5-2	0.5 -2	24-80	107-294	Bialek et al., 2014
Synthetic dairy wastewater	MBR	25	a	NR	10-11	37-56	140[c]	Al-Malack and Aldana, 2015
Skim milk	UASB + aerobic MBR	17-25	NR	2.4-1.6	0.4 -0.6 (total 0.6-0.8)	95 UASB, 99 total	110-310	Buntner et al., 2013
Dairy wastewater	UASB + Activated sludge	20	0.26 ± 0.023b	3.4	1+ 0.08	60	NR	Tawfik et al., 2008

ASBR Anaerobic sequencing batch reactor, MBR Membrane bioreactor, SGBR Static granular bed reactor, AFBR Anaerobic fixed bed reactor, IFB Inverted fluidized bed
NR Not reported, GS Granular sludge
a <1 % fat in substrate
b Units gVS/d
c maximum methane yield
d Continuation of previous system
e Oil and grease (mg/L), ie 760 ± 66 mgCOD/L (1 g/L oil and grease equivalent to 2.89 gCOD/L)

Due to the combined advantages of treatment systems, hybrid reactors have been used for low-temperature anaerobic treatment as well. EGSB-AF hybrid reactor (pilot) was operated at a mean temperature of 17°C, and, was reported to achieve higher than 90% COD removal during the treatment of dairy wastewater at HRT ranging from 0.33-1 d (O'Flaherty 2013). Membrane coupled-UASB reactor treating skim milk was used at low ambient temperatures (17-25°C) for treating synthetic dairy wastewater at short HRTs of 0.6-0.8 d (OLR 2.4 gCOD/L·d), and, achieved methane yields of 150-240 mL-CH_4/gCOD (Buntner et al., 2013). The studies that were undertaken so far for the treatment of dairy

wastewaters often employed synthetic dairy wastewater formulated from skimmed milk powder which has a low-fat content (<1% fat) (Table 2.5).

Real dairy wastewater was treated in activated sludge section-coupled UASB at low ambient temperatures at HRT of 1 d and achieved 60% COD removal during the anaerobic treatment (Tawfik et al., 2008). However, a regular sludge discharge (amounting to about 20% of total influent COD) was maintained, which would have contributed to the removal of COD and lipids (lipid fraction constituted 14% of influent COD) from the UASB. Therefore, the biodegradation of lipid fraction in this study remains unconfirmed, more so, as the methane production was not reported (Tawfik et al., 2008). Thus, despite the number of studies performed on the treatment of dairy wastewaters, the methane production from FOG and LCFA-rich dairy wastewaters has not been studied at low ambient temperatures. Furthermore, although a wide range of reactor options are available for anaerobic treatment, granular sludge reactors and their hybrid configurations have been preferred so far for the anaerobic treatment of wastewaters at low temperatures.

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3 ACETOTROPHIC ACTIVITY FACILITATES METHANOGENESIS FROM LCFA AT LOW TEMPERATURES: SCREENING FROM MESOPHILIC INOCULA

Abstract

The inoculum source plays a crucial role in the anaerobic treatment of wastewaters. Lipids are present in various wastewaters and have a high methanogenic potential, but their hydrolysis results in the production of long chain fatty acids (LCFAs) that are inhibitory to anaerobic microorganisms. Screening of inoculum for the anaerobic treatment of LCFA-containing wastewaters has been performed at mesophilic and thermophilic conditions. However, an evaluation of inocula for producing methane from LCFA-containing wastewater has not yet been conducted at low temperatures and needs to be undertaken. In this study, three inocula (one granular sludge and two municipal digester sludges) were assessed for methane production from LCFA-containing synthetic dairy wastewater (SDW) at low temperatures (10 and 20°C). A methane yield (based on mL-CH_4/g-COD_{added}) of 86-65% with acetate and 45-20% with SDW was achieved within 10 days using unacclimated granular sludge, whereas the municipal digester sludges produced methane only at 20°C but not at 10°C even after 200 days of incubation. The acetotrophic activity in the inoculum was found to be crucial for methane production from LCFA at low temperatures, highlighting the role of *Methanosaeta* (acetoclastic archaea) at low temperatures. The presence of bacterial taxa from the family *Syntrophaceae* (*Syntrophus* and uncultured taxa) in the inoculum was found to be important for methane production from SDW at 10°C. This study suggests the evaluation of acetotrophic activity and the initial microbial community characteristics by high-throughput amplicon sequencing for selecting the inoculum for producing methane at low temperatures (up to 10°C) from lipid-containing wastewaters.

3.1 Introduction

High rate anaerobic treatment is an efficient solution to treat wastewaters without expending energy for aeration and to simultaneously produce a bioenergy source as methane. Mesophilic conditions have been commonly applied for the anaerobic treatment of wastewaters due to the optimal growth temperature range of the microbial consortia (Lettinga et al., 2001). However, numerous wastewaters, including domestic and industrial, are discharged at temperatures of 20°C or lower, and their anaerobic treatment at these low temperatures could improve the net energy gain from the treatment process.

Over 700 million tonnes of milk is produced annually worldwide, which leads to the generation of huge volumes of dairy wastewaters (Faye and Konuspayeva, 2012; Karadag et al., 2015). These dairy effluents constitute high amounts of lipids (35-500 mg/L) along with carbohydrates and proteins (Perle et al., 1995), and the presence of each of these constituents poses specific challenges. Lipids have a higher methanogenic potential than carbohydrates or proteins (Alves et al., 2009), but their anaerobic treatment is challenging due to the synergistic inhibitory effects of their hydrolysis by-products (Chen et al., 2014; Koster and Cramer, 1987; Lalman and Bagley, 2002; Lalman and Komjarova, 2004; Shin et al., 2003). Single compounds (Nedwell, 1999; Nozhevnikova et al., 2003) as well as industrial wastewaters (Bialek et al., 2014; Connaughton et al., 2006a; Sheldon and Erdogan, 2016; Zhang et al., 2012) have been treated anaerobically at low temperatures. Yet, the anaerobic treatment of lipids at low temperatures (≤20°C) remains understudied.

During lipolysis, the lipids hydrolyze to long chain fatty acids (LCFAs) which are inhibitory to acidogens, acetogens, and methanogens, even at low concentrations (Kim et al., 2004; Koster and Cramer, 1987; Lalman and Bagley, 2002; Shin et al., 2003). For example, at 21°C, 30 mg/L of linoleic acid, 30 mg/L of oleic acid, and 10 mg/L of stearic acid inhibit methane production from 100 mg/L of acetate (Lalman and Bagley, 2001, 2000). Additionally, LCFA impedes methane production by imposing mass-transfer limitations and preventing aggregation of acetogens and methanogens (Daffonchio et al., 1995; Pereira et al., 2005). Although the individual and synergistic inhibitory effects of LCFA (oleic, stearic, and linoleic acids) on hydrogen, glucose, and butyrate fermentation have been studied in batch at 21°C, the methane production was not reported (Lalman and Bagley, 2002) which obscures the potential use of LCFA as a methanogenic precursor under psychrophilic conditions. Investigations on the anaerobic treatment of dairy wastewaters (4 g-COD/L) with a low fat content of 40 mg-COD/L (typically <1% of the total chemical oxygen demand) have been conducted previously at temperatures as low as 10 and 15°C (Bialek et al.,

2012). Very recently, the knowledge on lipid treatment has been extended to low temperatures for the assessment of lipase activity at reduced temperatures (4, 8, and 15°C) in domestic wastewater (Petropoulos et al., 2018). Yet, methane production from LCFA mixtures has not been reported at low temperatures and warrants further investigation.

The inoculum source plays a crucial role in anaerobic treatment and more so at low temperatures, as the microbial community in the inoculum affects the substrate-degradation potential and the methanogenic activity (Kettunen and Rintala, 1997; Sun et al., 2016). Both suspended and granular sludges have been used in low temperature anaerobic digestion (LTAD) studies to treat various wastewaters (Bialek et al., 2012; Connaughton et al., 2006a; El-Kamah et al., 2010; Enright et al., 2005; Fia et al., 2012; Luostarinen and Rintala, 2005; McHugh et al., 2006; Trzcinski and Stuckey, 2010; Zhang et al., 2012). Previous comparisons of granular to suspended sludges at low temperatures for methane production have contrasting conclusions, and their suitability appears to be case-specific. At 15°C, when Xing et al. (Xing et al., 2010) compared inocula from permafrost sites (lake sediment, pond silt, and wetlands) to mesophilic granular sludge, a higher methane production rate (71 mL-CH_4/gVSS·d) from glucose of around 2 times was achieved using the waterfowl lake sediment (suspended sludge) compared to the other inocula. Conversely, at 15°C when Enright et al. (Enright et al., 2005) compared a sludge mixture (waste-activated sludge, cattle manure, and non-granular anaerobic sludge) to two granular sludges, more methane was produced by the granular sludges from acetate, H_2/CO2, and ethanol.

In the anaerobic treatment of lipid-containing wastewater, both suspended sludge and granular sludge have been used as inoculum at mesophilic and thermophilic conditions. Granular sludge has been reported to be suitable for anaerobic LCFA treatment due to its lower specific surface area (Hwu et al., 1996), a higher methanogenic activity (acetoclastic, hydrogenotrophic, propionate, and ethanol fermentation), and a lower oleate toxicity compared to suspended sludge while treating oleate (OLR 2-8 g-COD/L·d) (Pereira et al., 2002b, 2002a). However, usage of suspended sludge as an inoculum source has been suggested for treating oleate-containing wastewater due to its higher LCFA sorption capacity (Pereira et al., 2002b, 2002a), as LCFA sorption is required for its degradation based on a sequential sorption-desorption mechanism (Hwu et al., 1998b). Therefore, an evaluation of different inocula is needed for assessing the methane production from LCFA-containing wastewaters at low temperatures.

Apart from the physicochemical characteristics, the microbial community composition of the anaerobic sludge is affected by the high lipid or LCFA concentration and by the low temperature. LCFA degradation

proceeds through the removal of 2 carbons in each β-oxidation cycle usually resulting in the production of acetate. In a microbial consortium, this β-oxidation requires the syntrophic coupling of acetogenic bacteria to the hydrogenotrophic methanogens to maintain low hydrogen partial pressures. The microbial communities involved in codigesting fat, oil, and grease (FOG) or LCFA at mesophilic and thermophilic conditions have been deciphered using high-throughput amplicon sequencing, and syntrophs have been concluded to also play a significant role in their degradation (He et al., 2018; Regueiro et al., 2016; Ziels et al., 2017b, 2015). Only 7 acetogenic bacteria belonging to the families *Syntrophomonadaceae* (class *Clostridia*) and *Syntrophaceae* (class *Deltaproteobacteria*) (Alves et al., 2009; Sousa et al., 2009) are currently known to degrade LCFA, and the bacteria from the family *Clostridiaceae* (class *Clostridia*) have been suggested to degrade LCFA (Palatsi et al., 2010; Sousa et al., 2007b), signifying the importance of bacterial taxa from these 3 families in LCFA degradation. Furthermore, the predominance of hydrogenotrophic methanogens has not been established at low temperatures in the anaerobic treatment of wastewaters with low-lipid and LCFA content (Bialek et al., 2014; Lettinga et al., 2001; McKeown et al., 2009a; Regueiro et al., 2014; Seib et al., 2016; Siggins et al., 2011) due to the thermodynamic preference for hydrogen utilization than acetate utilization at low temperatures (Kotsyurbenko, 2005). An investigation of the microbial consortia involved in degrading the lipid- or LCFA-containing wastewaters at low temperatures through high-throughput amplicon sequencing has not yet been applied to the microbial consortia involved in degrading the lipid- or LCFA-containing wastewaters at low temperatures and could provide novel insights about the key taxa involved.

As the choice of inoculum for LTAD depends on its unique physicochemical characteristics and microbial community composition, the objective of this study was to assess the effect of the inoculum source on methane production from LCFA-containing synthetic dairy wastewater (SDW) in batch assays at low temperatures of 20 and 10°C. Amplicon sequencing was applied to characterize the microbial communities before and after the batch operation to study the changes in microbial community composition during incubation.

3.2 Materials and Methods

3.2.1 Inoculum and substrate

Three inocula obtained from local sources were used in this study—two suspended sludges and one granular sludge. Suspended sludges from mesophilic anaerobic digesters of municipal wastewater treatment plants, Rahola (RD) and Viinikanlahti (VD), Tampere, Finland, were collected, sieved with a

16 mm mesh, and stored for 4 weeks under nitrogen-purged atmosphere at 7°C. Granular sludge (GS) was obtained from a mesophilic upflow anaerobic sludge blanket (UASB) reactor treating wastewaters from an integrated production of beta-amylase enzyme and ethanol from oat (Jokioinen, Finland) and stored for 3 weeks in nitrogen-purged atmosphere at 7°C. The sieved suspended and granular sludges were characterized (Table 3.1).

SDW and acetate were used as substrates in this study. SDW simulated constituents of dairy wastewater and contained a protein source (casein hydrolysate), a carbohydrate source (lactose monohydrate), and a fat source (LCFA mixture) (Table 3.2). The LCFA mixture consisted of palmitate, stearate, oleate, and linoleate in a COD ratio of 30 : 15 : 45 : 10 (Table 3.2) based on LCFA concentrations frequently found in dairy wastewaters (Kim et al., 2004; Sousa et al., 2009).

Table 3.1. Characteristics of the three inocula used in the assays at 10 and 20°C.

Parameters	Granular Sludge (GS)	Rahola Digestate (RD)	Viinikanlahti Digestate (VD)
Soluble COD (mg/L)	525 ± 10	2520 ± 95	1080 ± 40
Volatile Fatty Acids (mg/L)	147 ± 2.0	-*	-*
pH	6.8 ± 0.2	7.3 ± 0.1	6.9 ± 0.1
Alkalinity (mM)	44.0 ± 0.1	78.0 ± 0.1	94.0 ± 0.1
TS (g/L)	42.0 ± 5.0	51.0 ± 1.5	20.1 ± 1.0
VS (g/L)	36.0 ± 4.0	28.0 ± 1.0	11.1 ± 0.2
TSS (g/L)	39.0 ± 2.0	51.0 ± 2.0	20.0 ± 0.2
VSS (g/L)	34.0 ± 1.6	27.0 ± 0.15	11.0 ± 0.2
SO_4^{2-} (mg/L)	4.4 ± 0.01	184.0 ± 0.8	67.0 ± 0.1
PO_4^{3-} (mg/L)	6.6 ± 0.01	57.0 ± 0.3	1.5 ± 0.03
Cl^- (mg/L)	17.0 ± 0.1	9.0 ± 0.01	29.0 ± 0.05

* Below detection limits

Table 3.2. Composition of the synthetic dairy wastewater (SDW) used as a substrate in the assays with VD, RD, and GS at 10 and 20°C.

Substrate component	LCFA	% of COD	Concentration (mg/L)
Casein		25	348
Lactose		42	730
LCFA, total		33	229
	Palmitate	10	69
	Stearate	4.9	34
	Oleate	13.2	91
	Linoleate	4.9	35

3.2.2 Methane production in batch assays

Batch studies were performed using 120 mL serum bottles, with a liquid volume of 60 mL. In all assays, 15 mL of substrate stock solution (SDW or acetate) and 10-30 mL of inoculum (GS, RD, or VD) were added into the bottle, to ensure 2 g-COD/L of the substrate and 6 g-volatile solids (VS)/L of inoculum. 2 mL of stock nutrient solution was added to the bottles consisting of the following (g/L): $MgSO_4 \cdot 7H_2O$ (6), NH_4Cl (16.8), $KH_2PO_4 \cdot 3H_2O$ (0.24), $Na_2HPO_4 \cdot 2H_2O$ (0.2), $CaCl_2 \cdot 2H_20$ (6), yeast extract (6), $FeCl_2 \cdot 4H_2O$ (0.12), H_3BO_3 (0.003), $ZnCl_2$ (0.003), $CuCl_2 \cdot 2H_2O$ (0.002), $MnCl_2 \cdot 4H_2O$ (0.03), $Na_2MoO_4 \cdot 2H_2O$ (0.001), $CoCl_2 \cdot 6H_2O$ (0.12), $NiCl_2 \cdot 6H_2O$ (0.005), $Na_2SeO_3 \cdot 5H_2O$ (0.01), EDTA (0.06), and resazurin that was acidified with 1 mL of 36% hydrochloric acid prior to usage (Kettunen and Rintala, 1995), and the volume was adjusted to 60 mL with distilled water. Subsequently, the pH in the assays was adjusted to 7.0 by adding 0.1 M sodium hydroxide or 0.1 M hydrochloric acid solutions. The headspace was flushed with nitrogen gas for 10 min and closed with a butyl rubber stopper to ensure anaerobic conditions after which it was sealed with aluminium crimp caps. The experiments were performed in triplicates and incubated at 20 or 10°C without shaking. Assays without substrates were prepared similarly, to act as blanks. Supernatant and sludge from the bottles were sampled at the end of the trial (200 d) for soluble COD (sCOD) and volatile fatty acid (VFA) measurements and for microbial community analysis, respectively. Assays prepared with the granular sludge as inocula will be referred to as GS, and the assays prepared with the municipal digester sludges—RD and VD—will be referred to as RD and VD, respectively, in the subsequent sections. A long assay duration (200 d) was undertaken due to the possibility for diauxic behaviour as seen previously in the anaerobic treatment of LCFA-containing wastewater at 20°C in our study (data not published) and of fat-containing dairy effluent at 37°C (Cavaleiro et al., 2008).

3.2.3 Analytical methods and calculations

The biogas content (CH_4, CO_2, and H_2) was analyzed using a Shimadzu GC-2010 gas chromatograph, equipped with a Porapak N column and a thermal conductivity detector. Helium was used as the mobile phase at a flow rate of 20 mL/min. Injector, oven, and detector temperatures were set at 110, 80, and 110°C, respectively. The volume of the gas was measured (Owen et al., 1979), and reported at standard temperature and pressure (STP). Liquid supernatant from the bottles at the end of 200 d was filtered through 0.45 µm polyethersulphone syringe filters for sCOD and VFA measurements. For VFA measurement, a Shimadzu GC-2010 having ZB-Wax column was used for analysis, with helium as carrier gas at a flow rate of 19.6 mL/min. Injector and FID detector temperatures were both 250°C. The oven

temperature was programmed to heat as follows: at 40°C for 2 min, then heated up to 160°C with 20°C/min and up to 220°C with 40°C/min, after which temperature was held at 220°C for 2 min.

The sCOD was measured using the potassium dichromate titrimetric method according to the Finnish standard SFS 5504. Total solids (TS), VS, total suspended solids (TSS), and volatile suspended solids (VSS) were measured according to APHA 2005 and alkalinity according to the Finnish standard SFS 3005. The concentrations of anions (SO_4^{2-}, PO_4^{3-}, and Cl^-) in filtered samples were measured by a Dionex ICS-1600 Ion Chromatography System, equipped with an IonPac AS4A-SC anion exchange column, a DS6 heated conductivity cell, and 1.9 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ as the eluent at a flow rate of 1 mL/min. Salinity measurements of the substrates were used for calculating dissolved gas (methane and carbon dioxide) concentrations and were carried out using a handheld conductivity meter WTW Cond 3210. Bunsen solubility coefficients were obtained using constant values from Yamamoto et al. (Yamamoto et al., 1976) for equation 1, and were applied to estimate solubilized methane following equation 2 (Breitbarth et al., 2004).

$$\ln\beta = \mathrm{A1} + \mathrm{A2}\ (100/T) + \mathrm{A3}\ln(T/100) + \mathrm{S}[\mathrm{B1} + \mathrm{B2}(T/100) + \mathrm{B3}(T/100)^2] \quad (1)$$

where β is the Bunsen solubility coefficient, A and B are constants (A1 = -67.1962, A2 = 99.1624, A3 = 27.9015, B1 = -0.07291, B2 = 0.041674, B3 = -0.00646), *T* is the temperature in Kelvin, and S is the salinity in parts per thousand.

$$\mathrm{n}(\mathrm{CH_4})_{\mathrm{aq}} = (\mathrm{pgT} * \alpha * \mathrm{V_{aq}})/(\mathrm{R} * \mathrm{T_o}) \quad (2)$$

where n_{aq} denotes the total amount of gas (mol) dissolved in the aqueous phase, pgT is the partial pressure of the gas in the headspace (atm), α is the Bunsen coefficient, V_{aq} is the volume of aqueous phase (L), and R is the gas constant (0.082 atm·L/mol·K). Methane production was determined by adding the methane dissolved in the liquid phase to the methane volume in the gaseous phase and subtracting the methane production in the assay blanks from this value. The methane yield (%) was calculated from the ratio of methane produced in each assay (mL/g-COD) to the theoretical methane volume produced per gram COD at standard temperature and pressure (STP) (350 mL/g-COD). Overall mass balances were performed in terms of COD at the beginning (Table 3.2) and at the end of the 200 d experiment for all the assays. The cumulative methane production from the assays was fitted with the modified Gompertz equation:

$$M(t) = P.\exp\left[-\exp\{\frac{Rm.e}{P}\ (\lambda - t) + 1\}\right], \quad (3)$$

where, M(t) is the cumulative methane production (mL-CH_4) at time t (d), P is the methane production potential (mL-CH_4 or mL-CH_4/gVS), R_m is the maximum methane production rate (mL-CH_4/d), and λ is the lag-phase for methane production (d). The curve fitting tool in Matlab R2017b was used to calculate the lag-phase (λ) and the maximum methane production rate (R_m).

3.2.4 DNA extraction, quantification and 16S rRNA sequencing

The microbial samples (three inocula and from all the assays after 200-day incubations) were stored at -20°C immediately upon sampling. After thawing the samples at 4°C, the total DNA was coextracted along with RNA from the microbial samples (Griffiths et al., 2000). The concentration of extracted DNA was quantified with a Qubit Fluorometer (Life Technologies), and the DNA purity was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The DNA samples were sent to FISABIO (Spain) for PCR amplification of the V4 region of the 16S rRNA gene with universal primers 515f and 806r (Caporaso et al., 2011) and amplicon sequencing with the Illumina MiSeq platform.

3.2.5 Bioinformatics and Statistical tools

Computational analysis of the sequenced data was done using the Quantitative Insights Into Microbial Ecology (QIIME v1.9) pipeline (Caporaso et al., 2010). The average length of the forward and reverse reads was 291 and 294 bp, respectively. The paired-end reads were joined using a fastq-join method (Aronesty, 2013) with a min overlap of 50 bp and a perc_max_diff of 15%, after which quality filtering was performed using the split_libraries_fastq.py script in QIIME (Caporaso et al., 2010). The sequences were clustered into operational taxonomic units (OTUs) using the open-reference OTU picking with the BLAST method, and taxonomy assignment was performed with the Silva 128 consensus taxonomy at all levels (Glöckner et al., 2017; Pruesse et al., 2007). Chimeric sequences were identified using ChimeraSlayer, and the final OTU table was generated from the nonchimeric sequences using the script make_otu_table.py.

The dataset consisted of 5743805 sequences in total, which clustered into 12326 OTUs at 97% similarity level. There were 127 abundant OTUs that formed 99.9% of the community and were subsampled to an even sequencing depth of 26511 (based on the number of reads in the smallest sample) for alpha diversity metrics (diversity within samples). Community diversity and species richness were estimated using Chao1 and Shannon indices and the number of observed OTUs. The alpha diversity indices for the inoculum duplicates and assay triplicates are presented as the averaged values of the microbial community composition within the inoculum duplicates or within the experimental assay triplicates after 200 days.

The subsampled data set consisting of sequences representing the 127 most abundant OTUs was used in subsequent analyses. The subsampled OTU table was fourth-root-transformed for even distribution, and a resemblance matrix was constructed using Bray-Curtis similarity in the Plymouth Routines In Multivariate Ecological Research (PRIMER) V7 (Clarke et al., 2014). Cluster analysis was performed to discern patterns in the microbial community of the different inocula in the low-temperature treatment of LCFA-containing wastewater. Cluster analysis through hierarchical clustering (group average method) was performed at the taxonomic class level on the operational taxonomic units (OTUs) and separately on the assay samples to plot the dendrograms. The fourth-root-transformed OTU table was used for representing the microbial community composition in a shade plot with dendrograms.

3.3 Results and Discussion

3.3.1 Inoculum characteristics and microbial community composition

3.3.1.1 Physico-chemical characteristics

Among the three mesophilic inocula, the granular sludge (GS) had a higher VS : TS ratio (0.86) compared to the municipal digester sludges—RD and VD (0.55)—while GS had lower sCOD than the municipal digester sludges (520 vs. 1100-2500 mg/L) (Table 3.1). GS contained some VFA (147 mg/L) whereas the VFA was lower than the detection limit in both of the municipal digester sludges. The two municipal digester sludges differed in several properties; e.g., the TS, sCOD, and PO43- concentrations were several-fold different (Table 3.1) suggesting the impact of inflow wastewater characteristics and plant operation, as the two municipal wastewater treatment facilities have similar unit processes, which are the primary sedimentation and activated sludge processes, followed by mesophilic anaerobic digestion of the excess sludge generated.

3.3.1.2 Microbial community composition of the inocula

High-throughput amplicon sequencing was used to investigate the microbial community composition of the three inocula. The municipal digester sludges (RD and VD) had a higher microbial community diversity (Chao1: 104-108, Shannon: 4.53-4.73, observed_otus: 103-105) compared to the GS inoculum (Chao1: 93, Shannon: 3.69, observed_otus: 79) (Table 3.3), although the three inocula were obtained from mesophilic reactors. The bacterial classes *Anaerolineae* (relative abundance 7.4-23.25%), *Clostridia* (relative abundance 0.1-0.6%), and *Synergistia* (relative abundance 6.3-10.6%) and the archaeal

class *Methanomicrobia* (relative abundance 7.8-19%) were present in all the three inocula. Although the municipal digester sludges (RD and VD) had a similar microbial community composition, the relative abundance of *Methanomicrobia* (13 vs. 7%) and *Anaerolineae* (23 vs. 13%) was higher in RD than in VD inoculum. Overall, GS inoculum had a high relative abundance of *Methanobacteria* (21.5%), uncultured *Aminicenantes* (25%), and *Deltaproteobacteria* (5%) compared to that of the municipal digester sludges.

The microbial community composition and diversity in the three inocula were different. The higher microbial community diversity in the municipal digester sludges (Table 3.3) was likely due to the wide variety of substrates received by the municipal wastewater treatment plants. In comparison, GS was sourced from an UASB treating carbohydrate and alcohol-based wastewater, which potentially narrowed the diversity of the microbial community.

Table 3.3. Alpha diversity metrices (diversity within samples) – Chao 1 indices, Shannon indices and number of observed OTUs for all the OTUs achieving 99.9% cut-off in the overall relative abundance. Detailed information on the sample names is shown in Table S3.1.

	Chao1	Shannon	Observed_otus
VD Inoculum	108	4.73	105
$VD_{Blank}10$	106	4.38	104
$VD_{Acetate}10$	108	4.62	106
$VD_{SDW}10$	109	4.31	105
$VD_{Blank}20$	116	5.13	109
$VD_{Acetate}20$	115	5.06	109
$VD_{SDW}20$	115	4.79	109
RD Inoculum	104	4.53	103
$RD_{Blank}10$	108	4.76	105
$RD_{Acetate}10$	113	4.49	107
$RD_{SDW}10$	114	4.61	104
$RD_{Blank}20$	112	4.46	110
$RD_{Acetate}20$	113	5.12	111
$RD_{SDW}20$	111	4.8	109
GS Inoculum	93	3.69	79
$GS_{Blank}10$	82	3.8	70
$GS_{Acetate}10$	77	3.86	66
$GS_{SDW}10$	80	3.78	66
$GS_{Blank}20$	88	4.02	78
$GS_{Acetate}20$	87	3.93	85
$GS_{SDW}20$	77	4.04	76

To date, only 7 species from the classes *Clostridia* or *Deltaproteobacteria* are known to degrade LCFA (carbon atoms > 12) of which only 4 species (*Syntrophomonas sapovorans, Syntrophomonas curvata*, *Syntrophomonas zehnderi*, and *Thermosyntropha lipolytica*) from the class *Clostridia* are currently known to degrade the unsaturated LCFAs, e.g., oleate and linoleate (Alves et al., 2009), and have also been found in various LCFA-fed digesters (Grabowski et al., 2005; Gunnigle et al., 2015; Hatamoto et al., 2007; Ziels et al., 2017a, 2016). Moreover, at low temperatures, the Deltaproteobacterial class plays

a significant role along with the archaeal classes of *Methanobacteria* and *Methanomicrobia* (Bialek et al., 2012; Gunnigle et al., 2015; McKeown et al., 2009; Regueiro et al., 2014; Seib et al., 2016; Smith et al., 2013). Therefore, monitoring the bacterial and the archaeal taxa belonging to the classes *Deltaproteobacteria* and *Clostridia*, and *Methanobacteria* and *Methanomicrobia*, respectively, was considered of special interest while investigating anaerobic LCFA treatment at low temperatures in this study.

3.3.2 Methane production at low temperature from SDW and Acetate

The potential of the three different inocula for methane production from SDW and acetate was studied in batch assays at 10 and 20°C (Fig. 3.1). Blank assays without any added substrates were prepared to subtract the methane production from their corresponding assays with the added substrates. The cumulative methane production curves were fitted with the Gompertz equation (-square: 0.9-0.99) and were used for calculating the lag time and the maximum methane production rate (R_m).

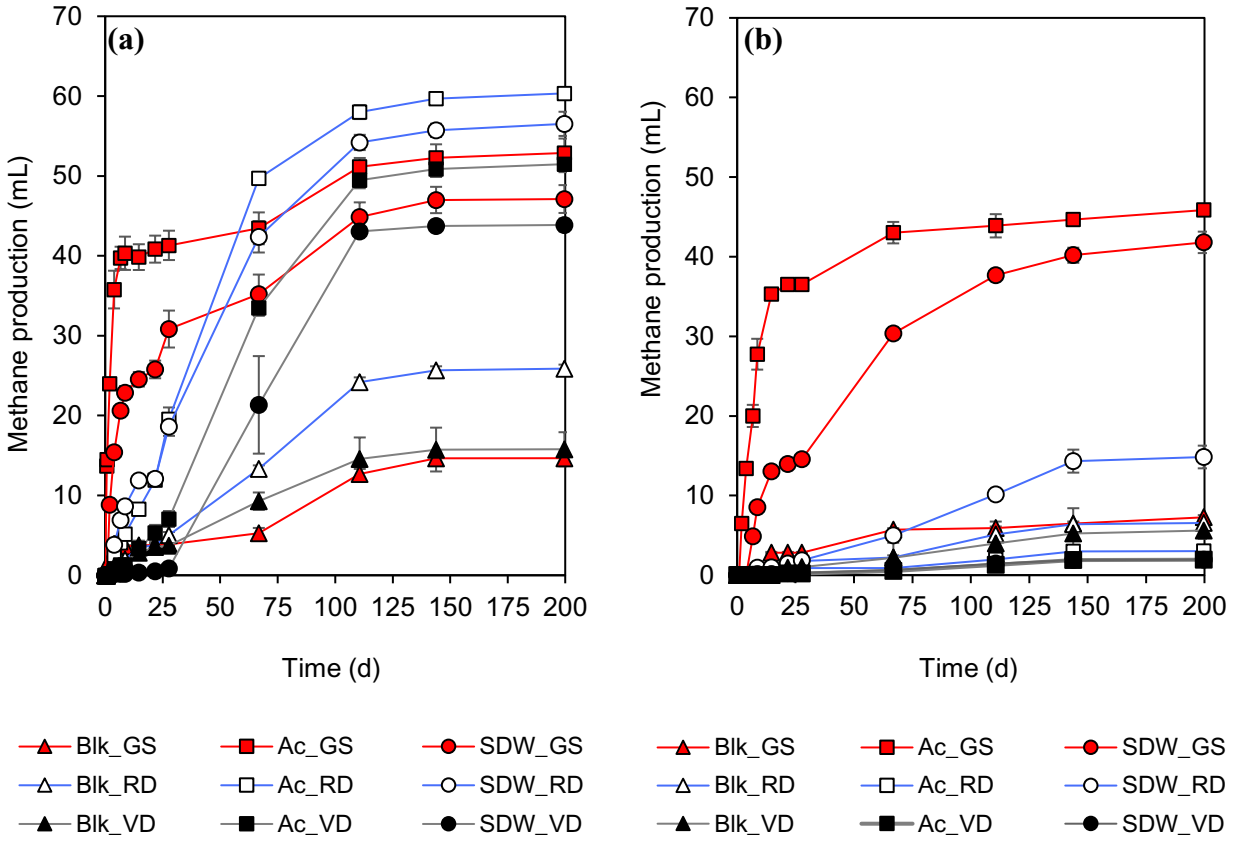

Fig. 3.1. Methane production (mL) at (a) 20°C and (b) 10°C from different inocula (GS: granular sludge; RD: Rahola Digestate; VD: Viinikanlahti Digestate) incubated with no substrate (Blk), acetate (Ac), and synthetic dairy wastewater (SDW).

Table 3.4. Lag phase, maximum methane production rate and methane yield with granular sludge and the municipal digester sludges from SDW and acetate in batch assays at 10 and 20°C.

