

Ground-Truthing ADM1: Establishing Methodologies for Data Gathering in Anaerobic Digestion for Parameterization.

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ABSTRACT

Growing global populations equate to increased human waste, water consumption and energy demands. Anaerobic digestion may serve as the solution to resolving the dilemma of a reliance on finite non-renewable energy resources (i.e., fossil fuels) and an inexhaustible source of human waste such as food waste, sewage, and agricultural by-products. Anaerobic digestion uses microorganisms to degrade waste and convert it into methane (biogas). The Anaerobic Digestion Model 1 (ADM1) was published in 2001 as a widely applicable tool to promote the utilisation of anaerobic digestion, however the ADM1 was created from a consolidation of data from numerous studies which may not have focused on the production of biogas. A methodology utilising the globally unique System-60 AD research facility in the Centre of Excellence for Anaerobic Digestion (CEAD) was developed for optimised data acquisition to improve ADM1 parameters, thereby allowing for the ground-truthing of ADM1. Methodologies include the use of a starved system and monitoring of gas production rates alongside hourly sampling for twenty four hours, samples analysis was conducted to observe concentrations of fatty acid, chemical oxygen demand, total solids, and volatile solids percentage post inoculation. The protocol was conducted to investigate the effects of butyric, hexanoic, and octanoic acid inoculation on gas production in anaerobic digesters. Preliminary data gathered indicates that the methodology devised employing System-60 was successful in observing changes within an anaerobic digester, and improvements or modifications can be made for use of System-60 in future experimentation to investigate anaerobic digestion optimization and other projects focused on anaerobic digestion.

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Author's Declaration:

I, Anyi Pulford, declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

All work in this thesis is my own with the following exceptions:

- a) The configuration, set up, troubleshooting and methods within the GC-FID were done by Dr.Yumechris Amekan (section 2.2.1)**

- b) Disassembly, de-contamination, reassembly, gas flow monitor calibration, troubleshooting, and maintenance of System-60 anaerobic digester systems (section 2.1.1) were performed by Kimberley Barnes.**

- c) The values of VS% of digestate inoculated with hexanoic and octanoic acid were gathered by Kimberley Barnes.**

- d) Bioinformatic analysis of gas data and the subsequent figures generated (Fig.7-9) were conducted by Annabel Cansdale using R.**

1.0 INTRODUCTION

The growth in global populations equates to increased waste production, in addition to escalating energy and water demands. Unavoidable human waste such as sewerage, food, and agricultural by-products may be the answer to the diminishing non-renewable energy resources, and biogas production from anaerobic digestion serves as the intermediary between waste and renewable energy. To understand and promote wider applications of anaerobic digestion the Anaerobic Digestion Model 1 (ADM1) was published in 2001 and the development was a collaborative effort between experts in anaerobic digestion process analysis, simulation, and modelling. As stated by the authors of the ADM1 'this model should not be regarded as the only or the best way to describe anaerobic processes¹'. The ADM1 was created from data obtained from multiple sources which may not have been focused on optimisation of biogas production. The aim of this thesis is to establish a methodology to optimise data acquisition of digestate properties and biogas production with the use of anaerobic research facility System-60 a globally unique AD research facility located within the Centre of Excellence for Anaerobic Digestion (CEAD) at the University of York to produce data sets which would aid in furthering models such as the ADM1; thereby ground-truthing the ADM1. The aims of this project therefore are:

- To investigate acetogenesis, the downstream processes of biogas production in anaerobic digestion of digestate upon inoculation of three even chained fatty acids, butyric, hexanoic, and octanoic acid.
- To create a starved system to exclude other potential organics which could be utilised by digester microbial communities for biogas production, and the introduction of fatty acid to starved systems to observe its effects on digester characteristics.
- To sample digesters inoculated with fatty acids every hour for a 24-hour period, and observe the subsequent gas production, and digestate properties following fatty acid inoculum. Digestate properties are elucidated by sample analysis for chemical oxygen demand, total solids, total volatile solids percentage, and fatty acid concentration.
- To assess use of the methodology in System-60 and its potential in future studies for the gathering data of AD processes to be used in modelling.

1.1 Wastewater Treatment

An estimated volume of 150 L of wastewater is produced per person per day², with a total of 11 billion LL of wastewater collected in UK sewers per day³. The UK population of mid-2019 was estimated to be 66.8 million, an increase of 7.8% was projected for mid-2024 with population estimates of 72 million in the UK⁴. With these projections, the processing of 'waste' must be considered. Although wastewater and subsequent by-products of wastewater treatment are often considered to be 'waste' it is a utilitarian commodity with the means to overcome many problems faced today. The following sections will outline wastewater treatment, the associated energy demand (i.e., water energy nexus), as well as the importance and processes of anaerobic digestion which can help achieve a circular economy.

1.1.1 Overview of wastewater treatment effluent

1.1.2 Preliminary and primary treatment

Wastewater enters wastewater treatment plants (WWTP) as raw sewage (**Fig.1 (1)**) and undergoes stepwise purification treatments. Equipment (e.g., screens, grit) and methods can vary from plant to plant, however the wastewater treatment procedures generally follow the scheme seen in **Fig.1**. Depending on the wastewater management infrastructure, rainwater which carries various debris can also enter WWTP as part of raw sewage. First wastewater will undergo preliminary treatment, WWTP will use screens (**Fig.1 (2)**) to remove the solid, suspended solids and floating debris^{3,5,6} which may include sanitary products, other non-flush items, colloids⁵, or debris carried by rain water such as branches. Upon removal of larger solid debris, the waste stream will go through a grit chamber (**Fig.1(3)**). Grit chambers minimise head loss for further downstream processing⁶. Grit is traditionally classified as particles larger than 0.15mm⁶ (solid inert particles e.g., sand and gravel). The preliminary treatment's objective is to ensure downstream processing of wastewater will not be hindered and decrease inefficiencies or damage to decontamination equipment^{3,5,6}.

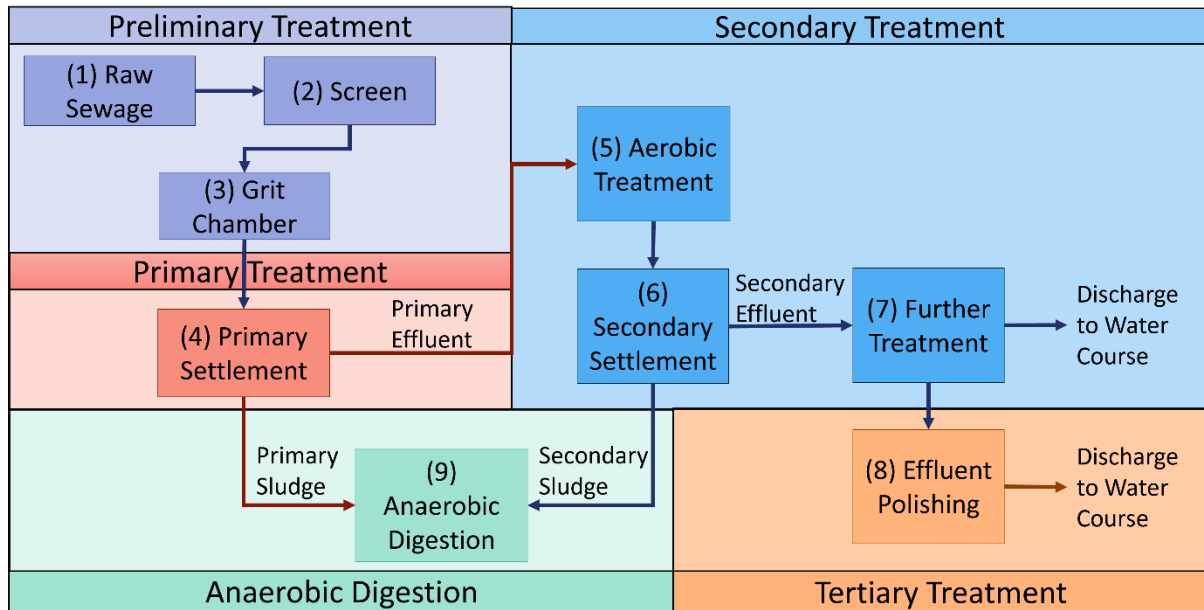


Fig.1 A flowchart illustrating the process wastewater undergoes in wastewater treatment plants adapted from Waste treatment and Disposal⁷ sewage and industrial]

Upon completion of preliminary treatment, wastewater will be ready for primary treatment. Primary treatment usually occurs in a settlement tank (**Fig.1(4)**) and requires passive or chemically enhanced settlement (e.g., coagulation/flocculation⁵) of suspended solids not removed during preliminary treatment. The suspended solids which sink to the bottom of settlement tanks are predominantly organic and are commonly referred to as primary sludge. The primary sludge travels through an outflow pipe typically located near or at the bottom of the settlement tank and will either undergo further treatment by anaerobic digestion ([section 1.2](#)) or disposal. The primary effluent is the liquid remaining in the settlement tank once suspended solids have precipitated. The primary effluent will proceed to secondary treatment.

1.1.3 Secondary Treatment

Secondary treatment's objective is to reduce residual organic matter/biochemical oxygen demand (BOD) through biological means (i.e., bacterial degradation). Discharge of waters with high levels of BOD (i.e. anthropogenic nutrient pollution) into bodies of water and associated streams can lead to eutrophication (excessive algal or aquatic plant growth) with knock-on effects leading to ecological degradation due to oxygen depletion^{3,8}. Hence secondary treatment is a crucial component of wastewater treatment.

1.1.3.1 Aeration Tanks

Secondary treatment begins with an aeration tank which is a vessel containing aerobic microorganisms with primary effluent transported in. Primary effluent is subjected to aeration (**Fig.1(5)**) which involves actively introducing oxygen to primary effluent to allow aerobic microorganisms to thrive and breakdown the remaining organic impurities^{3,5}. The methods of oxygen injection and microorganism inoculation may vary from plant to plant; however, the primary principle (based on activated sludge models^{9,10}) is to provide microorganisms with surplus oxygen to enhance bacterial consumption of organic materials.

The aerobic treatment outflow enters a second settlement tank which facilitates suspended aerobic microorganisms to settle to the bottom. The settled aerobic microorganisms form secondary sludge/waste-activated sludge^{3,5,9}. Similar to primary sludge, secondary sludge may either be pumped away to undergo anaerobic digestion or for disposal. The further clarified liquid within aeration tanks, known as secondary effluent, will be subjected to disinfection by varying methods (e.g., chlorination or UV-irradiation; sometimes considered tertiary treatment). The disinfectant and discharge of post-treatment wastewater is dependent on the classification of the watercourse and municipal regulations.

1.1.3.2 Trickling Filter

Trickling filters similar to aeration tanks utilise further biodegradation by microorganisms^{10,11}. Although trickling filter configurations may differ, the principles are as described below. An influent pipe carrying primary effluent which is pumped through a rotating elongated sprinkler arm allowing primary effluent to be evenly dispersed onto the surface of a fixed media bed (the composition of the media bed varies^{10,11,12}; support medium in **Fig.2**). The enclosure containing the highly permeable medium/filter bed promotes proliferation of biofilm onto the packing medium. Most trickling filters are exposed and freely ventilated to the atmosphere, however in covered enclosures aerobic conditions are maintained through differing methods¹⁰. Although the process is named trickling filter, the primary objective if the packing medium is not the sieving action, but to allow for the formation of biofilm. The trickle of primary effluent allows microorganisms established onto the medium to degrade organic matter present in the wastewater thereby decreasing BOD¹¹. The effluent of trickle filter enters a secondary settlement tank, from this step onward the effluent and sludge are treated similarly to that of aeration tanks. It's important to note that some WWTP utilise trickling filters as a tertiary treatment following secondary treatment.

1.1.4 Tertiary Treatment

Tertiary treatment is a treatment process most subjected to variation as it addresses different pollutants (e.g. pharmaceutical, industrial, and others) encountered. Watercourses designated as 'sensitive areas may not deem secondary treatment effluent to be discharged, therefore requiring tertiary treatment to protect water bodies. Tertiary treatments may include but are not limited to ultrafiltration, reverse osmosis, adsorption using activated carbons, and constructed wetlands⁵.

1.1.5.0 Energy Demand

Having established a general overview of wastewater treatment, the energy demand of these plants should be addressed. An estimated average of 1%-3% of a country's total electrical energy output is required for WWTPS¹³. On a world-wide scale 20% of electrical energy consumption designated to public utilities was attributed to wastewater treatment operations¹⁴. According to US congressional research 'energy is the second highest budget item for municipal drinking water and wastewater facilities, after labour cost¹⁵. Within the next decade certain area's energy demand of these utilities are projected to increase significantly, for instance Australia's projected 25% population growth by 2030 will cause water utilities energy consumption to escalate between 130%-200% above current levels¹⁶.

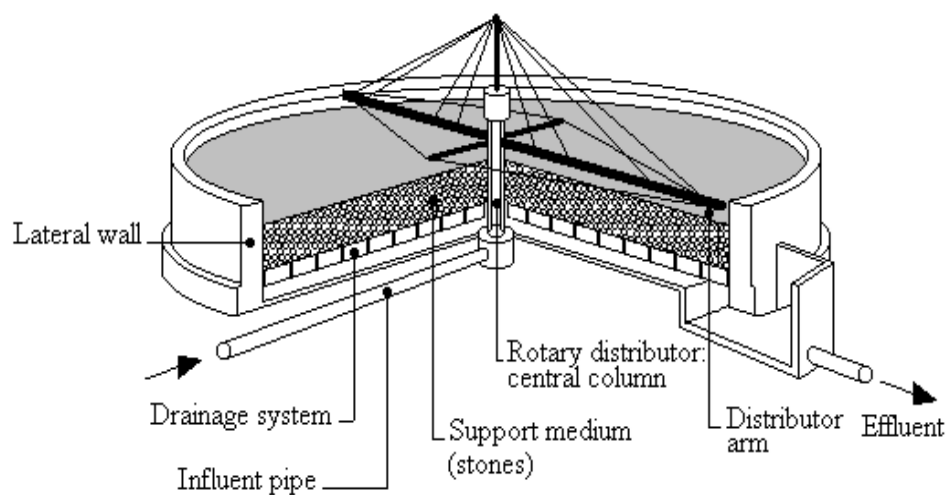


Fig.2 A standard trickling filter utilised in WWTPs adapted from Jordão and Pessoa (2009)¹⁷

A factor to be taken into consideration regarding energy demand is that there are no standardised methods of measuring energy consumption of WWT¹⁸. Multiple studies^{14,15,19,20} addressed the inconsistent communication of water-related energy as well as proposed methodologies to mitigate the distortion of research and the application in government

reports, media and policy¹⁴. A study¹⁴ determined significant confusion and distortion of water-related energy communication and provided steps for improved global protocol and policy through improved definition and analysis. As a result of the recent European Union and other countries' mandates/policies on greenhouse gas (GHG) emissions¹⁹, (of which WWTP are a significant contributor of GHGs) there will be improvements and stringent policies on energy monitoring enacted.

1.1.5.1 Water Energy Nexus and a Circular Economy

Energy usage is intrinsic to wastewater treatment, however sizable quantities of water availability are necessary to meet energy requirements. The water availability would be required for thermoelectric power plant cooling, fuel production, and mineral extraction and mining¹⁵. Similarly in the water sector, energy is required for pumping, transportation, and every step mentioned in wastewater treatment (section 1.1). This intersectional relationship is known as the water energy nexus. Currently there is not a formal definition of the water energy nexus the term is coined to refer to the principle of the inherent relationship between the two. WWTPs are an exceptional demonstration of this nexus and can be central to transforming the nature of water and energy demand into a circular economy. The central principle of a circular economy is to juxtapose contemporary linear and open-ended economies as a closed/looped economic system with emphasis to act as a regenerative system by minimising waste, emission, and resource usage through deceleration, closure, and constricting material and energy loops; which can be achieved within long-lasting design, reuse, remanufacturing, maintenance, repair, refurbishment, and recycling²¹. Water and energy are natural resources which influence the economy, using the principles underpinning circular economy, wastewater can be exploited for its embedded energy through biomethanisation, creating a circular economy within water and energy sectors, the key to harnessing the potential within wastewater is anaerobic digestion.

1.2.1.0 Anaerobic Digestion

Processes which make use of anaerobic microbes have had an omnipresence in history; although fermentation is the more commonly known process, using microbes in the absence of oxygen to break down substrates to produce soy sauce, bread, cheese, alcohol, and many other dietary staples. Similarly, anaerobic digestion also applies the use of microbial communities under anaerobic conditions to harness the energy potential embedded in waste.

The focus of this thesis is on the application of anaerobic digestion to wastewater sludge and digestate. The establishment of methodologies to further enhance AD and optimisation of AD parameters may also facilitate an improved application of AD to other waste streams such as agricultural and food waste.

AD has been used in municipal wastewater treatment for sludge disposal, as AD enables relatively inert and stable organic or inorganic residue to be converted from putrescible organic matter while generating biogas (methane)²². Existing models such as the Anaerobic Digestion Model 1 (ADM1)¹ utilised data from multiple works and models. The ADM1 was achieved through a collaborative effort put forth by a collective of international experts in simulation, modelling, and anaerobic process analysis. The ADM1 was originally published in 2002 and has stated that it was made with the intent of broad application, IWA has also published Activated Sludge Models (ASM), however in contrast to the ADM1 the ASM1 was followed by ASM2 and ASM3²³, whereas there has not been any further editions for the ADM1. Advances have been made in the field of anaerobic digestion and by establishing a methodology to observe the systematic changes within AD upon inoculation of individual components within the model (**Fig.3**) e.g., volatile fatty acids, monosaccharides, and amino acids, data generation focused on improved parameterization for improved biogas production can be achieved.

1.2.2.0 Anaerobic digestion processes

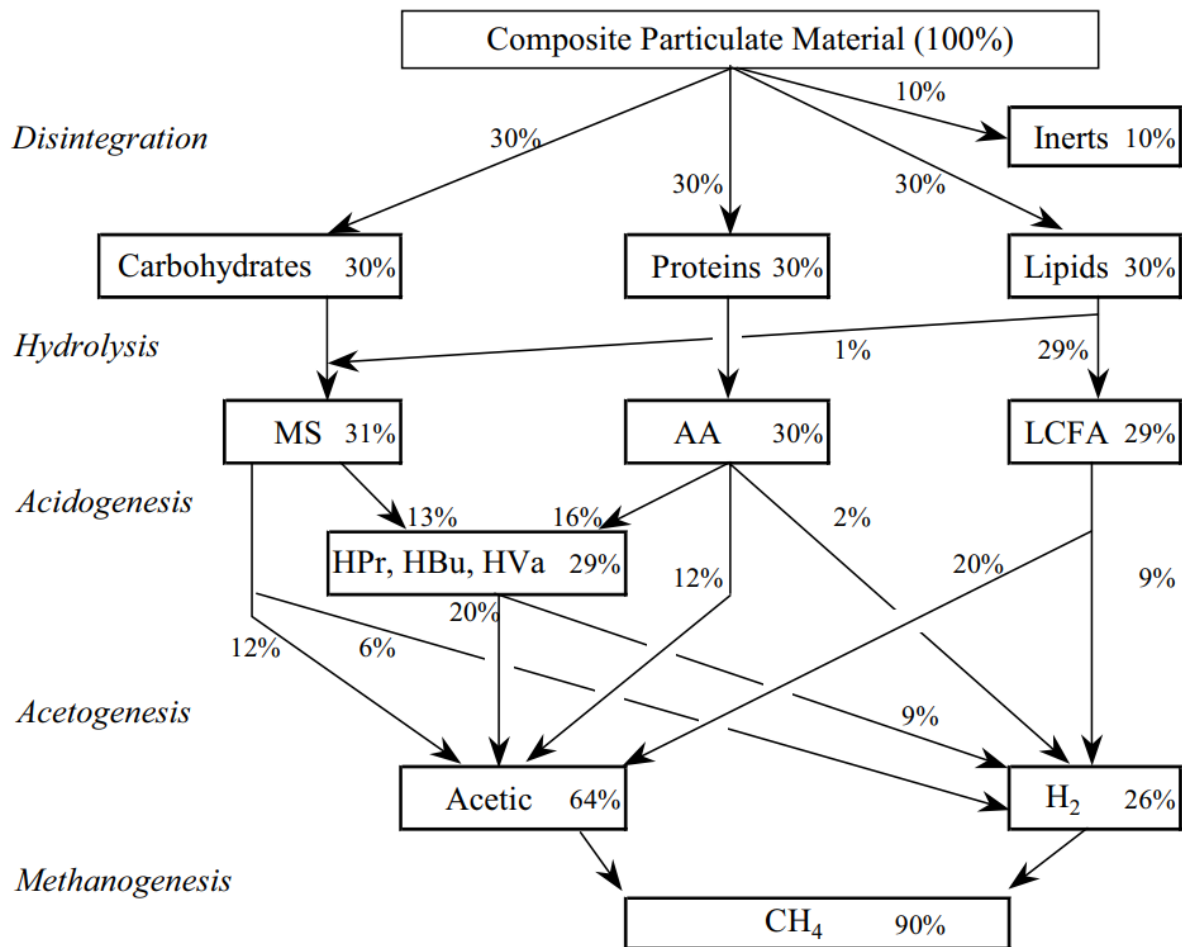


Fig.3 Flow chart representing COD flux and degradation process in anaerobic digestion (Figure taken from ADM1^[1]).

The breakdown of organic material begins with disintegration followed by hydrolysis, acidogenesis, acetogenesis, and concludes with methanogenesis (**Fig.3**). Disintegration and hydrolysis may occur as non-biological and extracellular biological processes, disintegration was noted to be the separation of a composite particulate material into its constituents and are included as the first process to enable widespread application. Disintegration is important predominantly to represent whole cell (as composite particulate material) lysis, or recycling of dead anaerobic biomass and composite separation for digestion of primary sludge and waste activated sludge (WAS) digestion¹. Hydrolysis is defined in the ADM1 as the enzymatic degradation of composite particulate matter or macromolecule depolymerization into soluble monomers; organisms which directly benefit from soluble products most likely produce the hydrolytic enzymes¹. The task group concludes that the organism growing on the surface of a particulate should be considered the effective catalyst rather than the enzymes produced;

another conceptual model of enzyme secreted by organisms into the bulk liquid and absorbed onto a particulate was dismissed¹.

The soluble monomers considered in ADM1 are monosaccharides (MS), amino acids (AA), and long chain fatty acids (LCFA) produced from carbohydrates, proteins, and lipids respectively. Mixed product acidogenesis occurs with sugars and amino acids as acidogenesis is a microbial degradative process which does not require additional electron donors or acceptors; LCFA degradation is considered in acetogenesis as it is an oxidation reaction requiring external electron acceptors^[1]; organisms present in AD are able to produce several products from monosaccharides and amino acids; however ADM1¹ was focused on the production of volatile fatty acids as a downstream metabolite.

Upon conversion of MS, and AA into VFAs, LCFAs and VFAs will undergo 'syntrophic hydrogen-producing acetogenesis and hydrogen utilising methanogenesis'¹. Syntrophic hydrogen-producing acetogenesis is undertaken in oxidation steps by bacteria to degrade LCFA or higher organic acids to acetate. As an internal electron acceptor is not present, hydrogen ions or carbon dioxide are utilised as electron acceptors to produce hydrogen gas or formate respectively¹. The hydrogen gas and formate produced by acidogenic bacteria are used by methanogenic organisms for methanogenesis.

1.2.3 Fatty acids in anaerobic digesters.

A previous study²⁴ into the roles of VFA (or short chain fatty acids) of C₁-C₆ in highly efficient AD focused on the VFAs as a substrate for biomethanisation in pre-treated and untreated waste activated sludge (the study of this thesis is digestate rather than WAS), the study concluded that straight chain VFAs had undergone a more rapid degradation than their respective isomers²⁴. The study also concluded when non-inhibitive concentrations of VFA were used as an inoculum VFAs were an efficient substrate used by methanogenic microorganisms²⁴. Reciprocal isomerisation of butyrate and isobutyrate observed in a separate study²⁵ on anaerobic bacterium (WoG13²⁵) was also observed in anaerobic digestion²⁵. The catabolism of butyrate was therefore the most rapid as they can be degraded via β -oxidation and isomerisation²⁵. A study has demonstrated that the addition of butyric acid to an AD running with low energy substrate such as cattle manure will yield a methane production which doubles that of a reactor without butyric acid as an additive²⁶.

Medium chain fatty acids (MCFA) are fatty acids with 6-12 carbons. The number of studies into MCFA degradation in anaerobic digestions are not in abundance however there are studies²⁷⁻²⁹ which investigate the use of AD and addition of alcohol to divert metabolic processes to produce high value MCFAs to achieve a circular bioeconomy²⁷⁻²⁹. In contrast to MCFAs there is a relative abundance of studies into long chain fatty acids (LCFAs) and its effect on AD. Long chain fatty acids are classified as fatty acids with carbon chains exceeding 12 carbons, when comparing SCFA and MCFAs long chain fatty acids are an inhibitory factor³⁰ in AD and are seen as a barrier to overcome to achieve a more efficient AD process³¹ LCFAs were observed as inhibitory to multiple vital reactions in AD due to its toxicity to the microorganisms which carry out the reactions^{30,32}. In addition to the toxicity of LCFA to microorganisms it was observed to create a prolonged lag phase to the degradation of LCFA and butyrate^{30,32}. A study into the bactericidal effect of long chain fatty acids in AD³², with capric acid as the model substrate observed the LCFA: biomass ratio was less relevant than the lethal threshold level (6.7 - 9.0 mol/d³) of LCFA concentration. LCFAs are also known to cause

The performance of anaerobic digesters has been linked to VFA concentrations^{24,33,34}, as seen in **Fig.3** VFAs are the intermediary compound in biomethanisation metabolic pathway in AD; however, high concentrations of VFA cause microbial stress because of a decrease in pH which results in anaerobic digester failure^{24,33,34}. Therefore by examining VFAs and the effects of their concentration we can extrapolate optimal conditions and AD efficiencies in biomethanisation. A study²⁴ states it is widely known that as fatty acid chain length increases the presence of VFAs in anaerobic digestors decrease. Butyric, hexanoic, and octanoic acid were the VFAs chosen as the fatty acid for this methodology (however, other chain lengths or inoculum under differing conditions may be used in future studies). The justification for choosing VFAs is because they are the precursor to acetogenesis and are further downstream in the degradation process towards biomethanisation within AD (**Fig.3**).

1.2.3.1 Fatty acid degradation in AD

Fatty acid degradation has been well characterised under aerobic conditions of β -oxidation, however the anaerobic counterpart for fatty acid degradation is still ongoing. Studies^{35,36} have shown that an analogous version of β -oxidation occurs without the use of

molecular oxygen in pure cultures. **Fig.4** illustrates a proposed anaerobic β -oxidation degradation of fatty acids in *fad* regulon of *Escherichia coli*³⁶.

The ADM1's self-described 'core'¹ can be divided into two portions, with its first segment focused on the biochemical parameters followed by the second portions which is directed towards the physicochemical aspects of AD¹. The gathering of data for the bioprocesses is what the methodology established in this thesis aims to do. As an initial point of investigation processes further downstream in AD degradation will be examined, the acetogenesis of VFA (interchange and known as SCFA) and MCFAs in anaerobic digestion of digestate from secondary treatment. Butyric (**iii**), hexanoic (**ii**), and octanoic acid (**i**) were chosen as the fatty acid inoculum as they are saturated, even chain length fatty acids which undergo β -oxidation in a more simplistic fashion than unsaturated, odd chain length fatty acids.

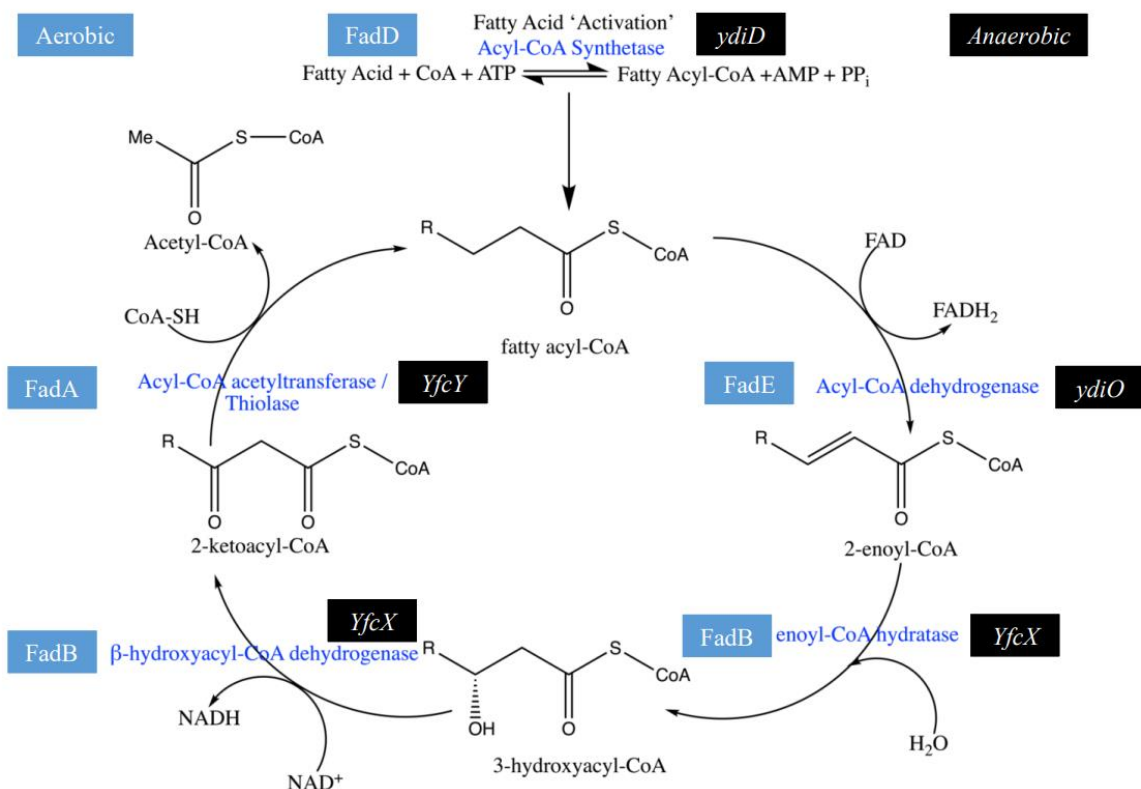
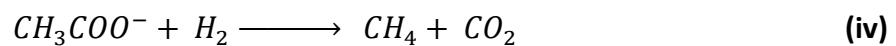
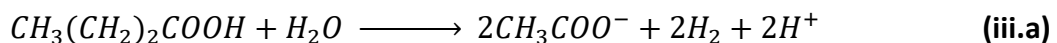
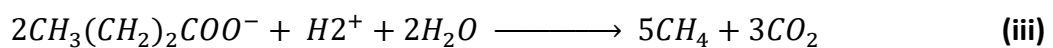
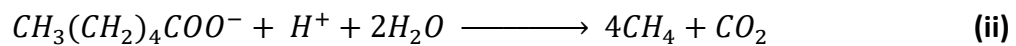
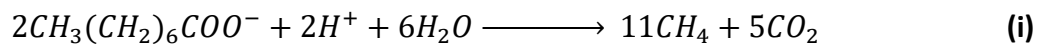


Fig.4 Diagram illustrating proposed anaerobic fatty acid degradation pathways in the context of known aerobic pathway counterparts.

This diagram was designed by collating known and accepted aerobic β -oxidation with proposed anaerobic β -oxidation counterparts^{37,38}. Acyl-CoA dehydrogenase/FadE (first step in β -oxidation) & 3-hydroxyacyl-CoA dehydrogenase/FadB (catalyses 3rd step in β -oxidation) were repressed under anaerobic conditions. Suggesting alternative pathways for fatty acid degradation under anaerobic conditions³⁵.

Two MCFAs were selected, octanoic acid with 8 carbons **(i)** was the longest fatty acid chain, and hexanoic **(ii)** acid with 6 carbons. Studies have mostly been into the production of MCFA in AD²⁷⁻²⁹ rather than the degradation and gas production of MCFAs in AD, hence they were chosen as an inoculum. As fatty acid chain lengths increase gas production is predicted to occur over a longer period and steadily increase as octanoic acid is degraded through β -oxidation cycles into hexanoic and butyric acid. Hexanoic acid is more commonly categorized as a MCFA, however depending on the study the definition of a VFA could be fatty acids with 1-4 or 1-6 carbons; notwithstanding the classification of hexanoic acid with 6 carbons was chosen as part of this project as it is the intermediary between octanoic and butyric acid. The changes in gas production volume and digestate characteristics because of hexanoic acid inoculum were expected to be more observable within the 24 h timeline relative to octanoic acid. Butyric acid **(iii)** the precursor to acetic acid **(iii.a)** was chosen for its rapid turnover into acetic acid for methanogenesis **(iv)**, the effect of butyric acid on biogas production and digestate characteristics were expected to be the most impactful with immediately following inoculation; the effect of butyric acid inoculation was also predicted to be the most brief relative to hexanoic and octanoic acid.



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| Table 1 Chemical equations of each fatty acid degradation and acetic methanogenesis. |
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1.2.4 Significance

Some urban water infrastructures are either past or close to their appropriate design lifespan of 50-75 years therefore they are due for a considerable refurbishment, there have been proposals for wastewater treatment plants which substitute aerobic processes with anaerobic ones¹⁸.

The hindrances of AD implementation include but are not limited to the large capital investment required, regulatory policies, and a lack of economic incentives³⁹. However, the efforts towards reducing anthropogenic carbon emission's impact in the endeavour to

combat climate change is becoming increasingly prevalent as a global priority e.g. The Paris Agreement⁴⁰, the net-zero coalition, and COP (Conference of Parties) which focuses on updates in climate actions undertaken by members of the United Nations every five years (the Paris agreement was established during COP21). The hindrances of AD implementation may be offset by the global initiatives striving towards green energy.

Further understanding of anaerobic digestion will aid in the development and implementation of biogas production as a source of renewable energy which has the potential to offset greenhouse gas emissions and implement an energy self-sufficiency to adapters of AD facilities. As stated by the authors of ADM1, the ADM1 is a model from an amalgamation of studies, the methodologies using System-60 enable consistent measurements to yield dataset focused on parametrisation and investigation of anaerobic digestion process. The success in employing this methodology with the use of System-60 means future studies into more upstream processes within AD such as LCFAs, amino acids, and carbohydrates (hydrolysis stage of degradation) or how specific composite particulates undergo disintegration can be achieved (See [3.6.0 Future Studies](#)). This will lead to a robust dataset focused on acquiring data for modelling and optimised parametrisation of ADM1, or by extension the formulation of an ADM1 modified for biogas production; which as stated by its authors the intentions of the ADM1 was 'merely a common platform from which simulation applications for a wide range of specific processes should be developed^[1]'.

2.0 METHODS

2.1 Observing volatile fatty acid degradation in anaerobic digestion of digestate.

Volatile fatty acids (VFAs) were chosen as it is a component found in the later stages of AD process cascade (acetogenesis) and was a more favourable inoculum than monosaccharides and amino acids due to its higher conversion efficiency into biogas (**Fig.3**). Increasing chain lengths of fatty acids were used as an investigative means to observe the changes in AD processes with increased fatty acid chain length ([section 1.2.3.1](#)). The preliminary data generated and visualised as a result ([section 3.0](#)) signifies that the procedures established and carried out with the use of System-60 was a reliable approach to dissecting AD processes.

2.1.1 System-60 anaerobic digesters

Six separate 5 L digesters (F1-F6) within System-60 were used throughout experimentation. Digesters were disassembled, cleaned, and dried to ensure no cross contamination, sanitised and reassembled digesters were sealed ensuring an anaerobic environment, then inspected for gas leaks followed by calibration of gas monitors, the aforementioned tasks and maintenance of digesters and gas monitors were all carried out by Kimberley Barnes, as the training required could not be undertaken due to Covid-19 restrictions at the time of experimentation Digesters were stirred at 15 r.p.m to ensure mixing, and temperatures were kept constant at 35°C with heating sleeves .

Two, 30 L barrels of digestate were collected on-site from Yorkshire Water's Naburn Wastewater Treatment Works and transported back System-60 5 L of digestate were decanted per digester. Once digesters were filled, the digestate was left (with constant stirring and temperature) for approximately 2-2.5 weeks to 'starve' microbial communities present in the sludge. Gas produced from digesters were recorded with a gas flow metre which utilised tip buckets, when an amount of gas has been produced the bucket will tip, each bucket tipped were automatically registered and recorded digitally; the programmable logic controller accounts for the atmospheric pressure and temperature of the room which caused the amount of gas which caused the tip The extent of starvation was determined by gas flow rate (mL/h). Once gas flow rates were close to 0 mL/h, the assumption was that organic remnants which could be used by microbial communities were sufficiently depleted, which in turn allowed assurance that any observable effects were caused by inoculation of VFA.

2.1.2 VFA addition and sampling

Three even chained fatty acids, butyric, hexanoic, and octanoic were used as feedstock for digester microbial communities (DMC) (justification in section.1.2.3.1). Each fatty acid required 6 digesters; hence 3 separate identical experiments (with different VFAs) were carried out. A single dose of VFA was used to inoculate a digester through a specialised sampling/feeding port and serological pipette. A low VFA concentration of 5 mM were used for digesters F1-F3, while a higher concentration of 10 mM were used for digesters F4-F6. Throughout experimentation gas volumes were measured, mixing and temperature were kept constant. Samples were collected every hour for 24 h with triplicates taken at 0 and 8 h (Fig. 5)

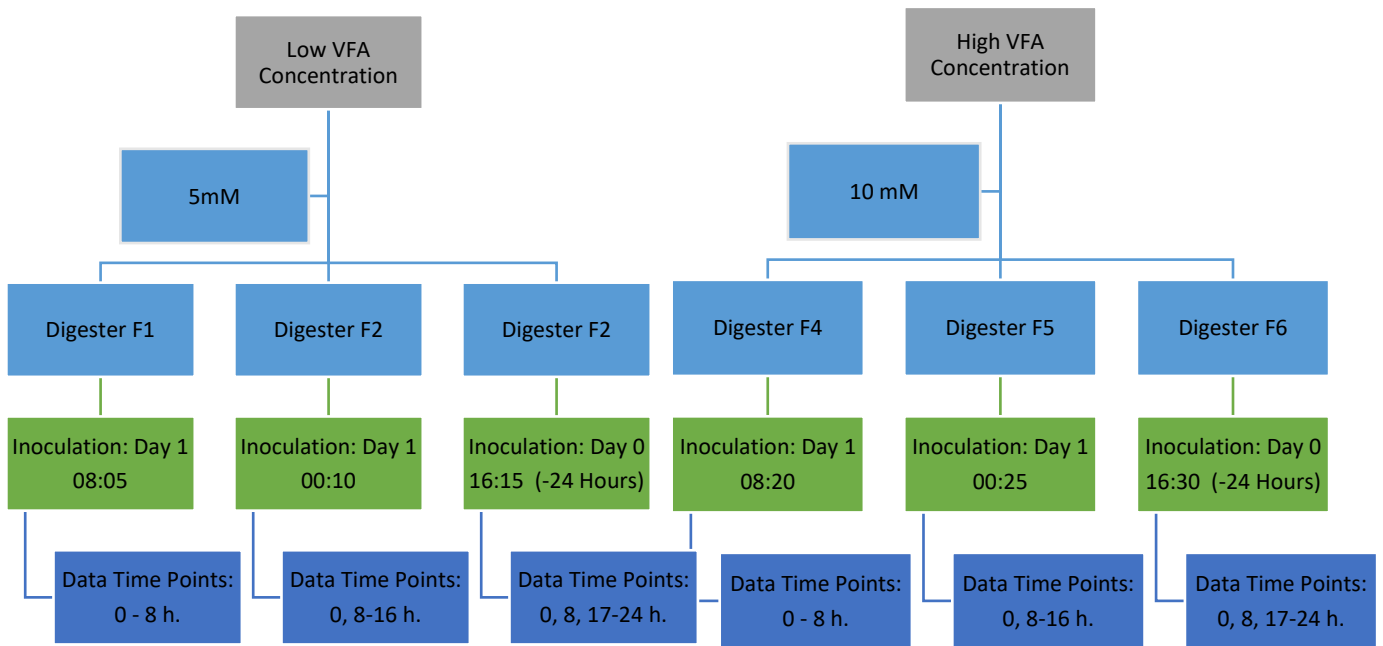


Fig. 5 Diagram summarising the inoculation and sampling process of anaerobic digesters F1-F6.

Inoculations were staggered by 8 h; this allowed sample data points encompassing 24 h to be collected within an 8 h period. Digesters F3 and F6 were given the inoculum a day prior to sampling at 16:05 with 5 mM and 16:30 with 10 mM butyric acid respectively; following this F2 and F4 were inoculated at 00:10 with 5 mM and 00:25 with 10 mM butyric acid respectively. On the following day (sampling day) F1 and F4 were given the VFA at 8:05 with 5 mM and 8:10 with 10 mM respectively (**Fig.5**).

Digesters F1-F6 were sampled every hour for 8 h with 5 minutes between each digester sampled. 35 mL samples were taken with sterile 10 mL open ended serological pipette through the sampling port. The samples were aliquoted into the appropriate vessels and volumes (Fig. 6), then frozen at -4°C until further analysis.