Temperature (°C)	Substrate	Lag phase, λ (d)			Maximum methane production rate, R_m (mL-CH_4/d)			Methane yield (%)		
		GS	RD	VD	GS	RD	VD	GS	RD	VD
20	Acetate	0	8.9	18.1	9.7 ± 6.1	1.01 ± 0.09	0.7 ± 0.05	89 ± 6	81 ± 3	84 ± 9
	SDW	0	4	51.1	1.3 ± 0.7	0.77 ± 0.13	1.4 ± 0.4	76 ± 6	72 ± 3	67 ± 8
10	Acetate	0.75	20.1	21.7	3.5 ± 1.15	0.02 ± 0.01	0.03 ± 0.01	93 ± 2	<1	<1
	SDW	1.5	22.9	20.9	0.6 ± 0.2	0.13 ± 0.02	0.02 ± 0.01	82 ± 3	19 ± 4	<1

sCOD removal at 20°C was similar with all three inocula, 78-81% with SDW and 81-90% with acetate (Fig. S3.1), but at 10°C, a higher sCOD removal was obtained with GS (82% in SDW-addition and 91% in acetate-addition) than with the municipal digester sludges. Despite the sCOD removal of 29-53% in the municipal digester sludges at 10°C (Fig. S3.1), methane production was low (methane %), which suggests that the sCOD removal was apparently due to the sorption by inoculum biomass (Hwu et al., 1998b). Moreover, after 200 days, VFAs were detected only with the municipal digester sludges at 10°C (total VFAs of 290-780 mg/L with SDW and 760-1070 mg/L with acetate). With SDW, the most abundant VFA was acetate (110-580 mg/L), and in the acetate-fed assays, only acetate (760-1070 mg/L) was found (Fig. 3.2), which suggests an inhibition of acetotrophic activity. COD balance (Fig. 3.3) shows that 9-19% and 19-23% of the sCOD (non-VFA) remained in the acetate and SDW assays, respectively, after the 200 d period. This non-VFA sCOD might have been produced due to the release of endogenous decay products. The unaccounted COD in the acetate and SDW assays increased at 10°C compared to 20°C (Fig. 3.3) likely due to cell growth or substrate sorption to the biomass. The VFA accumulation did not affect the pH and was 6.9-7.1 in all the assays at 20 and 10°C at the end of the experiment.

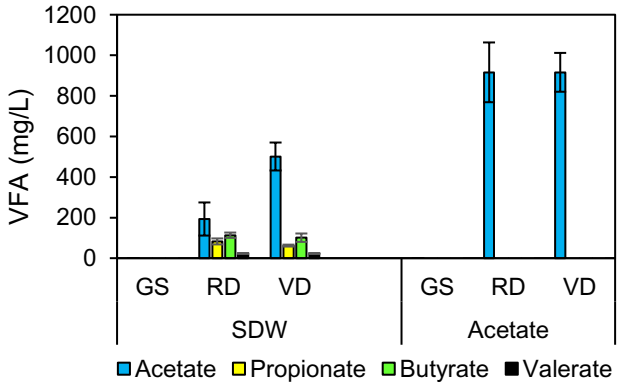

Fig. 3.2. Residual volatile fatty acid (VFA) concentration at 10°C with synthetic dairy wastewater (SDW) and acetate at the end of the 200 d experiment (GS: Granular Sludge; RD: Rahola Digestate; VD: Viinikanlahti digestate).

The methane production from acetate or SDW with GS at 20 and 10°C indicates that the COD from the carbohydrate (lactose), protein (casein), and LCFA (saturated and unsaturated) fractions was metabolized by the anaerobic consortia in GS (Fig. 3.3). On the contrary, the decrease in temperature diminished methanogenesis in RD and VD assays. Moreover, at 10°C, the fraction of SDW hydrolysed (sum of the VFA-COD and the CH_4-COD in Fig. 3.3) was <50%, indicating that the hydrolysis and acidification of carbohydrate and protein fractions were also diminished at 10°C. Even in the acetate-fed assays at 10°C, less than 50% of the substrate uptake was detected in RD and VD assays. The presence of high hydrogen partial pressures limits the syntrophic LCFA degradation by β-oxidation, and LCFA further inhibits the trophic groups in anaerobic microbial consortia, which could have affected the SDW uptake at 10°C. However, substrate uptake (acetate and SDW) at 10°C was not energetically limited by hydrogen accumulation/increased hydrogen partial pressure in RD and VD, as hydrogen was not found in the gas phase. Therefore, the lack of substrate uptake was related to the inhibition in acetotrophic activity in the digestate inocula at 10°C.

Previously, single LCFAs—linoleic acid (30 mg/L), oleic acid (30 mg/L), and stearic acid (10 mg/L)—have been reported to inhibit methanogenesis from 100 mg/L of acetate at 21°C (Lalman and Bagley, 2001, 2000). In this study, however, a combination of LCFAs (34.7 mg/L of linoleate, 91.2 mg/L of oleate, and 33.8 mg/L of stearate) present in SDW was converted to methane by GS at 20 and 10°C and by the two municipal digester sludges at 20°C (Fig. 3.1). In spite of a higher LCFA load in the current experiment (38 mg LCFA/g·VS) than the inhibitory concentration reported elsewhere with a single LCFA (20 mg LCFA/g·VS and 6.67 mg LCFA/g·VS at 21°C (Lalman and Bagley, 2001, 2000)), methane was produced from a mixture of saturated and unsaturated LCFAs in this study. To the best of our knowledge, this is the first report of methane production from a LCFA mixture containing unsaturated LCFAs (oleate and linoleate) at 10°C, wherein the methane production was driven by the inoculum origin. These results can be used for understanding and developing anaerobic processes for the low-temperature treatment and methane production from lipid-containing wastewaters.

3.3.3 Effect of Low Temperature and SDW on Microbial Community Composition

3.3.3.1 Microbial Community Diversity after 200 days

Due to the differences in the relative abundance of the microbial community composition in the three inocula, and the differing methane production in the assays at different temperatures and substrates, an underlying shift in the bacterial and archaeal taxa was envisaged after the 200 d incubation period. High-throughput amplicon sequencing was used to investigate the microbial community composition in the assays after 200 days. A total of 12326 OTUs were found, of which 99.9% of the microbial community

belonged to 127 OTUs consisting of 33 bacterial and 4 archaeal classes. Therefore, a small portion of the taxa (0.01% of total OTUs) comprised a 99.9% portion of the microbial community.

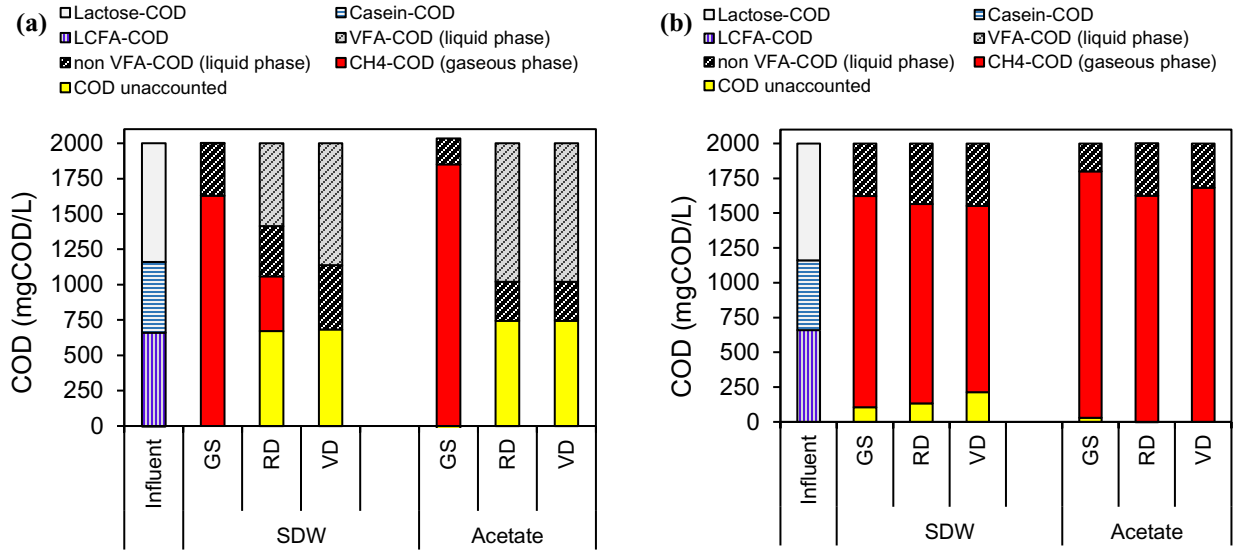

Fig. 3.3. Mass balance of COD at (a) 20°C and (b) 10°C in the assays with different inocula (GS: granular sludge; RD: Rahola Digestate; VD: Viinikanlahti digestate) and with the substrate synthetic dairy wastewater (SDW) or acetate.

Moreover, similar to their inoculum, even after the 200 d, the municipal digester sludges (RD and VD) had a higher microbial diversity (Chao1: 106-116, Shannon: 4.31-5.13) compared to the GS (Chao1: 93, Shannon: 3.69, observed_otus: 79) (Table 3.3). Based on the Chao1 and the number of observed OTUs, the community diversity increased in VD and RD but decreased in GS during the 200-day period, independent of the temperature or the substrate.

3.3.3.2 Relative abundance of the Archaeal Classes after 200 days

The bacterial and archaeal taxa that changed with the substrate and temperature after the 200 d incubation period were assessed by their change in relative abundance. Only the OTUs with relative abundance above 0.1% are further discussed. In all the assays, 9 archaeal OTUs were found belonging to *Methanobacteria* (3 OTUs), *Methanomicrobia* (3 OTUs), *Thermoplasmata* (1 OTU), and WCHA1-57 (2 OTUs). The class *Methanobacteria* was found at a higher relative abundance in the GS assays (13-19%) compared to VD and RD (less than 7%) (Fig. 3.4), but its relative abundance decreased over the incubation period compared to the GS inoculum (21%) bearing no temperature-specific or substrate-specific trends. The class *Methanomicrobia* was found in all the assays after the 200 d incubation, with a higher relative abundance in GS (22-28%, increased from 19%) compared to VD (increased from 13% to 19-23% at 10°C) and RD (increased from 8% to 15-20% at 20°C) (Fig. 3.4). Within the class *Methanomicrobia*, the relative

abundance of the hydrogenotrophic *Methanolinea* increased in the GS (0.8-6.5% vs. 0.3% in inoculum), while the hydrogenotrophic ARC26 (ambiguous taxon) had a higher relative abundance in VD and RD (0.1-2%) than in GS (Fig. 3.5). *Methanosaeta* was the only acetoclastic archaeal genus found after the 200 days, and its relative abundance increased at 20°C in the acetate-fed assays compared to blank assays in GS (from 20 to 25%), VD (from 8.3 to 9.1%), and RD (from 5.1 to 13.8%). At 10°C, in the acetate-fed assays, the relative abundance of *Methanosaeta* remained high in GS (20-22%) and increased only in VD (from 11 to 24%) (Fig. 3.5). The members of the class *Methanobacteria* and *Methanomicrobia* have previously been detected in psychrophilic environments (Wei et al., 2014) and in anaerobic LCFA degradation assays (Amha et al., 2018) and were also found to be prevalent in this study.

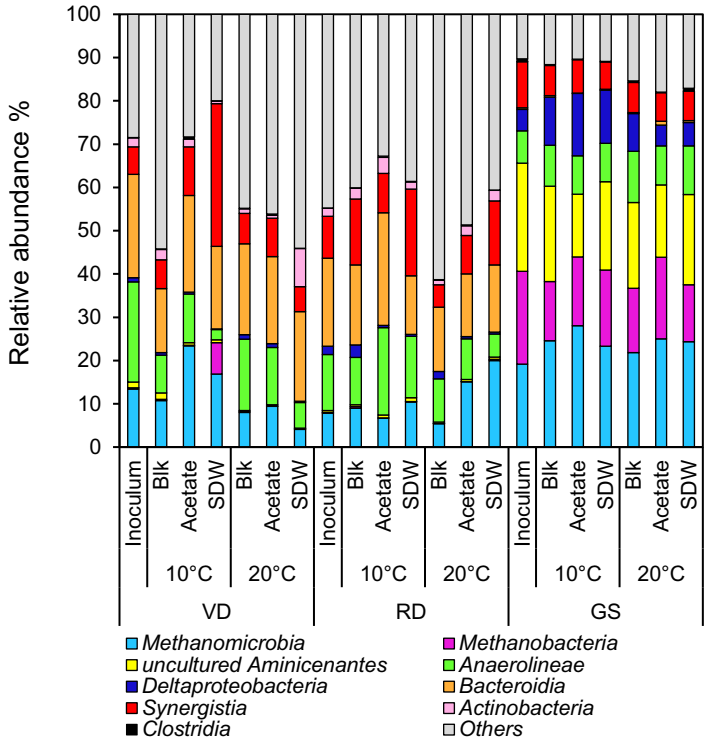

Fig. 3.4: Relative abundance (%) of the bacterial and archaeal classes found in the 16S rRNA amplicon libraries in the samples during the 200 d experimental period from different inocula (GS: granular sludge; RD: Rahola Digestate; VD: Viinikanlahti Digestate) incubated at 10°C and 20°C with no substrate (Blk), acetate, and synthetic dairy wastewater (SDW). Detailed information on the sample names is shown in Table S3.1.

VFA accumulation (predominantly acetate) was observed with RD and VD assays at 10°C when fed with SDW. In addition, in the RD and VD assays at 10°C fed with acetate, only 50% of acetate was consumed with negligible methane production (Fig. 3.3). Even with a high relative abundance of *Methanosaeta* (only acetoclastic taxa found in this study) in VD and of hydrogenotrophic taxa in VD and RD at 10°C, negligible methane was produced in RD and VD assays at 10°C. This inhibition of acetotrophic activity at 10°C in RD and VD assays suggests acetate uptake by syntrophic acetate-oxidizing bacteria (SAOB). SAOB growth can

be energetically feasible at lower temperatures close to the thermodynamic equilibrium, and their presence has been confirmed at temperatures as low as 7°C (Dolfing, 2014; Gies et al., 2014; Nüsslein et al., 2001). SAOB are slow-growing and have been isolated in the presence of strong selection pressures (Westerholm et al., 2018, 2016). In our study, the presence of stressors, such as low temperature, likely imparted a competitive advantage to the acetate oxidizers for acetate uptake compared to the acetoclastic methanogen, *Methanosaeta*. This advantage is conferred to the acetate oxidizers from syntrophic coupling with hydrogenotrophic methanogens, due to more favorable energetics of the hydrogenotrophic methanogenic pathway at lower temperatures (10°C). A shift in the methanogenic pathway from acetotrophic to hydrogenotrophic has been observed previously with a temperature drop (Lettinga et al., 2001; McKeown et al., 2009a; Siggins et al., 2011), and LCFA presence has been known to inhibit acetoclastic methanogens more than hydrogenotrophic methanogens (Alves et al., 2009; Lalman and Bagley, 2002), thereby suggesting a need for maintaining high hydrogenotrophic activity for methane production at low temperatures. In contrast to the previous studies at low temperatures, this study highlights the need for maintaining high acetotrophic activity for LCFA utilization at low temperatures through methanogenic archaea and/or syntrophic acetate oxidation bacteria (SAOB) (further discussed in Section 3.3.3).

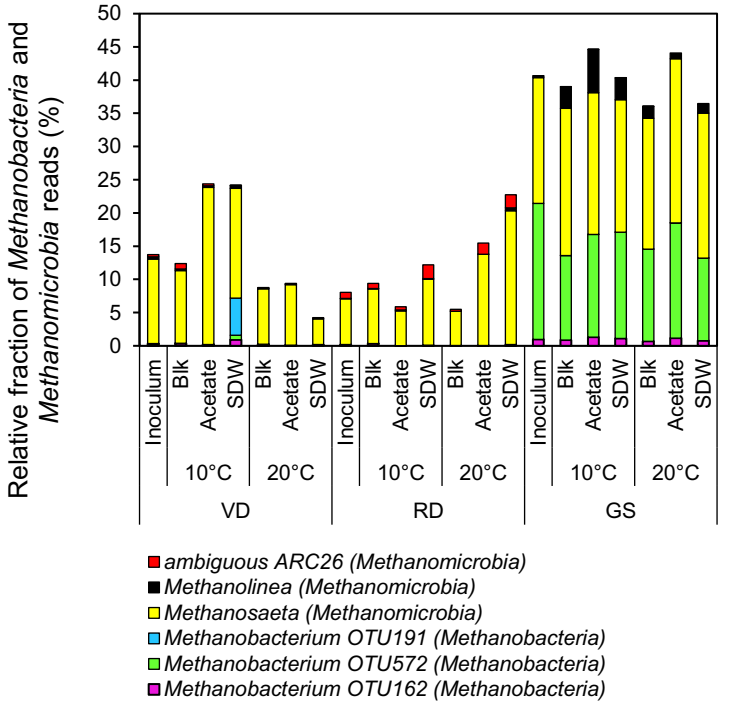

Fig. 3.5: Relative fraction (%) of the archaeal genera belonging to the classes *Methanobacteria* and *Methanomicrobia* found in the 16S rRNA amplicon libraries in the samples during the 200 d experimental period from different inocula (GS: granular sludge; RD: Rahola Digestate; VD: Viinikanlahti Digestate) incubated at 10°C and 20°C with no substrate (Blk), acetate, and synthetic dairy wastewater (SDW). Classes are mentioned within brackets. Detailed information on the sample names is shown in Table S3.1.

3.3.3.3 Relative abundance of the bacterial classes after 200 days

In all the assays, the bacterial classes *Anaerolineae* (relative abundance 2.4-20%), *Clostridia* (0.1-0.7%), and *Synergistia* (6-33%) were found at the end of the batch incubation. The classes *Bacteroidia* and *Actinobacteria* were present in higher abundance in both municipal digester sludges than in GS (*Bacteroidia* 15-26% vs. <1%, respectively, and *Actinobacteria* 1-9% vs. <0.1%, respectively), while GS had a higher relative abundance of uncultured *Aminicenantes* (15-22% vs. 0.2-1.4%) and *Deltaproteobacteria* (5-14% vs. 0.2-2.8%) (Fig. 3.4). The large variation in relative abundance of different classes is due to the changes in temperature and substrate and are discussed below.

After the 200 d incubation period, 6 OTUs were found in the class *Deltaproteobacteria*, among which 4 belonged to the order *Syntrophobacterales* (families *Syntrophaeceae* (3 OTUs), *Syntrophobacteraceae* (1 OTU)) and two to the order *Desulfuromonadales* (family *Geobacteraceae*) (Fig. 3.6). The relative abundance of the acetogenic bacterium *Syntrophus* and of an uncultured taxon (from family *Syntrophaceae*) increased in the GS at 10°C (from 3.3% to 9.6-12.3%) during the 200 d incubation (Fig. 3.6), highlighting the role of the acetogenic bacteria from the family *Syntrophaceae* in LCFA degradation at 10°C. The members of the family *Syntrophaceae* are known to degrade the saturated LCFAs—palmitate (C16:0) (Hatamoto et al., 2007), stearate (C18:0), and heptanoate (C17:0)—at mesophilic conditions (Grabowski et al., 2005), and only one known species from the family *Syntrophaceae*—*Syntrophus aciditrophicus*—has been found to degrade two saturated LCFAs (palmitate (C16:0) and stearate (C18:0)) (Jackson et al., 1999). *Syntrophus aciditrophicus* has a growth range at temperatures of 25-42°C; thus, a psychrotolerant growth mode at 10°C of *Syntrophus*-like taxa for metabolizing the unsaturated LCFAs (oleate and linoleate) is found in this study.

Furthermore, at 10°C, the GS had a methane yield of 82% from the substrate SDW (Table 3.2) that constituted of 33% LCFAs (18% unsaturated LCFAs (C18:2, C18:1) and 15% saturated LCFAs (C16:0, C18:0)). Therefore, this study confirms the possibility of methane production from the saturated and unsaturated C16 and C18 acids at low temperatures (10 and 20°C) with a crucial role played by syntrophs and acetotrophs. While the metabolic pathways involved in the degradation of saturated and unsaturated LCFAs individually and in mixture at low temperatures are not known yet, they should be elucidated with further studies.

Another important class *Synergistia* (16 OTUs) was found in all the samples (6.3-10.6%) (Fig. 3.4). Although the precise function of *Synergistia* in degrading LCFA remains unconfirmed, it has been found in the core microbiome of mesophilic and thermophilic LCFA-fed digesters (Amha et al., 2018). Of the 16

OTUs found in this study in the class *Synergistia*, 5 belonged to the thermophilic or mesophilic amino acid-degrading genera *Thermovirga* (Dahle and Birkeland, 2006), *Lactivibrio* (Qiu et al., 2014), or *Aminivibrio* (Honda et al., 2013). As casein constituted 25% COD in the SDW, the presence of amino-acid degraders is expected; however, few of the other 11 *Synergistia* taxa could have a role in methane production from SDW.

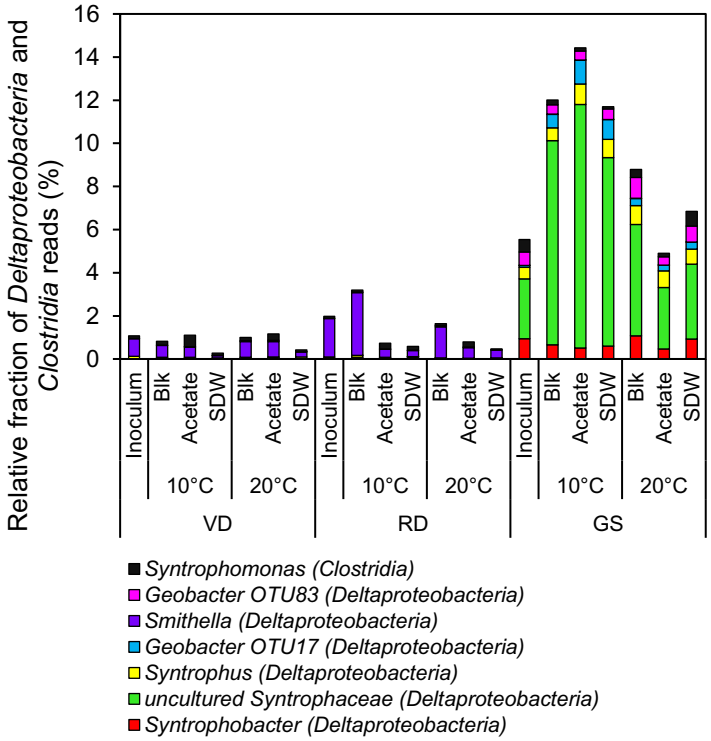

Fig. 3.6: Relative fraction (%) of the bacterial genera belonging to the classes *Deltaproteobacteria* and *Clostridia* found in the 16S rRNA amplicon libraries in the samples during the 200 d experimental period from different inocula (GS: granular sludge; RD: Rahola Digestate; VD: Viinikanlahti Digestate) incubated at 10°C and 20°C with no substrate (Blk), acetate, and synthetic dairy wastewater (SDW). Classes are mentioned within brackets. Detailed information on the sample names is shown in Table S3.1.

In GS, *Aminicenantes* clustered closely with the hydrogenotrophic *Methanobacteriales* (Fig. 3.7). Additionally, *Synergistetes* was found in a high relative abundance in RD and VD assays, and a recently found thermophilic SAOB, *Gelria* (order *Thermoanaerobacterales*) (Mosbæk et al., 2016), was found in a GS assay when fed with SDW at 20°C. The taxa from *Aminicenantes*, *Synergistetes*, and uncultured *Gelria* are putative SAOBs in this study due to their capacity and known role in syntrophic acetate oxidation (Gies et al., 2014; Ito et al., 2011; Mosbæk et al., 2016). However, as these species are uncultured, their specific functions cannot be confirmed. While syntrophic electron transfer by *Aminicenantes* has been suggested previously in a stratified lake at a low temperature of 7°C (Gies et al., 2014), the *Synergistetes* have been shown to perform syntrophic acetate oxidation with *Methanoculleus* and *Methanosarcina* in mesophilic anaerobic reactors (Ito et al., 2011).

Moreover, *Geobacter* (2 OTUs) were also enriched in GS assays at 10°C with concurrent high relative abundance of *Methanosaeta* in acetate and SDW assays. As *Geobacter* species can facilitate syntrophic electron transfer with *Methanosaeta* (Rotaru et al., 2014), there is also a possibility for *Geobacter*-mediated syntrophic acetate oxidation in this study. Furthermore, *Smithella* was found in the municipal digester sludges but not in GS and could have played a role in the LCFA degradation in SDW at 20°C due its known role in syntrophic acetate oxidation at 22°C (Gray et al., 2011). As the number of SAOB candidates has been increasing recently and many SAOB taxa remain unknown, the possibility of SAOB at lower temperatures in this study cannot be excluded. Advanced molecular techniques, such as metagenomics, along with quantitative methods like qPCR are required to evaluate the activity and functional role of *Syntrophus* and *Syntrophus*-like taxa in degrading the unsaturated and saturated LCFAs (palmitate, stearate, oleate, and linoleate) and for the identification of putative SAOB for syntrophic interspecies electron transfer at low temperatures of 20°C and 10°C.

3.3.3.4 Patterns in microbial community

The clustering of the microbial samples from different assays after 200 d was represented through dendrograms on a shade plot and principal coordinate analysis (PCoA) to discern patterns in the microbial community (Fig. 3.7 and S3.2). The PCoA analysis revealed that the first two axes explained approximately 80% (first axis 72.5% and second axis 9%) of the variation in the microbial community present in the VD, RD, and GS sludges (Fig. S3.2). Both the dendrograms in the shade plot and the similarity % in the PCoA plot show that the microbial community compositions in RD and in VD were similar to each other and clustered closely at 70% similarity in the blank and acetate, and SDW-fed assays. But the microbial community compositions of assays (blank, acetate, or SDW-fed) with GS inoculum had a higher similarity among themselves (85%) and clustered further from the RD and VD (Fig. S3.2), to which the GS shared a lower similarity (66% with RD and VD) (Fig. 3.7).

This clustering indicates that even after a prolonged incubation time of 200 days at a specific selection pressure (of temperature and substrate), the composition of the microbial communities did not converge. The only instance where an overlap in microbial classes was found between assays of different inoculum types was with the acetate-fed municipal digester sludges at 20°C (Fig. 3.7 and S3.2). This suggests the effect of acetate in converging the microbial communities in VD and RD, although even the initial microbial community composition of VD and RD was similar. A continuous reactor operation may further reveal the development of microbial community assembly occurring under strong selection pressures in various

inocula considering their dissimilar microbial composition. The similar performance (methane production) obtained with acetate or SDW with the three inocula at 20°C, irrespective of the differences in initial microbial community composition, suggests that functions were conserved in VD, RD, and GS at 20°C. However, this functional conservation was not effective at 10°C due to the strong abrupt selection pressures that hindered the metabolic functions and thus the methane production at 10°C compared to 20°C.

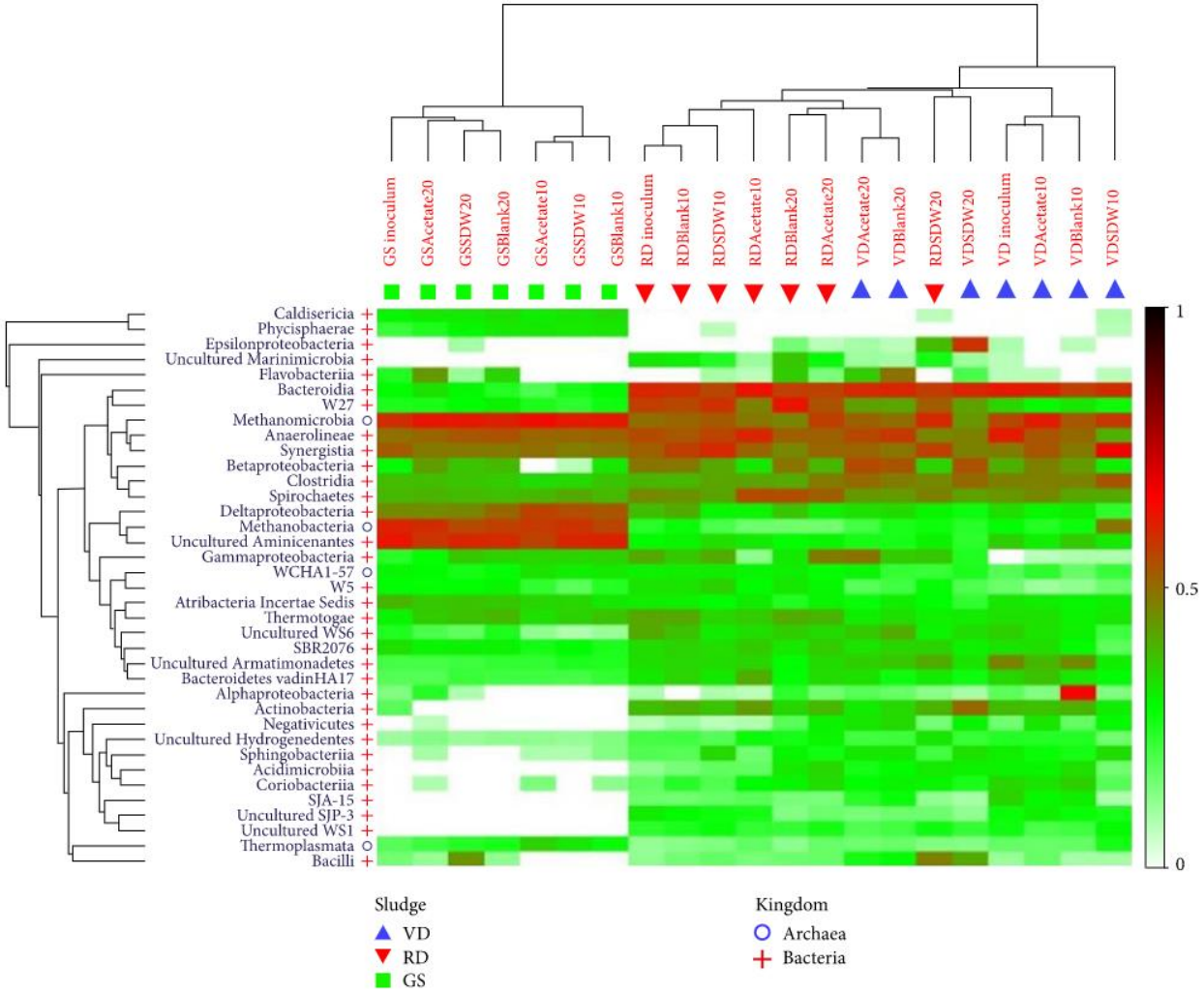

Fig. 3.7: Heat map showing the clustering and relative abundance of the bacterial and archaeal classes that formed 99.9% of the microbial community in the assays inoculated with VD, RD, and GS and fed with no substrate (blank), acetate, or synthetic dairy wastewater (SDW) at 10°C and 20°C. Symbols + and o indicate the kingdom bacteria and archaea, respectively, on the y-axis. Symbols ▲, ▼, and ■ represent the inoculum used in the assays as VD, RD, and GS, respectively, on the x-axis. Detailed information on the sample names is shown in Table S3.1.

The presence of syntrophic partners (acetogenic bacteria with methanogenic archaea) is crucial for the LCFA degradation and was evaluated through their cooccurrence through the dendrograms. The OTU clustering showed that the bacterial classes *Synergistia* and *Anaerolineae* grouped with the archaeal

class *Methanomicrobia* (the cluster was present in all samples) and conferred functional conservation to VD, RD, and GS at 20°C. Additionally, in GS, the bacterial classes Deltaproteobacteria and uncultured *Aminicenantes* grouped with the archaeal class *Methanobacteria* (the cluster present only in GS) (Fig. 3.7), which is indicative of functional redundancy in GS that was not present in the RD and VD. As optimal metabolite transfer is aided by close proximity between syntrophic partners (Schink, 1997; Sekiguchi et al., 1999), the formation of the distinct clusters in this study (Fig. 3.7) signifies structural proximity at a molecular level and indicates an underlying interactive functional role and putative ecological niche associations. Previously, Grabowski et al.(Grabowski et al., 2005a) had demonstrated the formation of close spatial associations of acetogens from *Deltaproteobacteria* (*Syntrophus*-related) with the methanogenic archaea—*Methanocalculus* and *Methanosaeta*—using in situ hybridization. In our study, the close clustering of *Deltaproteobacteria* and *Methanobacteria* in the GS could have facilitated the degradative capacity and the methane production with GS. An investigation using high-throughput sequencing (metagenomics) of closely clustered taxa involving bacterial acetogens and methanogenic archaea (such as *Methanomicrobia* with *Synergistia* and *Methanobacteria* with *Deltaproteobacteria* in this study) could confirm the functional role of the syntrophic partners involved in the clusters.