2.2.0 Sample analysis

Upon completion of butyric, hexanoic, octanoic acid inoculation and sampling; further analysis of samples followed. In total 174 samples were acquired, and each had undergone assays to investigate chemical oxygen demand, volatile solids percentage, and VFA concentration via gas chromatography flame ionisation detection.

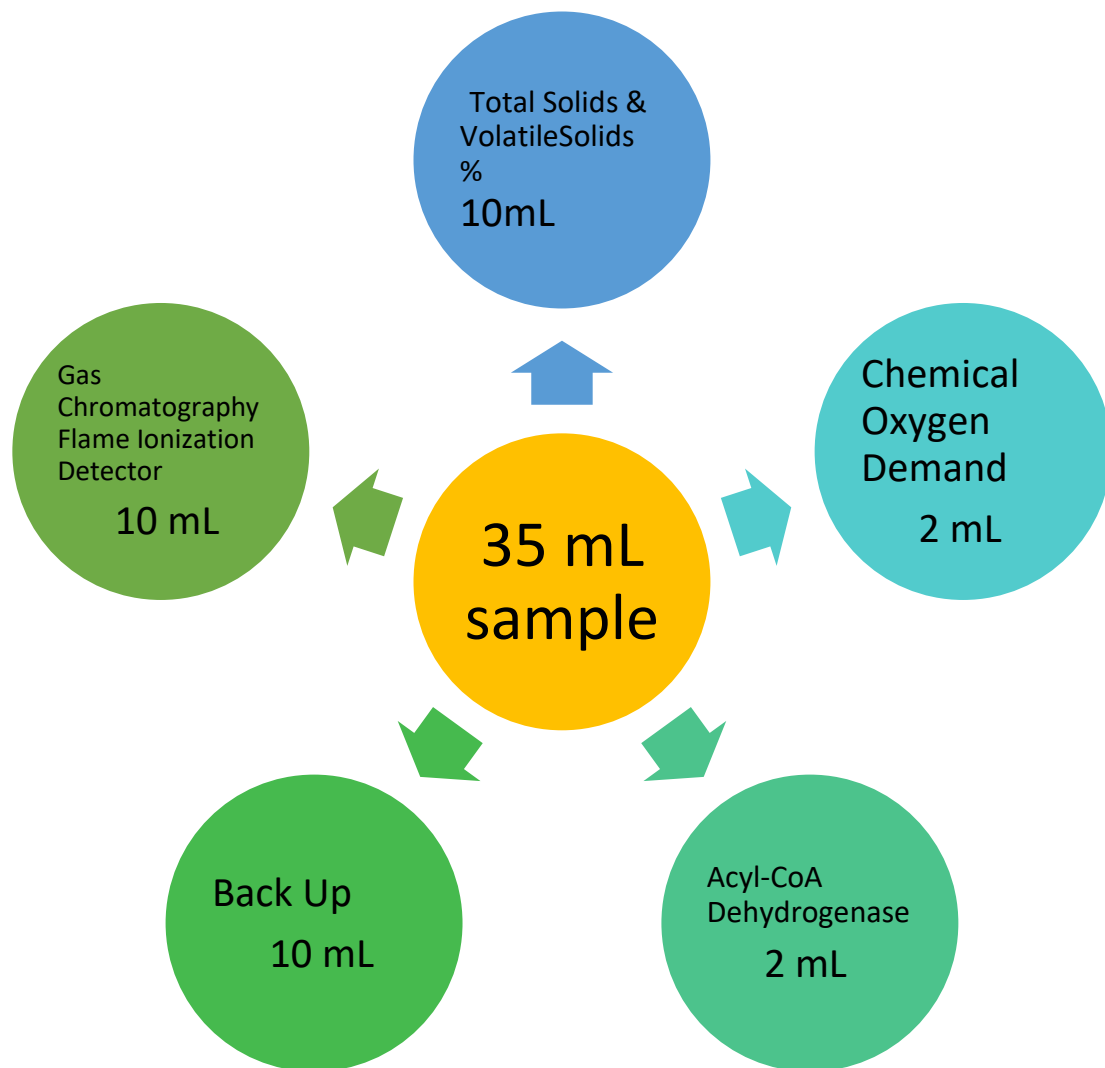


Fig.6 Diagram representing how samples taken from anaerobic digesters were separated and allotted for analysis.

2.2.1 VFA concentration via GC-FID

Gas chromatography flame ionisation detection (GC-FID) allowed for quantitative analysis of VFA degradation profile of DMCs; this channel of analysis presented changes in VFA concentration as well as downstream products of VFA degradation by DMCs. For minimised carry-over of previously injected samples, washes were conducted with water injected and ran through the GC-FID. Sample analysis sequences were performed in descending order from 24 to 0 h with increasing washes as VFA concentrations were suspected to increase nearer to 0 h, this was also done to diminish carry-over of previous samples.

The initial set-up, maintenance guidance, and troubleshooting of the GC-FID were managed by Yumechris Amekan as the training could not be undertaken due to covid-19

restrictions and time constraints. The standard used was volatile free acid mix (CRM46975) diluted with MilliQ water to concentrations of 10 mM, 5.0 mM, 1.0 mM, 0.5 mM, 0.1mM, and 0.005mM. 10 mL samples were defrosted and prepared for GC-FID. Samples were centrifuged at 4°C, 4000 r.p.m, for 15 m; the clarified supernatant aliquots were decanted into 2 mL tubes. If the supernatant required further clarification, repeated centrifugation at 10,000 r.p.m of 2 mL samples were conducted. Filter sterilisation of 2 mL samples were decanted into the syringe with 0.22 µm filter attached, the filtrate was directly discharged into sterile 2 mL Eppendorf tubes. Sterile samples were then pipetted into GC analysis vials and acidified with 7.5 µL of orthophosphoric acid. Data acquisition and analysis were conducted in Clarity™

To have minimised crossover contamination between sample analysis, washes were conducted before and after GC-FID sample analysis; samples estimated to have the lowest concentrations underwent analysis prior to samples suspected to have higher VFA concentrations. Therefore samples were grouped by their respective digesters and analysed in reversed sequential order, e.g. F3, F2, and F1, followed by F6, F4, and F3. Preceding digester-grouped sample analysis a standard was run through the GC-FID to mitigate the effects of room temperature or any conditions that may cause shifts to occur in chromatograms which could affect VFA concentration extrapolation.

2.2.4 Chemical oxygen demand

Due to the nature of digestate such as turbidity, colour and other attributes there was no way to quantitatively isolate and measure only the biomass concentration within digestate. Therefore the indirect approach of chemical oxygen demand for the measurement of biomass was taken.

COD kits Hach Lange 914 COD 5-60 g/L O₂ cuvette tube cell vial test were used to determine COD. 2 mL Eppendorf tubes containing samples were defrosted, 200 µL per sample were pipetted into the COD vial test. High speed digestion was carried out using Hach HT200S high temperature thermostat, samples underwent digestion for 15 m, including heating and cooling the total time is approximately 35 m. Once sufficiently cooled, cuvettes were read in the Hach TL23 series laboratory turbidimeter and results were given in O₂ g/L.

2.2.5 Total solids percentage

As stated previously, although biomass concentrations are unable to be extrapolated from digestate, volatile solids percentage allowed further increased confidence in the indirect approach we have taken to measure DMC biomass by using VS and TS²⁴.

Crucibles were washed and dried prior to conducting the assay, once weighed 10mL of defrosted samples were decanted into the crucible, once the weights of the crucibles and samples were collected, the samples were placed into a Memmert Beschickung loading modell 100-800 at 120°C, samples were left to dehydrate overnight (approximately 12-20 h). Upon complete dehydration the crucibles containing dried samples were placed in a concave tray containing silica gel desiccant and sealed to prevent condensation from rehydrating samples while crucibles cooled down to room temperature. Once cooled enough for handling and rehydration from condensation was prevented, the crucible and dried samples were weighed. Following this the volatile solids % were obtained by putting the crucibles containing samples into Milestone-Pyro Advanced Microwave Muffle and samples were heated to 550°C for 90 m. Table.2 was used to calculate volatile solids %.

| Sample Name | Empty crucible weight (g) | Wet crucible weight (g) | Dry crucible weight (g) | Ashed Crucible weight (g) | Fixed solids % (1) | Dry solids % (2) | Volatile solids % (3) |
|-------------|---------------------------|-------------------------|-------------------------|---------------------------|-----------------------|---------------------|--------------------------|
|-------------|---------------------------|-------------------------|-------------------------|---------------------------|-----------------------|---------------------|--------------------------|

$$\text{fixed solids \%} = \frac{\text{Ashed crucible weight (g)} - \text{Empty crucible weight (g)}}{\text{Wet crucible weight (g)} - \text{Empty crucible weight (g)}} \times 100 \quad (1)$$

$$\text{dry solids \%} = \frac{\text{Dry crucible weight (g)} - \text{Empty crucible weight (g)}}{\text{Wet crucible weight} - \text{Empty crucible weight (g)}} \times 100 \quad (2)$$

$$\text{volatile solids \%} = \text{dry solids \%} - \text{fixed solids \%} \quad (3)$$

Table.2 The table used to record and calculate volatile solids %.

3.0 RESULTS AND DISCUSSION.

The nature of the data gathered is preliminary due to the effects of COVID-19 and the one-year time allotment for this MSc research project. When the experimentation was expected to be carried out the U.K initiated a national lock down, and the University of York had to take mandatory safety precautions and shut down research facilities. When lock down restrictions were lifted or became less restrictive, facilities still required a booking system or restrictions on how many personnel were allowed in laboratory spaces. In addition to restricted laboratory access, the time required for certain consumables, chemicals, and equipment was significantly increased due to an abundance of factors such as staff shortages caused by COVID-19 in fulfilment centres, or product scarcity due to increased demands for COVID-19 related operations. COVID-19 restrictions also affected sample collection from Yorkshire Water's Naburn treatment site as Yorkshire Water had their own safety regulations and procedures to get on-site. Although preliminary, the data acquired provides insight as well as improvements to methodology and confirms that further repetition and application of improved methodologies will yield improved results.

Subsequent to digester inoculations and sampling, samples had undergone VFA concentration analysis via GC-FID, chemical oxygen demand assays and volatile solids percentage measurements. Triplicates were only done with 0 h- and 8-h samples (other time points only had 1 sample for analysis), the averages of the triplicated samples were taken and used for data visualisation. To clarify the gas data produced in digester F1-F3 and F4-F6 were not true triplicates (8 h inoculation time difference, and effects of sampling) of 5 mM and 10 mM fatty acid inoculation, samples collected then later analysed are **not** in triplicates except for 0 h and 8 h; sample analysis of hourly time points 1-7 h and 9-24 h were not in triplicates as the staggering of inoculation was done in order to sample all time points within 8 h (hence, only 1 sample was obtained per hour within 24 h). The timeframe to visualise gas rates were chosen as 48 h rather than the 24 h timeframe for all sample analysis, this was done to show that gas production occurred beyond the 24 h time frame. The 48 h time frame used to visualise gas data also illustrates the gas production of digesters without perturbances caused by sampling.

3.1.0 Gas production following VFA inoculation of anaerobic digesters

. As shown in **Fig.5** F3, and F6 were inoculated at 16:15 and 16:30 respectively on day 0, F2 and F5 were inoculated at 00:10 and 00:25 respectively on day 1, prior to sampling of all digesters F1 and F4 were inoculated at 08:05 and 08:20 on day 1 (day 1 was the day of sampling). The graphs (**Fig.7**) for all digesters except F1 and F3 were all shifted to visualise gas production at 0 h, as F1 and F3 captured 0-8 h, F2 and F4 were shifted by 8 h (digesters F2 and F4 were utilised to capture 8-16 h), digesters F3 and F6 (digesters that captured 16-24 h) were repositioned by 16 h, these shifts were requested to enable comparison of digesters upon inoculation. Some of the jagged patterns seen in corresponded with the eight hours of sampling conducted. The absence of gas rates was not due to the lack of gas production but a system error.

As seen in **Fig.7-9** shorter fatty acid chains had higher gas production earlier on, as fatty acid chain lengths increased gas production rates remained lower initially but steadily increased over time and had a more prolonged period of gas production than VFA chains of lesser chain lengths. Higher concentrations (10 mM) of VFA inoculation in digesters also yielded longer periods of gas production than lower concentrations. These results are indicative of microbial communities metabolising the fatty acid inoculum, shorter VFAs are more easily metabolised hence a more rapid response was seen in digesters with higher gas production rates for a shorter time frame. Increased VFA chain lengths required more metabolic processes (more β -oxidation cycles (**Fig.4**)) hence a lengthier process which led to prolonged periods of gas production. Although high amounts of gas rates were seen initially in digesters inoculated with shorter chain lengths, it is more likely that digesters injected with longer chain length VFAs produced more gas overall due to the increased amount of carbon and hydrogen in longer VFAs; a devised methodology to investigate is further discussed in [section 3.6.0](#).

3.1.1 Gas production in anaerobic digester inoculated with butyric acid.

Gas rates from initial butyric acid inoculation of F2, F3 and F6 exceeded 1000 mL/h, for gas data to be thoroughly examined the Y-axis maximum was fixed at 200 mL/h (**Fig.7**). Gas production followed immediately upon introduction of butyric acid.

Digesters with 5 mM butyric acid introduced immediately began to produce gas with rates of 50-100 mL/h, gas production abated after approximately 30 hours apart from F2 as

F2 had increased amounts of butyric acid injected. F1 gas production abated prior to other digesters inoculated with 5 mM butyric acid; this may be attributed to the sampling performed immediately following initial dosage. It could be presumed that the decrease in gas production was caused by completed degradation of butyric acid which was between 28-32 h for F1 and F3. F2 gas production steadily remained between 75-110 mL/h and began to decline at approximately 38 h post inoculation. F2's butyric acid dosage was higher than 5mM but possibly below 10 mM, however F2 did not respond identically to F5, this could be attributed to better inoculation technique done by supervisor Prof. James Chong with the entirety of the butyric acid being successfully introduced into the digester (A detailed description can be found in section [3.2.1.0](#))

Digester F4 behaved similarly to F1 as sampling occurred shortly after inoculation, gas production diminished sooner than digesters F2, F3, F5, and F6 which allowed for butyric acid degradation prior to sampling. Digesters F5 and F6 had continued but steadily declining gas production, after 24 h F5 gas production remained above 50 mL/h until 40 h but gas production continued. F6 gas production remained between 50-100 mL/h after 24h and persisted above F5 gas production, both digesters were inoculated with 10 mM butyric acid, this could be due to the 16 h allowed for F6 digesters to utilise the inoculum before sampling was conducted.

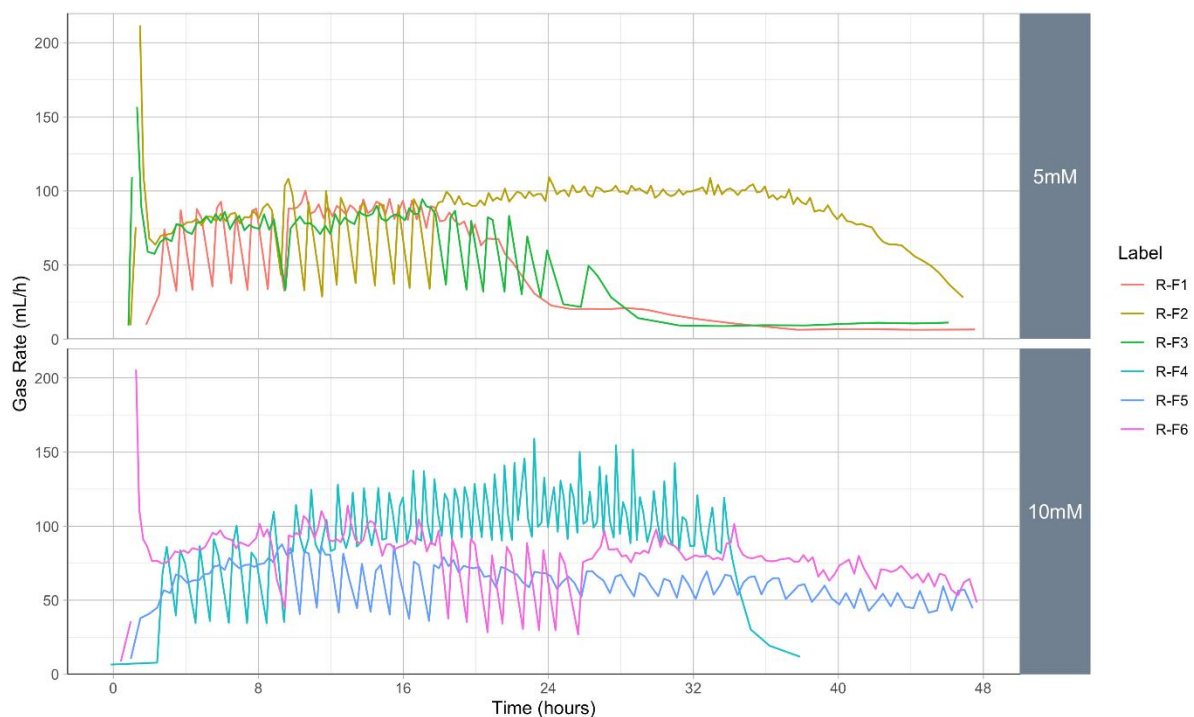


Fig.7 Effects on gas rates (mL/h) in anaerobic digesters (F1-F6) following a 5mM and 10mM dosage of butyric acid over 48 hours.

3.1.2 Gas production in anaerobic digesters inoculated with hexanoic acid.

Hexanoic acid inoculations did not yield gas production (**Fig.8**) as high as butyric acid inoculation (**Fig.7**), and the jagged pattern seen for sampling was not observed; however a decreased gas production was seen in digesters with their corresponding sampling time. This could be due to a sampling error which caused sampling to only be done in the sampling port. In contrast to butyric acid, gas production began to gradually increase over time, this could be due to the increased chain length. Digesters with 5 mM hexanoic acid inoculations had similar gas production profiles after 24 h with gas rates above 50 mL/h and gradually increased, a decrease is observed shortly before 48 h. The peaks seen in F3 during sampling (17-24 h) may have been due to dislodged gas in the tubing rather than a development within the digester. Digesters inoculated with 10 mM hexanoic acid had increased amounts of gas production in the first 4 h post inoculation. Gas rates after sampling period for 10 mM inoculation did not overlap as frequently as 5 mM inoculation, however the 10 mM inoculated digesters remained above 50 mL/h with no decrease seen near 48 h as seen in 5 mM inoculation.

As opposed to 5 mM inoculated digesters, sampling done immediately following inoculation did not cause an earlier cessation of gas production, nevertheless this may only be applicable within the 48 h window we observed in **Fig.8**, it is possible that F1, and F4 ceased gas production prior to other digesters but it may have transpired subsequent to 48 h.

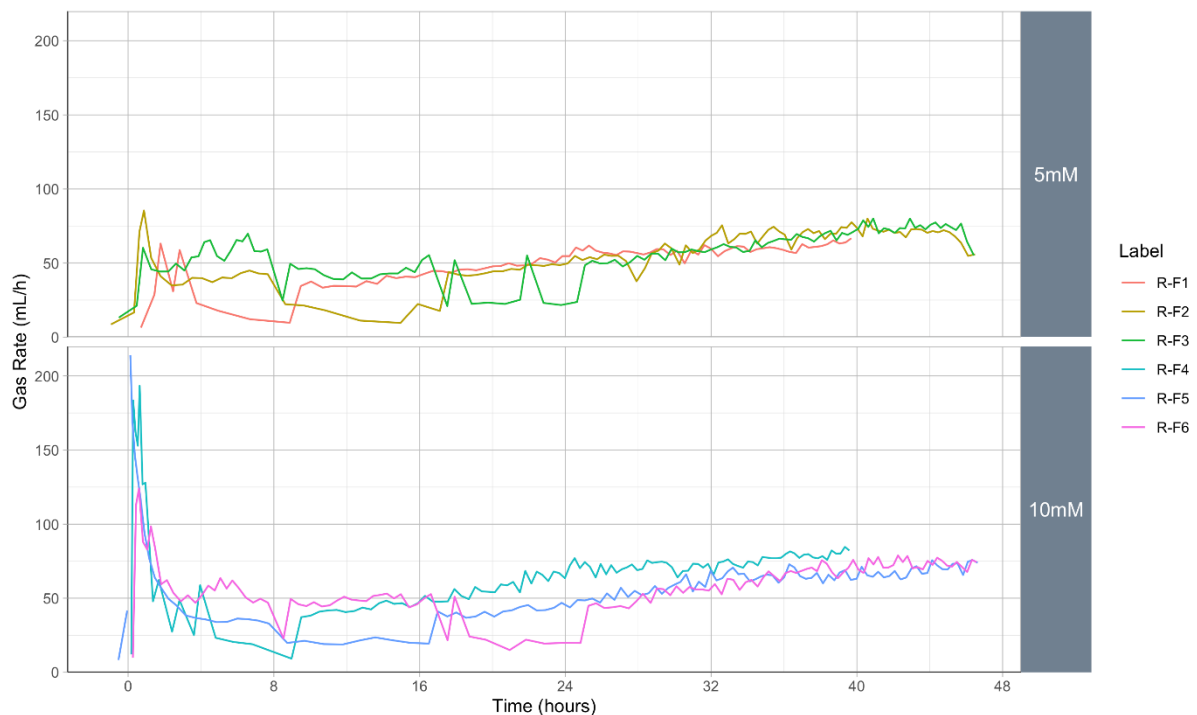


Fig.8 Effects on gas rates (mL/h) in anaerobic digesters (F1-F6) following a 5 mM and 10 mM dosage of hexanoic acid over 48 h.

3.1.3 Gas production in anaerobic digesters inoculated with octanoic acid.

Gas production within digesters are more prolonged as volatile fatty acid inoculum chain lengths were increased, rather than the greater amounts of gas produced in hours seen immediately following VFAs with shorter chain lengths. Octanoic acid with an 8-carbon chain length had the lowest amount of gas production immediately following digester inoculation (**Fig.9**). Only digester F6 responded with a gas production rate over 50 mL/h shortly after octanoic acid inoculation with rates which dipped below 50 mL/h and further decreased to below or lingered at 25 mL/h until approximately 48 h. Digesters F1-F3 gas decreased gas production correlative to when sampling was conducted, after 28 h gas rates began to have more overlap and steadily increased. Digesters F4-F6 did not have overlap comparable to F1-F3 after 28 h, only F5 and F6 had similar gas production profiles, however digester F4 which had sampling from 0-8 h began to have a higher gas rate than F5 and F6. The effects of sampling affected gas production in digesters inoculated with octanoic acid the most; gas rates approached 0 mL/h during sampling, whilst experimentation with butyric acid and hexanoic acid did not have gas rates which approached 0mL/h as closely as octanoic acid had. This may indicate that MCFA fatty acid with greater than 8 carbons can produce inhibitory characteristics within AD.

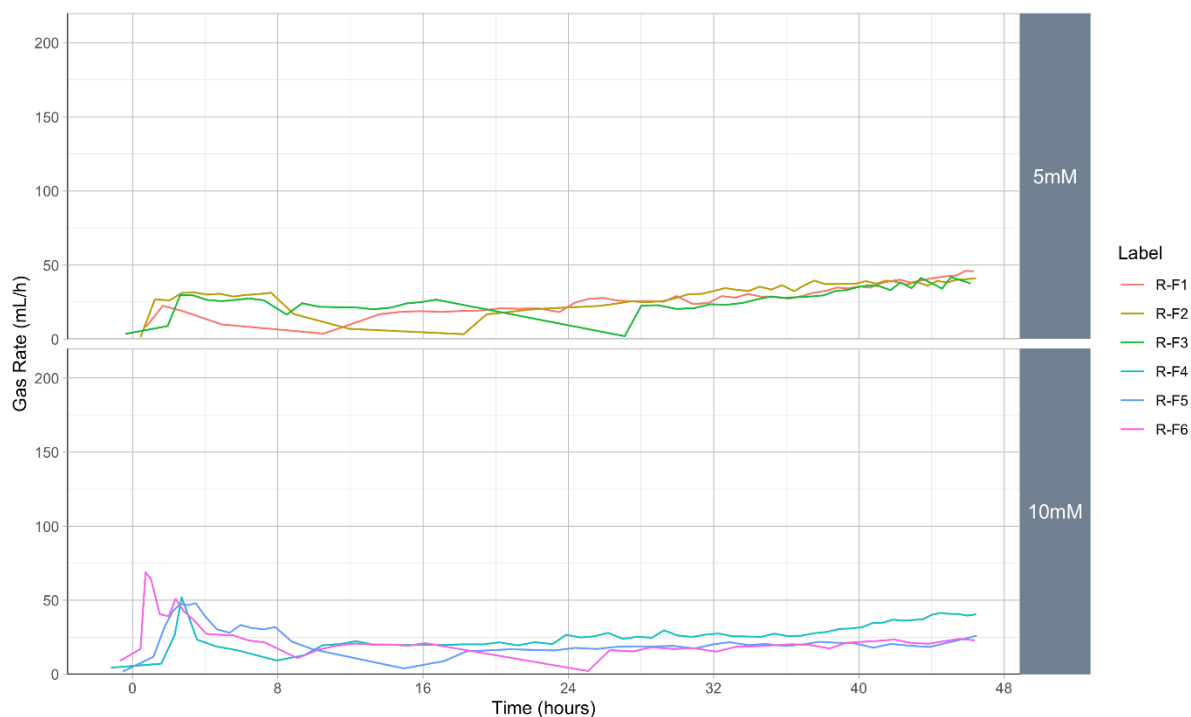


Fig.9 Effects on gas rates (mL/h) in anaerobic digesters (F1-F6) following a 5 mM and 10 mM dosage of octanoic acid over 48 h.

3.2.0 VFA concentrations

When observing the graphs of VFA concentrations, attention should be paid to the Y-axis, as the scales vary from each VFA inoculation, the use of a constant scale would cause a loss of features within the graph and cause trends to become indiscernible.

3.2.1.0 Butyric Acid Inoculation

It should be noted that during 5 mM inoculation of digester F2 at 00:05 designed to capture time points for 0 h and 8-16 h, digester F2 was not depressurised, and the intended 5 mM butyric acid dosage for the digester was ejected from the sampling port upon injection. There were no methods to discern the concentration of butyric acid ejected; therefore an additional 5 mM was delivered into the digester. Due to the scheduled timeline of this project as well as interruptions and possible interruptions of Covid-19, an additional 5 mM injection was the best way to ensure the butyric acid experimentations proceeded accordingly. From the results seen (**Fig.11[A][B]**) the butyric acid concentration introduced in digester F2 were between 5-10 mM, and digester F2 could then be seen as a replicate for digester F5 (F5 was designated to capture the same time points as F2 but instead with 10 mM butyric acid). As seen in (**Fig.11[A][B]**) the behaviour and values of VFA were analogous for F2 and F4.

3.2.1.1 Effects of 5 mM and 10 mM butyric acid inoculation on acetic acid concentrations

HOAc (Acetic Acid) concentrations (**Fig.10**) increased and stayed between 0.8-1.4 mM from 0-3 h, followed by a decrease in concentrations below 0.5 mM and remained below 0.5 mM until 6 h. An increase in HOAc concentration occurred after 6 h and continued to increase above 1.0 mM at 8 h. Subsequently from 8-17 h HOAc concentrations fluctuated between 1.0-1.5 mM. HOAc concentrations rose to 1.6 mM and 1.9 mM at 18 h and 19 h respectively and fell to 1.3 mM at 20 h. From 20-24 h HOAc concentrations began to increase.

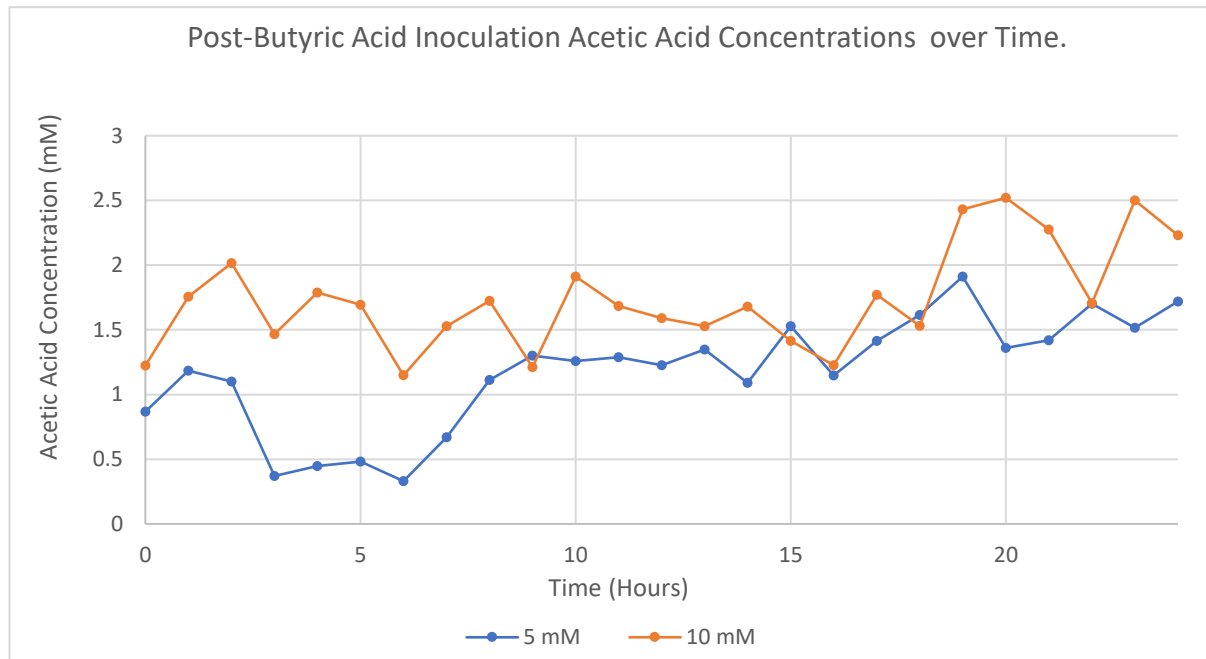


Fig.10 Graph illustrating the changes in acetic acid concentration (mM) after butyric acid inoculation over 24 h

From 0-2 h post 10 mM butyric acid inoculation HOAc concentrations started from 1.3-2.0 mM and fell to 1.5 mM from 2-3 h and remained between 1.5-2.0 mM from 3-18 h, concentrations only decreased below 1.5 mM at 6 h, 9 h, and 15-16 h, however concentrations never fell below 1 mM. An increased HOAc concentration was observed between 19-21 h and 23-24 h with concentrations ranging from 2.0-2.5 mM, however at 22 h HOAc concentration fell to 1.7 mM.

3.2.1.2 Effects of 5 mM and 10 mM inoculation of butyric acid and changes in inoculum concentration over time (Fig.11[A][B]).

Immediately following 5 mM butyric acid inoculation into digesters at 1 h butyric acid concentrations were recorded at 20 mM, a value which quadrupled the inoculation concentration (**Fig.11[A]**). From 3-7 h the butyric acid concentrations fell and remained below 5 mM, followed by an increase to 5 mM butyric acid at 9 h. From 9-17 h butyric acid concentration decreased to below 1.0 mM, from 17-20 h butyric acid concentrations fell to

below 0.5 mM, subsequently once 20 h passed butyric acid concentrations fell below detection levels. The supports the studies done previously as discussed in section. 1.2.3

Samples taken promptly after 10 mM inoculation (0 h) had butyric acid concentrations at 9.0 mM, at 1 h concentrations were recorded to be 46 mM (**Fig.11[B]**), similar to 5.0 mM butyric acid inoculum the concentration values were quadrupled one hour post inoculation (**Fig.11[A]**). Butyric acid concentration fell to 15 mM at 3 h and steadily decreased from 9-24 h butyric acid concentration remained below 10 mM and continually decreased.

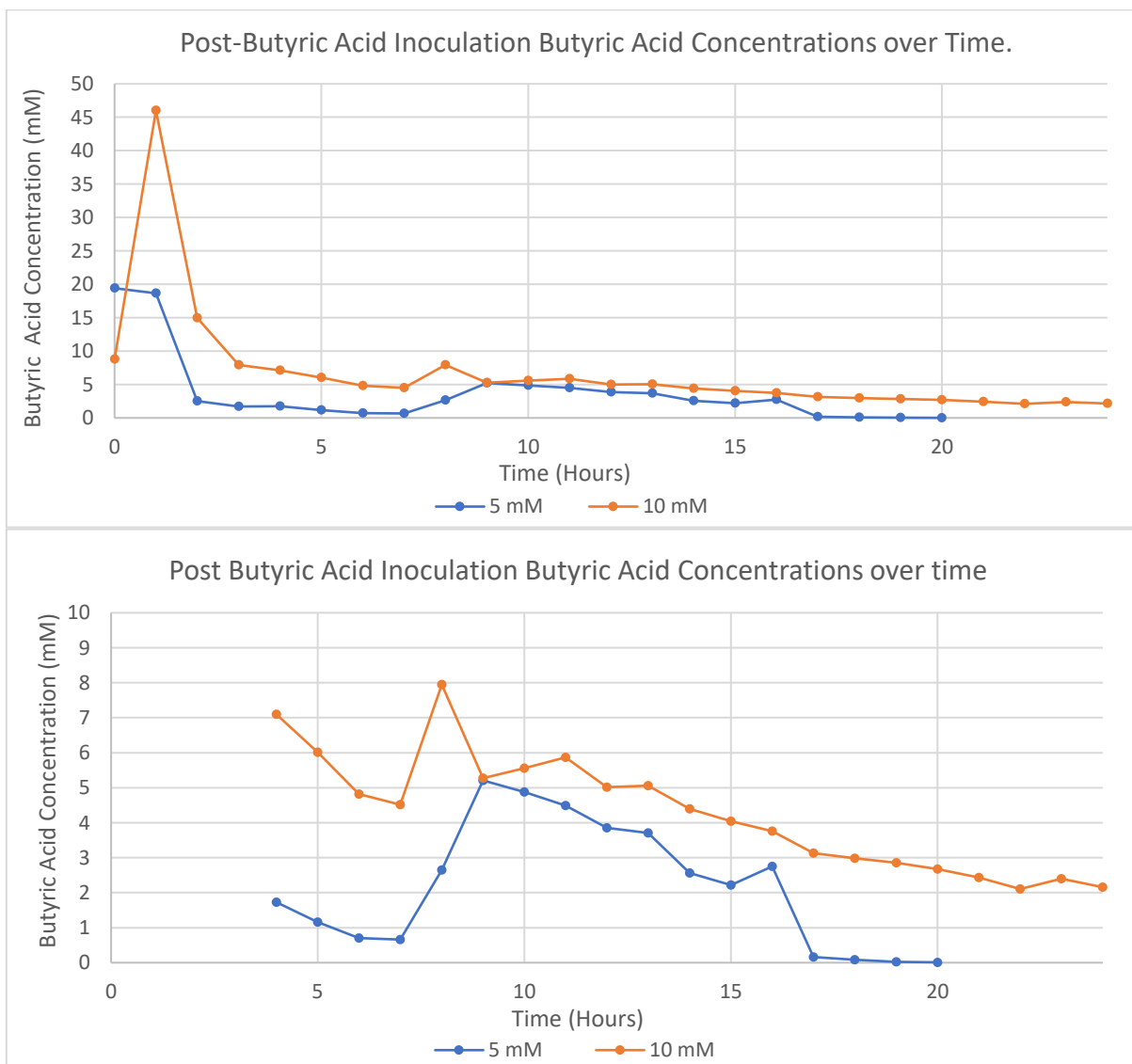


Fig.11[A] Graph illustrating the changes in butyric acid concentration (mM) after butyric acid inoculation over 24 h. **10 [B]** Graph illustrating the changes in acetic acid concentration (mM) after

3.2.1.3 Butyric Acid and Acetic Acid Concentrations Following a Lower and Higher Concentration of Butyric Acid Inoculation.

Overall for 5 mM inoculations HOAc concentrations remained between 0.3-1.5 mM and only 3 time points contained HOAc concentrations above 1.5 mM, no time points had concentrations above 2.0 mM. 10 mM inoculation HOAc concentrations predominately remained between 1.2-2.5 mM (**Fig.10**), most time points contained concentration above 1.5 mM with increased concentrations from 19 h onwards. Both 5.0 mM and 10 mM inoculated digesters had an upward trend of HOAc concentrations.

The specific trends of butyric acid concentration for both 5.0 mM and 10 mM butyric acid inoculations (**Fig.11[A]**) were obscured due to the effect of the high concentrations of butyric acid seen in the initial 3 h following inoculation affecting the y-axis scale (butyric acid concentration). When the Y-axis scaled for 4-24 h were used instead, an increase from 7-8 h were seen for both 5.0 mM and 10 mM inoculations, however 5.0 mM inoculation continued to increase until 9 h while 10 mM fell an hour after the peak at 8 h. The peak at 8 h could be due to the averaged triplicates across the three digesters inoculated with the same concentration of butyric acid; this does not account for the continued increase in butyric acid from 8-9 h for 5.0 mM inoculation.

This indicated that introduction of butyric acid up to 10mM was not inhibitive to AD processes and are efficiently degraded into acetic acid for methanogenesis. The increased HOAc over time also correlate with the gas production seen in **Fig.7**.

3.2.2.0 Hexanoic acid Inoculation

3.2.2.1 Effects of 5 mM and 10 mM hexanoic acid inoculation on acetic acid concentrations. (Fig.12)

Acetic acid concentrations were present at 0.5-1.0 mM immediately following 5 mM inoculation 0-3 h followed by an increased HOAc concentration of 1.0-1.5 mM from 3-5 h. A downward progression in HOAc concentration from 1.3 mM-0.4 mM (**Fig.12**) occurred from 5-10 h with an outlier peak of 1.3 mM at 7 h. A peak of 1.0 mM was observed at 11 h preceded by a fall to 0.5 mM at 12 h with continuation of diminished decrease in HOAc until 14 h. The decrease of HOAc alternated to an increase of HOAc after 14 h and continued until 19 h with a peak of 1.4 mM. HOAc concentrations gradually increased from 10-19 h with heightened concentration peaks at 11 h and 18 h of 1.0 mM and 1.4 mM respectively. HOAc

concentrations began to decrease at 19 h, once HOAc concentrations were below 0.5 mM from 20-24 h concentrations lingered within 0.4-0.5 mM.

Less variation in HOAc concentrations were seen in 0-8 h preceding 10 mM inoculation, HOAc remained between 0.5-0.9 mM. A sharp increase of 1.1 mM HOAc was seen at 9 h followed by a decline from 1.7 mM-0.8 mM at 9-11 h. Shortly after 11 h HOAc concentrations fluctuate within the range of 1.1-1.8 mM until 17 h. HOAc concentrations decreased at 17 h to 18 h from 1.4 mM to 0.8 mM and steadily increased for 3 h, similarly at 20-21 h another dip in HOAc occurred followed by another 3 h of steadily rising HOAc, from 23-24 h HOAc began to decrease.

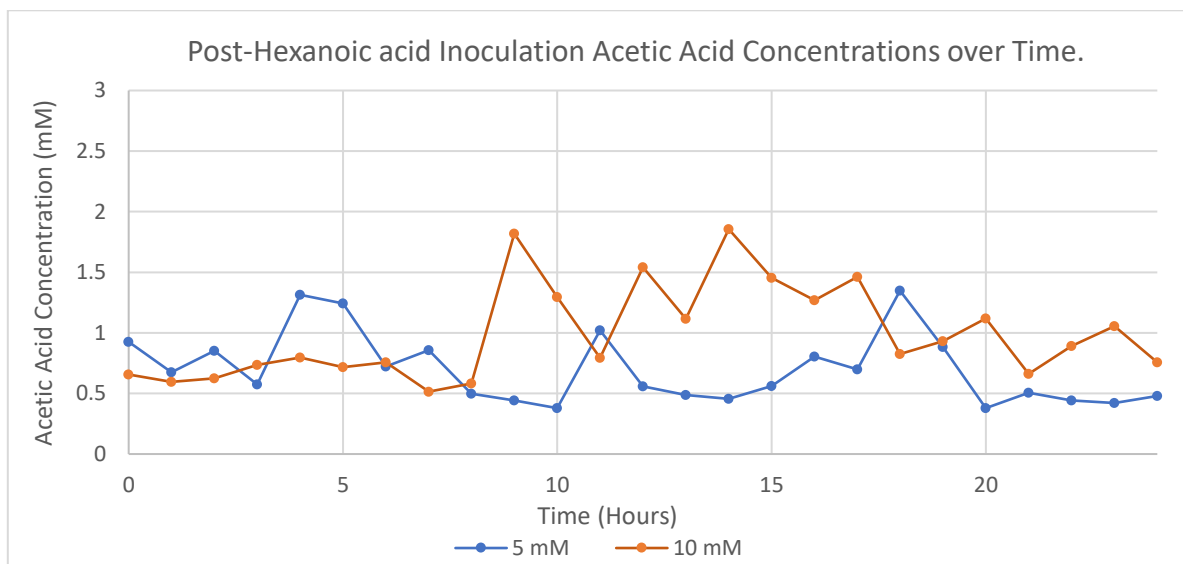


Fig.12 Graph illustrating the changes in acetic acid concentration (mM) after hexanoic acid inoculation over 24 h.