3.4 Conclusions

For the first time, the anaerobic conversion of mixed LCFA (saturated as well as mono- and polyunsaturated) containing wastewater (SDW) to methane is demonstrated at low temperatures of 20 and 10°C. High-throughput amplicon sequencing revealed the crucial roles of the acetotrophic activity by *Methanosaeta* and putative SAOB and of the psychrotolerant bacteria from the family *Syntrophaceae* (*Syntrophus* and uncultured taxa) in LCFA degradation at 10°C. Unacclimated granular sludge achieved high methane yields (70-85%) with SDW and was found to be a suitable inoculum for the treatment of mixed LCFA containing wastewater at both 20 and 10°C. Unacclimated municipal digester sludges can be employed for treating the mixed-LCFA containing wastewaters at 20°C but not at lower temperatures. This study provides the basis for the inoculum selection by the evaluation of acetotrophic activity and the initial microbial community characteristics for producing methane from mixed LCFA-containing wastewater at low temperatures (up to 10°C).

Data availability

The 16S rRNA sequences used to support the findings of this study have been deposited in the NCBI Sequence Read Archive under project SRP164945.

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4 ANAEROBIC TREATMENT OF LCFA-CONTAINING SYNTHETIC DAIRY WASTEWATER AT 20°C: PROCESS PERFORMANCE AND MICROBIAL COMMUNITY DYNAMICS

Abstract

Facilitating anaerobic degradation of long-chain fatty acids (LCFA) is key for tapping the high methane production potential of the fats, oil and grease (FOG) content of dairy wastewaters. In this study, the feasibility of using high-rate granular sludge reactors for the treatment of mixed LCFA-containing synthetic dairy wastewater (SDW) was assessed at 20 °C. The effects of the LCFA concentration (33–45% of COD) and organic loading rates (2–3 gCOD/L·d) were determined using three parallel expanded granular sludge bed reactors. For the first time, long term anaerobic treatment of LCFA-containing feed at 20 °C was shown to be feasible and was linked to the microbial community dynamics in high-rate reactors. During a two-month operation, a soluble COD removal of 84–91% and COD to methane conversion of 44–51% was obtained. However, granular sludge flotation and washout occurred after two months in all reactors without volatile fatty acids (VFA) accumulation, emphasizing the need for sludge retention for long-term granular sludge reactor operation with LCFA-containing feed at low ambient temperatures. The temporal shifts in microbial community structure were studied in the high-rate treatment of SDW, and the process disturbances (elevated LCFA loading, LCFA accumulation, and batch operation) were found to decrease the microbial community diversity. The relative abundance of *Methanosaeta* increased with higher LCFA accumulation in the settled and flotation layer granules in the three reactors, therefore, acetoclastic methanogenesis was found to be crucial for the high-rate treatment of SDW at 20 °C. This study provides an initial understanding of the continuous anaerobic treatment of LCFA-containing industrial wastewaters at low ambient temperatures.

4.1 Introduction

Anaerobic granular sludge reactors such as upflow anaerobic sludge bed (UASB) reactors and expanded granular sludge bed (EGSB) reactors are feasible for the high-rate treatment of medium and high strength industrial wastewaters with chemical oxygen demand (COD) concentrations exceeding 1000 mg/L (Mao et al., 2015; van Lier et al., 2015). Applications of granular sludge reactors are common in the treatment of food and beverage industry wastewaters, such as sugar, confectionary, potato and malting wastewaters (van Lier et al., 2015), wherein carbohydrates typically contribute to most of the organic content. Contrarily, fewer granular sludge reactor applications exist for the anaerobic treatment of wastewaters with high lipid content (>20% of COD), such as those generated by the slaughterhouses, oil mills, and dairy industry.

As an example of lipid-containing wastewater, dairy wastewaters have the total COD typically ranging from 1 g/L to 5 g/L, of which 22%–45% is contributed by the fat, oil, and grease (FOG) fraction (Karadag et al., 2015). Hydrolysis of the FOG fraction results in a mixture of long chain fatty acids (LCFAs). In dairy wastewaters, palmitate, stearate, oleate, and linoleate are the commonly found LCFAs (Kim et al., 2004), whereas lactose (carbohydrate) and casein (protein) are the other major constituents. At mesophilic conditions, few laboratory and pilot-scale studies have suggested the potential use of granular sludge reactors (UASB and EGSB) for the treatment of FOG or LCFA-containing wastewaters (FOG 0.2–14.6 g/L) at hydraulic retention times (HRT) ranging from 0.7 to 5 d (Jeganathan et al., 2006; Leal et al., 2006; Passeggi et al., 2012; Saatci et al., 2003) while operational challenges of granular sludge flotation and washout have been encountered in other studies using granular sludge reactors (Gomes et al., 2011; Hwu et al., 1998b; Miranda et al., 2006).

Dairy wastewaters are discharged at low temperatures (~20 °C) and their economical treatment requires treatment processes at low temperatures. So far, to our knowledge no studies at discharge temperatures (~20 °C) of the anaerobic treatment of FOG or LCFA-rich industrial wastewaters, such as dairy wastewaters have been reported. Temperature impacts the physicochemical characteristics of individual LCFAs (solubility, sorption, diffusion and surface tension) besides the microbial kinetics and gaseous solubility (Bober and Garus, 2006; Lettinga et al., 2001). For example, oleate toxicity was higher in thermophilic than mesophilic conditions in batch assays (Hwu and Lettinga, 1997), while higher COD removal efficiencies and methane conversions were reported in continuous thermophilic than in mesophilic EGSB reactors treating LCFA mixtures (82% oleate, and 18% palmitate) (Hwu et al., 1998a). The high COD removal efficiencies at high organic loading rates (OLRs) obtained at low-temperatures (4 to 25 °C) upon

anaerobic treatment of various carbohydrate-rich and protein-rich wastewaters in granular sludge reactors (Bialek et al., 2012; Connaughton et al., 2006a; Esparza-Soto et al., 2013; McHugh et al., 2006; Sheldon and Erdogan, 2016) promote the interest to use granular sludge reactors for the treatment of LCFA-containing wastewaters at low temperatures. Moreover, the need for detailed investigations on low-temperature anaerobic treatment of dairy wastewater to improve the organic matter removal and methane production has been underlined in a recent review on granular sludge reactors (Karadag et al., 2015).

The anaerobic microbiomes involved in conversion of lipids or LCFAs to methane have been characterized in batch assays and high-rate reactors at mesophilic or thermophilic conditions (Duarte et al., 2018; Kundu et al., 2013; Salvador et al., 2013; Sousa et al., 2007a; Wagner et al., 2013; Ziels et al., 2017b). Bacterial groups that play a significant role in LCFA degradation have been identified, such as the taxa belonging to the families *Syntrophomonadaceae* (class *Clostridia*), *Clostridiaceae* (class *Clostridia*) and *Syntrophaceae* (class *Deltaproteobacteria*); including the genus *Syntrophomonas* that has been found in high concentrations in cultures achieving high LCFA conversion kinetics (Alves et al., 2009; Palatsi et al., 2010; Sousa et al., 2009, 2007b). Anaerobic LCFA-degrading cultures from low temperatures (at 10 and 20 °C) batch assays have been characterized as well (Singh et al., 2019a). Compared to mesophilic and thermophilic studies, *Syntrophus* and an uncultured *Syntrophaceae* taxa (psychrotolerant) and acetotrophic activity (mediated either by acetoclastic archaea or by syntrophic acetate oxidizing bacteria) were found to be crucial for methane production from LCFA-containing wastewater at the tested low temperatures (Singh et al., 2019a). As continuous feeding in high-rate reactors would administer selective pressure on the sludge microbiome distinct from the batch study, it is important to evaluate the effects of continuous operation on the key microbial taxa at low temperatures.

This study aimed to assess the feasibility of using high-rate anaerobic granular sludge reactors for treating LCFA-containing synthetic dairy wastewater (SDW) at low temperature. For that purpose, three laboratory EGSB reactors were operated using SDW at HRT, and, OLR in ranges considered feasible for applications at discharge temperature (20°C). The performance was assessed following sludge retention, COD removal efficiency, and methane production. In order to monitor microbial enrichment during reactor operation and to determine the key taxa that are putatively involved in the high-rate anaerobic LCFA degradation and methane production at low temperature, the microbial community composition of reactor sludges was determined at different times of reactor operation.

4.2 Materials and Methods

4.2.1 Inoculum and synthetic wastewater

Granular sludge was obtained from a mesophilic UASB reactor treating wastewater from the integrated production of the beta-amylase enzyme and ethanol from oat (Jokioinen, Finland). The granules were stored for 3 months at 7 °C in a nitrogen-purged atmosphere prior to usage. The granular sludge had a TS, VS and VS/TS ratio of 33.8 g/L, 29.3 g/L, and 86.6% respectively, with a pH of 6.8 ± 0.2.

Two SDWs consisting of fats (LCFA mixture), protein (casein hydrolysate) and carbohydrate (lactose monohydrate) were used in the experiments. Both SDWs had a COD of 2 gCOD/L, which contained of (1) 33% LCFA mixture (0.67 gCOD/L), 25% casein hydrolysate (0.5 gCOD/L) and 42% lactose monohydrate (0.82 gCOD/L), while the respective composition in (2) was 45% (0.9 gCOD/L), 19% (0.38 gCOD/L) and 36% (0.72 gCOD/L). The LCFA mixture in both wastewaters contained palmitate, stearate, oleate, and linoleate in a ratio of 30:15:45:10 on COD basis, according to their ratios and concentrations commonly found in dairy wastewaters (Karadag et al., 2015; Kim et al., 2004). Basal nutrient solution was added to both SDW (mg/L): $MgSO_4 \cdot 7H_2O$ (100), NH_4Cl (280), KH_2PO_4 (40), $Na_2HPO_4 \cdot 2H_2O$ (24), $FeCl_2 \cdot 4H_2O$ (2), H_3BO_3 (0.05), $ZnCl_2$ (0.05), $CuCl_2 \cdot 2H_2O$ (0.03), $MnCl_2 \cdot 4H_2O$ (0.5), $Na_2MoO_4 \cdot 2H_2O$ (0.008), $CoCl_2 \cdot 6H_2O$ (2), $NiCl_2 \cdot 6H_2O$ (0.092), $Na_2SeO_3 \cdot 5H_2O$ (0.164) and EDTA (1) (Kettunen et al., 1996). 2 g/L $NaHCO_3$ was supplemented to both SDW.

4.2.2 EGSB reactor experiments

Three identical EGSB reactors (acrylic, liquid volume 4.7 L, inner diameter 9 cm, height 81 cm) operated at room temperature (20 ± 2 °C) were used in this study (Fig. S4.1). The reactors contained a gas-solid separator on the top of the reactor and the reactor top was sealed with a rubber stopper (Fig. S4.1). The reactor liquor was recirculated using a peristaltic pump to maintain an upflow velocity of 2.2 m/h (Bialek et al., 2013) in the reactor. The feed was stored at 7 °C and pumped to the reactor with a peristaltic pump. Effluent from the EGSB reactors passed through a 500 mL gas-liquid separator that was connected to an effluent collection tank and a 5 L gas bag for biogas collection.

The three EGSB reactors were each inoculated with 50 gVS of granules, corresponding to a sludge bed height of about 20 cm. The three reactors were operated for 58 days at an OLR of 2 gCOD/L·d and 24 h HRT with SDW consisting of 33% LCFA-COD (Table 4.1). A major amount of sludge from one reactor was lost (84% of the inoculated VS) on day 42 due to technical failure, after which the remaining granules from the three reactors were mixed and divided equally over the three reactors, each having 27 gVS corresponding to 11 cm of granular sludge bed height. From day 59 to 80, one reactor ($R_{CONTROL}$) was

continued as before, while in another reactor (R_{OLR}) the OLR was increased to 3 gCOD/L·d and the HRT was shortened to 16 h. In the third reactor (R_{CONC}), the feed was changed to have a higher LCFA (45% LCFA-COD) content, while the OLR (2 gCOD/L·d) and HRT (24 h) were kept unchanged. On days 81–86 due to process instability in R_{OLR} and R_{CONC}, the OLR and feed composition were adjusted as previously operated (days 1–59). At the end of the experiments (days 87–98), the three reactors were operated in batch mode with recirculation and without feeding to assess if the declined methane production can be recovered.

Table 4.1. Summary of operational conditions in the three expanded granular sludge bed (EGSB) reactors ($R_{CONTROL}$, R_{OLR}, R_{CONC}) during the treatment of synthetic dairy wastewater (SDW) at 20°C during the different operational periods.

Parameter	Reactor	Period of operation			
		Normal LCFA load (start-up)	High LCFA load	Normal LCFA load (unstable)	Batch mode
Reactor operation (d)	$R_{CONTROL}$ R_{OLR} R_{CONC}	1-58	59-80	81-86	87-98
HRT (h)	$R_{CONTROL}$ R_{OLR} R_{CONC}	24	24 16 24	24	-
OLR (gCOD/L·d)	$R_{CONTROL}$ R_{OLR} R_{CONC}	2	2 3 2	2	0
LCFA-COD % in feed	$R_{CONTROL}$ R_{OLR} R_{CONC}	33	33 33 45	33	0
LCFA fed to reactors (gCOD/L·d)	$R_{CONTROL}$ R_{OLR} R_{CONC}	0.67	0.67 0.99 0.90	0.67	0

4.2.3 Methane production from granular sludge from the EGSB reactors

Upon completion of the reactor experiments, the methanogenic activity and lag phase from the flotation and settled layer granules of the three reactors were assayed in batch to assess the methane production. The assays were performed using 120 mL serum bottles in triplicate with a liquid volume of 60 mL. First, 10 mL of the granular sludge from the flotation (1.1–1.9 gVS/L) or settling (4.5–5.4 gVS/L) layer of the three reactors was added with 2 mL basal nutrient solution, and distilled water was added to have a liquid volume of 60 mL. The bottle headspace was purged with nitrogen for 10 min and incubated at 20 °C. The reactor inoculum (7.4 gVS/L) that had not been fed with LCFA acted as a reference and was assayed similarly.

4.2.4 Analytical methods and calculations

The CH_4 and CO_2 content of the biogas were determined using gas chromatography (Shimadzu GC-2014), fitted with a Porapak N column and thermal conductivity detector. Helium was used as the mobile phase at a flow rate of 20 mL/min. Injector, oven and detector temperatures were set at 110, 80 and 110 °C, respectively. The biogas volume was measured in the reactor studies with the water displacement method and according to Owen et al., (1979) in batch assays, and reported at Standard Temperature and Pressure

(STP). Methane conversion % was calculated based on the sCOD consumed (assuming a maximum of 350 mL-CH_4 per gram COD, at STP).

Samples for soluble COD (sCOD) and volatile fatty acids (VFAs) were filtered through 0.45 µm polyethersulphone syringe filters. The sCOD was measured using the potassium dichromate titrimetric method according to Finnish standard SFS 5504 (5504, SFS Finnish Standard Association, Helsinki, 1988). TS and VS were measured according to APHA, (2005). VFAs (acetate, propionate, butyrate, isobutyrate, and valerate) were measured by gas chromatography using Shimadzu GC-2010 (Singh et al., 2019a).

For measurement of the particle size distribution, 2–5 mL of the granules was pipetted on a graduated petri dish (minimum of 200 particles detectable). The petri dish was placed on a LED backlit light diffuser plate to achieve enhanced resolution. Computer controlled image acquisition was performed for every square occupied by particles in a sample taken using an AVT Marlin 145-B2 high-speed camera with a 1380 × 1090 resolution and a black and white CCD-cell. The particles were shown in the images as projections due to the background LED light, from which the projection outline and the area of the projection were determined. Particles smaller than 30 µm were not measured but constituted an insignificant fraction and did not affect the mean diameter. The images were processed using Matlab R2017b as explained in Tolvanen et al. (Tolvanen et al., 2013). d_{50}, referring to the particle diameter that contains 50% of the cumulative particle volume, and d_{max}, referring to the maximum diameter determined for the granule sample, were used as the parameters for inferring the effect of LCFA on the granular size at 20 °C.

4.2.5 16S rRNA amplicon sequencing and bioinformatics

Microbial samples from the three reactors were collected on days 0 (*n* = 2), 42 (*n* = 1) and 58 (n = 2) from the granular sludge (settled layer) and on day 98 (n = 2) from the flotation and settled sludge layer, and, were stored at −20 °C immediately upon sampling. DNA was extracted using the procedure introduced by Griffiths et al., (2000). The concentration of extracted DNA was quantified with a Qubit fluorometer (Life Technologies), and the DNA purity was measured using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The DNA samples were sent to FISABIO (Spain) for PCR amplification of the V4 region of the 16S rRNA gene with universal primers 515f and 806r (Caporaso et al., 2011), and used for amplicon sequencing with the Illumina MiSeq platform. The sequence data were analyzed using Quantitative Insights Into Microbial Ecology (QIIME v1.9) and taxonomy assignment was performed with Silva 128 consensus taxonomy at all levels (Aronesty, 2013; Caporaso et al., 2010; Glöckner et al., 2017; Pruesse et al., 2007) with the additional details mentioned in Singh et al., (2019).

This dataset was normalized through metagenomeSeq CSS matrix transformation (Paulson et al., 2013) in QIIME, and, subsequently used for the estimation of alpha diversity metrices (goods_coverage, shannon, ace, and chao1) that are presented as the average of the replicate samples. Seventy OTUs had a cumulative observation count higher than 0.01% (2303 sequences) in the samples and were used subsequently. The dataset was fourth-root transformed based on draftsman plot, and consequently, a resemblance matrix was constructed using Bray-Curtis similarity in Plymouth Routines In Multivariate Ecological Research (PRIMER) V7 (Clarke et al., 2014). The resemblance matrix was used to perform the principal coordinate analysis (PCoA) (Gower, 1966) to visualize the similarity patterns in different microbial samples. Cluster analysis through hierarchical clustering (group average method) was performed on the operational taxonomic units (OTUs), and separately on the reactor samples, to plot the dendrograms. The fourth-root transformed OTU table was used for representing the microbial community structure at class level in a shade plot along with the dendrograms.

Table 4.2. Summary of reactor performance in the three expanded granular sludge bed (EGSB) reactors ($R_{CONTROL}$, R_{OLR}, R_{CONC}) during the treatment of synthetic dairy wastewater (SDW) at 20 °C during the different operational periods.

Parameter	Reactor	Normal LCFA load (initial acclimation)	Normal LCFA load (Stable)	Normal LCFA load (after sludge redistribution)	High LCFA load	Normal LCFA load	Batch mode
Reactor operation (d)	$R_{CONTROL}$	1-28	29-42	43-58	59-80	81-86	87-98
	R_{OLR}						
	R_{CONC}						
pH	$R_{CONTROL}$	6.6 ± 0.2	6.6 ± 0.1	7 ± 0.1	7.1 ± 0.1	7 ± 0.1	7 ± 0.1
	R_{OLR}	6.6 ± 0.1	6.6 ± 0.1				
	R_{CONC}	6.6 ± 0.2	6.4 ± 0.2				
sCOD removal (%)	$R_{CONTROL}$	89 ± 7	83 ± 3	83 ± 4	91 ± 3	81 ± 11	-
	R_{OLR}	91 ± 4	90 ± 2	87 ± 4	88 ± 3	76 ± 7	-
	R_{CONC}	89 ± 5	83 ± 2	86 ± 3	79 ± 5	73 ± 11	-
Methane production (mL/d)	$R_{CONTROL}$	620 ± 655	1165 ± 270	1520 ± 410	1370 ± 310	930 ± 80	200
	R_{OLR}	1120 ± 750	1650 ± 240	1440 ± 420	1550 ± 500	310 ± 75	170
	R_{CONC}	1050 ± 680	1090 ± 270	1440 ± 400	800 ± 520	220 ± 6	nd
Methane conversion % ($mL\text{-}CH_4/gCOD_{consumed}$)	$R_{CONTROL}$	17 ± 16	35 ± 10	49 ± 9	39 ± 14	29 ± 1	-
	R_{OLR}	37 ± 18	52 ± 5	49 ± 10	36 ± 14	5 ± 2	-
	R_{CONC}	26 ± 18	34 ± 11	53 ± 8	21 ± 14	6 ± 1	-
Total VFA (mgCOD/L)	$R_{CONTROL}$	86 ± 90	77 ± 50	75 ± 14	0	4 ± 6	-
	R_{OLR}	78 ± 77	63 ± 30	74 ± 9	52 ± 59	0	-
	R_{CONC}	83 ± 86	225 ± 90	73 ± 13	280 ± 120	23 ± 18	-

nd – not detected

4.3 Results and Discussion

4.3.1 Anaerobic treatment of LCFA-containing SDW at 20°C

For the first 58 days, the triplicate EGSB reactors were operated identically. During the days 29 to 38, an average sCOD removal and COD to methane conversion of 82–90% and 20–40%, respectively, were achieved (Table 4.2). Some COD removal occurred apparently due to the accumulation of LCFAs on the granules (see below Section 3.3). After the sludge redistribution on day 42 (see Section 2.2) sCOD removal (84–91%) and methane production (up to 51% COD to methane conversion) remained high until day 58, while the VFA concentrations were <100 mg/L (Table 4.2, Fig. 4.1).

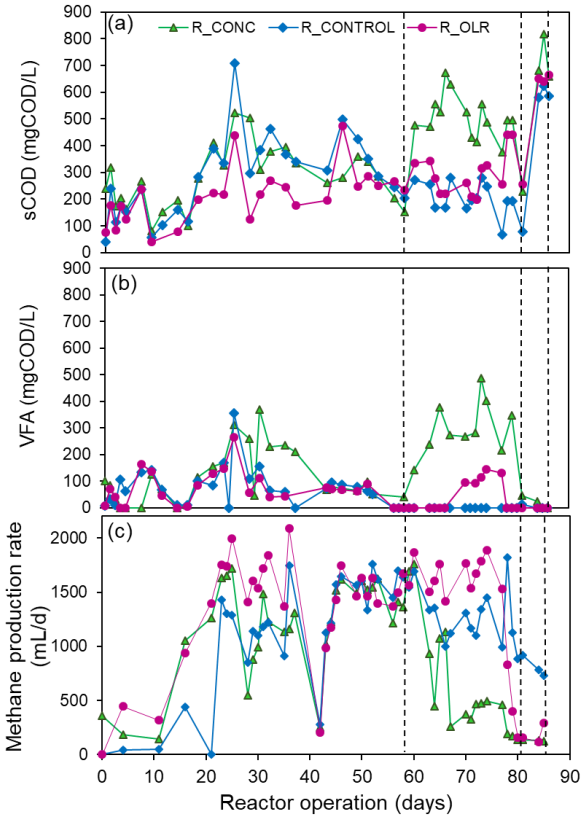

Fig. 4.1. Performance of the expanded granular sludge bed (EGSB) reactors - $R_{CONTROL}$, R_{OLR} and R_{CONC} for the treatment of synthetic dairy wastewater at 20 °C, (a) soluble chemical oxygen demand (sCOD) (b) and volatile fatty acid (VFA), in effluent and (c) methane production. Vertically dropped lines represent the change in long chain fatty acid (LCFA) loading rate (Table 4.1).

The results from the 58-d operation suggest that LCFA-containing dairy wastewater (with up to 33% COD as mixed-LCFA and an LCFA loading rate of 0.67 gCOD-LCFA/L·d) can be anaerobically treated in EGSB reactors at 20 °C. This is the first study reporting continuous low temperature anaerobic treatment of LCFAs with concentrations above 3% of the total COD, encouraging further investigations and testing with real industrial LCFA-rich wastewaters. In this study, the sCOD removal efficiency was higher (up to 90%) than the 60% COD removal reported during the treatment of real dairy wastewater in a UASB reactor at

20 °C (HRT 24 h) by Tawfik et al., (2008). Moreover, even with the application of over 3-fold higher OLR (2 gCOD/L·d vs. 0.6 gCOD/L·d) and over 25-times higher LCFA loading rate (0.6 gCOD/L·d vs. 0.022 gCOD/L·d) in the current study, comparable sCOD removal efficiencies (up to 80–90%) were achieved as reported previously in anaerobic sequencing batch reactor (ASBR) (Dague et al., 1998). Dague et al. (1998) reported a higher methane conversion at 20 °C than obtained in this study (80% vs. 48%, respectively). However, the methane conversion (against biologically degraded COD) in this study was likely higher than the 48% due to the accumulated substrate. An evaluation of the LCFA profiles in the effluent and LCFA sorbed on the sludge is recommended to accurately determine the methane production from individual LCFAs.

4.3.2 Effect of LCFA loading rate on anaerobic treatment of SDW

After 59 days, the effects of increasing LCFA loading rates were studied in two EGSB reactors through increasing the OLR from 2 to 3 gCOD/L·d (R_{OLR}) or increasing the LCFA concentration in the feed from 30 to 45% LCFA-COD (R_{CONC}) along with the operation of the third reactor as before ($R_{CONTROL}$) (Table 4.2). In all three reactors, granular flotation was initiated within 10 days, which intensified on prolonged SDW feeding. The sCOD removal efficiency in the three reactors remained ≥70%, whereas, acetate, propionate, and butyrate were the main VFAs in the effluent at individual concentrations below 150 mg/L. In R_{CONC} and R_{OLR}, the COD to methane conversion sharply decreased on day 60 (from 59 to 20%) and on day 78 (from 42 to 20%), respectively, while in $R_{CONTROL}$ the COD to methane conversion decreased from 45 to 65% to 20% in ca. 20 days.

In R_{CONC}, the methane production decreased, and granular flotation was observed within 2–3 days after the LCFA loading rate was increased. Granular flotation and washout were also observed in other reactors (Gomes et al., 2011; Hwu et al., 1998b; Miranda et al., 2006). After day 80, recovery of the methane production was attempted by decreasing the LCFA loading rate or operating the reactors as batch (for further details, see Materials and methods). However, the methane production could not be recovered, which highlights the importance of preventing sludge flotation and washout.

Our results at 20 °C and the previous research on LCFA at mesophilic or thermophilic conditions suggest that sludge flotation and granular washout occur in granular sludge reactors treating LCFA-containing wastewaters independent of the process temperature (Hwu et al., 1998b; Jeganathan et al., 2006; Pereira et al., 2002b; Rinzema et al., 1993). In this study, sludge flotation occurred after two months of operation in all the three reactors at LCFA loading rates of 0.67–0.99 gCOD-LCFA/L·d, wherein the sludge flotation was induced faster when feed constituted higher LCFA concentration (45% vs 33%), although complete sludge bed flotation was not observed in any of the three reactors during the 98 days. Therefore, LCFA loading rate and LCFA accumulation should be monitored while treating LCFA or FOG-rich wastewaters.

The use of real FOG-rich wastewater may bring additional complexities due to the presence of various organic and inorganic compounds that may affect the non-microbial (physico-chemical) and microbial characteristics during the treatment process, and necessitates further investigations evaluating these aspects at low temperatures.

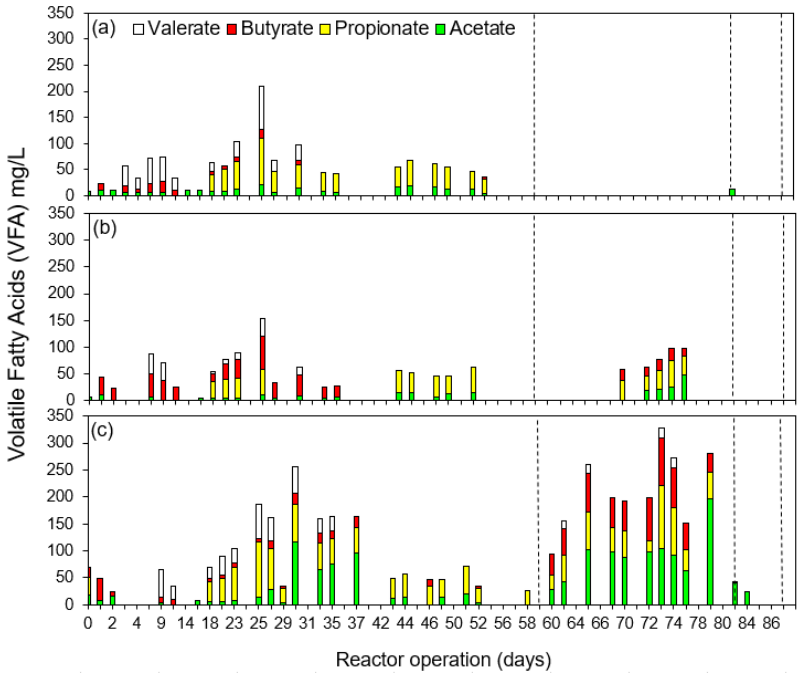

Fig. 4.2. VFA accumulation in the expanded granular sludge bed (EGSB) reactors for the treatment of synthetic dairy wastewater at 20 °C, (a) $R_{CONTROL}$, (b) R_{OLR} and (c) R_{CONC} at 20 °C. Vertically dropped lines represent the change in long chain fatty acid (LCFA) loading rate (Table 4.1).

4.3.3 Effect of LCFA loading rate on EGSB granule characteristics

The sludge bed height and sludge retention were monitored during the entire EGSB reactor operation, and granule sizes from the flotation and settled layers were determined at the end of the reactor operation (day 98). Initially, the three reactors had a sludge bed height of 20 cm, which decreased to 11 cm after sludge redistribution on day 43 and remained stable at 10.5 cm by the end of day 59 (Fig. S4.2). By the day 75, the granules encountered flotation resulting in flotation layer heights of 3.75–3.8 cm in $R_{CONTROL}$ and R_{OLR} and 6.5 cm in R_{CONC} (Fig. S4.2). As a result, the specific LCFA loading on the granular sludge in the settled layer increased. By the end of the reactor operation (day 98), the granule size had decreased compared to the inoculum granules (d_{50}, 1.7 mm) (Fig. S4.3). In $R_{CONTROL}$ and R_{OLR}, the d_{50} (1.3 mm) and the particle size distribution of granules from the settled and flotation layers was similar (Fig. S4.3). However, in R_{CONC} the granules in the flotation layer were considerably larger (d_{50} of 1.34 mm) than in the settled layer (d_{50} of 1.14 mm) (Table S4.1). As the equivalent diameter of granules larger than 1 mm may increase with LCFA accumulation up to a threshold value of 200 mgCOD/gVS, followed by the

migration of the granular aggregates to the flotation layer (Amaral et al., 2004), the R_{CONC} granules from the flotation layer had a larger size due to high substrate accumulation (presumably LCFA). Overall, the d_{50} of the granules from settled layers decreased by 24–33% in comparison to the inoculum. The broken granules in the effluent (of $R_{CONTROL}$) had a d_{50} of 0.28 mm, indicating that the disintegrated granules that had a 75–84% decrease in the d_{50} were washed-out from the reactor (Table S4.1). The results highlight the need for granular retention in LCFA-fed granular sludge reactors, and encourage investigations using alternate reactor configurations.

The sorption of substrate on the EGSB granules after reactor trials (day 98) was estimated from the methane production in batch assays without the addition of substrate. The characteristics of the granules used for the batch assays are shown in Table S4.2. The methane yield (mL-CH_4/gVS) from the flotation layer granules was higher than the methane yield from the corresponding settled layer granules (Fig. S4.4). Moreover, the flotation layer granules from R_{CONC} had a 3–6 times higher methane yield than the flotation layer granules from R_{OLR} and $R_{CONTROL}$ within 30 days (Fig. S4.4). The inoculum (unacclimated granules) had a short lag phase (<1 d) similar to the settled layer granules, but as expected had a lower methane yield (25 mL-CH_4/gVS) than the reactor granules (55–280 mL-CH_4/gVS) due to substrate accumulation on the reactor granules. The results indicate that the LCFA loading rate and LCFA concentration in the feed affected the substrate accumulation on the granules.

4.3.4 Microbial community evolution during SDW treatment

4.3.4.1 Microbial community diversity in the reactors during continuous SDW treatment

A total of 4464 OTUs were found, of which 70 OTUs comprised 99.9% of the microbial community. The species richness of the microbial communities decreased by day 98 compared to the inoculum and the microbial community in the reactors on days 42 and 58 (Table 4.3). Table 4.3 shows that the ace and chao 1 indices (Chao, 1984) decreased significantly in the reactor sludges (ace: p = 0.037, two-tailed t-test), (chao 1: p = 0.021, two-tailed t-test) on day 98 compared to the inoculum and reactor sludges on day 42 and 58, whereas the Shannon indices for the different reactor samples varied from 8.9 to 9.2 (p = 0.37, two-tailed t-test). Increased species richness and evenness have been linked to stable active communities (Naeem, 2009; Wittebolle et al., 2009), and the opposite has been linked to a lack of functional redundancy leading to long recovery durations post-perturbations (Spirito et al., 2018). Thus, the microbial communities in the reactors up to day 58 can be considered to be more active and stable than the microbial communities on day 98. The decrease in the microbial community diversity from days 58 to 98 can be linked to the disturbances in reactor operation (arising from elevated LCFA loading, sludge flotation and washout as well as from LCFA accumulation on the granules).

Table 4.3. Alpha diversity metrices (diversity within reactor sludges) – Ace, Chao 1, Good's coverage and Shannon indices for all the reactor sludges.