3.2.2.2 Effects of 5 mM and 10 mM inoculation of hexanoic acid and changes in inoculum concentration over time (Fig.13[A][B]).

Initial samples taken at 0 h post 5 mM inoculation had hexanoic acid concentrations six times higher than the initial 5.0 mM hexanoic acid dosage. With the exception of 2 h , hexanoic acid remained above 30 mM from 0-4 h. The graph excluding the higher hexanoic acid concentrations (**Fig.13[B]**) enabled a closer examination of changes in concentration. From 4-5 hexanoic acid concentrations dropped by 17 mM to 13.5 mM. A gradual decrease occurred from 5-6 h with a greater drop of 8 mM which took place from 6-7 h, followed by a 2 h gradual increase of 1.7 mM. Subsequent 9-24 h consisted of a slight oscillation but steady reduction in hexanoic acid concentration.

Samples taken from initial inoculation until 14 h post 10 mM inoculation were drastically higher than the 10 mM inoculation (**Fig.13[A][B]**). Samples taken immediately after inoculation resulted in hexanoic acid concentrations 19 times higher than the 10 mM injection. It was only until 6 h post inoculation where results only doubled the injection concentration. **Fig.13[B]** allowed for a better examination of the hexanoic acid degradation profile. Only samples after 14 h will be postulated upon due to the fact that samples prior to this are presumed to not be indicative of hexanoic acid degradation within the digester due to a sampling error (further discussed in the next section). From 14-15 h hexanoic acid concentrations dropped by 10 mM and another slight decrease followed by a 10 mM increase to 13 mM from 16-17 h and a decrease to 7.1 mM at 17-18 h. Samples from 18-23 h remained between 6.8-7.6 mM until 24 h where a 2 mM decrease was observed.

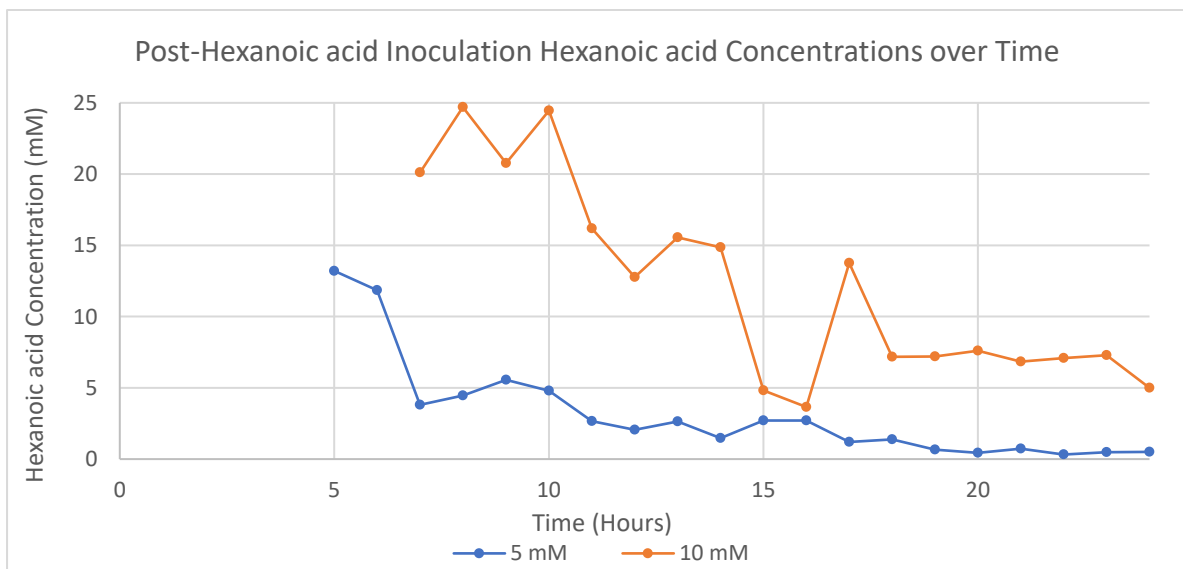
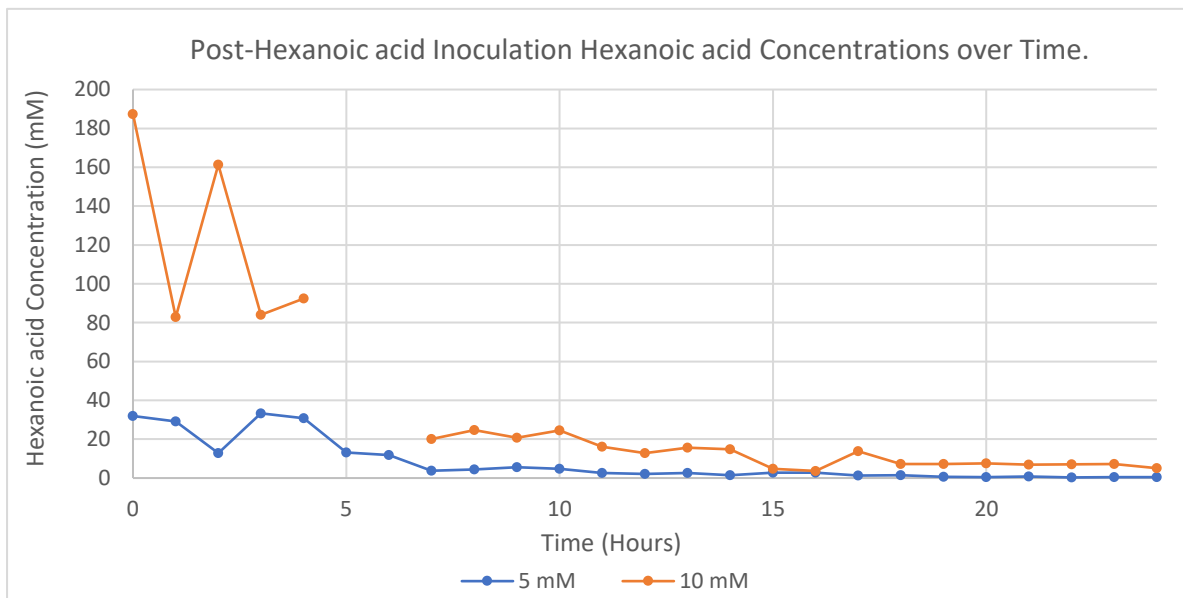


Fig.13[A] Graph illustrating the changes in hexanoic acid concentration (mM) after hexanoic acid inoculation over 24 h **[B]** changes in butyric acid concentration (mM) after butyric acid inoculation between 5-24 h

3.2.2.3 Hexanoic acid and Acetic Acid Concentrations Following a Lower and Higher Concentration of Hexanoic acid Inoculation.

The characteristic of hexanoic acid is oily and could be described as having a waxy quality. When digesters were inoculated, 10 mL pipettes were used and inserted down into the sampling port, however it caused digestate as well as VFA inoculations to be ejected out of the sampling port. Although the entire 10 mL serological pipette could have been inserted completely down the sampling port, the pipette was not inserted completely in order to circumvent the samples from being ejected. The aforementioned may have caused the viscous hexanoic acid to have stuck to the sampling port, therefore when and if the pipette was inserted into the sampling port it could have scraped some hexanoic acid and incorporated it into the sample. On the other hand, it is not uncommon for digestate to travel up the sampling port which may have caused the hexanoic acid to sit above the digestate rather than travel into the digester and become mixed. After several hours the sample as well as the digestate inside the sampling port could have been mixed or dislodged into the digester, which led to the drop in hexanoic acid concentrations. Improvements to methodologies established to mitigate the obstacles encountered will be further discussed within the discussion ([Section 3.5.1](#))

Although the sampling error may have affected the hexanoic acid degradation profile within the digesters, as hexanoic acid may have been retracted back into initial sampling post inoculation; however **Fig.13[A][B]** did indicate that some of the hexanoic acid introduced had been degraded in the digesters. 5.0 mM inoculation of hexanoic acid did not yield an increasing or decreasing trend of acetic acid concentrations, in conjunction with hexanoic acid concentrations of 24 h at 0.5 mM, this could be indicative of hexanoic acid being degraded into downstream metabolites such as butyric acid. This set of experimentations only allowed for a 24-hour frame of reference for VFA degradation of hexanoic acid in anaerobic digestion, acetic acid concentrations may have had a more similar concentration trajectory as the ones seen in digesters with butyric acid inoculum if adequate time had passed and hexanoic acid had been metabolised into butyric acid.

3.2.3.0 Octanoic Acid Inoculation

VFA concentrations for 10mM inoculations from 0-8 h (digester F4) were unable to be ascertained due to an unconfirmed GC-FID error (**Fig.14**, **Fig.15**). The error may have been caused by an extinguished flame, or an emptied gas cylinder. Samples for F4 (0-8 h) were analysed last as a preventative measure for carry-over of VFA from previous sample analysis due to the higher concentrations of VFA anticipated for samples taken for earlier timepoints. Due to F4 samples being analysed last in a set of 58 samples with varying number of washes as well as standard between each digester's samples, this would have exhausted one of the gases in the GC-FID. A line was able to be seen between 0-8 h because an average was taken for 0 h and 8 h. The efficiency of octanoic acid degradation by DMC could not be ascertained by only extrapolating HOAc and octanoic acid concentration over 24h; therefore possible downstream degradation products such as hexanoic (**Fig.15**) and heptanoic acid (**Fig.16**) were also visualised

3.2.3.1 *Effects of 5mM and 10mM octanoic acid inoculation on acetic acid concentrations*

Acetic acid concentrations were present between 0.5-1.0 mM during initial 0-4 h post 5 mM inoculation (**Fig.14**). HOAc rose to 1.6 mM between 4-6 h but decreased to 0.5 mM at 7 h, HOAc concentrations steadily increased to 1.3 mM at 7-10 h. HOAc concentrations remained between 0.5-1.0 mM from 11-24 h except for a 1.6 mM peak found at 22 h.

10mM inoculations of octanoic acid did not yield a predictable trend for acetic acid concentrations, from 8-10 h HOAc concentrations decreased and from 10-12 h concentrations increased. Data points for 13 h HOAc concentration were not acquired, however from 12-14 h HOAc concentrations had minimal fluctuations. From 14-15 h a decreased concentration occurred followed by an increased HOAc at 15-16 h. HOAc rose sharply to 2.8 mM at 17 h and decreased to 0.8 mM by 18 h an increase of 0.4 mM occurred, followed by 20-23 h HOAc concentrations which remained between 0.5-1.0 mM until 24 h where a decrease to 0.1 mM. This suggests that octanoic relative to butyric and hexanoic acid degradation was not as efficient, and a lag-period would be present in AD upon introduction of fatty acid chain lengths greater than 8 carbons.

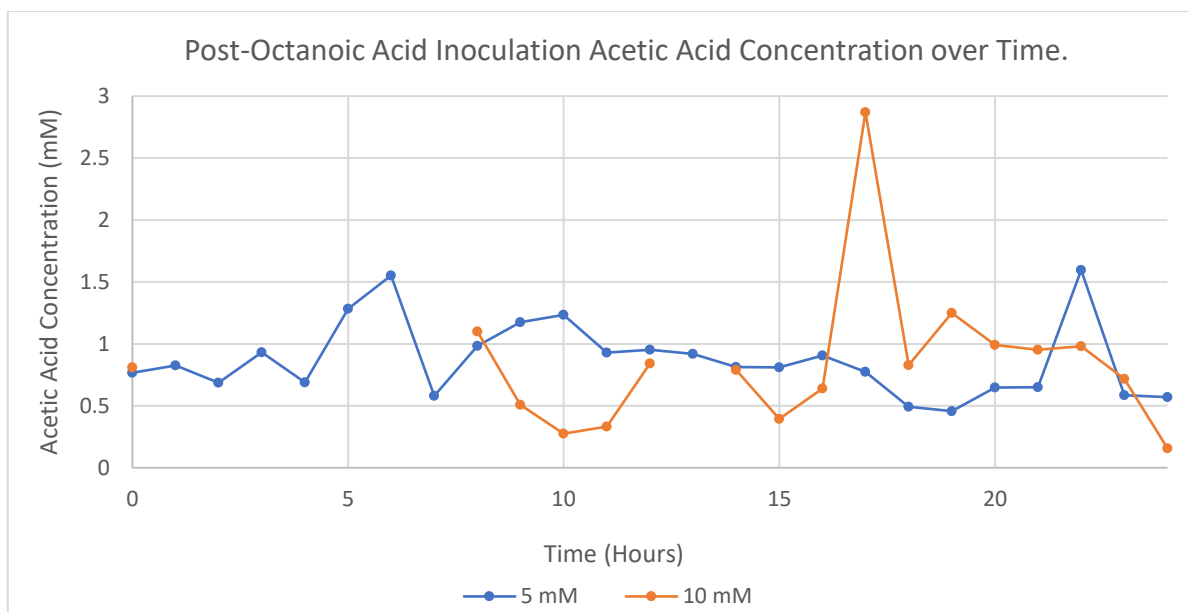


Fig.14 Graph illustrating the changes in acetic acid concentration (mM) after octanoic acid inoculation over 24 hours

3.2.3.2 Effects of 5mM and 10mM octanoic acid inoculation on hexanoic acid concentrations

An average of 0.2 mM was seen for hexanoic acid concentrations following 5 mM octanoic acid inoculation (**Fig.15**), this may be residual of previous hexanoic acid inoculation 2 weeks prior to octanoic acid experimentations. Hexanoic acid concentrations remained approximate to 0.2 mM until 4 h, apart from a dip to 0.07 mM at 3 h. An increased concentration to 0.3 mM was observed at 5 h and hexanoic acid concentrations remained at 0.3 mM until 7 h. Subsequently hexanoic acid concentrations began to incrementally increase by 0.1 mM to 0.7 mM at 10 h and remained at 0.7 mM until 11 h followed by an increase to 0.9 mM at 12 h then a decreased concentration towards 0.8 mM at 14 h and remained until 15 h. A sharp increase to 1.5 mM occurred at 16 h followed by a precipitous decrease at 17 h to 0.1 mM, subsequently at 18 h concentration rose back towards 0.7 mM and began to increase towards 1.4 mM from 18-23 h, at 23-24 h hexanoic acid began to decrease.

Hexanoic acid concentrations following 10 mM from 0 h and 8 h were recorded to be under 0.1 mM, from 8-10 h concentrations increased towards 0.3 mM followed by concentrations which remained approximate to 0.2 mM from 11-12 h, a dip towards 0.1 mM at 13 h after which hexanoic acid levels began to increase towards 0.7 mM from 13-22 h with a deviation of a small dip seen 18 h. A peak of 1.1 mM was detected at 23 h with a subsequent dip back to 0.7 Mm. Although hexanoic acid concentrations gradually increase from 0 h, a more visible increasing trend in hexanoic acid concentrations following both concentrations

of octanoic acid inoculation was seen after 8 h. This suggests although octanoic acid may be inhibitive to DMC, octanoic acid degradation occurs approximately 8 h following inoculation. The lower concentration of octanoic acid (5 mM) showed a higher capability for degradation by DMC relative to 10 mM.

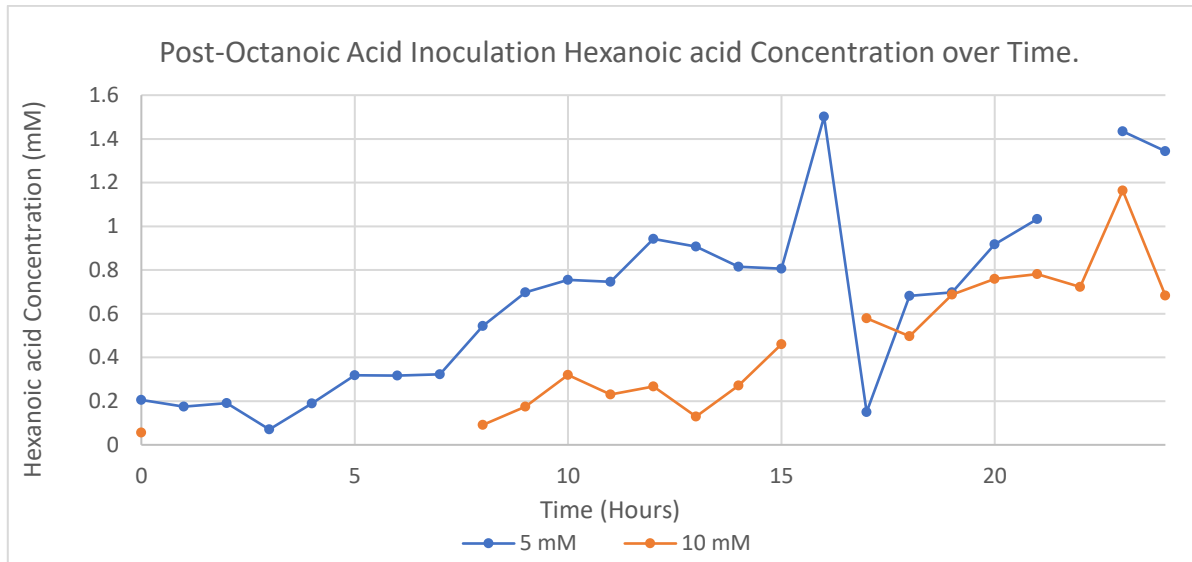


Fig.15 Graph illustrating the changes in hexanoic acid concentration (mM) after octanoic acid inoculation over 24 hours

3.2.3.3 Effects of 5mM and 10 mM octanoic acid inoculation on heptanoic acid concentrations

Post 5 mM octanoic acid injection, no notable shifts in heptanoic acid concentrations were observed from 0-15h as concentrations remained between 0.05-0.2 mM with a deviation at 8 h where heptanoic concentrations were recorded at 0.6 mM (**Fig.16**). A peak was observed at 16 h where concentrations were registered at 1.6 mM next a decrease in concentration to 0.3 mM from 16-18 h. Heptanoic concentration time points were unable to be ascertained after 18 h except for 23 h where the concentration was 2.0 mM.

Digesters inoculated with 10mM octanoic acid had heptanoic concentrations that were not dissimilar to the pattern observed in digesters inoculated with 5 mM octanoic acid, from 0-15 h concentrations remained between 0.05-0.2 mM (with the exclusion of sample concentration at time points 1-7 h), a peak of 1.9 mM at 16 h similar to 5 mM inoculation was seen, similarly the concentration decreased back down to 0.5 mM where it remained from 16-22 h, subsequently from 22-24 h heptanoic acid concentration began to rise and concentrations were recorded to be 1.5 mM at 24 h. Heptanoic concentrations were not detectable for certain time points, and the trend for heptanoic acid concentrations **Fig.16**

were not as discernible as the trend observed in hexanoic acid concentrations **Fig.15**. It is possible for heptanoic acid concentrations to rise after 18 h post octanoic acid inoculation (versus the 8 h seen in **Fig.15**), however without the concentrations for 18-23 h post 5 mM octanoic acid inoculation it would not be guaranteed.

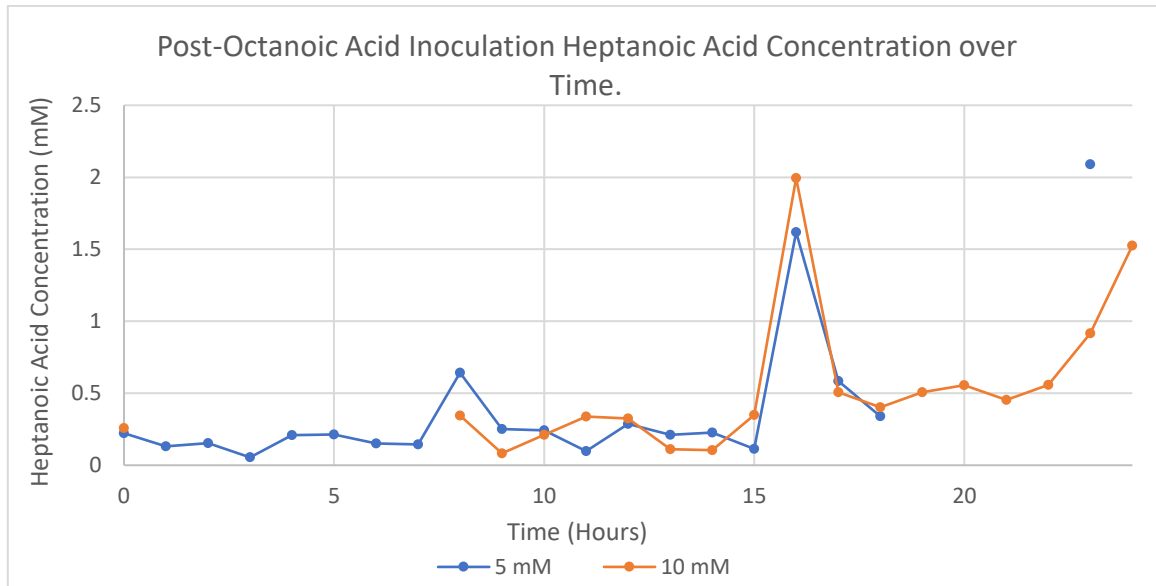


Fig.16 Graph illustrating the changes in heptanoic acid concentration (mM) after octanoic acid inoculation over 24 hours

3.2.3.4 Effects of 5 mM and 10 mM octanoic acid inoculation on octanoic acid concentrations

Post 5 mM octanoic acid inoculated digesters yielded a 0 h sample with 0.2 mM octanoic concentration which indicated that the inoculum was not drawn back into the pipette conversely it could also mean rather than the inoculum travelling into the digester it remained in the sampling pipe (**Fig.17**). 1-2 h had concentrations of 3.7 mM followed by a dip in concentration to 1.9 mM at 3 h, an increase to 5.5 mM by 4 h, then an incremental decrease of approximately 1 mM occurred until it reached 2.9 mM 7 h. At 8 h the concentration rose slightly to 4.6 mM followed by a sharp increase towards 14.0 mM by 9 h. A slight decrease shortly at 9 h then a greater decrease at 11 h of 4.1 mM, another sharp increase towards 11.0 mM at 12 h and a drop back down to 5.2 mM at 13 h where it remained near 5.0 mM for another hour. Decreased concentrations continued for an addition two hours until 16 h, an upsurge in concentration from 3.0 mM to 12 mM was detected at 17 h followed by a downturn in concentration to 2 mM where it remained until 20 h, however octanoic acid concentration were not extrapolated for 19 h. From 20-22 h concentrations decreased to 0.3

mM and after 22 h concentrations began to increase; concentrations were recorded at 2.3 mM at 24 h.

10 mM inoculation was observed to be less sporadic than the pattern seen for 5 mM inoculation and concentrations reported do not exceed the initial 10mM introduced into digesters. A decrease from 5.2 mM to 2 mM occurred at 8-9 h, an increase towards 6 mM happened in between 9-12 h. A decrease to 4.0 mM was recorded at 13 h, concentrations oscillated between 2.7-4.0 mM until 16 h. From 16-17 h an increase was recorded, and it remained at 5.7 mM until 18 h. An increase to 8.6 mM happened at 19 h, subsequently from 20 h octanoic acid concentrations began a downward trend with a concentration of 4.6 mM at 24 h. The peaks in octanoic acid concentration following 5 mM inoculations (**Fig.17**) indicates that an error may have occurred during inoculation (a fraction of inoculum was stuck within sampling port and sampled); hence the trends are not easily discernible. The concentrations of heptanoic (**Fig.16**) and hexanoic acid (**Fig.15**) therefore indicates that octanoic acid was introduced to DMC and subsequently degraded.

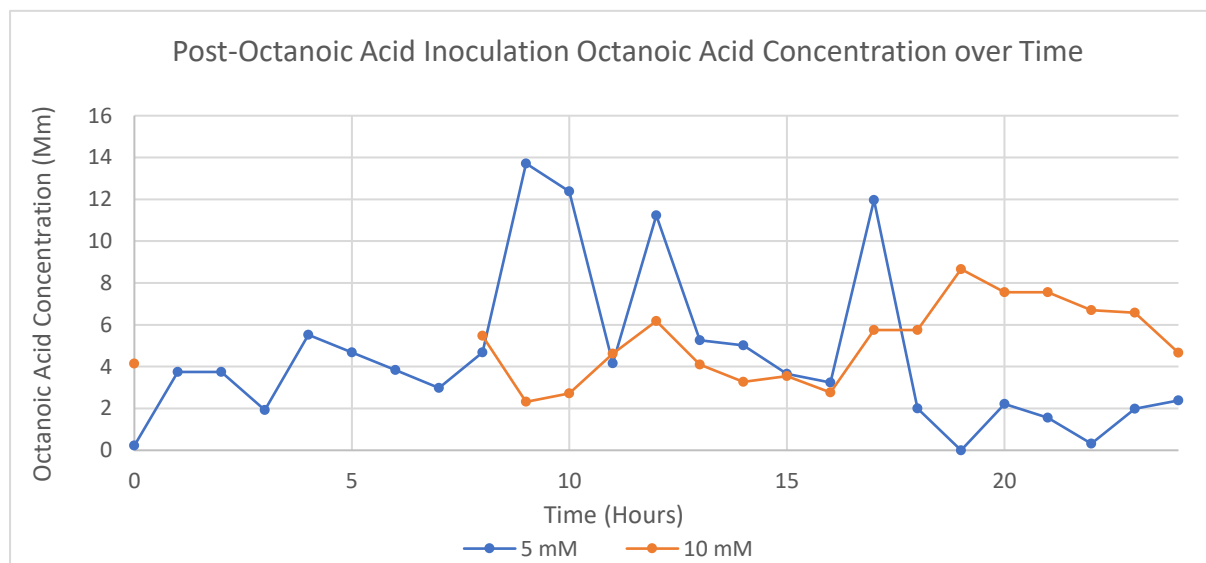


Fig.17 Graph illustrating the changes in octanoic acid concentration (mM) after octanoic acid inoculation over 24 hours

3.2.3.5 Volatile fatty acid concentrations following lower and higher concentrations of octanoic acid inoculation.

The incorporation of a graph of the butyric acid concentration over time following octanoic acid inoculation may have been advantageous, but the concentrations were all below 0.08 mM. This may not be indicative of an absence of butyric acid due to the rapid

turnover of butyric acid into isobutyric or acetic acid²⁴. 5 mM octanoic acid following inoculation had a sporadic degradation profile, the octanoic acid concentrations which were higher than the initial inoculation indicates that a partial concentration of octanoic acid was mixed into the digestate while some remained within the sampling port, the peaks seen may correspond with octanoic acid being dislodged from the sampling port in subsequent samplings.

The presence of heptanoic acid concentrations may be indicative of anabolism as VFA degradation occurs by cleaving 2 carbons off a fatty acid chain, therefore only hexanoic acid would be expected contrary to the heptanoic acid present. Another possibility could be microorganisms which would normally degrade octanoic acid through β -oxidation have been inhibited, therefore a separate microorganism not affected by the octanoic acid has begun degradation of octanoic acid through a different mechanism. It was also postulated that the detection of heptanoic acid may be caused by the cross contamination of the standard onto the syringe and thereby the water used for washes conducted between GC-FID runs. The peaks seen at 24 h, 16 h, and 8 h may be indicative of the cross contamination as samples were analysed in GC-FID in an inverse sequence and in sets of 24-17 h, 16-9 h, and 8-0 h, with a run of the standard between each group of time points.

Following analysis of standards in GC-FID three washes and runs were conducted with Milli-Q purified water. Nevertheless peaks of heptanoic acid were seen in 24 h, 16 h, and 8 h samples; the analysis of these samples was conducted immediately after the washes following standard analysis. Heptanoic acid concentration in digesters inoculated with 5mM octanoic acid may have been caused by a contamination of the water used for washing, however the 8h heptanoic acid peak was not as high as 16h and 24h, if it were a contamination caused by the standard a similar peak would be expected at 8h. This pattern of contamination was not seen in other sample analysis, or with any other VFAs, without further experimentation it could not be determined whether the presence of heptanoic acid was due to contamination or a biological cause.

3.2.4.0 Inoculation of anaerobic digesters with fatty acids.

Values quadrupling the initial inoculation concentrations could be due to an inoculation and human error, these higher than introduced concentrations of VFA were also seen with hexanoic acid. These results allowed an emphasis that while sampling, pipettes should be inserted entirely and the contents within the sampling port be pipetted and ejected into the digester. The trend seen with hexanoic acid may be due to hexanoic acid which remained in the sampling port. Although sampling errors had occurred the effect of VFA inoculations into anaerobic digesters on gas production can still be observed with the presumption that concentrations approximating the intended inoculation were still introduced into Digestate. Digesters inoculated with butyric acid showed decreased butyric acid concentration over time (**Fig.11[A][B]**) with increased amounts of acetic acid over time (**Fig.10**), acetic acid is a precursor to methanogenesis, these results complement the gas production seen (**Fig.7**). Both hexanoic acid and octanoic acid decreased over time, however it was not as effectively metabolised as butyric acid, and acetic acid did not exhibit an increased or decreased concentration over time. Gas production for both hexanoic and octanoic acid concentrations are comparable to their respective VFA concentration profiles, they both indicate that in order to obtain a more complete understanding of their effects, a longer time frame of sampling and observation must be conducted.

3.3.0 Chemical oxygen demand

Chemical oxygen demand (COD) is 'a measure of the oxygen equivalent of the organic content of a sample that is susceptible to oxidation by a strong chemical oxidant'^[24]. Chemical oxygen demand is normally deployed to measure the number of organic pollutants present in water, in our experimentation COD was utilised in conjunction with VS% to determine the change in biomass within the digesters. COD results did not exclusively reflect the amount of biomass within digesters as an inoculum of VFAs was introduced. VFAs also contain oxidisable carbons (**Table.3[A][B]**) therefore COD is not a direct measurement of the biomass within digesters. However as seen in **Table.3[A]** the COD of each fatty acid introduced to digesters are negligible as the values are all below 0.05 O₂ g/L while the COD measured from samples were all recorded to be above 10 O₂ g/L. The COD would not be expected to rise drastically over time, as the utilisation of inoculum by microbial organisms would decrease oxidisable carbon of fatty acids, this however would increase biomass which would lead to an increase in COD, therefore COD would be expected to remain stable or steadily increase slightly. The sampling

error which affected VFA concentration results would also affect COD results, as the samples taken were aliquoted after sampling and sampling did not occur independently for individual assays.

| Inoculum | Concentration (mM) | Chemical Equation | Chemical Oxygen Demand (O ₂ g/L) |
|---------------|--------------------|-------------------|---|
| Butyric Acid | 5 | (a) | 0.009 |
| Butyric Acid | 10 | (a) | 0.018 |
| Hexanoic Acid | 5 | (b) | 0.011 |
| Hexanoic Acid | 10 | (b) | 0.022 |
| Octanoic Acid | 5 | (c) | 0.012 |
| Octanoic Acid | 10 | (c) | 0.024 |

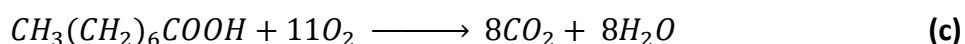
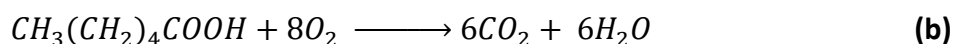
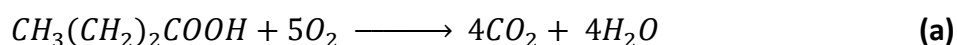


Table 3 [A] A table showing the inoculum and the concentrations used, alongside corresponding chemical equation (**[B]**) and the chemical oxygen demand for each inoculum. **[B]** Chemical equation to illustrate the chemical oxygen demand of each inoculum throughout experimentation.

3.3.1 Effects of 5 mM and 10 mM butyric acid inoculation on chemical oxygen demand.

Post inoculation O₂ g/L from 0-3 h O₂ g/L decreased to below 20 O₂ g/L and began to rise from 3-5 h. From 5-14 h COD oscillates between 20-25 O₂ g/L, a spike of approximately 7 O₂ g/L occurred from 14-16 h followed by COD oscillations within 20-25 O₂ g/L. O₂ g/L dropped below 20 O₂ g/L at 23 h post inoculation and began to rise to approximately 25 O₂ g/L at 24 h.

Following 10 mM butyric acid inoculation COD an increase of O₂ g/L can be seen from 0-1h, 1 h post inoculation O₂ g/L decreases from 1-3 h comparable to 5 mM inoculation. Following the reduction in COD similar to 5 mM inoculations O₂ g/L began to rise, in contrast to pattern seen in 5 mM inoculation, O₂ g/L rose to nearly 35 O₂ g/L. Subsequently COD drops to 20 O₂ g/L at 9 h, followed by oscillations between 9-14 h A peak of 6 can be seen from 14-16 h. At 16 h COD rose by 12.5 O₂ g/L and remained above 30 O₂ g/L until 19-21 h

where COD dropped to 22 O₂ g/L. From 21-23 h a peak of 34 O₂ g/L occurred and fell to 24 O₂ g/L. Similar to 5 mM inoculation COD began to rise after 23 h to 27 O₂ g/L.

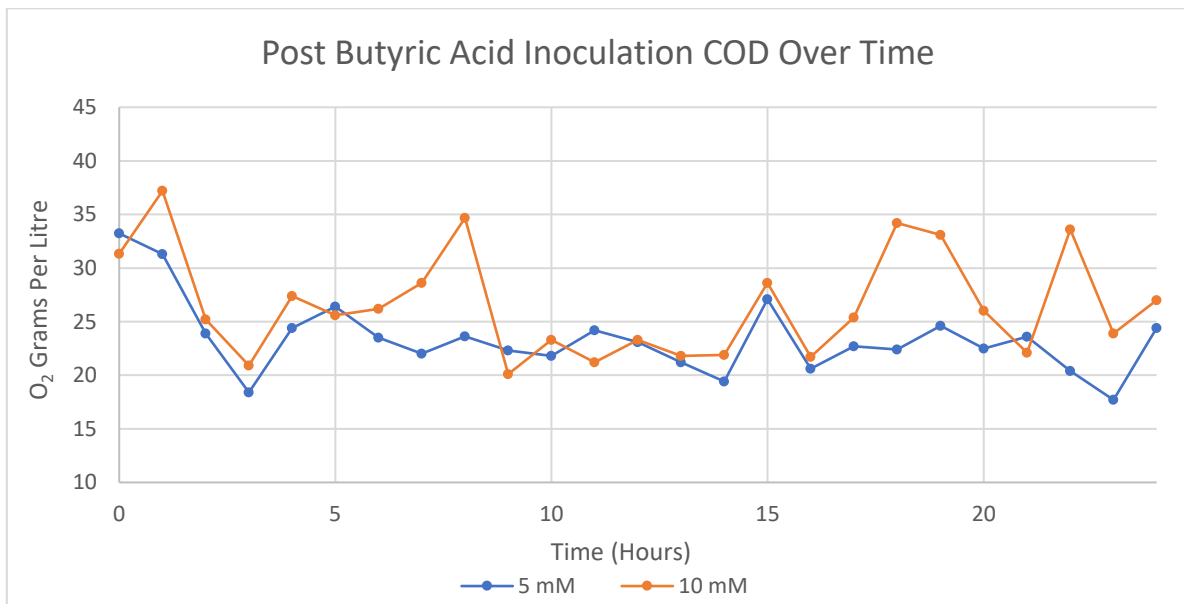


Fig.18 Changes in chemical oxygen demand O₂ g/L of digestate inoculated with butyric acid over 24 hours.

3.3.2 Effects of 5 mM and 10 mM hexanoic acid inoculation on chemical oxygen demand.

COD of digestate in digesters inoculated with 5mM hexanoic acid (**Fig.19**) did not have values above 26 O₂ g/L, an increase from 22 O₂ g/L at 0h to 26 O₂ g/L at 1 h was followed by continuous decrease towards 11 O₂ g/L at 5 h. COD began to increase until 8 h where it reached 23 O₂ g/L, from 9-10 h COD remained at approximately 20 O₂ g/L and decreased to 13 O₂ g/L at 11 h, from 11-15 h COD increased towards 26 O₂ g/L followed by a minor decrease to 23 O₂ g/L at 16 h. COD at 17-24 h steadily remained between 24-26 O₂ g/L apart from 19 O₂ g/L at 20 h.

The highest COD was observed for digestate inoculated with 10 mM hexanoic acid at 0 h, it had a COD of 44 O₂ g/L (**Fig.19**). One hour post inoculation COD decreased down to 26 O₂ g/L, then increased to 33 O₂ g/L at 2 h. COD was reduced to 17 O₂ g/L at 3 h and COD remained between 16-19 O₂ g/L until 5 h. COD reached 25 O₂ g/L at 6 h, from 6-13 h COD remained between 19-28 O₂ g/L. COD exhibited 34 O₂ g/L at 14 h a drop to 19 O₂ g/L at 15 h a smaller increase at 16 h to 26 O₂ g/L, COD dropped back down to 20 O₂ g/L at 17 h and an increase back to 34 O₂ g/L at 18 h. Subsequently at 18 h COD was continually reduced to 11 O₂ g/L, at 22 h COD was recorded at 19 O₂ g/L, followed by a slight increase to 21 O₂ g/L at 23 h and an increase to 26 O₂ g/L at 24 h.

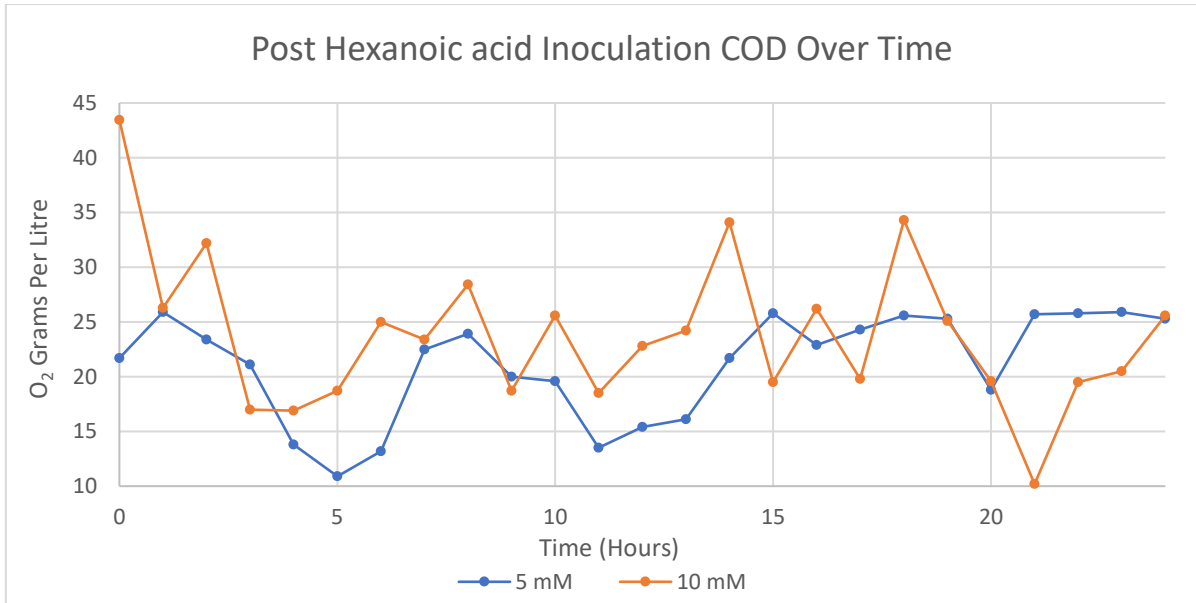


Fig.19 Changes in chemical oxygen demand O₂g/L of digestate inoculated with hexanoic acid over 24 hours.

Overall digester inoculated with 5 mM hexanoic acid had a COD which did not surpass 26 O₂ g/L with drops in COD, whereas 10 mM inoculated digestate exhibited more drastic peaks and troughs. The pattern of COD seen with 10 mM inoculation may have been influenced by a sampling error.

3.3.3 Effects of 5 mM and 10mM octanoic acid inoculation on chemical oxygen demand.

Digestate inoculated with 5 mM octanoic acid yielded a COD of 32 O₂ g/L at 0 h, followed by a decrease towards 21 O₂ g/L from 1-2 h, COD then fluctuated between 21-24 O₂ g/L from 2-5 h. COD decreased to 19 O₂ g/L at 5-7 h, then increased until it reached 27 O₂ g/L at 10 h. COD dropped at 11 h to 17 O₂ g/L then increased to 25-27 O₂ g/L where it remained from 12-13 h until COD dropped back down to 17 O₂ g/L at 14 h. An increased COD of 24 O₂ g/L occurred at 15 h, COD remained between 20-24 O₂ g/L for 3 h until COD dropped to 8 O₂ g/L at 18 h followed by an increase back towards 22 O₂ g/L at 19 h. COD began to decrease from 19 h for 3 hours until COD began to increase at 21 h until it reached 27 O₂ g/L at 22 h, after the peak at 22 h COD began to decrease until it reached 18 O₂ g/L at 24 h. A pattern was observed for digestate inoculated with 5 mM octanoic acid, a drop in COD seemed to be followed by an increased COD which lasted from one to three hours which would subsequently lead to another drop in COD.