Reactor (days of operation)	Ace	Chao1	Shannon	Good's coverage	Shannon (99.9%)
Inoculum (0)	2159	2186	9.32	0.72	5.76
$R_{CONTROL}$ (42)	2570	2413	9.68	0.70	5.79
R_{OLR} (42)	1943	2305	9.27	0.71	5.79
R_{CONC} (42)	956	934	8.75	0.81	5.81
$R_{CONTROL}$ (58)	1352	1362	9.19	0.8	5.77
R_{OLR} (58)	2081	1929	9.59	0.77	5.8
R_{CONC} (58)	1057	982	8.93	0.82	5.8
$R_{CONTROL}$ (S) (98)	1229	1089	9.17	0.81	5.58
R_{OLR} (S) (98)	966	927	9.04	0.85	5.47
R_{CONC} (S) (98)	1086	999	9.22	0.85	5.55
$R_{CONTROL}$ (F) (98)	1018	950	8.99	0.83	5.55
R_{OLR} (F) (98)	1212	1084	9.24	0.83	5.44
R_{CONC} (F) (98)	1232	1114	9.23	0.81	5.58

(F) and (S) represent the flotation and settled layer sludges on day 98 in the reactors $R_{CONTROL}$, R_{OLR} and R_{CONC}

4.3.4.2 Microbial community dynamics and composition

The 70 OTUs that formed 99.9% of the taxa consisted of 4 archaeal and 66 bacterial OTUs. The PCoA plot of the reactor sludges shows that 81% of the variation was explained by two axes (54% by first and 27% by the second) (Fig. 4.3). The microbial community composition after the perturbations (increased LCFA loading, sludge flotation, and washout, and the consequent batch operation) on day 98 was significantly different from the microbial community composition of the reactor sludges on days 42 and 58, as well as, the inoculum ($p < 0.05$, PERMANOVA, single factor design, 999 unique permutations). This means that the microbial community composition was affected by the perturbations and also by prolonged SDW feeding, as observed from the reactor sludges of $R_{CONTROL}$ on days 42, 58 and 98 (Fig. 4.3).

The bacterial classes *Bacteroidia* and *Clostridia* had a high relative abundance up to 58 d of the reactor operation and decreased by day 98 due to prolonged SDW feeding and the increased LCFA loading rates (Fig. 4.4). Furthermore, the bacteria belonging to the class *Synergistia* were enriched from day 0 to day 58. Bacteria belonging to classes *Bacteroidia* and *Clostridia* are functionally diverse and often include fermentative bacteria (Amani et al., 2010; Venkiteshwaran et al., 2017), whereas bacteria from class *Synergistia* are syntrophic acido- and acetogens involved in amino-acid fermentation (Jumas-Bilak et al., 2015; Singh et al., 2019a). Therefore, the presence of bacteria belonging to these classes in anaerobic consortia treating complex LCFA-containing wastewater seems reasonable. High abundances of taxa from the microbial classes - *Bacteroidia*, *Clostridia*, *Synergistia*, *Methanomicrobia* and *Methanobacteria* in the

reactor sludges indicate their role in anaerobic treatment of complex LCFA-containing wastewater in the high-rate reactors at 20 °C. At thermophilic and mesophilic conditions (35–54 °C), the above-mentioned microbial classes, with the exception of *Methanomicrobia*, formed the core community of the LCFA-degrading microbiomes in reactors that were operated either in batch or continuous-mode (Amha et al., 2018), signifying the importance of the *Methanomicrobial* taxa in this study.

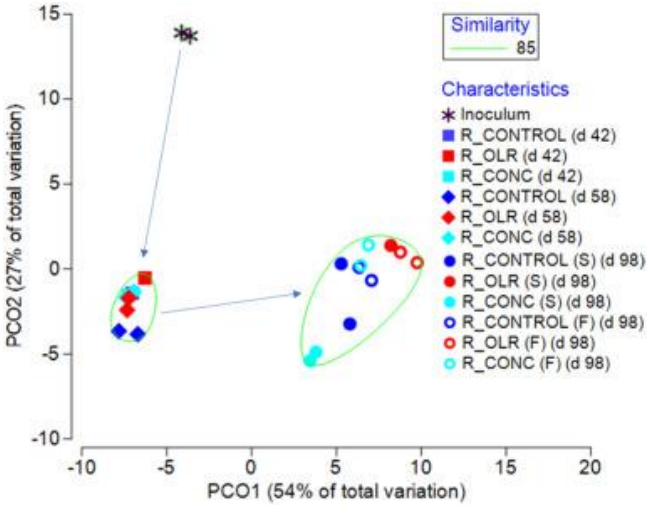

Fig. 4.3. Principal coordinate analysis (PCoA) plot of the bacterial and archaeal classes that formed 99.9% of the microbial community in the reactor sludges on days 0, 42, 58 and 98. The x and y-axes represent 54% and 27% of the total variation, respectively. Ellipses are drawn for the reactor sludges having a high similarity (85%) in the microbial community. (F) and (S) represent the sludges from the flotation and the settled layer, respectively, from the corresponding reactors.

The 4 archaeal OTUs that formed 99.9% of the abundant microbial taxa in the reactor sludges belonged to theclasses *Methanobacteria* and *Methanomicrobia* (Fig. 4.4), which constituted solely of the hydrogenotrophic genus – *Methanobacterium*, and the acetoclastic genus – *Methanosaeta*, respectively (Fig. 4.5). The relative fraction of the *Methanobacterial* and *Methanomicrobial* reads, corresponding to the genera *Methanobacterium* and *Methanosaeta* is presented in Fig. 4.5, and the *Methanosaeta*: *Methanobacterium* ratio was used to evaluate the major methanogenesis pathway. The *Methanosaeta*:*Methanobacterium* ratio demonstrated that although hydrogenotrophic methanogenesis was predominant in the inoculum (0.74), acetoclastic methanogenesis was more prominent by day 42 (0.8–1.1) and was the major methanogenesis pathway by day 58 (1.5–1.9) in the three reactors.

Hydrogenotrophic methanogenesis has been reported to be favourable at lower temperatures, due to the increased hydrogen solubility resulting in increased substrate availability, and, the increased thermodynamic favourability for hydrogen utilization at low temperatures resulting in the availability of free energy for microbial growth (Kotsyurbenko, 2005; Lettinga et al., 2001). The methanogenesis pathway has been found to be predominantly hydrogenotrophic during the high-rate anaerobic treatment

of various wastewaters, including the comparison of the treatment of VFA-based wastewater at 37 °C to 15 °C (Connaughton et al., 2006b), the temperature drop from 37 to 16 °C during the treatment of VFA- and glucose-based wastewaters(McHugh et al., 2004), during the treatment of raw domestic wastewaters at ambient temperatures ranging from 14 to 21 °C (Álvarez et al., 2008), and, with a temperature drop (37 to 25, and 25 to 15 °C) during treatment of synthetic dairy wastewater (Bialek et al., 2012). Furthermore, hydrogenotrophic methanogens (*Mehanomicrobiales*) were also found to be marginally dominant to acetoclastic methanogens (*Methanosaetaceae*) during treatment of lipid-rich domestic wastewater (lipids:carbohydrates:proteins = 0.45: 0.35: 0.2) at 4 and 8 °C in fed-batch reactors (Petropoulos et al., 2017). In addition, during the high-rate treatment of LCFA at mesophilic conditions, hydrogenotrophic methanogens such as *Methanobacterium* were found at a higher relative abundance than the acetoclastic methanogens such as *Methanosaeta* (Duarte et al., 2018), and were less sensitive to hydraulic and organic shocks than *Methanosaeta* (Kundu et al., 2013). In contrast, the findings of this study show the prevalence of acetoclastic methanogenesis as the predominant pathway during the continuous high-rate treatment of mixed-LCFA wastewater at 20 °C.

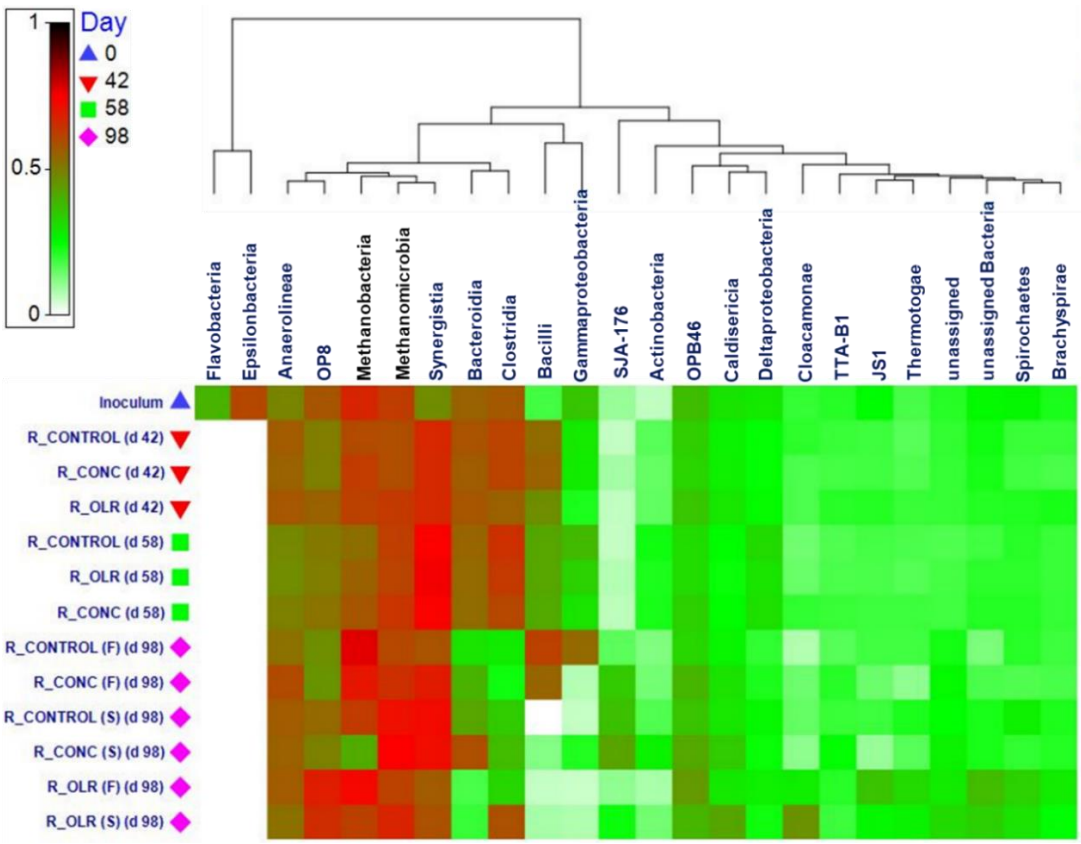

Fig. 4.4. Shade plot showing the clustering and relative abundance of the bacterial and archaeal classes that formed 99.9% of the microbial community in the reactor sludges during the 98-d experimental period. (F) and (S) represent the sludges from the flotation and the settled layer respectively from the corresponding reactors.

At the end of the reactor operation (day 98), the *Methanosaeta:Methanobacterium* ratio increased (0.74, 0.51, and 0.37 in R_{CONC}, R_{OLR}, and $R_{CONTROL}$) in the flotation layer sludges, with the increased LCFA concentration (45%, 33%, 33% in R_{CONC}, R_{OLR}, and $R_{CONTROL}$) and LCFA loading (0.9, 0.99 and 0.67 gCOD-LCFA/L·d in R_{CONC}, R_{OLR}, and $R_{CONTROL}$). In the settled layer sludges on day 98, the *Methanosaeta*:*Methanobacterium* ratio was similar in $R_{CONTROL}$ and R_{OLR} (1.7 and 1.5 respectively) and 10-folds higher in the settled layer sludge of R_{CONC} (11.3).

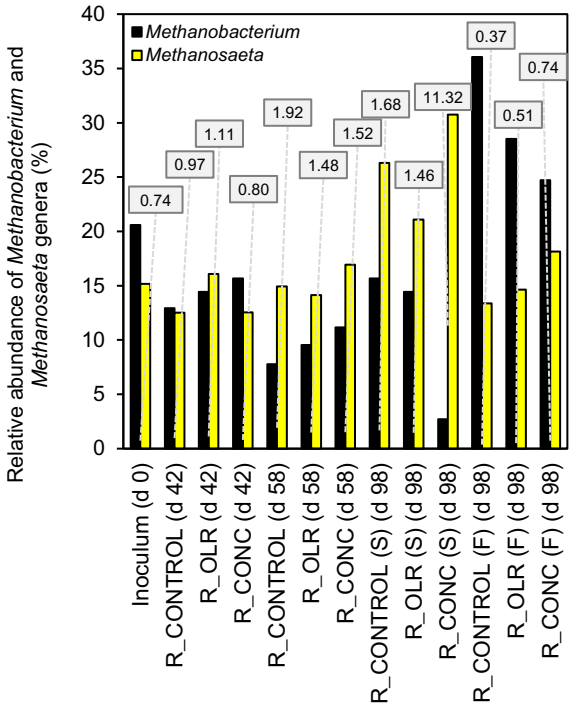

Fig. 4.5. Relative abundance (%) of the archaeal genera *Methanobacterium* and *Methanosaeta* found in the 16 s rRNA amplicon libraries obtained from the reactor sludges during the 98-d operational duration. The ratio of relative abundances of *Methanosaeta* to *Methanobacterium* are presented as values for each of the samples. (F) and (S) represent the sludges from the flotation and the settled layer, respectively, from the corresponding reactors.

As the LCFA loading rates in the EGSB reactors in this study were higher than that of batch and fed-batch reactors operated at low temperatures (Lv et al., 2015; Petropoulos et al., 2017), production of acetate at higher concentrations is envisaged from the beta-oxidation of LCFAs as the terminal end-product. Thus, acetate uptake facilitated by *Methanosaeta* plausibly drove the LCFA degradation in this study, since the methane production from LCFAs is determined by the intermediate acetate concentration and acetate uptake kinetics (Schink, 1997; Ziels et al., 2017a). This suggests the continuous SDW feeding shifted the predominant methanogenesis pathway from hydrogenotrophic to acetoclastic (Fig. 4.5). The acetogens and methanogens in the reactor sludges were inhibited by the increase in LCFA loading rate and LCFA concentration in feed, as observed from the VFA accumulation (Fig. 4.1, Fig. 4.2) and declined methane production from days 58 to 98 (Fig. 4.1). As acetoclastic methanogens are sensitive to the presence of

LCFAs, the retention and activity of acetoclastic methanogens (here, *Methanosaeta* (Fig. 4.5)) can be considered as crucial for the LCFA degradation at discharge temperatures of about 20 °C from an application point of view. The results from this study show that the continuous anaerobic treatment of LCFA-containing dairy wastewaters is possible at low ambient temperatures, and, encourages further systematic studies on the high-rate anaerobic treatment of LCFA-containing industrial wastewaters at temperatures lower than 20°C.

4.4 Conclusions

The potential of high-rate anaerobic EGSB reactors for treatment of mixed-LCFA containing dairy wastewater at low temperature using relatively short treatment times (24 h HRT) and reasonable loading rates (OLR of 2 gCOD/L·d) is demonstrated. In EGSB reactor treatment, high sCOD removal efficiencies (84–91%) can be achieved, of which around 50% of the removed COD is converted into methane, while another 50% is sorbed on the reactor granular biomass. The development of operation strategies of EGSB reactors and reactor design are required to confirm the long-term process performance, as granular flotation and washout occurred after two months apparently due to LCFA sorption and disintegration of granules. Continuous operation at low temperature with mixed-LCFA resulted in enrichment of the bacterial classes *Bacteroidia*, *Clostridia* and *Synergistia*, which include several known fermentative and/or syntrophic acido- and acetogenic bacteria. At low temperature, for the first time the shift in dominant methanogenesis pathway from hydrogenotrophic to acetoclastic (mediated by *Methanosaeta*) is demonstrated and suggests that retention of acetoclastic methanogens is crucial for the continuous high-rate treatment of mixed-LCFA at low temperatures of 20 °C.

Data availability

The 16S rRNA sequences used to support the findings of this study have been deposited in the NCBI Sequence Read Archive under project PRJNA525245.

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5 RAPID GRANULATION AND ENHANCED METHANISATION AT 20˚C IN A NOVEL, DYNAMIC-SLUDGE-CHAMBER - FIXED-FILM (DSC-FF) BIOREACTOR TREATING LCFA WASTEWATER

Abstract

Lipid-containing wastewaters, such as those arising from dairy processing, are frequently discharged at temperatures ≤ 20°C and their valorization offers a large opportunity to expand the application of high-rate anaerobic wastewater treatment and resource recovery. Lipid hydrolysis generates long chain fatty acids (LCFA), which hinder stable reactor operation by inducing the flotation of sludge and its subsequent washout. Therefore, a combined approach of a novel reactor configuration, a specific inoculum and de novo granulation was applied for the treatment of mixed-LCFA containing dairy wastewater at 20°C. The Dynamic Sludge Chamber Fixed Film reactors (DSC-FF) facilitated settled granular sludge, floating sludge zone and fixed-film phase within a single module. Reproducibly high chemical oxygen demand (COD) removal efficiencies (87-98%) were achieved in three replicated DSC-FF reactors during the continuous treatment of LCFA-containing dairy wastewater (with 33% COD as LCFA) for the first time (HRT: 0.5-3 d, LCFA loading rate: 220-1333 mgCOD-LCFA/L·d) at 20°C. Granulation was achieved from a combination of inoculum mixtures in less than 10 d at 20°C, even with a high mixed-LCFA concentration (33% COD-LCFA). The results demonstrate the feasibility of the approach for treating LCFA-containing wastewaters at discharge temperatures and offer the potential for expanded and more energetically productive applications of high-rate anaerobic wastewater treatment.

5.1 Introduction

High-rate anaerobic treatment is a sustainable option for the treatment of a variety of wastewaters, both municipal and industrial, where reactors are normally operated at mesophilic or thermophilic conditions. The extension of anaerobic digestion (AD) technologies to lower temperatures (≤ 20°C) is crucial for improving the net energy recovery from wastewater treatment, particularly in temperate climates. Several lipid-containing wastewaters, such as dairy wastewaters, are energy-rich and are emitted in large quantities at low ambient temperatures. Thus, they could be utilized for anaerobic treatment to utilize their high methane production potential, i.e theoretically, 1.43 L-CH_4/g-lipid (Alves et al., 2009).

The anaerobic treatment of lipid-rich streams is problematic, since their hydrolysis produces long chain fatty acids (LCFA), compounds that can cause serious operational issues in anaerobic treatment. In high-rate anaerobic reactors, such as upflow anaerobic sludge bed (UASB) reactors, lipids pose operational challenges due to sludge flotation, washout, scum layer formation and substrate transfer limitations arising from the encapsulation of sludge by LCFA (Pereira et al., 2005; Rinzema et al., 1994). Moreover, the LCFAs behave as surfactants at neutral pH (Sam-Soon et al., 1991) and disrupt the granular structure, thus further intensifying the sludge washout. Therefore, developing high microbial activity and sludge retention in high-rate reactors is crucial for the treatment of lipid or LCFA-containing wastewaters, especially at low temperatures due to the decrease in microbial growth rates with decreasing temperature. As a result, the development and retention of granular sludge has proven difficult and a bottleneck in anaerobic treatment of LCFA-containing wastewaters.

Lipid or LCFA-treatment at low temperature necessitates improved reactor design for enhancing the sludge retention. Strategies applied include biofilm formation on support material in fixed-film reactor configurations (anaerobic filter, fluidized-bed reactor, fixed-bed reactor, moving bed biofilm reactor (MBBR)), sludge flotation in specialized reactor designs such as the anaerobic flotation reactor (AFR) (Paques, the Netherlands) and inverted anaerobic sludge bed (IASB) reactor (Alves et al., 2007), or usage of the settling characteristics of granular sludges in granular sludge reactors, i.e. UASB and static granular bed reactor (SGBR), or the usage of membrane bioreactor systems (Dereli et al., 2015; Jensen et al., 2015; Ramos et al., 2014). Granular sludge reactors have been used to treat lipid-containing wastewaters at mesophilic conditions (Jeganathan et al., 2006; Leal et al., 2006). Furthermore, reactor configurations consisting of both single or two-stages have been frequently used at low temperatures (5-20°C) for the treatment of dairy wastewaters containing low concentrations of lipid and LCFA in various granular sludge and fixed-film reactors (Table 5.1) (Bialek et al., 2014; Buntner et al., 2013; Dague et al., 1998; Luostarinen

and Rintala, 2005; Nikolaeva et al., 2013; Park et al., 2012; Ramasamy and Abbasi, 2000; Tawfik et al., 2008; Toldrá et al., 1987; Viraraghavan and Kikkeri, 1990).

Table 5.1 Process performance of anaerobic dairy wastewater treatment in fixed-film and granular sludge reactors at psychrophilic and low ambient temperatures.

Substrate	Reactor	Temp (°C)	Influent pH	LCFA loading rate (gCOD/L.d)	OLR (g-COD/L.d)	HRT (d)	sCOD removal (%)	Methane yield at STP (mL/g-COD.d)	Reference
Non-fat dry milk	ASBR	5-25	7	a	0.6-2.4	0.25- 1	60-98	95-290	Dague et al., 1998
Dairy processing wastewater	SGBR	10-25	5.8 ± 0.7	NR	0.6-9.7	0.38- 4	>90	90-340	Park et al., 2012
Synthetic dairy parlour wastewater	Two phased-UASB septic tank	10-20	6.1 ± 0.4	NR	0.1-0.24	3.5+ 1.5	33-62	NR	Luostarinen and Rintala, 2005
Dairy wastewater	UASB + Activated sludge	20	7.9 ± 1.2	0.75 ± 0.06 (22%)[b,c]	3.4	1(+ 0.08)	69	NR	Tawfik et al., 2008
Skim milk	UASB + aerobic MBR	17-25	7	NR	2.4-1.6	0.4 -0.6 (total 0.6-0.8)	95 UASB, 99 total	110-310	Buntner et al., 2013
Dairy wastewater	FBR (Sand)	20 (35)	9.05, adjusted to 7	NR	0.6-6	0.33,0.25, 0.167, 0.083	20-42%	NR	Toldrá et al., 1987
Synthetic skimmed dairy wastewater	IFB (ExtendospheresTM , light mineral material of silica and traces of aluminium)	10	7	a	0.5-2	0.5 -2	24-80	107-294	Bialek et al., 2014
Dairy washing wastewater	AFBR (fixed-bed) Tire rubber + zeolite	22-26	7.2 ± 0.3	NR	4.4-24	1 -5.5	28-82	70-180	Nikolaeva et al., 2013
Dairy wastewater	AF (plastic)	21	7-7.3	NR	4	1	55	180	Viraraghavan and Kikkeri, 1990
Synthetic dairy wastewater	Nylon mesh-biofilm support system in CSTR	Room temp	NR	NR	NR	15,14,13,12,11,10	46-59%	NR	Ramasamy and Abbasi, 2000
Synthetic dairy wastewater	DSC-FF	20	7.1 ± 0.1	0.22-1.33	0.66-4	0.5-3	87-97%	24-360	This study

ASBR Anaerobic sequencing batch reactor, SGBR Static granular bed reactor, UASB Upflow anaerobic sludge blanket, MBR Membrane bioreactor, FBR Fluidized bed reactor, IFB Inverted fluidized bed, AFBR Anaerobic fixed bed reactor, AF Anaerobic filter, CSTR Completely stirred tank reactor, DSC Dynamic sludge chamber, FF Fixed-film
NR Not reported, GS Granular sludge
a <1 % fat in substrate
b Oil and grease (mg/L), ie 750 ± 66 mgCOD/L (based on 0.8g-LCFA/L from 1 g/L oil, 2.89 gCOD-FOG/L from 1g/L LCFA)
c Lipid % in brackets

Thus, these reactors show potential for the low temperature anaerobic treatment of LCFA-containing wastewaters. However, in our previous study (Singh et al., 2019a), the treatment of synthetic dairy wastewater (SDW) (33% LCFA-COD) at 20°C in expanded granular sludge bed (EGSB) at operational durations ranging from 60-70 d led to sludge flotation and washout, which was influenced by the LCFA loading rate and the LCFA concentration in the feed. Hybrid reactors combining retention principles of the above-mentioned reactor types offer suitable reactor design possibilities, for example, the addition of a

fixed-film compartment to an EGSB reactor improved process performance (COD removal and propionate degradation) at 20°C during treatment of whey wastewater (McHugh et al., 2006). Lower-temperature studies have mostly been performed using non-fat dry milk substrates (≤ 3% lipid-COD), however, it is not representative of the typical dairy wastewater which is full non-treated.

The development of a specialized microbial community is crucial for maintaining high methanogenic activity and a high syntrophic-LCFA degrading activity at relatively low ambient temperatures. Various inocula from permafrost and Arctic regions (< 7°C) have been investigated for lipid hydrolysis at 4-15°C to develop inocula for psychrophilic anaerobic treatment of lipids (Petropoulos et al., 2017), but are difficult to avail. Locally available inocula are often mesophilic and hence, should be investigated for the start-up of the low temperature AD process. In our previous study, the use of mesophilic inocula was demonstrated to be feasible for the batch treatment of LCFA-containing dairy wastewaters at temperatures as low as 20 and 10°C (Singh et al., 2019b). Hence, the use of a mixture of different sludges, each possessing a unique substrate or recalcitrant-degrading capacity can confer distinct metabolic characteristics to the engineered sludge microbiome, and, offers possibilities for the low temperature treatment of LCFA-containing wastewaters. Microbial aggregation is prevented by the surfactant behaviour of LCFAs (Daffonchio et al., 1995), often leading to the disintegration of the granular structures and the subsequent sludge washout. In our previous study, the mean diameter of the inoculum granules decreased by 24-33% (from 1.7 mm to 1.14-1.3 mm) when fed with a mixed-LCFA containing SDW at 20°C (Singh et al., 2019b). Therefore, the use of sludge mixtures may promote sludge granulation by enabling a wider scale of biotic and abiotic interactions between the microbes and nuclei for granulation.

The objective of this study was to achieve the anaerobic treatment of mixed LCFA-containing SDW at low temperature (20°C) by using a novel reactor design, along with the use of an inoculum mixture for granulation. The reactor configuration was designed by the fabrication of a fixed film (FF) compartment above the dynamic sludge chamber (DSC). Three laboratory-scale DSC-FF reactors were operated with SDW (33% LCFA-COD) at 20°C, and the effects of increasing LCFA loading rates on the COD removal efficiency, LCFA degradation and methane production were studied by gradually shortening the hydraulic retention time (HRT) in DSC-FF reactors. The reactors were seeded with an inoculum mixture having distinct metabolic activities (high LCFA-degradation capacity, and high acetotrophic activity) to engineer a microbial consortium for low temperature treatment of SDW.

5.2 Material and Methods

5.2.1 Inoculum and synthetic wastewater

Two anaerobic sludges were sourced - granular sludge from a Lt-AD® reactor (NVP Energy Limited) which treated dairy wastewater in Arrabawn Dairies, (Kilconnell Ireland), and a flocculent sludge from ADI-BVF® reactor (ADI Systems, Evoqua) which treated fat, oil and grease (FOG)-containing dairy effluents in Arrabawn Dairies, (Kilconnell Ireland). The two anaerobic sludges were mixed in a 1:1 ratio (by volatile solids (VS)) based on the findings from Holohan et al., (unpublished results) and inoculated to triplicate DSC-FF reactors (to a final value of 10 gVS/L).

SDW (2 gCOD/L) was made with skimmed milk powder (67% COD, 1.33 gCOD/L) and a LCFA mixture (33% COD, 0.67 gCOD/L) having palmitate, stearate, oleate, and linoleate in a ratio of 30:15:45:10 on COD basis. 2 g/L $NaHCO_3$ and 1 mL/L basal nutrient solution were supplemented to SDW. The composition of the LCFA mixture and basal nutrient solution were similar to our previous study (Singh et al., 2019b).

5.2.2 Reactor design and experimental set-up

Three similar laboratory-scale reactors (glass, volume 7 L) (Fig. 5.1) were designed to contain three operational sections - a granular sludge phase (liquid volume, 3.65 L), an anaerobic flotation zone and an FF compartment to aid in biofilm growth and sludge retention. This reactor design was based on the reactor configuration developed by Holohan et al., (unpublished results), and modified through the extension of the FF compartment to enhance its ability. The reactor liquor was recirculated through an outlet underneath the flotation zone using a peristaltic pump to maintain an upflow velocity of 2 m/h and expansion of granular sludge bed for improved contact between sludge and feed. Hence, this reactor design has been referred to as a DSC-FF, based on these characteristics.

The influent feed was stored at 7°C and pumped to the reactor with a peristaltic pump. An outlet after the FF section discharged the effluent to a collection tank. The top of the reactor was connected to a 10 L gas bag for biogas collection. The three DSC-FF reactors were each inoculated with inoculum mixture (10 gVS/L) and were fed with SDW at 20°C for the entire experimental duration (150 d) under identical operational conditions. The HRT was decreased gradually from 72 to 12 h (72, 42.5, 24, 18, 12 h). In each of the three reactors, the HRT was reduced when a similar performance (sCOD removal %) was achieved consecutively for durations equivalent to 3 HRTs, except at 72 h HRT where process performance (sCOD removal %) from durations equivalent to 2 consecutive HRTs was used.

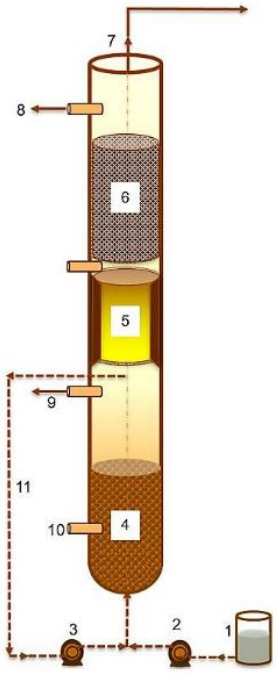

Fig. 5.1 Schematic representation of the components of the Dynamic Sludge Chamber Fixed Film (DSC-FF) reactor design. (1) Influent tank, (2) Peristaltic pump (feed), (3) Peristaltic pump (recirculation), (4) Granular sludge bed, (5) Anaerobic flotation zone, (6) Anaerobic fixed-film (FF) compartment, (7) Biogas collection, (8) Effluent outlet and sampling, (9) Sampling port (for post-granular chamber samples), (10) Sampling port (for granular sludge), and (11) Liquid recycle.

Liquid samples from ports present after the FF and after the granular sludge bed (Fig. 5.1) were taken 3-4 times a week for the measurement of pH, total COD (tCOD) and soluble COD (sCOD), and volatile fatty acids (VFA). At certain time points, these liquid samples were used for LCFA estimation. Gas samples from the gas bags connected to headspace were also collected 3-4 times a week to estimate the methane composition and the methane volume. The development of biofilm was observed visually and under scanning electron microscopy (SEM) in the granular sludge bed and on filter bed material on inoculation of the sludge mixture.

5.2.3 Analytical methods and calculations

The methane content of the biogas was determined using gas chromatography (Varian), equipped with a glass column and a flame ionization detector. Nitrogen was used as the mobile phase at a flow rate of 25 mL/min. The pH was measured with a HI 2210 pH meter, the analysis of total solids (TS) and VS was performed gravimetrically using standard methods (APHA, 2005). Liquid samples were centrifuged at 8000 rpm for 10 minutes and the supernatant was used for COD (sCOD) measurements by the potassium dichromate colorimetric method using commercial COD digestion tubes on Hach Lange DR 5000 TM UV-Vis Spectrophotometer according to the USEPA digestion method 8000. Further, the supernatant was

mixed with 50 µL of 30% orthophosphoric acid and filtered with 0.22 µm Minisart® syringe filters for VFAs analysis. 2-ethylbutyric acid was added as an internal standard in all the samples and standards prior to the quantification of VFAs (acetate, propionate, butyrate, valerate, caproate and caprylate) by gas chromatography on Varian Saturn 2000 GC having a BP 21 FFAP capillary column (SGE analytical science) and a flame ionization detector with helium as the carrier gas at a flow rate of 1 mL/min. Injector and FID detector temperatures were 250°C and 300°C, respectively. The oven temperature was programmed to heat as follows: held at 60°C for 10 s, heated from 60°C to 110°C at 30°C/min, then heated up to 200°C at 10°C/min after which temperature was held at 200°C for 2 min. The LCFA analysis was performed according to Gelder et al., (unpublished data) which was a modified protocol from Neves et al., (Neves et al., 2009), and Ichihara et al., (Ichihara and Fukubayashi, 2010).

5.3 Results

5.3.1 Reactor performance: COD removal and methane production

The three replicate DSC-FF reactors were fed with SDW at 20°C at HRTs from 72 h to 12 h (OLR: 0.67-4 gCOD/L·d) for 150 d. The treatment achieved by the three reactors was similar during the entire experimental duration, therefore, the results for only one of the reactors are presented. The results of the other two reactors are provided as supplementary data (Fig. S5.1-S5.3). Initially, the reactor was operated at 72 h for 8 days resulting in tCOD and sCOD removal efficiency of 88-94% and 96-98% with a methane yield of 4-5% (Fig. 5.2). At an HRT of 42.5 h, the tCOD and sCOD removal remained high at 94-98% and 97-98%, and the methane yield increased to 25-30%. At 24 h HRT, the tCOD removal fluctuated (82-94%) but the sCOD removal efficiency high from 90-98% whereas the methane yield increased further to 48-58%. After the HRT decrease to 18 h, the tCOD removal was 88-91%, with a sCOD removal and methane yield of 96-98% and 47-64% (average 55%), respectively (Fig. 5.2). Further, a gradual HRT decrease was employed from 18 h to 12 h to replicate the conditions of HRT change in a wastewater treatment plant. With this HRT decrease, the tCOD and sCOD removals decreased to 63-72% and 84-89%, respectively, by the end of reactor operation at 12 h HRT, with a high methane yield ranging from 80-103%.