Immediately after digestate was inoculated with 10 mM octanoic acid, a COD of 26 O₂ g/L was observed (0 h) followed by a decrease to 18 O₂ g/L at 1 h. From 2-3 h COD increased

towards 23 O₂ g/L at 3 h. COD was reduced to 16 O₂ g/L at 4 h and rose to 32 O₂ g/L at 6 h followed by a decreased COD of 23 O₂ g/L at 7 h. COD remained between 22-24 O₂ g/L from 7-10 h, COD rose towards 28 O₂ g/L and remained between 24-28 O₂ g/L from 11-16 h. At 17 h COD was 31 O₂ g/L, from 17-19 h, COD decreased to 19 O₂ g/L and increased until it reached 23 O₂ g/L at 21 h followed by a reduction of COD for 2 h down to 16 O₂ g/L. The final COD value taken was 23 O₂ g/L at 24 h. COD appeared to be higher overall in digestate inoculated with 10 mM octanoic acid when compared to 5 mM octanoic acid inoculations, however 5 mM inoculated digestate had greater COD values in the initial 5 h. Gas data (**Fig.9**) and fatty acid concentrations (**Fig.17**) indicated that octanoic acid may be inhibitive to DMC however **Fig.20** did not illustrate any drastic decreases of COD not followed by an increase in COD; this could be indicative of inhibition and not bactericidal effects of octanoic acid to DMC.

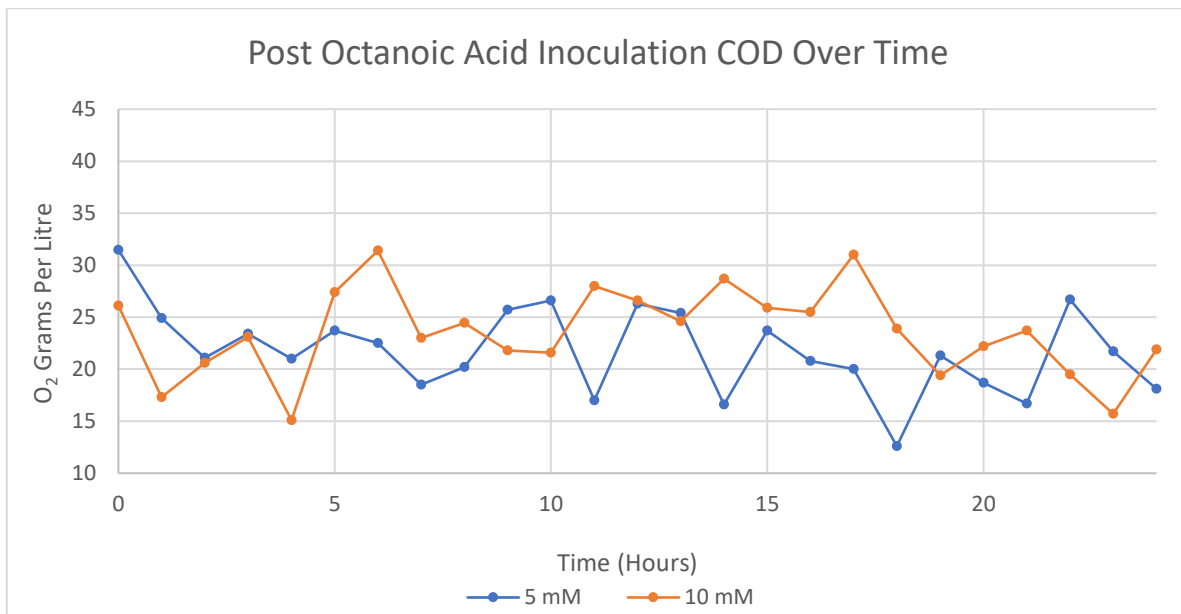


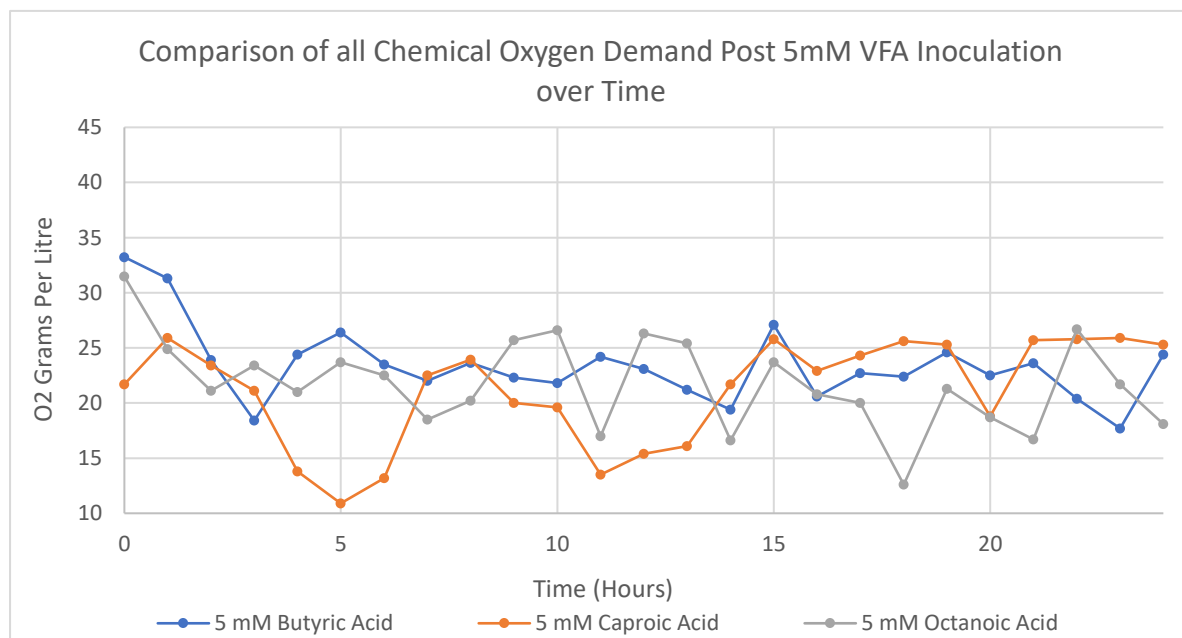
Fig.20 Changes in chemical oxygen demand O₂/g/L of digestate inoculated with octanoic acid over 24 hours.

3.3.4 Changes in chemical oxygen demand of digestate in anaerobic digesters inoculated with 5 mM and 10 mM butyric, hexanoic, and octanoic acid.

Digesters inoculated with 10 mM of VFA yielded COD values greater than digestate inoculated with 5 mM of VFA (**Fig.18-20**). Most samples collected from 10 mM VFA inoculations had COD values between 20-30 O₂ g/L and samples which reached COD values greater than 30 O₂ g/L; whereas most samples collected from 5 mM fatty acid inoculations had a COD of 20-25 O₂ g/L Only 3 samples from digesters inoculated with 5 mM fatty acid obtained values greater than 30 O₂ g/L which were only observed in initial time points

(Fig.21[A][B]). COD results for hexanoic acid inoculations produced samples with the largest increased and decreased COD across all fatty acid inoculums; in contrast to butyric (Fig.18) and octanoic acid (Fig.20) which remained relatively steady for 24 h. The stable and/or the drop in COD followed by an increase trend in Fig.18, and Fig.20 could indicate that biomass within the digester were not severely influenced (growth or decay) bar the inoculum except for inhibition cause by octanoic acid.

Abrupt peaks and drops seen in COD results for hexanoic acid (Fig.19, Fig.21[A][B]), relative to butyric and octanoic acid may not accurately reveal the oxidisable organics or biomass present in digestate. Samples were frozen following sampling as analysis could not be carried out on the day of sampling, digestate that were frozen and defrosted tended to form a layer of solids at the bottom and liquid on top. If samples had not been shaken rigorously, adequate mixing would not have occurred and therefore inconsistently low or high COD samples would have been recorded. Another factor which impacts hexanoic COD is the viscid/sticky, and waxy nature of hexanoic acid which may have clung to the inside of the sampling port, therefore when sampling pipettes may have scraped any residual hexanoic acid within the sampling port leading to the drastic peaks and troughs seen in Fig 19 or Fig.21[A][B].



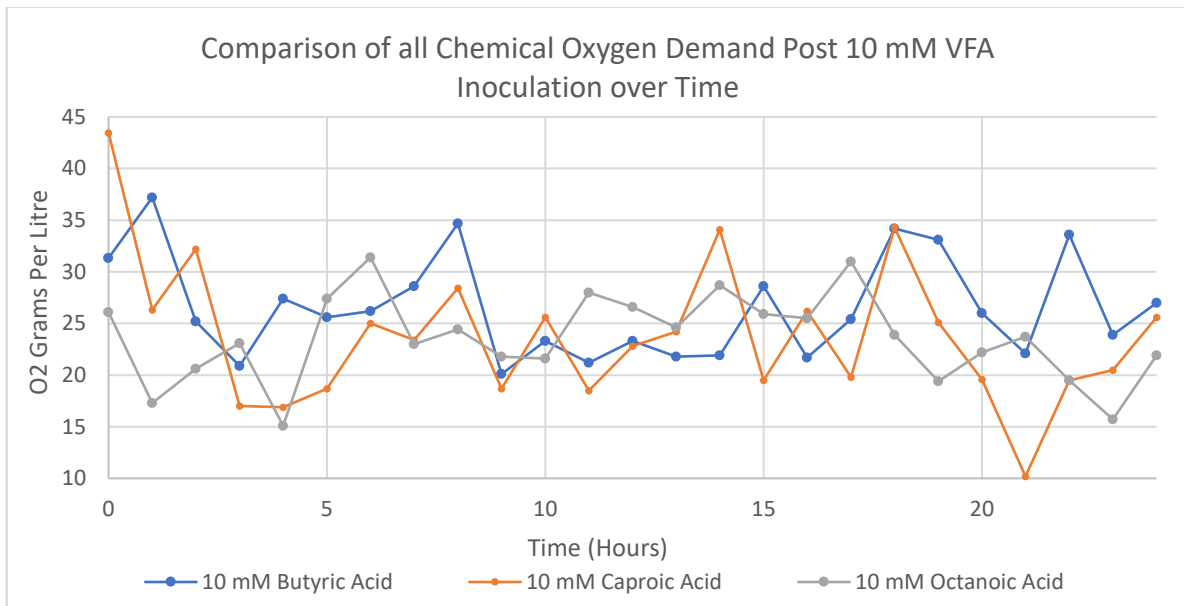


Fig. 21[A][B] Changes in chemical oxygen demand O₂g/L of digestate inoculated with 5mM and 10Mm[B] butyric, hexanoic, and octanoic acid over 24 hours.

3.4.0 Volatile solids percentage

The DMC is starved prior to each experiment so we can infer that the increase in organics is associated with an increase in biomass and the fatty acid used to inoculate digesters [F]. COD + VS% stay at a baseline, however fatty acid concentrations begin to decrease; this could be due to an increase in biomass while fatty acids were degraded.

3.4.1 Effects of a 5 mM and 10 mM inoculation of butyric acid on volatile solids percentage in anaerobic digestion.

Immediately following inoculation of 5 mM and 10 mM butyric acid (0 h) to digesters volatile solids % (VS%) values were both ≈1.20%, however a drop to 0.79% followed by a rise to 1.21% was observed in digesters with 5 mM butyric acid at 0-1 h and 1-2 h respectively (**Fig.22**). At 2 h and 3 h post inoculation VS% was similar for both 5 mM and 10 mM digesters. A parallel trend was detected in VS% was from 3-8 h in both dosages of butyric acid, however digesters with the lower butyric acid dosage had lower VS% (**Fig.22**).

From 8-9 h post 5 mM inoculation of butyric acid VS% reached 1.47%, from 9-11 h VS% remained between 1.40%-1.47%, a sharp increase to 1.65% and a drop to 1.02% were observed at 12 h and 13 h respectively. Following the drop to 1.02% at 13 h VS% rose to 1.59% from 13-15 h. Following the peak at 15 h, VS% decreased to 1.26% at 16 h. VS% steadily increased until 19h when 1.70% was reached, upon reaching 1.70%, VS% began to decrease

towards 1.18% until 21 h. At 22 h VS% peaked at 1.87% and fell to 1.04% and 1.43% at 23 h and 24 h respectively.

From 8 h onwards digestate with 10 mM inoculum had VS% decrease to 1.12% at 9 h then an increase to 1.40% at 10 h, following the increase VS% continually decreased until it reached 1.01% at 11 h remained at \approx 1.00% until 13 h. At 13 h post inoculation VS% began to rise to 1.30% at 15 h followed by a decrease to 1.16% from 15-17 h. The greatest VS% fluctuations occurred between 17-24 h the reasoning for these fluctuations were not clear. 17-24 h with 10 mM inoculum were captured by digester F6, there were no readily apparent cause for this as no problems were encountered with F6. Conclusions drawn from VS% of 17–24 h in 10 mM butyric acid inoculated digesters would be ill advised unless this experiment as well as the fluctuations seen were repeated.

Although there were discernible differences in VS% of digesters inoculated with 5 mM and 10 mM butyric acid, there are some points in which the VS% overlap, such as 2-3 h, and 13-14 h. There have also been time points where VS% of both dosages are close to one another such as 10 h, 16 h, 22 h, and 24 h. Overall VS% for both 5 mM and 10 mM butyric acid in digester appear to have slight increases in VS% over 24 h.

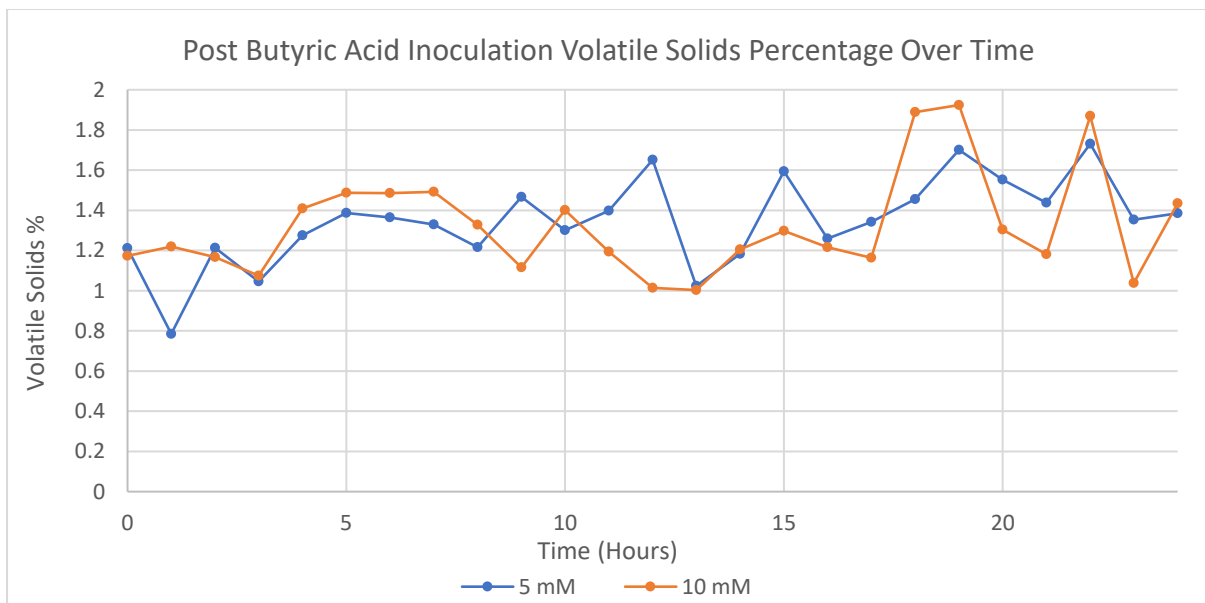


Fig.22 Changes in volatile solids % of digestate inoculated with butyric acid over 24 hours.

3.4.2 Effects of a 5 mM and 10 mM inoculation of hexanoic acid on volatile solids percentage in anaerobic digestion

Initial VS% (samples taken immediately following hexanoic acid inoculation) were lower in 5 mM inoculations than 10 mM inoculations; after 1 h, digesters inoculated with 5 mM hexanoic acid remained greater than VS% of 10 mM hexanoic acid inoculation until 4 h. Both digesters dosed with 5 mM and 10 mM had a decreasing trend of VS% until 4 h (**Fig.22**).

After 3 h digesters with a 10 mM dosage sharply rose to 1.21% and remained between 0.86%-1.21% until 9h where VS% increased to 1.46% at 10 h. Following the peak at 10 h VS% fell to 0.99% where it remained from 11-12 h. Subsequently from 12-13 h VS% increased to 1.39%, following this peak VS% remained between 1.10-1.36% until 20 h. Samples for VS% beyond 20 h for 10 mM hexanoic acid inoculations were lost therefore the data points are missing.

4 h following inoculation of digesters with 5 mM butyric acid, VS% slightly increased (relative to 10 mM) from 0.48%-0.66% then decreased back to 0.48% from 4-6 h. The rapid increase mirroring the increase seen at 3-5 h for 10 mM inoculation were seen at 6-8 h for 5 mM hexanoic acid inoculation. Following the rise to 1.39% at 8 h, values began to decrease after 8h and continued decrease until it reached 0.70% at 12 h; the steepest decrease occurred between 9-10 h. VS% began to increase towards 1.46% from 12-14 h. Following this increase VS% steadily remained between 1.39%-1.46% from 14-17 h. Another rise and fall in VS% were noted from 17-20 h, a peak of 1.88% was recorded for 18 h followed by a decrease towards 1.25% from 18-20 h. After 20 h VS% increase to 1.52% and remained between 1.47%-1.52% until 24 h was reached.

5 mM and 10 mM dosages of hexanoic acid into anaerobic digesters had some similarities, both has VS% which fell after one hour however they both recovered in varying degrees after 4 h. The rise in VS% occurred earlier for 10 mM vs. 5 mM; the subsequent dip in VS% occurred similarly after 10 h, however the dip in VS% for 5 mM inoculated digesters was more prolonged. The decreased VS% only lasted from 10-12 h for 10 mM digester, however in 5 mM digesters the dip of VS% lasted between 8-12 h until it began to increase. Although 5 mM had a prolonged decrease in VS% the increased VS% which followed was greater than 10 mM; VS% for 5 mM inoculated digesters remained higher than 10 mM digesters after 14 h apart from a minor difference seen at 16 h.

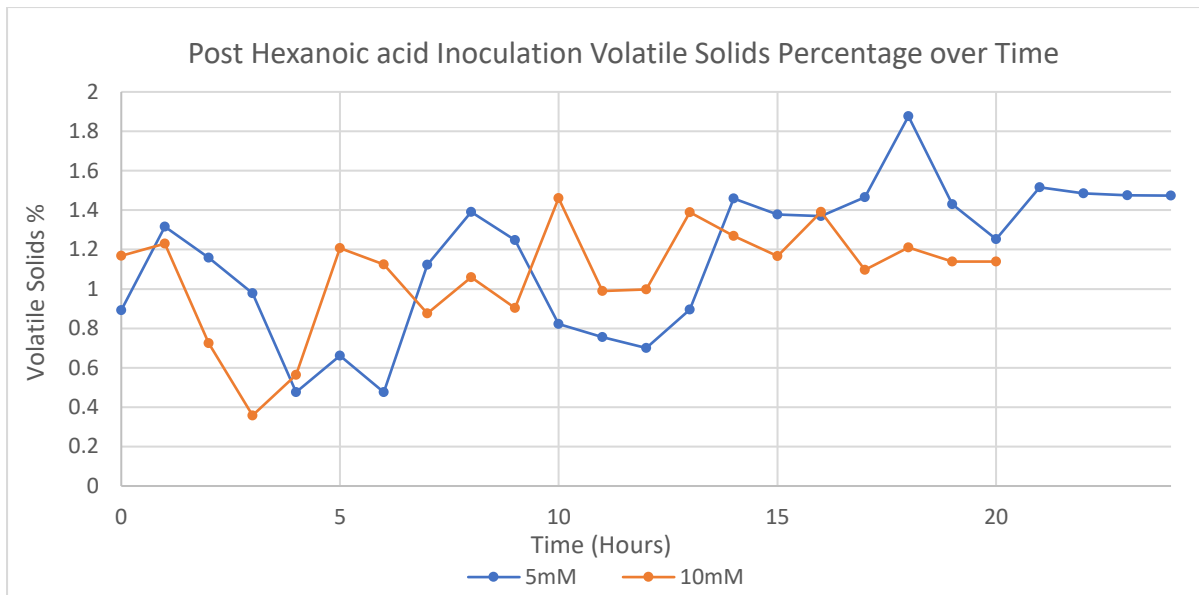


Fig.23 Changes in volatile solids % of digestate inoculated with hexanoic acid over 24 hours.

3.4.3 Effects of a 5 mM and 10 mM inoculation of Octanoic Acid on volatile solids percentage in anaerobic digestion

The initial VS% for both 5 mM and 10 mM dosage of octanoic acid into digesters were not similar butyric acid, although the difference is greater the initial VS% is more like that of hexanoic acid. The trends visualised in VS% of octanoic acid inoculated digesters (**Fig.24**) are easier to discern like butyric acid and contrary to hexanoic acid.