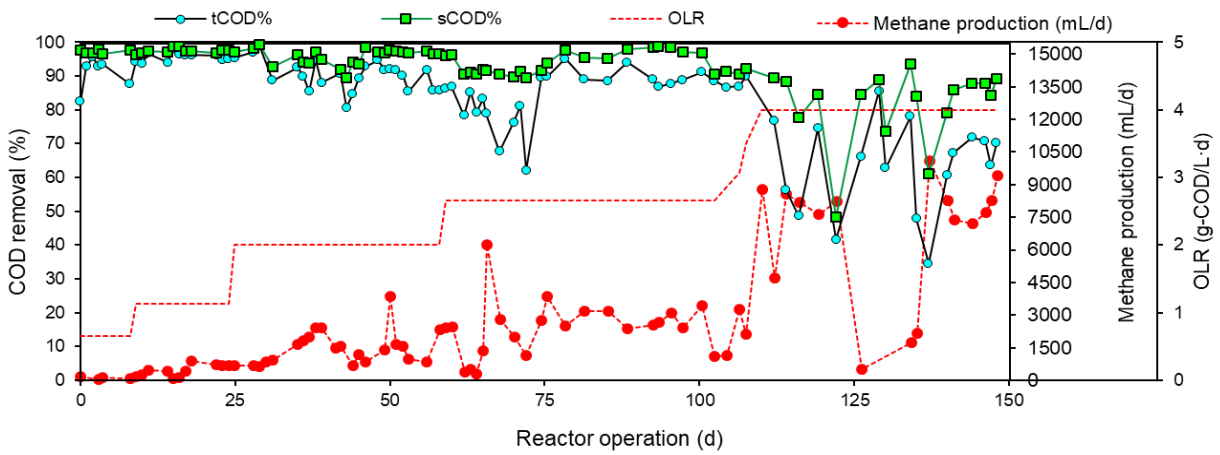

Fig. 5.2. Total COD (tCOD) and soluble COD (sCOD) removals in the Dynamic Sludge Chamber Fixed Film (DSC-FF) reactor effluent and daily methane production at the different HRTs of 72, 42.5, 24, 18 and 12 h from the DSC-FF reactor.

Table 5.2. Operational conditions and process performance of the Dynamic Sludge Chamber Fixed- Film (DSC-FF) reactor and separately by the DSC at the different HRTs of 72, 42.5, 24, 18 and 12 h.

Duration (d)	**0-8**	**9-24**	**25-58**	**59-100**	**110-148**
HRT	72	42.5	24	18	12
OLR (gCOD/L.d)	0.66	1.13	2	2.67	4
LCFA loading rate (mgCOD-LCFA/g-VS.d)	41	68	120	180	240
LCFA loading rate (mgCOD/L.d)	220	377	667	890	1333
pH (effluent)	7.9 ± 0.3	7.7 ± 0.5	7.8 ± 0.1	7.2 ± 0.1	6.9 ± 0.1
pH (DSC)	6.9 ± 0.1	6.8 ± 0.1	6.9 ± 0.2	6.8 ± 0.1	6.8 ± 0.1
tCOD removal % (DSC-FF)	93 ± 0.3	95 ± 1	87 ± 3	89 ± 1	68 ± 3
sCOD removal % (DSC-FF)	97 ± 1	97 ± 0.5	97 ± 0.4	98 ± 1	87 ± 2
tCOD removal % (DSC)	81 ± 3	90 ± 2	75 ± 8	76 ± 1	53 ± 3
sCOD removal % (DSC)	95 ± 2	97 ± 1	86 ± 8	95 ± 1	86 ± 4
tCOD (mgCOD/L) (DSC-FF)	142 ± 2	94 ± 12	327 ± 49	207 ± 31	621 ± 27
sCOD (mgCOD/L) (DSC-FF)	57 ± 17	50 ± 9	84 ± 6	48 ± 11	251 ± 25
tCOD (mgCOD/L) (DSC)	406 ± 82	194 ± 30	620 ± 92	469 ± 19	925 ± 82
sCOD (mgCOD/L) (DSC)	97 ± 37	66 ± 20	331± 167	93 ± 13	280 ± 64
VFA (mgCOD/L) (DSC-FF)	32 ± 7	44 ± 6.5	2 ± 2	0 ± 0	208 ± 43
LCFA (mgCOD/L) (DSC-FF)	138 ± 10	74 ± 5	123 ± 10	95 ± 7.5	352 ± 6
VFA (mgCOD/L) (DSC)	2 ± 1	43 ± 11	0	3.3 ± 1	100 ± 40
LCFA (mgCOD/L) (DSC)	160 ± 20	140 ± 10	109 ± 3	115 ± 14	360 ± 18
Methane concentration (%)	32 ± 1	62 ± 2	73 ± 2	74 ± 10	75 ± 1
Methane yield (sCOD added)	24 ± 9	95 ± 5	186 ± 15	190 ± 25	360 ± 30
Methane yield (sCOD consumed)	25 ± 9	97 ± 5	210 ± 10	200 ± 30	420 ± 30
Methane yield (%) (sCOD added)	7 ± 2	27 ± 1	53 ± 4	55 ± 7	103 ± 9
Methane yield (%) (sCOD consumed)	7 ± 2	28 ± 1	60 ± 3	58 ± 8	121 ± 9

The methane production increased with the decrease in HRT. While at 72 h HRT (OLR 0.67 gCOD/L·d), the methane production in the gaseous phase was low (82 ± 33 mL/d), it increased to 693 (± 29), 1405 (± 675) and 2990 (± 415) mL/d at HRTs of 42.5, 24 h and 18 h, respectively (OLR: 1.13, 2 and 2.67 gCOD/L·d, respectively) (Table 5.2). Moreover, the decrease in HRT to 12 h (OLR: 4 gCOD/L·d) resulted in an increased methane production of about 8477 (± 700) mL/d and methane yield of 103%. Although the OLR and LCFA loading rate at the different HRTs were applied consistently, the substrate was accumulated on the granular sludge and biofilm during the reactor operation. At particular durations observed at 12 h HRT,

tCOD removal was low but with high methane production (e.g., days 113-119) which was followed by higher tCOD removal but had low methane production (e.g., days 126-134); and subsequently again had a lower tCOD removal but increased VFA and methane production (e.g., days 135-140). During the high COD removal, sorption of the substrate presumably occurred on the granules and biofilm, exerting a substrate overload beyond the intended OLR; whereas in the subsequent duration, methanization of the accumulated substrate occurred. This trend is referred to as the cyclical alternation in the organic overloads.

5.3.2 Profiles of metabolic intermediates from LCFA degradation

The VFA profiles (C2-C6: acetate, propionate, butyrate, isobutyrate, valerate and caproate) and LCFA profiles (C10-C18: caprate, laurate, myristate, palmitate, stearate, oleate and linoleate) were monitored periodically in the effluent. With HRTs of 72 and 42.5 h, the total VFA concentration in the effluent varied between 20 and 120 mg COD/L consisting mainly of acetate, propionate, butyrate and low concentrations of valerate. Whereas at HRTs of 24 and 18 h the VFA concentrations were negligible, with low presence of acetate (Fig. 5.3). At HRTs of 18h and higher, all the LCFAs added to the reactor were degraded and mainly lauric acid was detected in the effluent (25-53 mg/L) (Fig. 5.4). As the HRT was reduced from 18 to 12 h, the VFA concentrations in the effluent increased up to 190 mg/L, due to the marked increase in acetate and propionate concentrations up to 60 and 130 mg/L, respectively, and the VFA concentration decreased to 97 mg/L towards the end of the reactor operation (Fig. 5.3). Simultaneously, at 12 h HRT, the concentrations of palmitate (15-41 mg/L) and stearate (21-42 mg/L) fed to the reactor increased in the effluent, while oleate and linoleate were completely degraded also at this HRT (Fig. 5.4). Caproate (26-45 mg/L), an intermediate fatty acid produced from the β-oxidation of even-chained LCFAs, was found for the first time at a 12 h HRT even after a prolonged operation for 20 d. The concentration of caproate, palmitate and stearate did not decline by the end of 150-d reactor operation (Fig. 5.3, 5.4), whereas the LCFA intermediates caprate and myristate were not found at any of the HRTs.

5.3.3 Comparative role of DSC and FF compartments in organics (COD) fractionation

At 72 and 42.5 HRT, treatment by FF reduced the tCOD by half (from 329 to 177 mgCOD/L at 72 h HRT, and from 177 to 102 mgCOD/L at 42.5 h HRT) compared to the sludge chamber, accompanied by LCFA degradation from 158 to 138 mgCOD/L at 72 h HRT, and from 143 to 74 mgCOD/L at 42.5 h HRT (Fig. 5.5). Specifically, the FF degraded the 10 carbon LCFA intermediate – caprate at 72 h and 42.5 h HRT compared to the DSC (Fig. S5.4). As the HRT decreased to 24 h, the FF section was able to remove the increased incoming tCOD and sCOD by 50% and 75%, respectively, as the tCOD and sCOD removal by the sludge

chamber deteriorated compared to the longer HRTs of 72 and 42.5 h. At 18 h HRT, the FF section produced low concentrations of VFA although the sludge chamber degraded most VFAs. At both 24 and 18 h, the total LCFA concentration before and after FF was similar, but the FF degraded palmitate and stearate incoming from the sludge chamber and the effluent (Fig. S5.4). With an applied decrease in HRT to 12 h, about 50% and 86% of the incoming tCOD and sCOD was treated in the sludge chamber, and the FF further reduced the tCOD (925 to 620 mgCOD/L) (Fig. 5.5).

The sCOD removal by FF remained similar to that of the sludge chamber (350 vs 340 mgCOD/L) at 12 h HRT (Fig. 5.5). More VFAs were produced at a 12 h HRT by the sludge chamber than at longer HRTs, and the mix constituted of propionate, valerate and caproate, whereas after the FF, acetate and butyrate were also found (Fig. S5.5). The VFA concentration in the final effluent increased compared to the sludge chamber. The FF removed up to 15% of tCOD and 10% of sCOD at the different HRTs and played an important role in managing the fluctuations from increased particulate (tCOD) and LCFA loads.

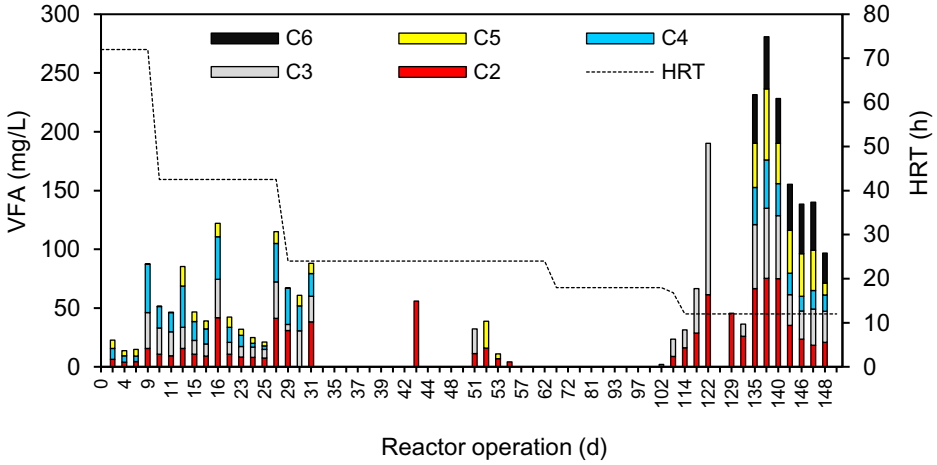

Fig. 5.3. Volatile fatty acids (VFA) – acetate (C2), propionate (C3), butyrate and iso-butyrate (C4), valerate (C5) and caproate (C6) in the Dynamic Sludge Chamber Fixed Film (DSC-FF) reactor effluent at the different HRTs of 72, 42.5, 24, 18 and 12 h.

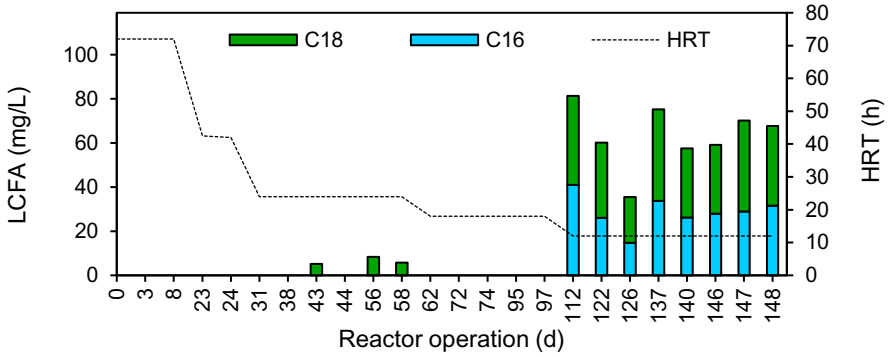

Fig. 5.4. Long chain fatty acids (LCFA) – caprate (C10:0), palmitate (C16:0), and stearate (C18:0) in the Dynamic Sludge Chamber Fixed Film (DSC-FF) reactor effluent at the different HRTs of 72, 42.5, 24, 18 and 12 h.

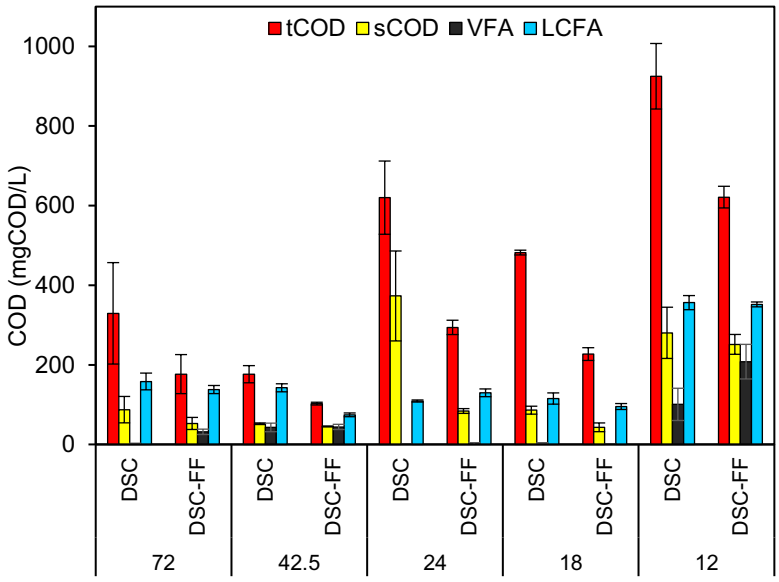

Fig. 5.5. Organics COD fractionation as tCOD, sCOD, VFA-COD and LCFA-COD after treatment by the dynamic sludge compartment (DSC), and further by the fixed-film (FF) compartment (shown as DSC-FF), at the different HRTs of 72, 42.5, 24, 18 and 12 h.

The COD removal in the sludge chamber indicated its importance for the overall degradation of the organics, and more specifically of the LCFAs. Initially, the FF compartment was uncolonized, and thus the organics removal (tCOD, sCOD, VFA and LCFA) was likely due to entrapment by the support matrix in the FF section. The sludge chamber treated organics at an HRT of 18 h (LCFA loading rate of 890 mgCOD/L·d, specific loading rate 180 mgCOD/gVS·d) with a COD removal efficiency of > 75% (both tCOD and sCOD). However, COD removal decreased upon shortening the HRT to 12 h (LCFA loading rate of 1.33 gCOD/L·d, specific LCFA loading rate 240 mgCOD-LCFA/gVS·d), especially the tCOD (53%), leading to a higher inflow of particulates to the FF compartment. Moreover, at 12 h HRT the FF section received a higher amount of stearate and palmitate compared to the longer HRTs. At the 12 h HRT, the FF contributed appreciably to the removal of particulate COD: tCOD removal 68% vs 53% in the sludge chamber (Table 5.2), but further degradation of palmitate and stearate was not observed in the FF.

5.3.4 Sludge washout and flotation

No sludge washout was observed at the HRTs of 72 h, 42.5 h and 24 h. Upon a further decrease in the applied HRT from 24 to 18 h, and from 18 to 12 h, the effluent became more turbid initially but after prolonged operation at the HRTs became subsequently clearer. The average effluent VS at 18 h and 12 h HRT were 0.6 (±0.1) gVS/L and 1.5 (±0.1) gVS/L, respectively. This sludge washout presumably resulted from the sloughing-off from the biofilm due to the increased effluent flow rate. Small broken granules were observed in the flotation layer (less than 10% of sludge in DSC at any time) upon the decrease in HRT

from 42.5 to 24, 24 to 18, and 18 to 12 h. However, the sludge flotation at these different HRTs did not vary, despite the increase in specific LCFA loading rate.

5.3.5 *De novo* granulation in DSC

The two sources seeded as inoculum mixture - flocculent and granular sludges - were at first distinguishable visually due to their different colors (Fig. 5.6 a,c), as well as under SEM (Fig. 5.6 b,d). Subsequently, the flocculent sludge started encapsulating the granular sludge. Within 3-4 d, the differences in the two sludges could not be perceived visually and had an overall greyish appearance due to continuous mixing of the two inocula, in contrast to the earlier distinct yellow (Fig. 5.6 a) and black (Fig. 5.6 c) colors of the sludges. By the end of reactor operation, distinct granules were observed in the DSC (Fig. 5.6 e,f).

5.4 Discussion

5.4.1 Advancement from the state-of-the art

To the best of our knowledge, previous studies on anaerobic treatment of dairy wastewaters at lower temperatures (5-20°C) have used wastewaters with almost a 10-times lower lipid or LCFA content (3% COD-LCFA) (Table 5.1). The anaerobic treatment of LCFA at temperatures ≤ 20°C has been investigated for the first time in this study and the performance and operation stability of the reactors is determined. This study shows that high rate anaerobic treatment is indeed a feasible option for LCFA-containing wastewaters at discharge temperatures. This is promising as in our previous study the anaerobic treatment (> 60 d) of a similar synthetic dairy wastewater in lab-scale EGSB reactors at 20°C led to a deterioration in the COD removal efficiency and methane production at an OLR of 2 gCOD/L·d (LCFA loading rate 0.67 gCOD-LCFA/L·d), and the process performance did not recover even after the feeding was stopped (Singh et al., 2019b). Furthermore, the current results accomplished wastewater treatment even at the HRTs of 0.5-0.75 d in the DSC-FF reactors, compared to the previous treatment of lipid-containing dairy wastewater (22% COD-lipid) where a COD removal efficiency of 46-69% was achieved at HRT of 1 d at 20°C (Tawfik et al., 2008). Even compared to a previous batch treatment of lipid-containing municipal wastewater at 4-15°C, where a COD removal efficiency of 38-47% was achieved at lipid loading rates of 0.1-0.13 gCOD/L·d (38-45% COD-lipids) at an OLR of 0.29 gCOD/L·d (Petropoulos et al., 2018), in this study a higher COD removal efficiency was achieved with the continuously operating DSC-FF reactors with 2-10 times higher LCFA loading rates (0.22-1.33 gCOD-LCFA/L·d).

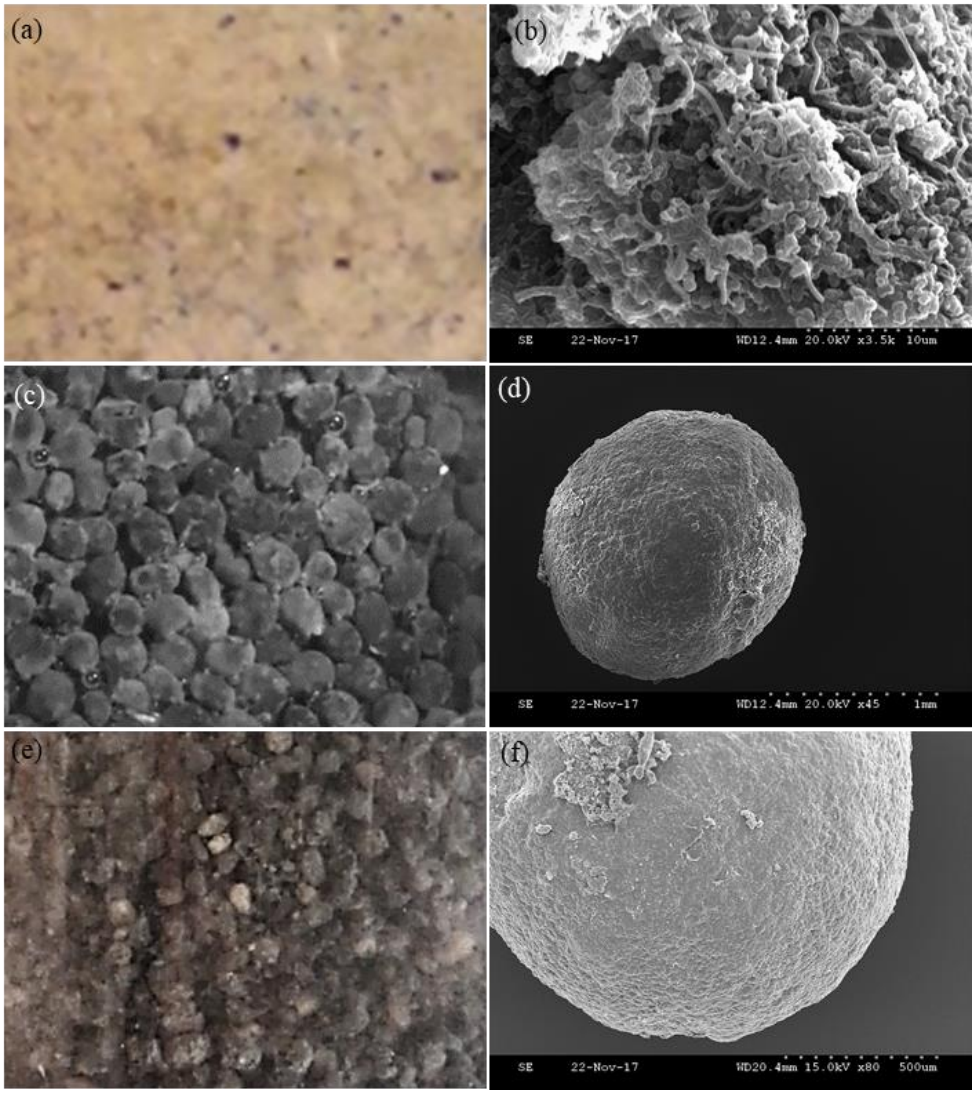

Fig. 5.6. Schematic representation of rapid granulation from inoculum mixture. Sludges prior to inoculation, flocculent sludge on (a) visual observation, and (b) under Scanning Electron Microscopy (SEM) at 10 µm; granular sludge on (c) visual observation, and (d) under SEM at 1 mm. De novo granulation in the dynamic sludge compartment (DSC) after 8 days on (e) visual observation, and (f) under SEM at 500 µm.

5.4.2 Novel reactor configuration: proof-of-concept

The process performance of the triplicate DSC-FF reactors during the 150-d trials validates the design rationale and demonstrates the suitability of the reactor for the treatment of LCFA-containing dairy wastewater at low temperature (Table 5.2). Furthermore, it offers proof-of-concept that LCFAs can be degraded efficiently at discharge temperatures. The considerations from the reactor design facilitated a high contact between the sludge and substrate in the sludge chamber due to the use of dispersion nozzle and mixing, and yet prevented any suction of the floating granules by the recycle pump (Fig. 5.1), which sometimes is the reason for process failure in laboratory scale studies due to increased sludge washout (Yoda and Nishimura, 1997). The upflow velocity effectuated the separation of gas bubbles from the

granular sludge surface and therefore, prevented any incidental lifting of the granular sludge bed. The anaerobic flotation zone compartment in the sludge chamber was able to accommodate granular sludge flotation, although minimal sludge flotation (< 10%) was observed, even up to at the LCFA loading rate of 1333 mg COD-LCFA/L·d (specific LCFA loading rate of 240 mgCOD-LCFA/gVS·d). Previously sludge flotation has been reported at LCFA loading rates lower than in our study – 86-203 mgCOD-LCFA/gVS·d (Hwu et al., 1998), or 80 mgCOD-FOG/gTS·d (Macarie et al., 2018), wherein flotation of the entire sludge bed resulted in reactor process failure. The design rationale in devising the anaerobic flotation zone was to first enable the slow degradation of the lipids or LCFA from the LCFA-encapsulated granules (having intact granular structure), followed by settling of the granules to the sludge bed. This dynamism maximized the microbial activity in the sludge chamber while continuously treating LCFA-containing wastewaters. The FF included as a secondary phase in the DSC-FF design, received a lower particulate matter and LCFA concentrations due to their prior conversion in the sludge chamber. Consequently, the impacts of high lipid concentrations, such as, biofilm-thinning or filter clogging were not encountered in this study as seen previously in anaerobic filter reactors treating oleate (Alves et al., 2001). The FF also had the ability, especially when acting at lower temperatures, to trap and degrade palmitate that came out of solution and floated through the sludge bed. The main results show that the FF compartment assisted primarily in the particulate removal and VFA conversion owing to the development of acetogenic and acidogenic capacity.

Clogging in the FF was not observed during the 150-d period suggesting the capacity of DSC-FF reactors to handle an LCFA loading rate up to 1.33 gCOD-LCFA/L·d (OLR 4gCOD/L·d, 33% mixed-LCFA). However, it also suggests that the application of higher LCFA loading rates should be applied with caution as it may result in particulate entrapment and cause filter clogging. Tawfik et al (2008) prevented sludge washout by maintaining a regular sludge discharge (20% of total influent COD) at an LCFA loading rate of 0.75 gCOD-LCFA/L·d (specific LCFA loading rate 95.4 mgCOD-LCFA/gVS·d) while treating dairy wastewater in a UASB reactor at 20°C, though, such daily sludge disposal means a loss of energy-rich organic fraction from the reactor. In comparison, in the current study, sludge disposal was not needed as there was no excess sludge build-up observed up to an LCFA loading rate of 1333 mgCOD/L·d (specific LCFA loading rate 240 mgCOD-LCFA/gVS·d). In our previous work, the disintegration of granules to smaller particles, and their subsequent flotation and washout were encountered on operating EGSB reactors for 60-70 days at LCFA loading rates of 667-990 mgCOD/L·d (Singh et al., 2019b). Contrarily, the operational challenges associated with the treatment of LCFA-containing wastewaters such as flotation, disintegration and washout were handled in this study with no sludge disposal requirements.

5.4.3 Methane yields and dissolution

The methane yield (7 to 103%) increased temporally during the 150-d DSC-FF reactor operation. The high methane yield (> 100%) at 12 h HRT cannot be attributed only to the increased methane concentration in the biogas (Table 5.2), as similar methane concentrations (73-75%) were obtained at the HRTs of 24 and 18 h as well. The two-fold increase in methane yield, 103% at a 12 h HRT compared to 55% at 18 h HRT, suggests a pseudo methane yield from the conversion of the accumulated substrate, and the actual methane yield was lower than 103% at a 12 h HRT. The cyclical alternating organic loads experienced by DSC at 12 h HRT was presumably due to the LCFA accumulation on granular sludge, as LCFAs have a high sorption propensity. Cavaleiro et al., (2009) overloaded sludge with an LCFA-rich feed at 37°C in feed cycles (20-30 d) followed by react cycles with no feeding for methanization of the accumulated substrates. This strategy of alternating organic loads had enhanced the methane yield from 67% to 91% wherein, LCFA accumulation increased in the reactor up to 60 d (2 feed cycles) but was methanized subsequently resulting in increased COD removals and methane yields due to specialization of the microbial community (Cavaleiro et al., 2009). In our study, the alternating organic loads likely enriched the LCFA degraders and could be employed as a strategy to improve methanization of LCFA-rich wastewater at 20°C, as previously demonstrated using the discontinuous feed-react mode or pulse feed mode at 35-37°C for oleate treatment (Cavaleiro et al., 2009; Ziels et al., 2017). The substrate accumulation in the DSC-FF reactors as shown by the fluctuation in the methane yield was independent of the feed rate and COD removal, which further incurred multiple cycles of alternating OLR on the reactor sludges. As LCFAs have a high sorption propensity and were primarily adsorbed in the granular sludge bed, ing LCFA loading rate in continuous DSC-FF reactors likely enriched the microbiome involved in LCFA degradation, and could be employed as a strategy to improve methanization of LCFA-containing wastewater at 20°C, as previously demonstrated using the discontinuous or pulse feed modes at 35-37°C for oleate treatment (Cavaleiro et al., 2009; Ziels et al., 2017). Moreover, the ability of DSC-FF reactor to accommodate this substrate accumulation demonstrates its ability in handling shock loads.

The methane yields at 20°C in the DSC-FF reactor at 72-18 h HRT were lower compared to the high-performing continuous anaerobic reactors (80%) (van Lier et al., 2015), likely due to the inadequate development of the biofilm and increased methane dissolution due to low temperature (20°C). The temperature-dependent Henry's equilibrium constants and diffusion kinetics are altered by a decrease in temperature, leading to a decreased bubble formation propensity at 20°C compared to 35°C (Hikita and Konishi, 1984), which results in a lowered liquid-to-gas methane transport and a higher methane dissolution at 20°C. Therefore, the operation of the DSC-FF reactor is recommended at conditions

increasing the liquid-to-gas methane transport for improving the methane recovery at 20°C, while simultaneously controlling the slough-off from the biofilm.

5.4.4 Degradation of LCFAs at 20°C

The DSC-FF reactors were able to consistently remove the mixed-LCFAs in the influent to LCFA concentrations below 50 mg/L in the effluent at HRTs as short as 18 h. Effluent stearate and palmitate concentrations increased at the 12 h HRT. During the treatment of wastewaters with high lipid loads, LCFA accumulation is often encountered in different reactor types and constituted mainly of palmitate or stearate (Cavaleiro et al., 2009; Dereli et al., 2015; Duarte et al., 2018; Pereira et al., 2005; Ziels et al., 2015, 2017) due to the fast conversion of unsaturated LCFAs (linoleate, oleate) to palmitate independent of the methanogenic activity (Cavaleiro et al., 2016). Furthermore, effluent caproate concentrations increased at 12 h HRT after treatment by both the sludge chamber and the FF sections. Therefore, the increased concentrations of caproate, palmitate and stearate at 12 h HRT could be attributed to the inhibition of specific LCFA degraders at the increased flow rate, thereby highlighting the limits of the slow-growing LCFA degraders in the microbiome at 12 h at the LCFA loading rate of 1.33 gCOD-LCFA/L·d.

5.4.5 Granulation and development of distinct metabolic activity

The sludge inoculation approach involved de novo granule formation from two different inoculated sludges (a flocculent sludge with high LCFA-degradative capacity and a granular sludge with high methanogenic activity, respectively) and the maintenance of granular structure at HRTs of 72 to 12 h. In our previous study, granular sludge was used for treating mixed-LCFA containing dairy wastewater at 20°C with similar trace element supplementation but the sludge granules disintegrated (Singh et al., 2019a). Therefore, in this study, the role of divalent cations as granulation nuclei can be overruled and the nuclei for granulation was provided presumably by the inoculum mixture (broken granules and the flocculent sludge). The biotic and abiotic interactions involved in the de novo granule formation were promoted due to the combination of biogas production, energy-rich wastewater and selective pressure. Previously, sludge granulation has been achieved while treating lipid-containing dairy wastewater (47% FOG-content) in mesophilic UASB reactors inoculated with flocculant sludge, but after a prolonged duration (> 200 d) (Passeggi et al., 2009). This study demonstrates rapid granulation and consequent negligible sludge washout while treating mixed-LCFA containing wastewater (33% COD-LCFA) at 20°C.

Overall, the twin approaches of a suitable reactor configuration and the inoculation of a well-adapted microbial sludge in this study were able to handle the challenges expected in the anaerobic treatment of LCFA-containing wastewater at 20°C. The process performance of the novel reactor configuration (Table

5.2) in the 150-d period demonstrated the potential suitability of the DSC-FF reactor and use of mesophilic inocula for the anaerobic treatment of LCFA-containing dairy wastewater up to LCFA loading rate of 1.33 gCOD/L·d (specific LCFA loading rate of 240 mgCOD-LCFA/gVS·d) at discharge temperature. Further studies with single LCFA and with industrial dairy wastewater at pilot-scale are needed to assess the process boundaries of the low-temperature anaerobic treatment at normal and shock lipid and LCFA loads.

5.5 Conclusions

This study evaluated the anaerobic treatment of mixed LCFA-containing dairy wastewater at low temperature (20°C) by using the DSC-FF reactor design. High sCOD removal and methane production were achieved with mixed LCFA-containing dairy wastewater up to OLR of 4 gCOD/L·d (LCFA loading rate 1.33 gCOD-LCFA/L·d) at 20°C. Rapid sludge granulation from an inoculum mixture of granular sludge and flocculent sludge in DSC, and, the formation of biofilm in FF were achieved during the treatment of mixed-LCFA wastewater even with high LCFA concentration of 33% (COD-basis) and LCFA loading rates of 0.22-1.33 gCOD-LCFA/L·d to allow successful treatment of LCFA-containing wastewater at 20°C. The results from this study demonstrate the feasibility of high-rate treatment of LCFA-containing wastewaters at discharge temperature using the combined approaches of suitable reactor configuration and inoculation of a sludge mixture.

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6 DYNAMICS AND ASSEMBLY IN ACTIVE MICROBIOME OF GRANULES AND BIOFILMS TREATING LCFA-RICH WASTEWATER IN HIGH-RATE REACTORS

Abstract

Microbial community dynamics and assembly in anaerobic microbiomes are influenced by the operational parameters, and high selection pressures uniquely shape the microbial community structure and assembly. The active microbiomes from the granular sludge (from DSC), biofilm (from FF) and effluent were studied over a 150-d duration by using 16S rRNA sequencing during the treatment of long-chain fatty acids (LCFA)-rich synthetic dairy wastewater (SDW) at high hydraulic flows (hydraulic retention time (HRT) 0.5-3 d). High initial microbial diversity facilitated the efficient conversion of SDW. High microbial diversity in the granular sludge enabled efficient treatment (COD removal, LCFA removal), despite the wide fluctuations in the relative abundances of the granular sludge consortia. The microbial (bacterial and archaeal) diversity differed in the reactor sections - the biofilm microbiome was less diverse than the granular sludge microbiome, as the taxa sloughed-off from the granular microbiome contributed to the formation of biofilm (on FF). *Methanomicrobia, Deltaproteobacteria, Clostridia, Bacteroidia, and Actinobacteria* were found in high relative abundances both in the granular sludge and biofilm microbiomes, with a prominent presence of *Syntrophus* and *Methanospirillum* in biofilm, in comparison to the presence of *Geobacter, Desulfobulbus*, and, *Syntrophobacter* in the granular sludge microbiome. A deterministic microbial community assembly was found in the granular sludge, biofilm and effluent microbiomes over the 150-d operational duration, despite transient stochasticity under the strong selection pressures. *Methanosaeta*-mediated acetoclastic methanogenesis was found to be the predominant methanogenesis pathway involved in the methanization of LCFA-rich wastewaters at discharge temperatures (20°C) in different microbial retention modes (granular sludge, biofilm) in the DSC-FF reactors. This study brings forth novel insights into the microbial community dynamics (diversity, abundance) and assembly in anaerobic microbiomes under strong selections pressures, viz., inhibitory substrate (LCFA), high hydraulic flow and low ambient temperatures (20°C).

6.1 Introduction

Anaerobic digestion (AD) has proven to be a robust and sustainable technology for the production of energy-rich biofuel, i.e. methane. This microbially-mediated process involves a sequential conversion of the organic fractions, that majorly constitute of carbohydrates, proteins, and lipids, to methane. The lipid fraction rapidly hydrolyses to long-chain fatty acids (LCFA) which consist of over 90% of the COD from lipids (Hanaki et al., 1981). Despite the high methane production potential of the lipids, numerous challenges have been reported in their anaerobic treatment that are operational (blockage of pipes, sludge flotation, and washout) (Alves et al., 2009), physicochemical (mass transport limitation and microbial disaggregation) (Daffonchio et al., 1995; Pereira et al., 2005), and microbial (inhibition of different trophic groups, fermentative, acidogenic, acetoclastic and methanogenic) (Koster and Cramer, 1987; Lalman and Bagley, 2002; Lalman et al., 2004; Lalman and Komjarova, 2004; Shin et al., 2003).

Previously culture-independent molecular methods, such as denaturing gradient gel electrophoresis (DGGE), quantitative polymerase chain reaction (qPCR) and catalyzed reporter deposition fluorescence in-situ hybridization (CARD-FISH) have been used to investigate granular sludge microbiomes treating fat, oil, and grease (FOG)-rich milk wastewater (0.15% fat), and molasses-based and glucose-based wastewaters in hybrid anaerobic reactors at 37°C (HRTs 0.75-5 d) (Kundu et al., 2013). The authors concluded that the substrate type (carbohydrate-rich vs fat-rich) shaped the relative abundances of the microbial community but not the diversity (Kundu et al., 2013). However, numerous taxa are found in low relative abundances (0.1-1%) and monitoring of these rare taxa would provide a more accurate representation of the shifts in microbial community diversity (De Vrieze et al., 2018; Qin et al., 2019) by using high-throughput next-generation sequencing technologies, such as 16S whole amplicon rRNA sequencing.

Moreover, the microbial taxa in an anaerobic consortium may diversify, or enrich based on the interactions among the microbial species that co-occur in time and space, and their responses to the applied selection pressures (Nemergut et al., 2013). Within an anaerobic microbiome, the microbial taxa may be driven to co-exist after undergoing selection, resulting in a deterministic community assembly; or undergo random changes, resulting in a stochastic community assembly (Yuan et al., 2019). The microbial assembly mechanism is indicative of the stability of a microbial community (Werner et al., 2011), thus, the robustness and replicability of processes under high selective pressures, for example, the high-rate anaerobic treatment at low temperatures, may be inferred based on an understanding of the microbial community assembly mechanisms.

A recent study evaluated anaerobic treatment of synthetic dairy wastewater (SDW) containing mixed LCFAs at 20°C in a novel reactor configuration consisting of a dynamics sludge chamber (DSC) and a fixed-film (FF) compartment, and showed that anaerobic treatment was successful at short HRT (down to 12 h) (Singh et al., (unpublished results)). The reactors facilitated the development and retention of different sludge types within a single reactor - settled granular sludge and floating sludge zone in the DSC, and fixed-film phase in the FF. These sections may have contributed to the distinct substrate degradation (chemical oxygen demand (COD) removal, LCFA removal) in the two sections. Hence, sequencing the active microbiomes from the granular sludge (from DSC) and biofilm (from FF) may bring novel insights into the dynamics (diversity, abundance), and assembly of the microbial taxa actively involved in high-rate treatment of LCFA-rich wastewaters; and, may stipulate the major methanogenesis pathway in the different microbial retention modes (granular, biofilm, planktonic) engineered in this study.

The objective of this study was to determine the key microbial taxa actively involved in the degradation of LCFA-rich SDW in the high rate DSC-FF reactors. 16S rRNA sequencing of the active microbiomes obtained from granular sludge (from DSC), biofilm (from FF), and, effluent at the different HRTs (72, 42.5, 24, 18, 12 h) was performed to evaluate the microbial community dynamics and assembly. Additionally, a comparison of the microbiome in the DSC and FF section to the effluent microbiome was performed to evaluate the washed-out taxa at different HRTs.

6.2 Materials and Methods

6.2.1 DSC-FF reactor operation and analytical methods

Two anaerobic sludges were sourced and mixed in a 1:1 ratio (by volatile solids (VS)) for the inoculation of triplicate DSC-FF reactors (to a final value of 10 gVS/L). The three laboratory-scale DSC-FF reactors (volume 7 L) were fed with SDW by gradually decreasing the HRT from 72 to 12 h (72, 42.5, 24, 18, 12 h), and were operated identically for 150 d at 20°C (Fig. S6.1). The composition of SDW and the basal nutrient solution were similar as reported in section 5.2.1. In each of the three reactors, the HRT was reduced when a similar performance (95% similarity) was achieved consecutively for 2-3 HRTs. Liquid samples for pH, tCOD, sCOD, and VFA measurements were taken 3-4 times per week after the DSC and after the FF sections; and was used for LCFA analysis during the periods considered stable. Gas samples from gas bags connected to reactor headspace were also collected 3-4 times per week for the measurement of methane concentration and biogas volume. The analyses were performed as described previously (section 5.2.3).

The tCOD and sCOD from the liquid samples were measured by the potassium dichromate colorimetric method on Hach Lange DR 5000 TM UV Vis spectrophotometer. The VFA and LCFA were measured using

Varian Saturn 2000 gas chromatograph and the methane content was measured using gas chromatography (GC)-flame ionization detector (Varian) with the GC parameters and sample preparation details outlined in section 5.2.3. TS, VS, and pH were measured according to APHA, (2005). Analysis of the methane volume and the methane content was performed as outlined in section 5.2.3. The methane yield (%) was calculated from the ratio of methane produced to g-COD added or g-COD consumed (assuming 350 mL-CH_4/g-COD at STP as 100%).

6.2.2 Sampling, nucleic acid extraction and 16S rRNA gene amplicon sequencing

Microbial samples were collected from the granular sludge layer in DSC (at the end of each HRT), and the biofilm in the FF compartment and the effluent (at the end of the HRTs from 42.5 to 12 h). At the start of the experiment, the inoculum mixture was collected. For the collection of granular sludge samples, the DSC-FF reactors were purged to prevent sludge stratification and obtain representative samples of granular sludge. Subsequently, the FF material was collected manually using sterile forceps, from the middle of the FF section. The FF material was sonicated for 5 min at 20°C with 10 mL of phosphate buffer saline (PBS) (pH 7.2), and the resulting supernatant was centrifuged at 8000 rpm for 10 minutes at 4°C. Simultaneously, 25 mL of the effluent obtained from each of the reactors was centrifuged at 8000 rpm for 10 min at 4°C. The microbial pellets from the FF and effluent samples, the granular sludge samples from DSC, and the inoculum mixture were immediately flash-frozen in liquid nitrogen upon collection and stored at -80°C.

The microbial samples were thawed on ice, and co-extraction of nucleic acids (DNA and RNA) was performed (Griffiths et al., 2000). The concentrations of the extracted DNA and RNA were quantified with a Qubit fluorometer (Life Technologies), and the DNA purity was measured using a Nanodrop (NanoDrop Technologies, Wilmington, USA). Further, DNase treatment was performed for DNA removal using Invitrogen Turbo-DNase kit (Thermo Fisher, USA) following the recommended procedure. The DNA-free samples consisting of RNA were converted to cDNA using M-MuLV Reverse Transcriptase kit (New England BioLabs, USA) according to the instructions provided by the supplier. These cDNA samples were sent to FISABIO (Spain) for PCR amplification of the V4 region of the 16S rRNA gene using universal primers 515f and 806r (Caporaso et al., 2011) and amplicon sequencing of the active microbiome on the Illumina MiSeq platform.

6.2.3 Computational sequence analyses and statistical tests

The sequence data was analysed using Quantitative Insights Into Microbial Ecology (QIIME v1.9) and taxonomy assignment was performed with Silva 123 consensus taxonomy at all levels (Aronesty, 2013;

Caporaso et al., 2010; Glöckner et al., 2017; Pruesse et al., 2007), with the details published previously (Singh et al., 2019a). The dataset consisted of 8366619 sequences in total, which clustered at 97% similarity-level into 35562 OTUs. This dataset was normalized through metagenomeSeq CSS matrix transformation (Paulson et al., 2013) in QIIME, and used for the estimation of alpha diversity metrics (ace, shannon, chao1, and goods coverage). 122 OTUs had a cumulative observation counts higher than 0.01% in the samples and were used subsequently as the 99.9% dataset, and, are presented as the relative abundances. The high good's coverage achieved for the 99.9% dataset (0.98-1) confirmed sufficient sequencing depth for the investigation of the microbial communities in this study. The 99.9% dataset was fourth-root transformed based on draftsman plot, and a resemblance matrix was constructed using Bray-Curtis similarity in Plymouth Routines In Multivariate Ecological Research (PRIMER) V7 (Clarke et al., 2014). The resemblance matrix was used to perform the principal coordinate analysis (PCoA) (Gower, 1966) to visualize the similarity patterns in microbial community compositions in the different sections (granules, biofilm, and effluent), and to perform the distance-based redundancy analysis (db-RDA) to visualize the effect of environmental variables on microbial community composition at different HRTs. Cluster analysis through hierarchical clustering (group average method) was performed on the operational taxonomic units (OTUs) and separately on the reactor samples to plot the dendrograms. The fourth root transformed OTU table was used for representing the microbial community composition at class level in the shade plot.

The significance of the changes in microbial community diversity with the decrease in HRT was assessed by performing statistical analysis with a t-test (two-sample assuming unequal variances) separately for the granular sludge, biofilm and effluent microbiomes. Furthermore, the significance of the shifts in microbial community composition with the decrease in HRT was evaluated by performing statistical testing in single-factor design (with 999 unique permutations) using the HRT and reactor replicates as factors with permutational multivariate analysis of variance (PERMANOVA). For the statistical tests, $p < 0.05$ was considered as significant.

6.3 Results

The temporal shifts in the active microbiomes of the granular sludge bed from DSC, the biofilm from FF and the effluent from replicated novel high-rate reactors were studied by 16S rRNA sequencing. The summary of the operational conditions, and process performance during the anaerobic treatment of SDW at 20°C at different HRTs (72 to 12 h) in the DSC and FF sections are presented in Table 6.1 and Table 6.2, respectively. Reproducible sCOD removal and methane production were achieved in anaerobic SDW

treatment during the 150-d operation. With a decrease in HRT from 72 to 12 h, there was an increase in the methane concentration (32-75%) and the methane yield (7-103%). At all the HRTs studied, the DSC received a higher OLR (0.66-4.00 vs 0.1-3.45 gCOD/L.d) and LCFA loading (0.22-1.33 gCOD-LCFA/L.d vs 0.05-1.01 gCOD-LCFA/L.d) than the FF (Table 6.1), and the DSC removed more tCOD, sCOD and LCFA than the FF (Table 6.2). At 12 h HRT, increased tCOD and LCFA loads were received by FF due to the fluctuations in SDW treatment by DSC, and VFA (C2 to C6 acids) concentrations increased in FF (Table 6.2). At HRTs of 72-18 h, the LCFAs were degraded completely, whereas, with the decrease in HRT to 12 h, the two unsaturated LCFAs – linoleate (C18:2), and oleate (C18:1) were degraded completely whereas the two saturated LCFAs – palmitate (C16:0) and stearate (C18:0) were partially degraded. Thus, the increased loading rate and hydraulic flows at 12 h HRT limited the degradative capacity of the granular sludge (in DSC), and the biofilm (in FF).

Table 6.1. Summary of operational conditions during synthetic dairy wastewater (SDW) treatment by dynamic sludge chamber (DSC) and fixed-film (FF) sections at hydraulic retention time (HRT) of 72, 42.5, 24, 18 and 12 h. Adapted from Singh et al., (unpublished results).

Operational parameters	**DSC**					**FF**				
HRT (h)	**72**	**42.5**	**24**	**18**	**12**	**72**	**42.5**	**24**	**18**	**12**
pH	7±0.1	7±0.1	7±0.1	7±0.1	7±0.1	7.02[a]	6.72[a]	7.71[a]	6.81[a]	6.77[a]
						7.02[b]	6.80[b]	7.67[b]	6.79[b]	6.78[b]
						6.91[c]	6.74[c]	7.57[c]	6.77[c]	6.72[c]
OLR (gCOD/L.d)	0.66	1.13	2	2.67	4	0.1[a]	0.09[a]	0.43[a]	0.69[a]	1.76[a]
						0.11[b]	0.11[b]	0.65[b]	0.63[b]	1.88[b]
						0.09[c]	0.15[c]	0.83[c]	0.79[c]	3.45[c]
LCFA loading rate (gCOD-LCFA/L.d)	0.22	0.37	0.67	0.89	1.33	0.05[a]	0.08[a]	0.2[a]	0.2[a]	0.72[a]
						0.05[b]	0.08[b]	0.11[b]	0.15[b]	0.73[b]
						0.06[c]	0.08[c]	0.11[c]	0.46[c]	1.01[c]
LCFA loading rate (mgCOD-LCFA/g-VS.d)	34	68	120	180	240					

a – Reactor 1, b – Reactor 2, c – Reactor 3.

Table 6.2. Summary of process performance during synthetic dairy wastewater (SDW) treatment by dynamic sludge chamber (DSC) and fixed-film (FF) sections at hydraulic retention time (HRT) of 72, 42.5, 24, 18 and 12 h in three parallel reactors (R1, R2, R3). Adapted from Singh et al., (unpublished results).

		After treatment by DSC					After treatment by DSC-FF				
HRT	Reactor	pH	tCOD (mgCOD/L)	sCOD (mgCOD/L)	Total VFA (mgCOD/L)	Total LCFA (mgCOD/L)	pH	tCOD (mgCOD/L)	sCOD (mgCOD/L)	Total VFA (mgCOD/L)	Total LCFA (mgCOD/L)
72	R1	7.02	289	73	16	157	8.00	177	58	44	133
	R2	7.02	329	87	2	158	8.00	177	53	31	158
	R3	6.91	261	87	1	174	7.87	131	63	8	128
42.5	R1	6.72	161	65	51	136	7.59	129	46	49	83
	R2	6.80	194	66	47	142	7.71	94	50	52	74
	R3	6.74	273	145	59	149	7.68	114	63	45	78
24	R1	7.71	433	240	0	197	7.73	260	112	0	150
	R2	7.67	651	411	0	112	7.79	300	82	1	188
	R3	7.57	825	263	0	114	7.83	633	342	1	115
18	R1	6.81	515	79	4	147	7.15	256	66	0	118
	R2	6.79	469	93	2	115	7.18	207	48	0	95
	R3	6.77	593	181	7	344	7.06	311	96	1	114
12	R1	6.77	879	346	113	361	7.06	663	249	275	306
	R2	6.78	938	325	93	364	6.93	621	251	225	361
	R3	6.72	1723	533	143	503	6.82	816	483	204	335

Of the 122 OTUs that constituted 99.9% of the abundant taxa in the sampled microbiomes, 16 were archaeal and 106 were bacterial. The microbial community diversity and composition of the sludge microbiomes (from the DSC and FF) and from the effluent of the three DSC-FF reactors are presented below (Fig. 6.1).

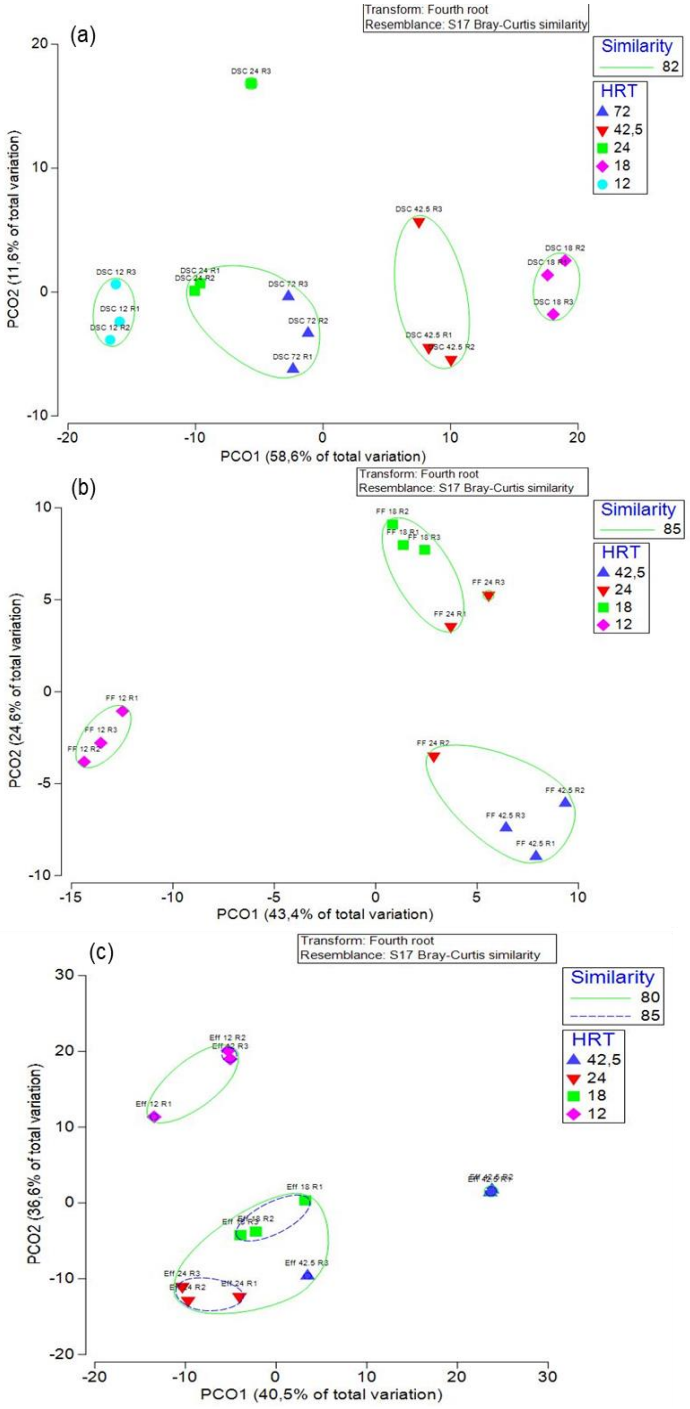

Fig. 6.1. PCoA of bacterial and archaeal taxa found at the HRTs 72, 42.5, 24, 18 and 12 h from the triplicate reactors R1, R2 and R3 in the (a) granular sludge microbiome obtained from the DSC, (b) biofilm obtained from FF, and (c) effluent.

6.3.1 Microbial community diversity in reactor sludges

The microbial diversity in the granules increased significantly along with structural reformation of the granules during the first 8 days of reactor operation based on the alpha diversity indices (Ace (p=0.02), Chao1 (p=0.053), and Shannon (p=0.02)) (Table S6.1). High community richness and evenness were established within the first 8 days of the reactor operation (Table S6.1), that provided a diverse pool of bacterial and archaeal taxa with the capability of metabolizing complex substrates; and presumably conferred adaptable metabolic potential needed for the degradation of SDW at the subsequent HRTs with increasing OLR and LCFA loading rates.

The microbial diversity in the biofilm microbiome followed shifts similar to that of the DSC microbiome from 42.5 to 24 h HRT, and, had lower diversity than the DSC microbiome as suggested by the Ace, Chao 1, and Shannon indices (Table S6.1). This association is envisaged as the bacteria and archaea from the DSC were upflown and entrapped into the FF support material, and subsequently proliferated in the biofilm.

The PCoA plots demonstrated that the microbial communities from the triplicate reactors at the different HRTs (72, 42.5, 18 and 12 h) were similar in the granular sludge (82% similarity among triplicate microbiomes) (Fig. 6.1a), and in the biofilm (85% similarity among triplicate microbiomes) (Fig. 6.1b). The microbial community compositions (at genus level) were significantly different at the different HRTs as substantiated by PERMANOVA (p=0.001).

The db-RDA demonstrated that in the granular sludges, the environmental parameters - fatty acid concentrations (of acetate, propionate, butyrate, valerate, caproate, palmitate), and sCOD explained 70% of the variations in the microbial communities at the different HRTs (Fig. S6.2 a), whereas, in the biofilms, the environmental parameters- fatty acid concentrations (of acetate, caproate, laurate, palmitate, stearate), tCOD, sCOD, and pH explained 68% of the variations in the microbial communities at the different HRTs (Fig. S6.2 b).

6.3.2 Microbial community composition in reactor microbiome

6.3.2.1 Microbial community composition in granular sludge

With the decrease in HRT from 72 h to 12 h, there was an increase in the OLR (from 0.67 to 4.00 gCOD/L.d) and the LCFA loading rates (from 0.22 to1.33 gCOD-LCFA/L.d) (Table 6.1) to the DSC. The bacterial classes of *Deltaproteobacteria*, *Bacteroidia*, *Actinobacteria*, and *Bacillli* and the archaeal class of *Methanomicrobia* (Fig. 6.2a) were abundant in the granular sludge. At the end of HRTs 42.5 h and 18 h,

the relative abundances of the classes *Deltaproteobacteria* and *Methanomicrobia* decreased, while that of the classes *Bacteroidia* and *Actinobacteria* increased (Fig. 6.2a). The acetoclastic archaeal taxa had lower relative abundances at the HRTs 42.5 and 18 h (49-64%) compared to the HRTs of 72, 24 and 12 h (74-84%) (Fig. 6.3a). Simultaneously, the hydrogenotrophic archaeal taxa had a higher relative abundance at the HRT of 42.5 and 18 h (33-49%) compared to at the HRTs of 72, 24 and 12 h (14-25%) (Fig. 6.3a). The archaeal community of the inoculum mixture was predominantly acetoclastic (63%) and had a lower relative abundance of hydrogenotrophic genera (35%) (Fig. 6.3a).

Furthermore, the increase in the relative abundances of the acetoclastic archaeal taxa *Methanosaeta* (23-29%) at HRTs 72, 24 and 12 h were accompanied with increased relative abundances of the bacterial taxa belonging to an *unassigned Syntrophaceae* taxa (0.87-1.5%), *Geobacter* (5-12%) and *Desulfobublbus* (2.8-9.7%) that was contrasted with their low relative abundances at the HRTs of 42.5 and 18 h (4.5-9.8%, <1%, <1%, respectively) (Fig. 6.4). The *unassigned Propionibacteriaceae* (10.4-15%) and *unassigned Bacteriodales* (14.6-18%) were abundant at HRTs 42.4 and 18 h than at the HRTs of 72, 24 and 12 h (1.5-2.4% and 5.5-10%, respectively) (Fig. 6.4). Additionally, at 12 h HRT, the relative abundances of *Methanospirillium* (2.2 vs <0.1%) and *Desulfobulbus* (9.8 vs 0.4-3%) increased in the granular sludge microbiome compared to the 18 h HRT (Fig. 6.5).

6.3.2.2 Microbial community composition in biofilm

In comparison to the identical influent received by the DSC section, the FF influent varied and was affected by the DSC treatment performance (Table 6.1). The decrease in HRT from 72 to 12 h increased the OLR in the FF section from 0.1 up to 3.5 gCOD/L.d, the LCFA loading from 0.05 up to 1 gCOD-LCFA/L.d and hydraulic flow through the FF section by 6-folds (Table 6.1). FF was involved in the tCOD removal and VFA production (Table 6.2), which suggests the involvement of the biofilm in the entrapment of the particulate COD and acidification of the incoming organics by the FF. However, with the decrease in HRT to 12 h, caproate (C6:0), palmitate (C16:0) and stearate (C18:0) were not completely removed by the FF section and accumulated at low concentrations in the effluent.

In the biofilm, the bacterial classes *Actinobacteria*, *Bacteroidia,* and *Bacillli* and the archaeal classes *Methanomicrobia* and *Methanobacteria* were abundant (Fig. 6.2b). With a decrease in HRT from 42.5 to 12 h, the relative abundances of *Methanosaeta* and *Geobacter* increased from 5.7 to 25.2% and from 0.1 to 2.3%, respectively. In particular, during the decrease in HRT from 18 to 12 h HRT, there was an increase in the relative abundances of the syntrophic bacteria - *Syntrophus* (0.3 to 2.6%), *Syntrophobacter* (0.5 to 2.6%), *Desulfobulbus* (0.3 to 1.1%), and *Geobacter* (1 to 2.3%); and of the archaeal taxa *Methanospirillium* (0.1 to 5%) (Fig. 6.5). The active microbiome in FF was primarily hydrogenotrophic at 42.5 h HRT,

transitioned towards acetoclastic at 24 h HRT and was acetoclastic (due to *Methanosaeta* presence) at HRTs of 18 h and 12 h (Fig. 6.3b, 6.5). The taxa *Syntrophus*, and, *Methanosaeta* were retained in the biofilm.

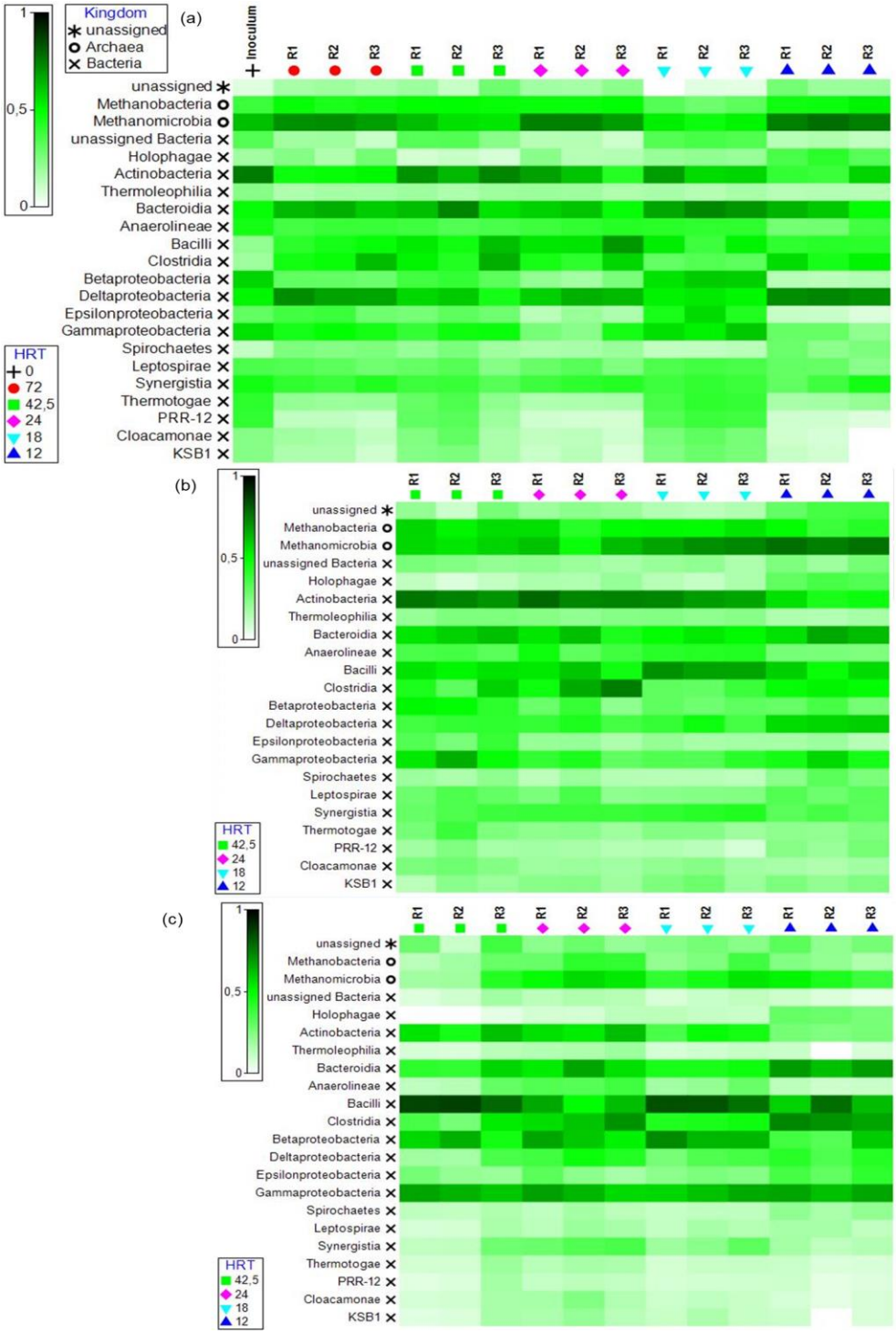

Fig. 6.2. Heat map showing relative abundances of the bacterial and archaeal classes found in the microbiome of the triplicate reactors R1, R2 and R3 at the HRTs 72, 42.5, 24, 18 and 12 h in the sections - (a) granular sludge obtained from the DSC, (b) biofilm obtained from FF, and the (c) effluent.

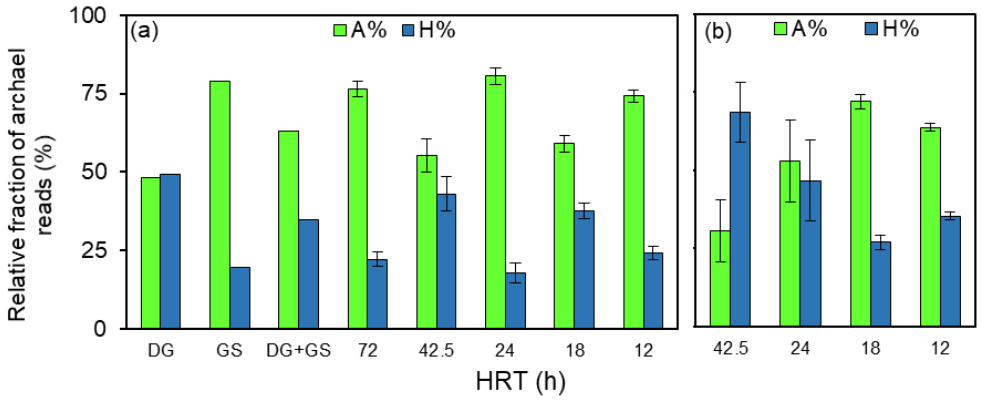

Fig. 6.3. Proportion of acetoclastic (A%) and hydrogenotrophic (H%) archaea found at the HRTs 72, 42.5, 24, 18 and 12 h from the triplicate reactors R1, R2 and R3 in (a) the granular sludge microbiome obtained from the DSC, and (b) the biofilm obtained from FF. Relative abundances of all of the hydrogenotrophic or acetoclastic taxa were summed up and divided by the cumulative relative abundances of entire archaeal taxa to obtain the percentage of hydrogenotrophic archaea (H%) and archaea (A%), respectively.

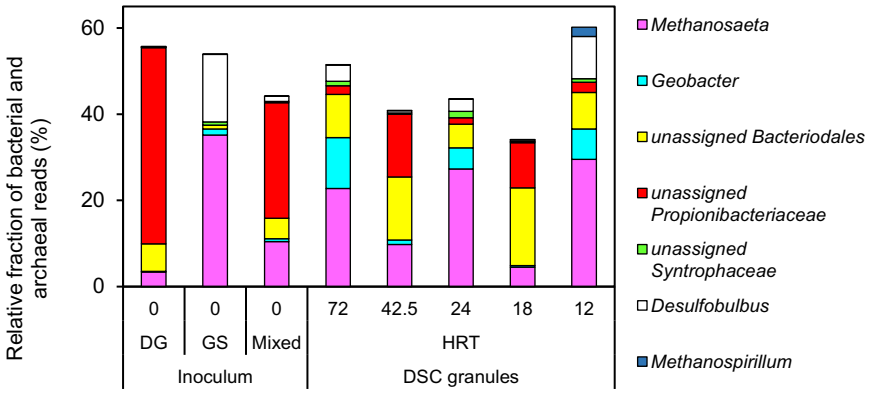

Fig. 6.4. Relative fraction (%) of the bacterial and archaeal genera with significant shift at either of the HRTs 72, 42.5, 24, 18 and 12 h from the triplicate reactors R1, R2 and R3 in the granular sludge microbiome obtained from DSC.

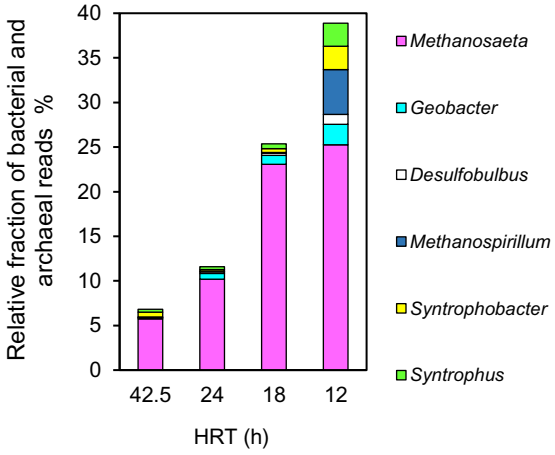

Fig. 6.5. Relative fraction (%) of the bacterial and archaeal genera with relative abundances >1% at either of the HRTs 42.5, 24, 18 and 12 h from the triplicate reactors R1, R2 and R3 in the biofilm microbiome obtained from FF.

6.3.2.3 Microbial community composition in effluent

The characteristics of effluent from the DSC-FF reactors altered with the decrease in HRT. Although, at 12 h HRT the effluent of DSC-FF reactors had an increase in tCOD, sCOD, VFA and LCFA concentrations, an increase in the methane concentration (up to 75%) and the methane yield (up to 103%) was also achieved (Table 6.1, 6.2). The effluent microbiome was abundant in the bacterial classes of *Actinobacteria, Bacteroidia, Bacilli, Clostridia, Betaproteobacteria*, and *Gammaproteobacteria* (Fig. 6.2c). The decrease in HRT from 72 to 12 h increased the washout of taxa from the bacterial classes *Bacteroidia* and *Clostridia* (Fig. 6.2c). The fermentative bacteria from class *Bacilli* (*unassigned Carnobacteriaceae* and *Lactococcus*) had higher relative abundances in the effluent at the HRTs of 42.5 and 18 h, than at the HRTs of 24 h and 12 h (Fig. 6.6). Contrarily, the relative abundances of *Anaeromusa* (class *Clostridia*) were higher at the HRTs of 24 h and 12 h, than at the HRTs of 42.5 h and 18 h (Fig. 6.6). Thus, an increase in the relative abundance of these above-mentioned taxa in the biofilm was followed by an increase of the same taxa in the effluent. *Acinetobacter* (class *Gammaproteobacteria*) was washed out from the reactors at the HRTs of 72 to 12 h, whereas the washout of unassigned *Aeromonadaceae* (class *Gammaproteobacteria*) was prominent only at the 12 h HRT (Fig. 6.6).

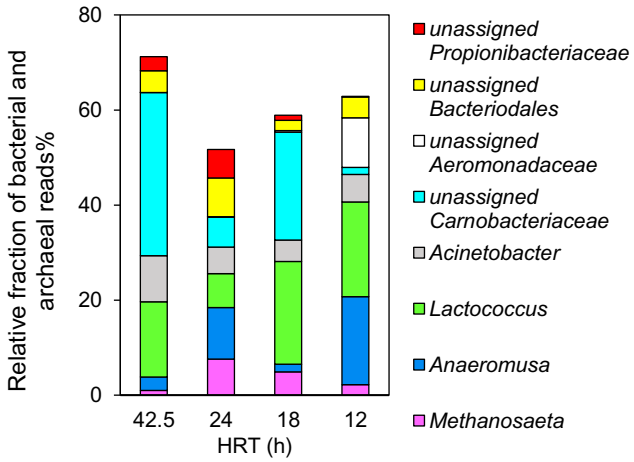

Fig. 6.6. Relative fraction (%) of the bacterial and archaeal genera with relative abundances >1% at either of the HRTs 42.5, 24, 18 and 12 h from the triplicate reactors R1, R2 and R3 in the biofilm microbiome obtained from the effluent microbiome.

6.4 Discussion

6.4.1 Synchronized microbial community dynamics in high-rate reactors

The active microbiomes from biofilm as well as granular sludge contained high relative abundances of the classes *Methanomicrobia (Methanosaeta, Methanospirillum), Deltaproteobacteria (Geobacter, Desulfobulbus, unassigned Syntrophaceae), Clostridia (Anaeromusa), Bacteroidia (unassigned Bacteroidetes), and Actinobacteria (unassigned Propionibacteriaceae)*. In the granular sludge microbiome

from DSC, two microbial clusters are envisaged - first constituting of *Methanosaeta*, *Geobacter*, and *unassigned Syntrophaceae* (at HRT 72, 24 and 12 h), and, the other comprising of *unassigned Propionibacteriaceae*, *unassigned Bacteroides* and the hydrogenotrophic methanogens (at 42.5 and 18 h). The presence of these different taxa at the same trophic level in each of the clusters signifies the potential for the various microbial taxa to process the same substrate (Allison and Martiny, 2008). Thus, the presence of these two different clusters likely offered alternate degradation pathways in the granular sludge microbiome for the metabolization of SDW fractions under the dynamic selection pressures (high-rate treatment at the low ambient temperature). In FF, the biofilm developed gradually on the support material with the decrease in HRT, and, was visually noticeable at the end of 18 h HRT (day 100) along with a simultaneous decrease in pH (from 7.8 to 7.1) (Table 6.2). The mature biofilm developed by the end of reactor operation had a prominent role of *Syntrophus* and *Methanospirillum* in the biofilm microbiome, in contrast to the prominent role of *Geobacter, Desulfobulbus*, and, *Syntrophobacter* in the granular sludge microbiome. The results demonstrate that synchronized anaerobic treatment (COD removal, LCFA removal) of LCFA-rich wastewater is feasible even if the relative abundances of the microbial consortia fluctuate when functionally redundant taxa are present.

Methanosaeta was the only acetoclastic genus among the 16 archaeal taxa found in the granular sludge microbiome in this study. Earlier *Methanosaeta* has been found in higher relative abundances in LCFA-rich SDW degrading anaerobic consortia at low temperatures from batch assays and continuous high-rate reactors (Chapter 3,4). However, the high relative abundance of *Methanosaeta* did not explicitly ascertain its activity in SDW degradation, as the microbes with low activity in the microbiome were also sequenced in these studies. The presence of *Methanosaeta* in the granules and biofilm in this study validates acetoclastic methanogenesis as the predominant pathway involved in the low-temperature anaerobic conversion of LCFA-rich wastewaters to methane. Moreover, the capability of *Methanosaeta* for retention and growth in the granules as well as in the biofilm at 20°C at high hydraulic flows and high LCFA loads is shown in this study.

The relative abundance of genus *Syntrophomonas* was low (<0.5%) in granules and biofilm, and did not increase with the increased OLR and LCFA loading rates during the 150 d operational duration. Previously, the β-oxidizers from the family *Syntrophomonadaceae* have been found (majority associated to *Syntrophomonas*) by using oligonucleotide probes in sludge treating lipid-rich industrial waste (relative abundances of 0.2-1%) (Hansen et al., 1999), and by using FISH probes in sludge treating edible tallow refinery waste (relative abundances of 3%) (Menes and Travers, 2006). More recently, increase in the 16S

rRNA gene concentration of *Syntrophomonas* has been found in mesophilic co-digesters while using lipid-rich co-substrates such as waste cooking oil (from >3% to 15%) (Ziels et al., 2016), and oleate (0.5% to 9%) (Ziels et al., 2017). Until now, the detection of taxa from *Syntrophomonadaceae* from FOG-, and LCFA-fed reactors has been variable. Additionally, the taxa from the family *Syntrophaceae* (genus *Syntrophus*) have been found in enrichments from low-temperature oil fields (Grabowski et al., 2005b) which suggests a more diverse LCFA degradative potential than *Syntrophomonas* that has been found frequently in numerous mesophilic and thermophilic LCFA degradation experiments (Hatamoto et al., 2007; Sousa et al., 2007; Ziels et al., 2017, 2016). *Syntrophomonas* has been found in low relative abundances (> 0.1%) during SDW treatment in our previous batch study at 10 and 20°C, and in continuous studies at 20°C (Chapter 3,4). Therefore, at 20°C, the LCFA degraders/β-oxidizers belong to *Deltaproteobacteria*, and *Syntrophomonas* may not have a prominent degradation role. Confirmation of the taxa involved in treating LCFA-rich feed at ambient temperatures and their metabolic interactions with methanogens is needed using, e.g., targeted qPCR and stable isotope probing (SIP)-metagenomics.

Until now, the role of *Syntrophus* has been established in the degradation of saturated and unsaturated LCFAs only in batch incubations (Grabowski et al., 2005b; Singh et al., 2019a), and this study shows for the first time their role in continuous reactors treating LCFA-rich wastewaters at high hydraulic flows at 20°C. *Syntrophus* presence in LCFA-degrading granular and planktonic cultivations has been reported previously (Grabowski et al., 2005a; Singh et al., 2019a), but this study shows the active role of *Syntrophus* in low-temperatures biofilm as well. The increase in relative abundances of *Syntrophobacter* (propionate-fermenting, acetogenic), *Desulfobulbus* (ethanol fermenting sulphur-reducing bacteria, acetogenic/acidogenic), and *Geobacter* (hydrocarbon-fermenting) in biofilm suggests their involvement in the interspecies metabolite transfer during anaerobic degradation of SDW. Overall, the significant increase of these taxa in the active microbiome of the biofilm can be related to the increased methane yield at 12 h HRT.

The washout of the bacterial classes *Bacteroidia* and *Clostridia* from the DSC-FF reactors with the shortening of HRT was likely due to the increased LCFA loading rate and hydraulic flow (Table 6.1). LCFA has a surfactant effect at neutral pH (Daffonchio et al., 1995), which may induce the sloughing-off of cells from the outer surface of biofilm particularly at high LCFA loading rates and hydraulic flows. The fermentative bacteria from *Bacteroidia* and *Clostridia* proliferated in the biofilm for VFA production, and, were washed-out resulting in their increased relative abundances in the effluent. Overall, the washout of the fermentative bacteria was likely affected by their growth and retention in the biofilm.

6.4.2 Microbial community assembly in granular sludge and biofilm microbiome

Despite the low microbial growth rates expected at 20°C, the microbial communities in the replicate DSC-FF reactors were able to maintain similar microbial community composition and demonstrated resilience by adapting to the selection pressures (high LCFA loading, high hydraulic flows). Even though stochasticity was introduced at 24 h HRT in the granular and biofilm microbiomes; the microbiomes in the three parallel DSC-FF reactors converged at the successive HRTs (18 h HRT). This demonstrates that the microbial community assembly of the granular sludges and the biofilms followed a deterministic assembly mechanism, although they had differences in the retention mechanism, i.e., by granule formation, and by adhesion within a biofilm. This signifies the robustness and replicability of the high-rate SDW treatment at 20°C.

Furthermore, various environmental parameters shaped the microbial community structure in the granular sludge and biofilms, that is, VFA, palmitate and sCOD concentrations in granular sludge microbiomes; and VFA, tCOD, sCOD concentrations and pH in the biofilm microbiomes. The sampling of microbial communities was conducted at the end of each HRT (in the period considered stable), and the environmental parameters for db-RDA analysis were used from the corresponding days. Thus, the effect of shock loads, or other disturbances on the microbial community dynamics from the period before achieving steady-state may have been disregarded. Thus, despite the knowledge gained on the temporal variations in microbial community with the shortening of HRT, the significance of the individual factors (tCOD, sCOD, LCFA, VFA, hydraulic flows, upflow velocity, granules vs biofilm retention, or pH) in shaping the microbial communities could not be deduced conclusively; and would need evaluation using single parameter for confirmation.

6.5 Conclusions

Specialized microbial communities with a high LCFA degrading and methanogenic activity were developed in novel high-rate DSC-FF reactors during anaerobic treatment at 20°C. Initially, the use of inoculum mixture provided high microbial diversity, which facilitated a diverse microbial community for the efficient conversion of SDW to methane. Synchronized anaerobic treatment (COD removal, LCFA removal) of SDW was feasible in the three reactors, despite wide fluctuations in the relative abundances of the granular sludge consortia owing to the functional redundancy that provided diverse microbial taxa capable of metabolizing same substrates. The biofilm microbiome was less diverse than the granular sludge microbiome, as the taxa sloughed-off from the granular microbiome would have partially entrapped onto the support material and formed the biofilm. As a result, the taxa from *Methanomicrobia,*

Deltaproteobacteria, Clostridia, Bacteroidia, and Actinobacteria were present in high relative abundances in the active microbiomes of the granular sludge and biofilm microbiome. However, by the end of reactor operation, the biofilm microbiome had a prominent role of *Syntrophus* and *Methanospirillum*, whereas, the DSC granular microbiome had a prominent role of *Geobacter, Desulfobulbus*, and, *Syntrophobacter.* Overall, the microbial communities in the granular, biofilm and effluent microbiomes converged upon prolonged operation (100 d) despite transient stochasticity, indicating an overall deterministic assembly mechanism even under the strong selection pressures. *Methanosaeta*-mediated acetoclastic methanogenesis was the predominant methanogenesis pathway involved in the methanization of LCFA-rich wastewaters at low ambient temperatures in active microbiomes from the granules as well as the biofilm. In addition, the washout of fermentative bacteria (from *Bacilli, Clostridia, Bacteroidia,* and *Gammaproteobacteria*) was affected dissimilarly at the different HRTs and depended on the growth and the retention of these fermentative bacteria in the biofilm.

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7 GENERAL DISCUSSION AND CONCLUSIONS

7.1 General discussion

In this thesis, the anaerobic treatment of LCFA-rich industrial wastewater was shown successfully at low temperatures in batch and continuous reactors, resulting in methane as the end-product. First, different inocula were evaluated in batch assays for their capability for anaerobic treatment of a LCFA-rich wastewater, at low operational temperatures of 20 and 10°C, and, the underlying microbial community shifts were deciphered using high-throughput sequencing (Chapter 3). The findings from the biochemical and microbiological investigations suggested the suitability of the granular sludge inoculum for the batch treatment of SDW at 10 and 20°C, supported by the presence of acetotrophic activity (from *Methanosaeta* and putative SAOB) and the LCFA degradative capacity (from *Syntrophus* and an uncultured *Syntrophaceae* taxon). The granular sludge inoculum found optimal from the batch assays were used for seeding three continuous EGSB reactors. The feasibility of SDW treatment, and, the effect of HRT and LCFA concentrations on treatment performance was assessed at a discharge temperature of 20°C by studying the biochemical, morphological, and temporal microbial community characteristics (Chapter 4). The continuous anaerobic treatment of SDW at 20°C was shown to be feasible in the EGSB reactors, and a *Methanosaeta*-mediated acetoclastic pathway was found to be the predominant methanogenesis pathway. The treatment of SDW for operational durations longer than two months resulted in granular sludge flotation and washout in the three reactors, promoting the need for sludge retention for long-term granular sludge reactor operation with SDW at low ambient temperatures. Thus, the comprehension

gained on the operational challenges and process limitations during the continuous operation of EGSB reactors was used for designing a novel reactor type, DSC-FF, for high-rate treatment of LCFA-rich wastewaters at discharge temperatures (20°C). The feasibility of this reactor design (DSC-FF) was evaluated at HRTs from 72 to 12 h, wherein high sCOD removals and methane yields were achieved (Chapter 5). Two sludges, with high acetoclastic activity and high LCFA degradative capacity each, were used as a sludge mixture to inoculate the triplicate DSC-FF reactors. The sludge mixture formed granules and enabled retention of the distinct substrate degradative capacities within the DSC, along with the development of biofilm in the FF section. The specific methanogenic activities of the DSC and FF sludges increased compared to that of inoculum at 37 and 20°C. The development of specialized microbial communities in DSC and FF was achieved during the 150 d (chapter 6). *Methanosaeta*-mediated acetoclastic methanogenesis was found to be the predominant methanogenesis pathway based on the active microbiomes from granules (from DSC) and biofilm (from FF). The washout of the fermentative bacteria (from classes *Bacilli, Clostridia, Bacteroidia*, and *Gammaproteobacteria*) were found to depend on the growth as well as the retention of these bacteria in the biofilm. The biochemical and microbiological results obtained during this research work provide an understanding of the continuous treatment process of LCFA-rich wastewaters at low ambient temperatures. Anaerobic treatment of LCFA-rich wastewaters was evaluated at low temperatures in batch assays and continuous high-rate reactors (Chapters 3-6), and the major findings obtained from this thesis are presented in Fig. 7.1.

The treatment efficiency (sCOD removal and methane conversion) of the two reactor designs (EGSB and DSC-FF) was comparable at 24 h HRT (OLR: 2gCOD/L·d) for treating SDW at durations of about 60 days. However, in the DSC-FF reactors, the flotation was negligible (lower than 5%) and the structure of granules was intact even at longer operational durations (150 d), whereas EGSB reactors encountered flotation and granular disintegration with a declined treatment efficiency at operational durations of 60-70 d. The presence of only saturated LCFAs (C16:0, and C18:0) in the DSC-FF effluent (at 12 h HRT) suggested that the conversion of the unsaturated LCFAs (C18:2, C18:1) was fast, and was not affected by the increased LCFA loading rate or the hydraulic flow even at the 12 h HRT. The LCFA saturation may have partially proceeded through the conventional LCFA degradation pathway involving obligate syntrophy between the hydrogen-producing acetogens and hydrogenotrophic methanogens for maintaining low hydrogen partial pressures (McInerney et al., 2008; Schink, 1997), and partially through the non-syntrophic hydrogenation as suggested by Cavaleiro *et al.*, 2016. During the treatment of wastewaters with high lipid loads, LCFA accumulation has often been encountered, constituting mainly of palmitate (C16:0) and stearate (C18:0) (Cavaleiro et al., 2009; Dereli et al., 2015; Duarte et al., 2018; Pereira et al., 2005; Ziels et

al., 2017a, 2015) due to the favourable energetics of hydrogenation of unsaturated LCFAs as opposed to the energetic constraints on β-oxidation cycles at standard conditions (Cavaleiro et al., 2016).

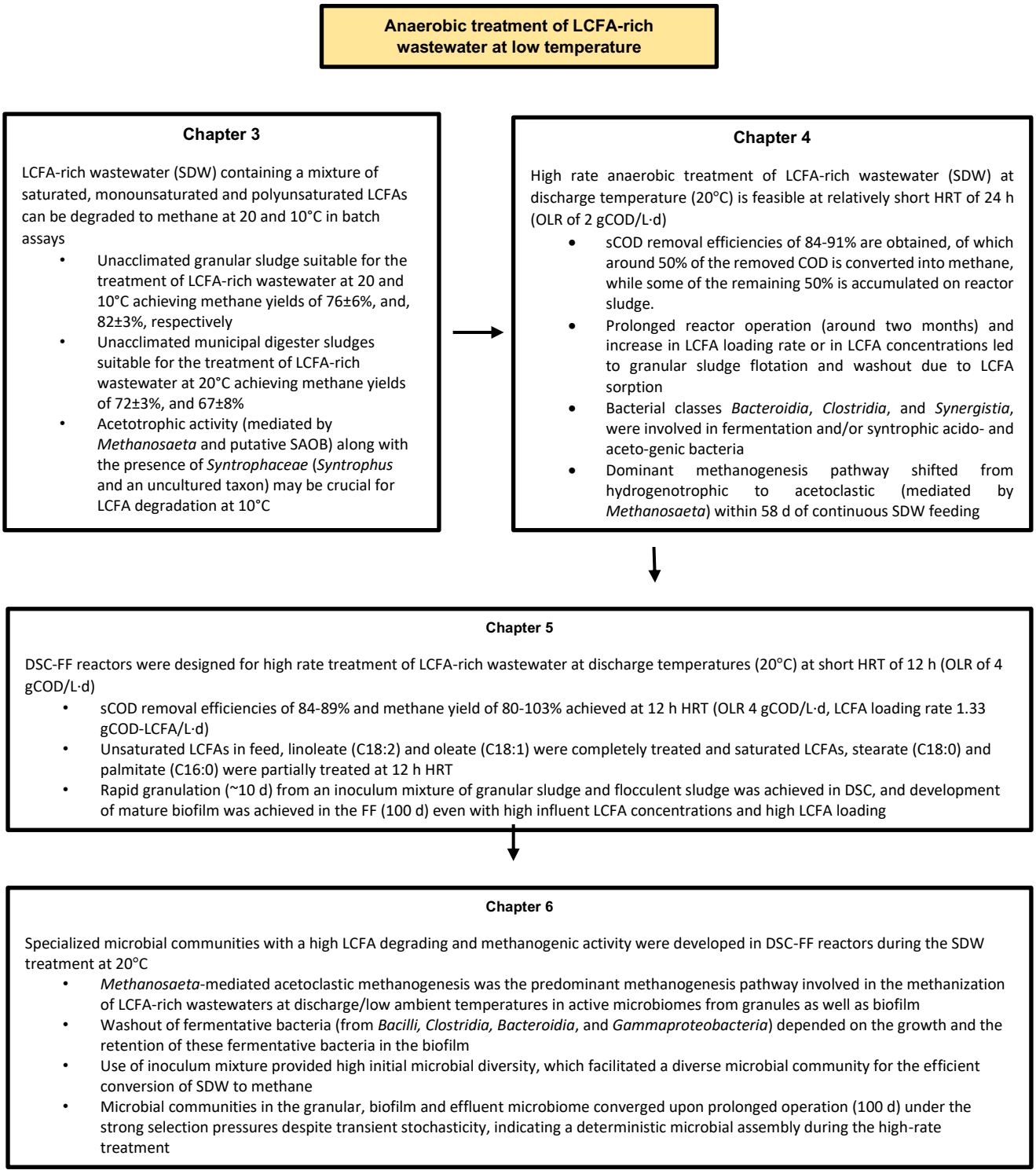

Fig. 7.1. Overview of major research findings in this thesis

The methane yield was calculated based on the daily methane production measurements from the triplicate DSC-FF reactors, which varied and responded to the conversion of the fed-substrate (SDW) and the accumulated substrate (on granules and biofilm), wherein the methane yield ranged from 80-103% (Fig. 7.1). Thus, despite the lower sCOD removal and lower saturated LCFA removal at 12 h HRT in the DSC-FF reactors, the microbial activity (acidogenic, acetogenic, methanogenic) was not impeded. The unavailability of sufficient sites (on granular sludge and biofilm) for LCFAs to sorb to may potentially have resulted in the incomplete degradation of the saturated LCFAs at 12 h HRT in DSC-FF reactors as the LCFA degrade through the sequential sorption-uptake-desorption mechanism (Hwu et al., 1998b).

During the treatment of SDW in batch and continuous reactors (Chapter 3,4,6), the most abundant taxa belonged to the bacterial classes *Bacteroidetes, Clostridia, Deltaproteobacteria*, and *Synergistetes*; and the archaeal class – *Methanomicrobia* (Fig. 7.2). Taxa from these bacterial classes had a role in hydrolysis and fermentation of the SDW substrate fractions (lactose and casein, or skimmed milk powder); and the acidogenesis and acetogenesis of the LCFAs (saturated and unsaturated). The bacterial classes *Anaerolineae* and *Aminicenantes* were found in high relative abundances in the batch assays (Chapter 3) and during EGSB trials (Chapter 4), but, were not found at similar high abundances in the active microbiomes from the DSC-FF reactors (Chapter 6) (Fig. 7.2) evidently due to the usage of dissimilar inoculum for the inoculating the DSC-FF reactors. It has been established that the substrate-degradation potential and methanogenic activity is influenced by the microbial community composition of the inoculum (Sun et al., 2016). The degradation of the mixed LCFAs formulating the SDW (33% LCFAs - 18% unsaturated LCFAs (C18:2, C18:1), and 15% saturated LCFAs (C16:0, C18:0)) by granular sludge inoculum even at 10°C was due to the microbial community composition in the inoculum.

The *Deltaproteobacterial* genera - *Geobacter, Desulfobulbus, Syntrophobacter, unassigned Syntrophaceae,* and, *Syntrophus*, and the *Methanomicrobial* genera – *Methanosaeta* and *Methanospirillium* can be considered as vital for SDW degradation due to their presence in the batch assays and the active microbiome from DSC-FF. This suggests the involvement of these archaeal genera in methane production from SDW and the LCFAs, viz., the uptake of LCFA terminal product, acetate by *Methanosaeta*; and the uptake of hydrogen by *Methanospirillium* while simultaneously facilitating the growth of syntrophic β-oxidizers (Chapter 3,6).

Until now, the role of *Syntrophus* or taxa from *Syntrophaceae* has been established in the LCFA degradation (saturated and unsaturated LCFAs) only in batch incubations (Grabowski et al., 2005a; Hatamoto et al., 2007a, 2007b). In this research, the presence of these two taxa for treating LCFA-rich

wastewaters at high hydraulic flows at 20°C was shown for the first time (Chapter 6). Moreover, the capability of *Syntrophus*, unassigned *Syntrophaceae* taxon, and, *Methanosaeta* to proliferate in a biofilm system was shown for the first time, as their presence has previously been reported only in granular and planktonic cultivations (Grabowski et al., 2005a). In previous studies, *Syntrophomonas* has been found to increase with LCFA loading and has been reported frequently as the LCFA degrader involved in β-oxidization (Alves et al., 2009; Sousa et al., 2009; Ziels et al., 2015). However, in this study, *Syntrophomonas* was found in low abundance (<0.5%) in the batch assays (Chapter 3), and EGSB reactor sludges (Chapter 4), and the relative abundances were found to decrease (to <0.1%) in the active microbiomes of granular sludge and biofilm (from DSC-FF reactors) during 150-d (Chapter 6). Thus, *Syntrophomonas* presumably was not involved in β-oxidation of LCFA at the low temperatures investigated during this research.

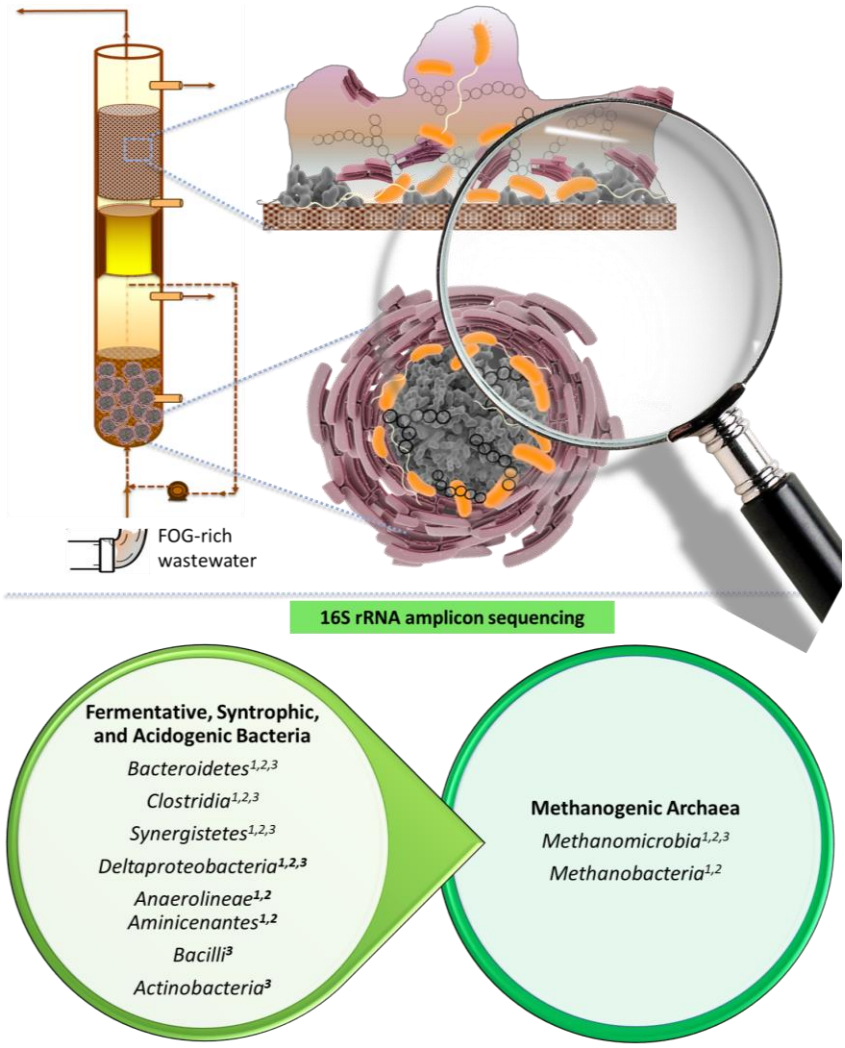

Fig. 7.2. Bacterial and archaeal classes present in high relative abundances in the microbiomes from (1) the batch assays at 10 and 20°C, (2) the EGSB reactor sludges at 20°C, and, (3) the active microbiomes of the DSC-FF reactor sludges at 20°C.

The methanogens from classes *Methanomicrobia* and *Methanobacteria* were abundant during all the trials, however, *Methanomicrobia* was more abundant than *Methanobacteria* reflecting the prominent role of *Methanomicrobial* taxa during high rate methanization of SDW at 20°C. In contrast, at thermophilic and mesophilic conditions (35-54°C) the microbial classes - *Methanobacteria, Clostridia, Bacteroidia, and Synergistia* were identified in the core microbiome of LCFA-degrading communities (Amha et al., 2018), thereby signifying the role of temperature in determining key microbial population during low-temperature LCFA methanization.

7.2 Recommendations for future research

The research work performed for this thesis brings forth new insights into the inoculum selection, treatment efficiency, and LCFA conversion and evolution of microbial community dynamics during the treatment of SDW at low temperatures. A reactor configuration (DSC-FF) was designed and evaluated for the high-rate treatment of SDW. The successful treatment of LCFA-rich feed at low temperatures presents numerous opportunities for the treatment of FOG-rich wastewaters.

Anaerobic-LCFA degraders obtained from engineered reactors were evaluated in this study, but, the limits of LCFA-degradation should be assessed further using psychrophilic inoculum sources to give a comparative analysis based on the temperature optima of the microbes. In the same vein, the evaluation of transient concentrations of long-chain, medium-chain and short-chain fatty acids with enriched and pure cultures should also be performed for the comprehension of metabolic pathways and possible identification of yet uncultured novel microbial taxa. Confirmation of the presence and the activity of taxa involved in treating LCFA-rich feed at ambient temperatures and their metabolic interactions with methanogens at low temperatures is still needed using a combination of methods, e.g., targeted qPCR and metatranscriptomics or stable isotope probing (SIP)-metagenomics.

Two reactors types – EGSB and DSC-FF, were investigated in this thesis work, but the LCFA degradation should be compared in alternate reactor configurations (such as ASBR, IASB) as well, at lower operational temperatures at LCFA loads higher than 0.9 gCOD-LCFA/L·d. It is recommended to use sludge retention and microbial activity as the desired target, and specific LCFA accumulation (gLCFA/gVS) as the controlling parameter while evaluating high-rate LCFA treatment at low temperatures. Acclimation considerably reduces the inhibitory effects, thus, microbial communities should be acclimated for achieving high LCFA loading rates at low temperatures; either by implementing the alternating periods of LCFA feed and no-

feed cycles as shown by Cavaleiro *et al.*, (2009) at 37°C, or, by LCFA pulse feeding as shown by Ziels *et al.*, (2017) at 35°C.

Due to the successful high-rate anaerobic treatment of SDW, the treatment of real dairy wastewater seems promising in the DSC-FF reactors at 20°C. Scaling up of DSC-FF reactors to pilot-scale with real dairy wastewater will provide a reasonable evaluation of the process parameters (substrate loading, HRT, temperature) for the treatment of FOG-rich dairy wastewaters at ambient temperatures. In this regard, the energy losses arising from the diurnal and seasonal fluctuations in the wastewater temperatures should also be considered while targeting towards an energy-neutral treatment process. The applications of this research work are in the anaerobic treatment of FOG-rich wastewaters (such as food and milk wastewaters, domestic wastewaters, and petrochemical wastewaters) globally produced in temperate regions for the production of biogas and the development of lipid-based biorefinery (carboxylate platform).

7.3 Conclusions

A mixture of saturated (C16:0, C18:0) and unsaturated LCFAs (C18:1, C18:2) within a dairy wastewater matrix were treated in batch at 10 and 20°C and in continuous anaerobic reactors at 20°C at HRTs up to 12 h using different anaerobic reactor configurations. LCFA-containing dairy wastewater (with up to 33% COD as mixed-LCFA and an LCFA loading rate of 0.67 gCOD-LCFA/L·d) can be anaerobically treated in EGSB at 20°C but the long-term continuous treatment of LCFA-rich wastewaters necessitate sludge retention for maintaining optimal methanogenic activity. A novel reactor, DSC-FF, designed during this research work was demonstrated to be feasible for the COD removal and methane production from SDW at discharge temperatures (~20°C) at HRTs from 72 to 12 h, and handle granular sludge disintegration and washout up to 150 d.

The LCFA loading rate and LCFA concentration in feed affected the process performance (sCOD removal, methane production), and, granular characteristics (flotation, granule size), due to the substrate accumulation (likely LCFA) on the granules. Acidogenesis and acetogenesis were inhibited at LCFA loads higher than 0.9 gCOD-LCFA/L·d, hence, the specific LCFA accumulation (gLCFA/gVS) and acetoclastic activity should be used as the controlling parameters for continuous treatment of LCFA-rich wastewaters at 20°C.

Inoculum mixture having distinct metabolic capabilities were coalesced by granulation for treating LCFA-rich wastewater, and, could be used for engineering inoculum for treating other challenging waste

streams. The biofilm development was achieved in the FF while treating SDW at 20°C, which contributed to the particulate COD removal.

New insights into the LCFA-degrading microbial community composition at low temperatures were obtained. The most important bacterial taxa involved in SDW degradation were from fermentative *Bacteroidetes* and *Clostridia,* β-oxidizers from *Deltaproteobacteria*, and VFA oxidizers from *Synergistetes*; particularly the acetogenic bacteria – *Syntrophobacter*, *Desulfobulbus, Geobacter,* and the β-oxidising bacteria belonging to *unassigned Syntrophaceae,* and, *Syntrophus* from the class *Deltaproteobacteria*. The archaeal classes *Methanomicrobia* and *Methanobacteria* were important, with a significant role of the acetoclastic methanogen, *Methanosaeta* and the hydrogenotrophic methanogen, *Methanospirillium.* The continuous SDW feeding to EGSB reactors and DSC-FF reactors at 20°C shifted the predominant methanogenesis pathway from hydrogenotrophic to acetoclastic. Moreover, the high relative abundance of acetoclastic methanogen *Methaosaeta* in the EGSB reactor sludges and the active microbiomes of the granules and biofilm from the DSC-FF reactors validated a prominent role of acetoclastic methanogens in the methanization of SDW. This research demonstrated that acetotrophic activity should be the basis of inoculum selection for LCFA treatment at low temperatures (up to 10°C).

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APPENDIXES: SUPPORTING INFORMATION

Supplementary Methods

The cumulative methane production in the batch assays was fitted with Modified Gompertz equation:

$$M(t) = P.\exp\{-\exp[\frac{Rm.e}{P}(\lambda - t) + 1]\}$$

where, M(t) is the cumulative methane production (mL-CH_4 or mL-CH_4/gVS) at time t (d),

P is methane production potential (mL CH_4 or mL CH_4/gVS),

R_m is maximum methane production rate (mL-CH_4/d or mL-CH_4/gVS·d), and

λ is lag-phase for methane production (d).

The curve fitting tool in Matlab R2017b was used to calculate P, R_m and λ.

Supplementary Figures

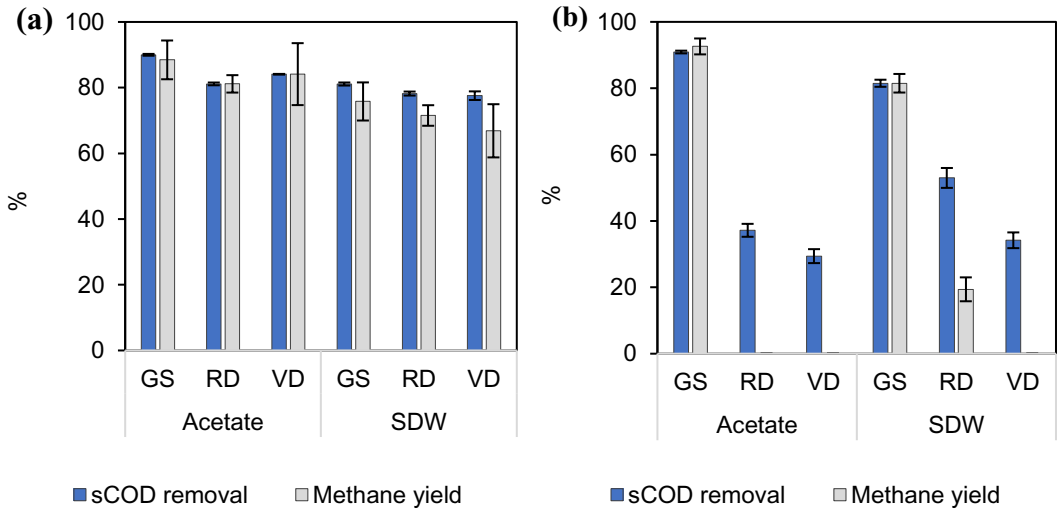

Fig. S3.1. Soluble COD (sCOD) removal and methane yield from acetate or synthetic dairy wastewater (SDW) at (a) 20°C and (b) 10°C with different inocula (GS=Granular Sludge, RD=Rahola Digestate and VD=Viinikanlahti Digestate).

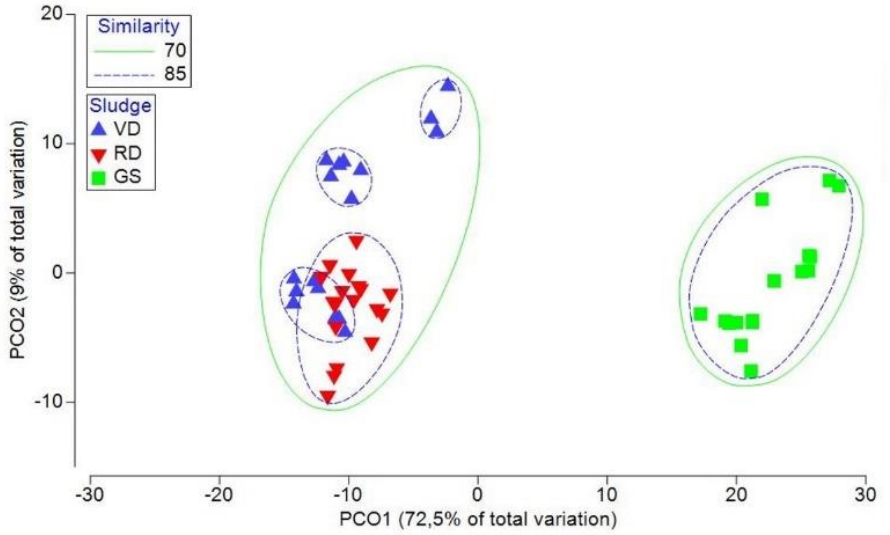

Fig. S3.2. Principle coordinate analysis (PCoA) plot of the bacterial and archaeal classes that formed 99.9% of the microbial community in the assays inoculated with VD, RD and GS and fed with no substrate (blank), acetate and synthetic dairy wastewater (SDW) at 10°C and 20°C. Solid line and dashed line indicate similarity among the samples at 70 and 85%.

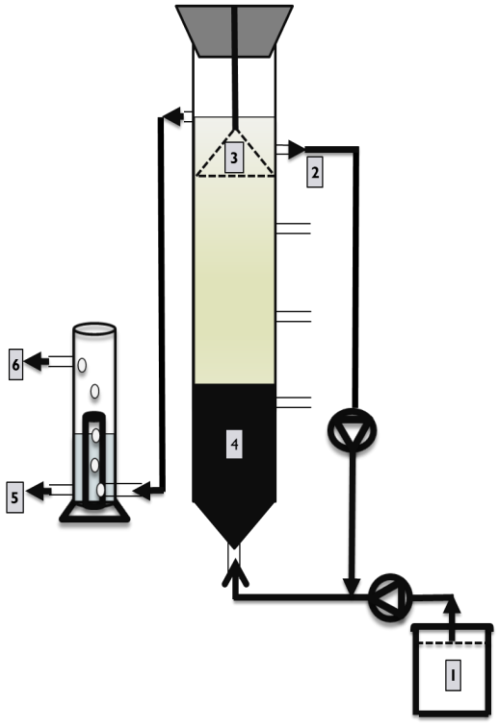

Fig. S4.1. Schematic representation of the expanded granular sludge bed (EGSB) reactor used for the treatment of synthetic dairy wastewater at 20°C (1) Influent tank, (2) Recirculation line, (3) Gas solid separator, (4) EGSB sludge bed, (5) Effluent tank, and (6) Biogas collection.

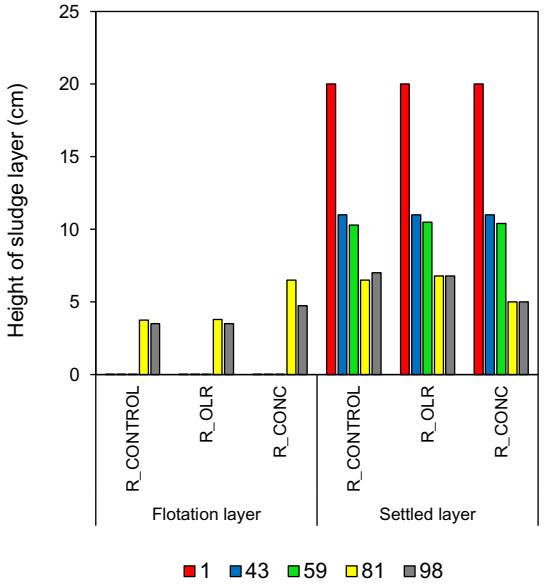

Fig. S4.2. Time-course representation of the physical segregation of granular sludge in the three expanded granular sludge bed (EGSB) reactors into two distinct layers: flotation layer and settled layer.

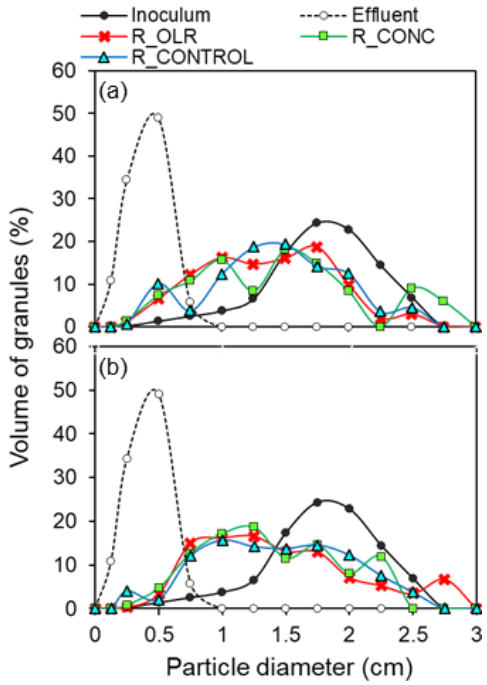

Fig. S4.3. Volume of granules (in percentage) comprised in different granular diameter range (cm) for the granules collected from the EGSB reactors (after the reactor study) in (a) flotation layer, and (b) settled layer. Particle size distribution of inoculum and the washed-out granules in the effluent of reactor are shown in both figures for comparison. $R_{CONTROL}$, R_{OLR}, and R_{CONC} represent the inoculum used as control at different conditions.

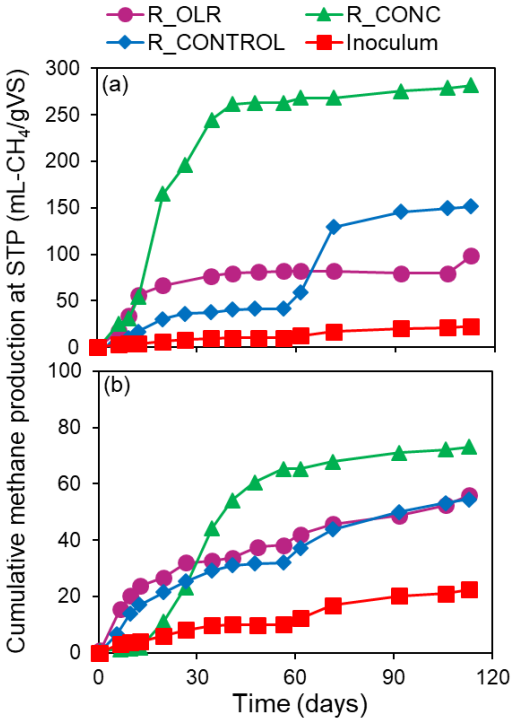

Fig. S4.4. Methane production (mL-CH_4/gVS) from granules collected from the EGSB reactors (after the reactor study) from (a) flotation layer, and (b) settled layer. Methane production from unacclimated inoculum (used as control) are shown in both figures for comparison.

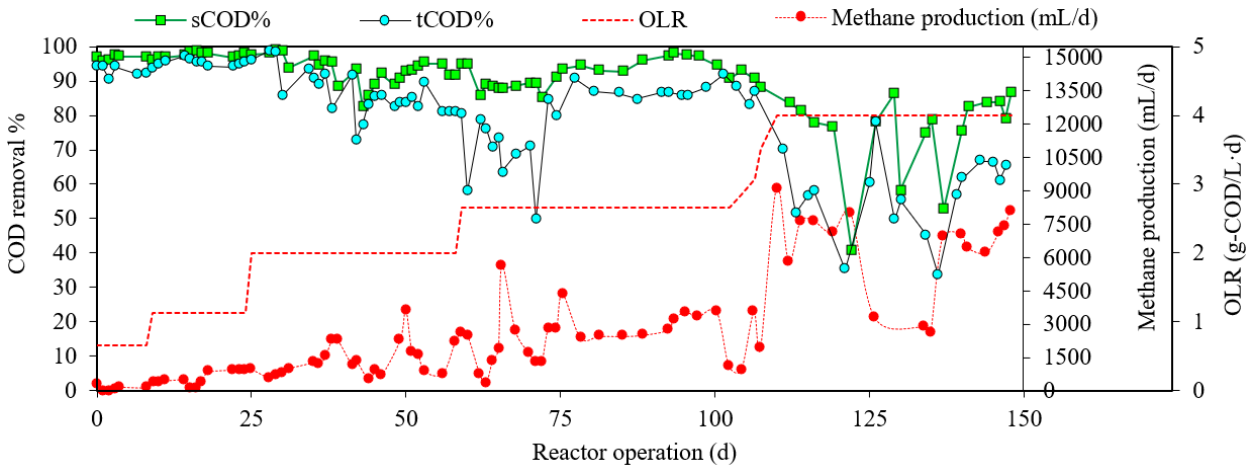

Fig. S5.1. Average total COD (tCOD) and soluble COD (sCOD) removals in the Dynamic Sludge Chamber Fixed Film (DSC-FF) effluents and average daily methane production at the different HRTs of 72, 42.5, 24, 18 and 12 h, from the three parallel DSC-FF reactors.

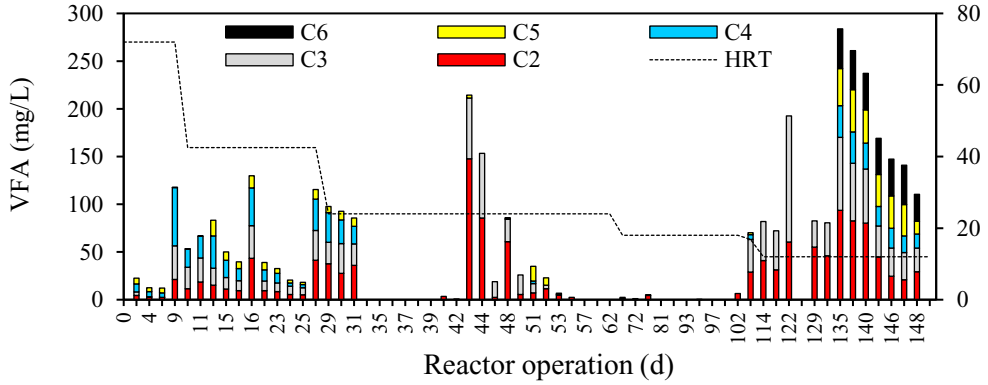

Fig. S5.2. Average volatile fatty acids (VFA) – acetate (C2), propionate (C3), butyrate and iso-butyrate (C4), valerate (C5) and caproate (C6) in the effluents of three parallel Dynamic Sludge Chamber Fixed Film (DSC-FF) reactors at the different HRTs of 72, 42.5, 24, 18 and 12 h.

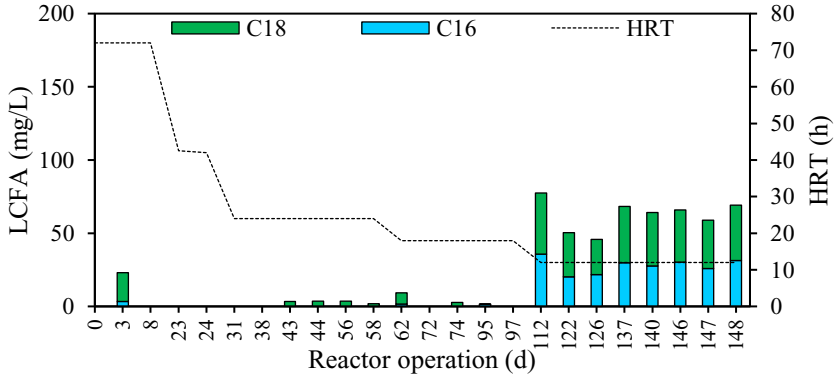

Fig. S5.3. Average long chain fatty acids (LCFA) – caprate (C10:0), palmitate (C16:0), and stearate (C18:0) in the effluents of three parallel Dynamic Sludge Chamber Fixed Film (DSC-FF) reactors at the different HRTs of 72, 42.5, 24, 18 and 12 h.

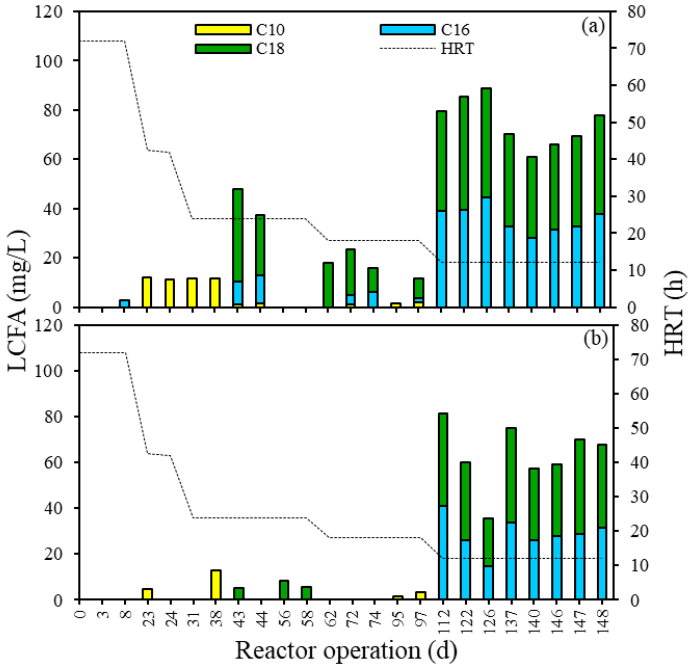

Fig. S5.4. Long chain fatty acids (LCFA) – caprate (C10:0), palmitate (C16:0), and stearate (C18:0) at the different HRTs of 72, 42.5, 24, 18 and 12 h after treatment by (a) DSC, and further (b) by FF.

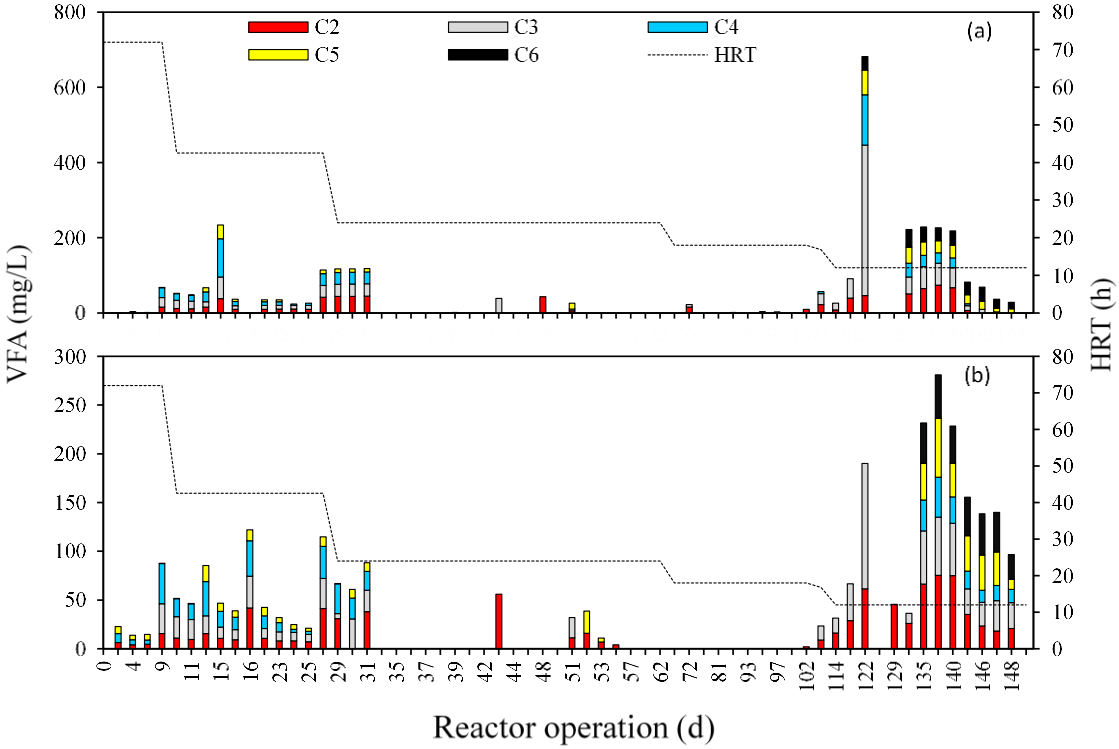

Fig. S5.5. Volatile fatty acids (VFA) – acetate (C2), propionate (C3), butyrate and iso-butyrate (C4), valerate (C5) and caproate (C6) at the different HRTs of 72, 42.5, 24, 18 and 12 h after treatment by (a) DSC, and further (b) by FF.

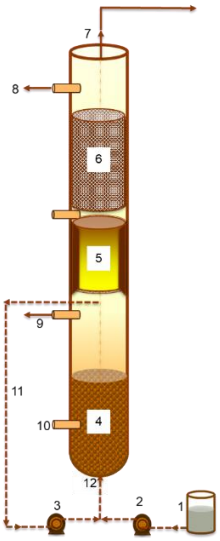

Fig. S6.1. Schematic representation of the DSC-FF reactor.

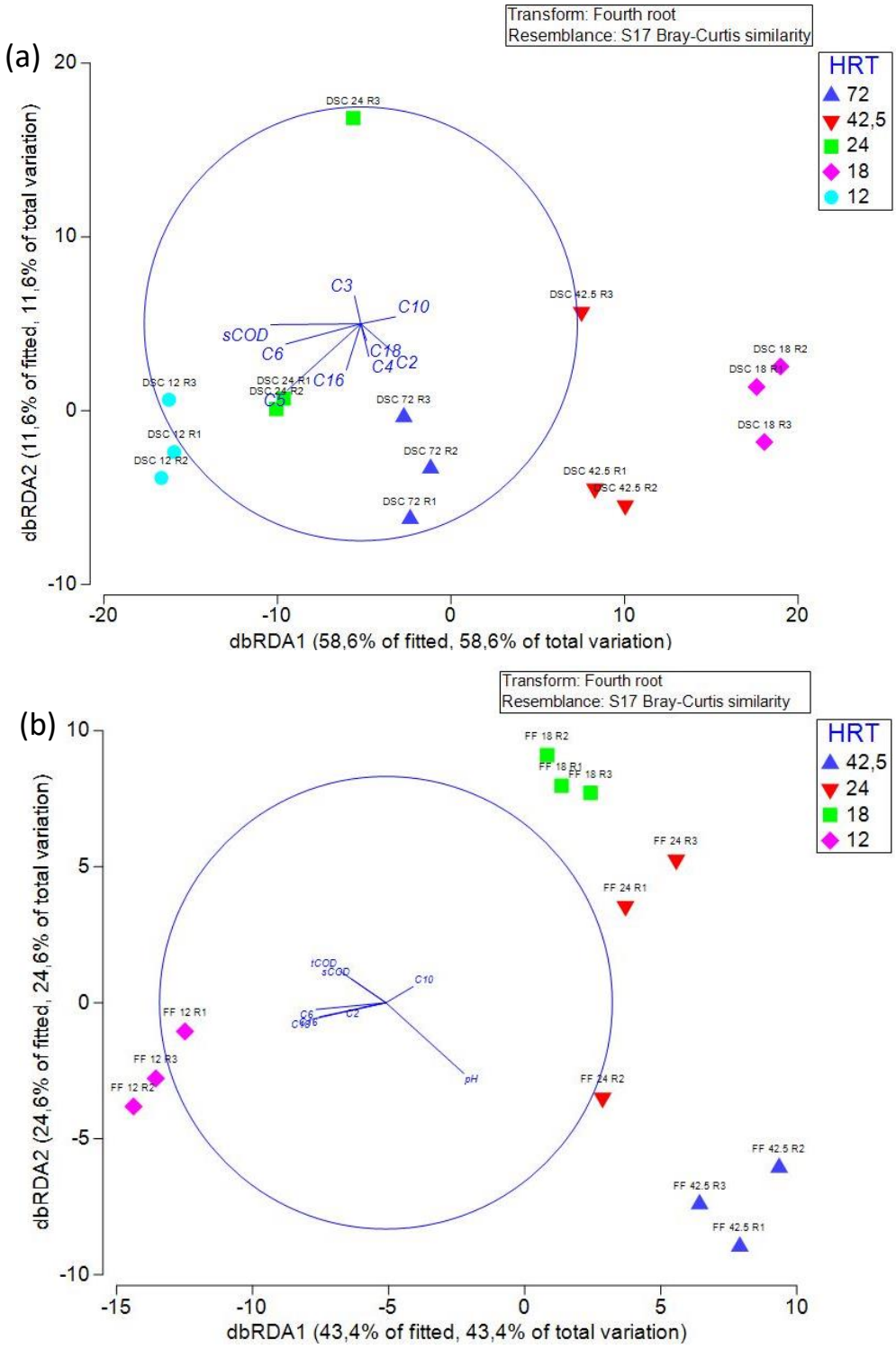

Fig.S6.2. db-RDA of bacterial and archaeal taxa found at the HRTs 72, 42.5, 24, 18 and 12 h from the triplicate reactors R1, R2 and R3 in the (a) granular sludge microbiome obtained from the DSC, and (b) biofilm obtained from FF.

Supplementary Tables

Table S3.1. Detailed information on the sample names used in the multivariate analysis plots.

	Inoculum	Temperature (°C)	Substrate
VD Inoculum	VD	-	-
VDBlank10	VD	10	Blank
VDAcetate10	VD	10	Acetate
VDSDW10	VD	10	SDW
VDBlank20	VD	20	Blank
VDAcetate20	VD	20	Acetate
VDSDW20	VD	20	SDW
RD Inoculum	RD	-	-
RDBlank10	RD	10	Blank
RDAcetate10	RD	10	Acetate
RDSDW10	RD	10	SDW
$RD_{Blank}20$	RD	20	Blank
$RD_{Acetate}20$	RD	20	Acetate
$RD_{SDW}20$	RD	20	SDW
GS Inoculum	GS	-	-
$GS_{Blank}10$	GS	10	Blank
$GS_{Acetate}10$	GS	10	Acetate
$GS_{SDW}10$	GS	10	SDW
$GS_{Blank}20$	GS	20	Blank
$GS_{Acetate}20$	GS	20	Acetate
$GS_{SDW}20$	GS	20	SDW

Table S4.1. Diameter d_{50} and d_{max} (mm) of the granules collected from the inoculum and from different sludge layers (flotation and settled layer) of the three expanded granular sludge bed (EGSB) reactors ($R_{CONTROL}$, R_{OLR}, R_{CONC}) at the end of the reactor operation.

	d_{50} (mm)				d_{max} (mm)			
	$R_{CONTROL}$	R_{OLR}	R_{CONC}	Inoculum	$R_{CONTROL}$	R_{OLR}	R_{CONC}	Inoculum
Settled Layer	1.29	1.23	1.14	1.7	2.48	2.55	2.16	2.49
Flotation Layer	1.3	1.24	1.34	-	2.39	2.27	2.56	-
Effluent	0.28				0.59			

Table S4.2. Methane production from the batch assays after 30 and 120 d and the characteristics of granules used in the batch assays collected from different sludge layers of the three expanded granular sludge bed (EGSB) reactors ($R_{CONTROL}$, R_{OLR}, R_{CONC}) at the end of the reactor operation.

Inoculum	TS (g/L)	VS (g/L)	VS/TS	Methane production (t=30 d) (mL)	Methane production (t=120 d) (mL)
Unacclimated granules	51.7 ± 1.6	44.1 ± 0.1	0.86 ± 0.04	2.89	10.12
R_CONTROL (F)	13.9 ± 0.9	11.4 ± 0.8	0.82 ± 0.04	3.15	19.35
R_CONTROL (S)	37.5 ± 0.1	32.4 ± 0.1	0.87 ± 0.01	6.1	17.06
R_CONC (F)	9.3 ± 0.2	7.1 ± 0.6	0.77 ± 0.08	16.09	19.36
R_CONC (S)	36.2 ± 0.1	32.2 ± 0.3	0.89 ± 0.01	10.58	22.82
R_OLR (F)	9.5 ± 1.1	7.7 ± 0.9	0.83 ± 0.19	6.11	6.27
R_OLR (S)	30.8 ± 0.8	26.9 ± 0.7	0.87 ± 0.04	6.12	13.61

Table S6.1. Alpha diversity metrices –Ace, Chao 1, Shannon indices and Good's coverage for the inoculum mixture, the DSC granular sludge microbiomes (HRTs from 72 to 12 h), the FF biofilm microbiomes, and the effluent microbiomes (HRTs from 42.5 to 12 h) from the three replicate reactors R1, R2, and R3, using the CSS normalized dataset.

	HRT	Reactor	Ace	Chao1	Shannon	Goods coverage	Goods coverage#
Inoculum mixture	0		1630	1716	9.53	0.78	0.96
DSC Granules	72	R1	2611	2543	9.91	0.74	1.00
		R2	2826	2850	9.89	0.71	1.00
		R3	2299	2266	9.79	0.75	1.00
	42.5	R1	2614	2599	10.03	0.75	0.99
		R2	2862	2886	9.98	0.74	0.99
		R3	2392	2390	9.94	0.76	0.99
	24	R1	3348	3478	10.07	0.69	0.99
		R2	3119	3065	9.98	0.70	0.98
		R3	1262	1242	9.34	0.83	1.00
	18	R1	2076	1999	9.75	0.77	1.00
		R2	1632	1629	9.53	0.80	1.00
		R3	2113	1985	9.83	0.79	0.95
	12	R1	3833	3588	10.22	0.68	0.93
		R2	1470	1363	9.37	0.80	1.00
		R3	1537	1516	9.40	0.79	1.00
Biofilm (FF)	42.5	R1	1397	1361	9.51	0.85	1.00
		R2	1213	1227	9.35	0.85	1.00
		R3	2183	2299	9.80	0.78	0.99
	24	R1	1950	1970	9.89	0.81	1.00
		R2	1417	1345	9.63	0.86	1.00
		R3	1978	2119	9.82	0.78	1.00
	18	R1	1918	1974	9.73	0.77	1.00
		R2	3274	3444	10.24	0.71	1.00
		R3	1959	2124	9.75	0.78	0.96
	12	R1	2802	2995	10.03	0.75	1.00
		R2	2353	2322	9.94	0.77	1.00
		R3	2332	2244	9.93	0.77	1.00
Eff	42.5	R1	1933	1910	9.64	0.75	1.00
		R2	2668	2609	9.95	0.71	1.00
		R3	3430	3299	10.22	0.69	0.99
	24	R1	2037	1953	9.72	0.74	1.00
		R2	2159	1996	9.96	0.78	1.00
		R3	1926	1821	9.72	0.76	1.00
	18	R1	1590	1555	9.56	0.77	0.99
		R2	2000	1978	9.76	0.75	0.99
		R3	3043	3350	9.97	0.70	0.99
	12	R1	2181	2063	9.81	0.74	1.00
		R2	1279	1252	9.36	0.81	1.00
		R3	1492	1571	9.45	0.79	1.00

99.9% dataset