At 1 h post 10 mM inoculation VS% increased to 1.52% followed by a sharp decrease towards 0.65% afterwards VS% began to increase in a linear fashion from 0.64%-1.50% between 2-7 h. At 8 h the peak decreased to 1.67%, then increased toward 1.42% at 10 h. A decrease in VS% towards 0.73% was observed to occur between 10-13 h followed by another sharp increase to 1.45% at 15 h. After 15 h VFA% remained between 1.30%-1.45% until 17 h. A sudden drop in VS% was detected at 18 h VS% remained between 0.75%-0.79% until it increased to 1.15% at 21 h and 22 h. Data for 23 h was lost, however at 24 h VS% increased to 1.47%.

In contrast to 1 h post 10 mM hexanoic acid inoculated digester, 5 mM digesters had a slight VS% decrease, then a small increase followed by a steep decrease towards 0.29% VS%. An increased in VS% towards 1.71% was observed after 3 h for a duration of 5 h. After the peak observed at 8 h a decreasing trend with slight peaks were notes to occur from 8 h to 14 h until a VS% of 0.75% was reached. An increase to 1.78% from 14-18 h followed by a steep

decrease to 1.08% at 19 h was seen. An increase from 1.11% to 1.45% occurred from 20-21 h, after 21 h VS% steadily remained between 1.45%-1.5%.

The trend of increasing VS% over 6 h occurred for both 5 mM and 10 mM octanoic acid inoculation (**Fig.24**), however the increased started at 3 h and 2 h respectively. Although the decrease in VS% was greater in 5 mM than 10 mM, 5 mM had a greater increase in VS% than 10 mM during the duration of increasing VS%. Both dosages of octanoic acid had a decreasing VS% after 10 h, the decreasing VS% concluded and began to increase at 14 h for 5 mM and at 13 h for 10 mM; after the second decreasing VS% trend 5 mM inoculated digesters had a greater VS% than 10 mM.

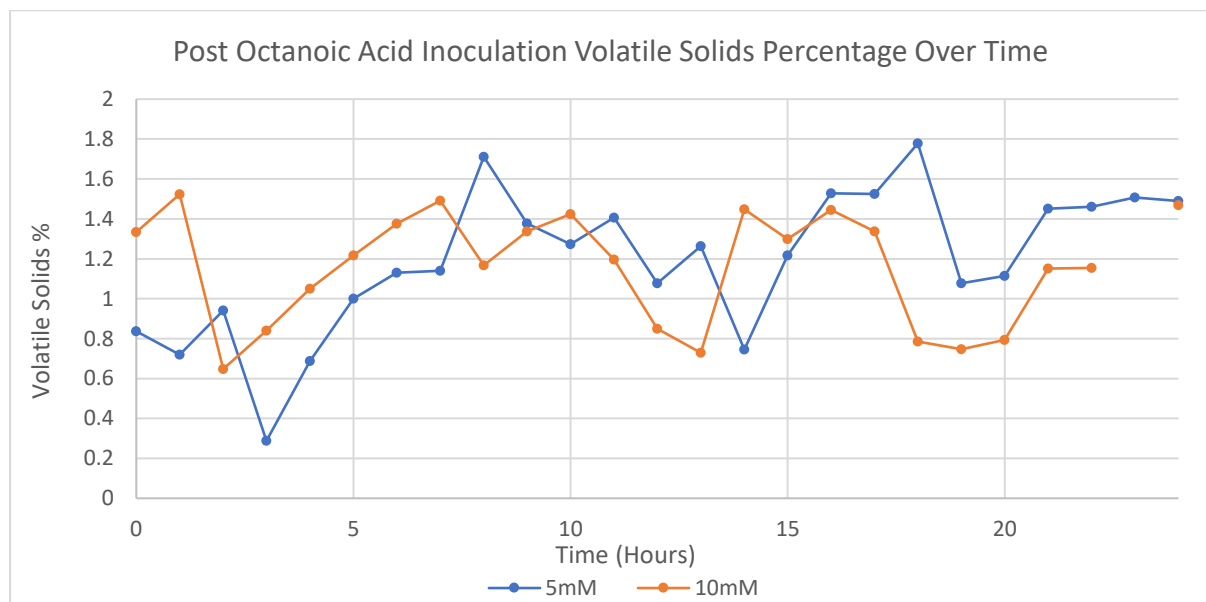


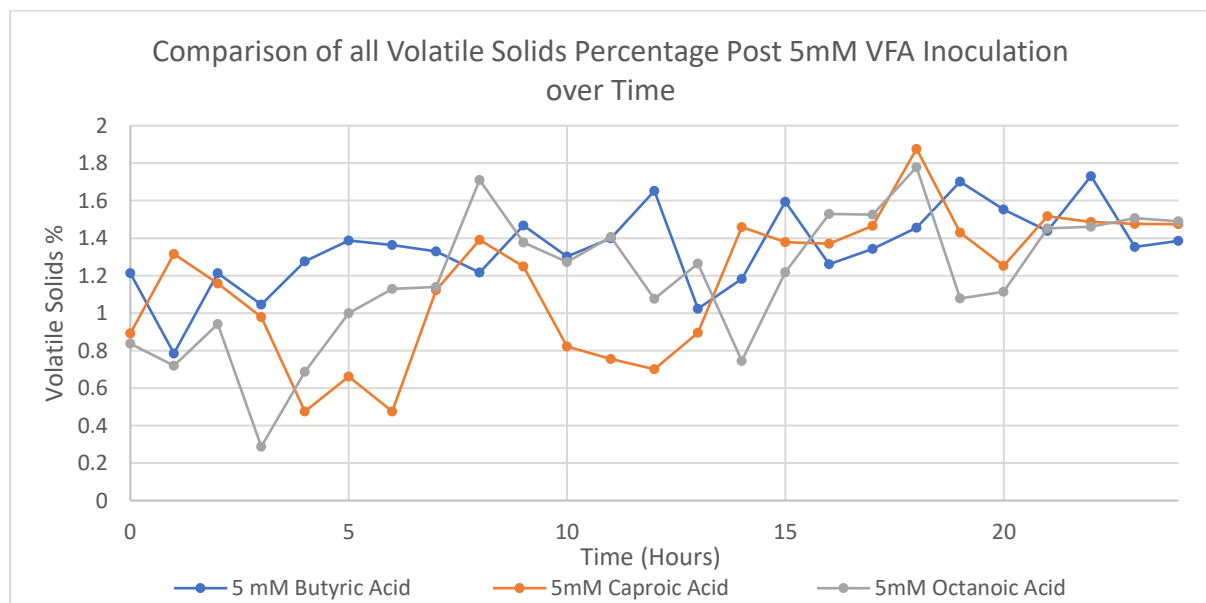
Fig.24 Changes in volatile solids % of digestate inoculated with octanoic acid over 24 hours.

3.4.4 Changes in volatile solids percentage of digestate in anaerobic digesters inoculated with 5mM and 10mM of butyric, hexanoic, and octanoic acid.

The visualisation of VS% in digesters inoculated with 5 mM or 10 mM inoculation of butyric, hexanoic, and octanoic acid (**Fig.25[A][B]**) illustrates that some trends can be observed. After 5 mM inoculations of butyric and octanoic acid (**Fig.25[A]**) a decrease then increase was observed in the first two hours, a greater decrease was seen in octanoic acid relative to butyric acid at 3 h. Following 3 h post 5 mM butyric and octanoic acid inoculations both butyric and octanoic acid VS% began to increase then stabilise, however butyric acid had a higher VS% than hexanoic and octanoic acid until 7 h. Prior to 15 h all three 5 mM inoculums had variations or similarities in their trends large or minor; however, after 10 hours except for butyric acid inoculum between 21-22 h, VS% seem to stabilise with less drastic variations.

The overlaid visualisation of 10 mM butyric, hexanoic, and octanoic acid inoculation in anaerobic digesters (**Fig.25[B]**) illustrates that more overlap and in VS% occurred in digesters inoculated with 10 mM than those with 5 mM (**Fig.25[A]**). At 0 h the initial VS% had a greater similarity between all 3 inoculums, with VS% of butyric and hexanoic acid having nearly identical values from 0-1 h (**Fig.25[B]**). Although octanoic acid had a greater VS% from 0-1 h the decreased VS% observed 1 h post inoculation was seen in all three fatty acids, butyric and hexanoic acid had an additional hour of decreasing VS% while octanoic acid VS% had already begun to increase after 2 h vs. increase after 3 h seen in butyric and hexanoic acid. Hexanoic acid VS% appeared to have remained the lowest and had VS% values which surpassed that of butyric and octanoic acid at 10 h and 13 h. At 10 h it seemed that the VS% for all 10 mM had values within proximity to each other. Beyond 10 h until 17 h the VS% indicates that the DMC behaved like one another, beyond 17 h the trends were less discernible.

The VS% could be an indication of how the biomass within the digesters are behaving (i.e. changing) an increase in VS% could indicate biomass growth, whereas a decrease in VS% could indicate biomass decay. Without further analysis such as how much the inoculum contributes to VS%, similar to what was done with the COD contribution of the inoculum (**Table.3 [A][B]**) the relationship between VS% change cannot be directly linked to changes in biomass.



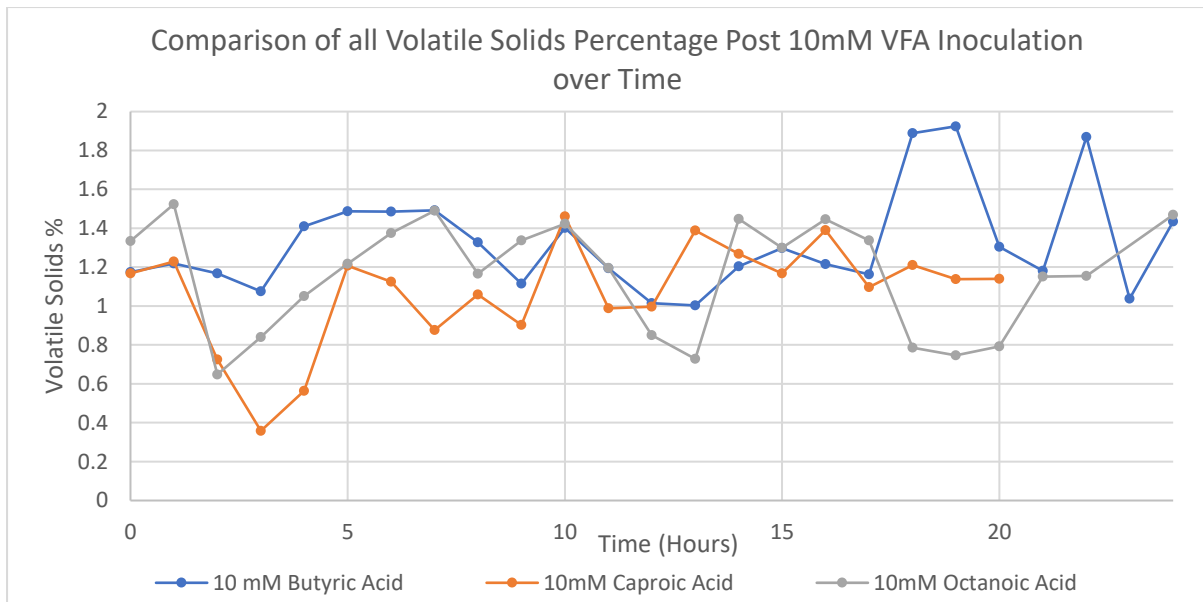


Fig.25[A][B] Changes in volatile solids % of digestate inoculated with 5mM and 10mM butyric, hexanoic, and octanoic acid over 24 h.

3.5.0 Improvements to methodology for future experimental repetitions and application.

3.5.1 Inoculation of anaerobic digesters with volatile fatty acids and subsequent sampling.

Each time sampling and inoculations are conducted, serological pipettes should consistently be fully inserted into the sampling port and into the digester (**Fig.26**). Liquids being ejected out the sampling port previously caused pipettes to not be fully inserted; future experiments will pipette excess liquids stuck in the sample port back into the digester. To ensure inoculation of VFA into digesters rather than being suspended in the sampling port, pipette containing fatty acid will be fully inserted, subsequently digestate and digestate within sampling ports should be pipetted up into the pipettes ensuring any residual fatty acid is introduced into the digester. Once experimentation and sampling has concluded for a fatty acid, the sampling port should be 'rinsed' with digestate to ensure no excess inoculum remains in the sampling port. An emphasis on sampling prior to inoculation (-1h) and subjected to the same analysis as samples post inoculation; this would allow a comparison to be made between pre- and post-inoculated digestate.

The amount of carbon introduced to the system should also be addressed and gas data should correspond and be visualised accordingly (e.g. 20 mmol and 40 mmol of carbon introduced for 5mM and 10 mM of butyric acid respectively). Similarly biogas produced

should and could also be normalised against VS% added. VS% of the inoculum should also be determined prior to experimentation.

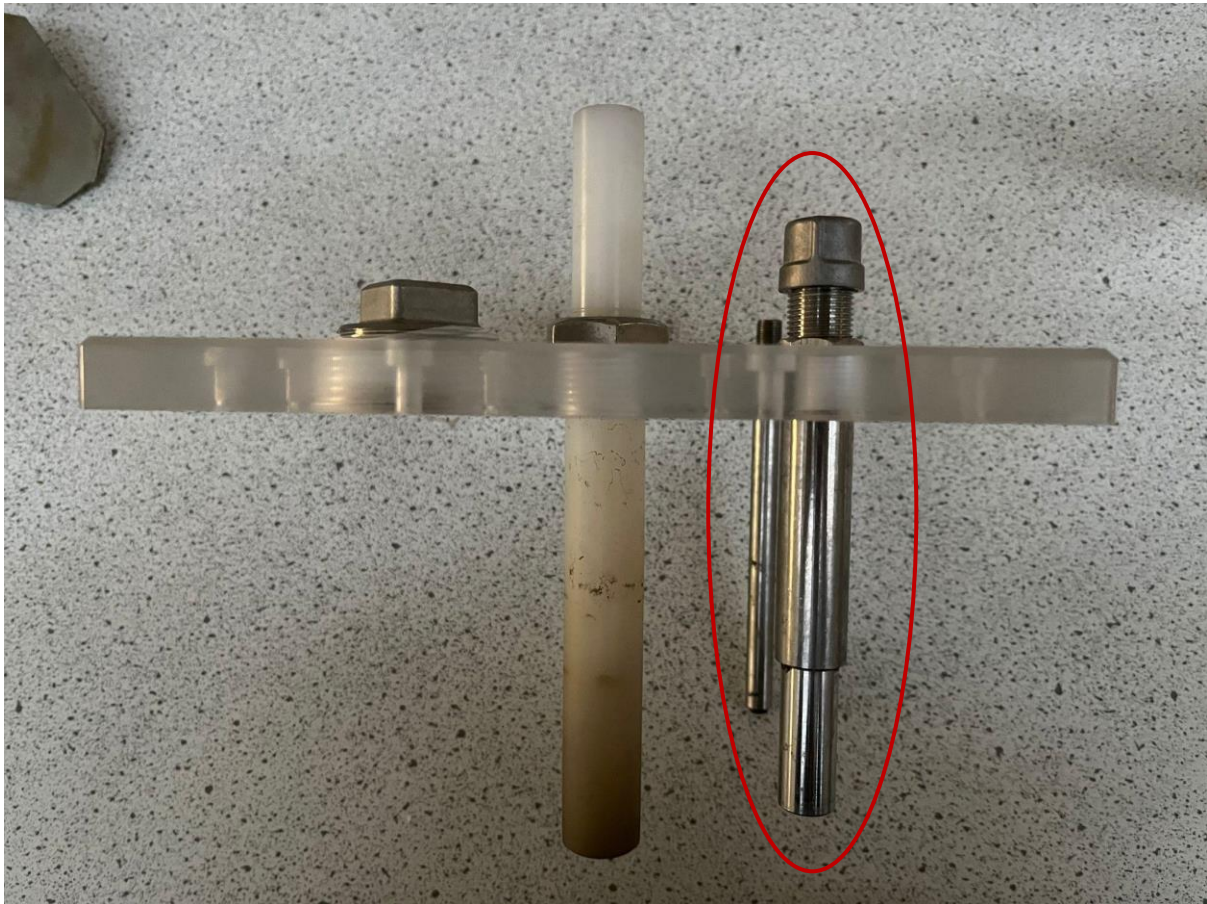


Fig.26 Image of anaerobic digestion lid and sampling port circled in red.

In addition to upgraded sampling and inoculation protocols, samples should be shaken vigorously prior to analysis due to the separations and layers produced that may skew the results; however this should not be applied to COD analysis as rigorous shaking could introduce oxygen into the sample. Measurements of pH in digester should also be included. GC-FID analysis also requires unused GC analysis bottles filled with dH₂O to be incorporated between sample groups to mitigate the cross contamination specified in [section 3.2.3.5](#). Upon repetition of experiments with improved methodologies implemented, the acquired data should be visualised with their corresponding fatty acid concentration data (e.g. VFA concentrations, COD, VS %, and gas rate visualised on one graph).

3.6.0 Future Studies

3.6.1 Monitoring gas composition of inoculated anaerobic digesters.

Gas production of anaerobic digesters inoculated with VFA were altered by sampling (section [3.1.0](#)). To observe the effects of fatty acid inoculation on gas production in anaerobic digesters sampling must be omitted; this can be achieved by inoculation with 5mM butyric, hexanoic, and octanoic acid in digesters F1, F2, F3, and 10mM of butyric, hexanoic and octanoic acid in digesters F4, F5, and F6 respectively without any sampling subsequent to inoculation. In addition to automatically recorded gas rates, CO₂ and CH₄ may also be monitored during experimentation, although System-60 has the necessary hardware for monitoring carbon dioxide and methane an unforeseen hardware error occurred and was unable to be resolved due to time constraints. Therefore future repetition of experiments will apply repaired hardware to enable gas composition monitoring and observation of volatile fatty acid inoculum effect on gas production rates without the interference of sampling. The experimentation with the omission of sampling will allow observation of how increasing lengths and increased concentrations of fatty acid affect gas production over time, as well as the time required for the degradation of each increased length of fatty acid in AD.

3.6.2 Acyl-CoA Dehydrogenase (ACAD) Assay

The aim of this enzymatic assay is to elucidate enzyme concentration following inoculation of anaerobic digester, this would allow examination of enzyme production rates when a singular source of energy is introduced. By using a proposed anaerobic fatty acid degradation pathway (**Fig.4**) in the context of known aerobic pathway counterparts [25,26]. Acyl-CoA dehydrogenase is the first step once fatty-acyl-CoA is committed into the β -oxidation cycle (transformation of fatty-acyl-CoA into 2-enoyl-CoA) (**Fig.4**); hence ACAD was chosen to be the primary focus, but also due to the availability of previous works done [25-30].

To the best of personal knowledge and investigations, ACAD assays have not been done with wastewater sludge. Gathering techniques from ACAD assays conducted with rat liver homogenate^[28,29] and specific ACAD assay based on spectrophotometric shift due to product formation (cinnamoyl-CoA)^[30], a protocol for conducting ACAD assays in wastewater sludge has been devised. The ACAD assay uses two pre-existing chromophores to be conjugated through a new double bond introduced between the phenyl and thioester functional group of 3-Phenylpropionyl-CoA (PP-CoA). The resulting product cinnamoyl-CoA

increases absorbance at 308nm. The following reaction (Fig.27) is dependent on the electron acceptor Phenazine Methosulfate (Fig.28).

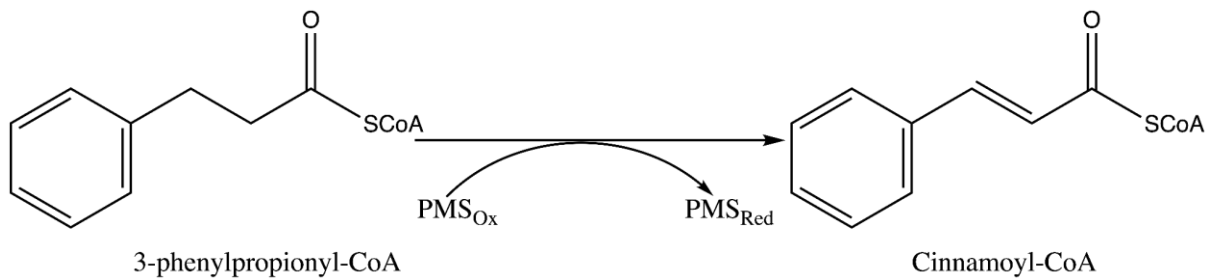


Fig.28 ACAD assay substrate 3-phenylpropionyl-CoA converted to cinnamoyl-CoA with PMS acting as electron acceptor^[30]

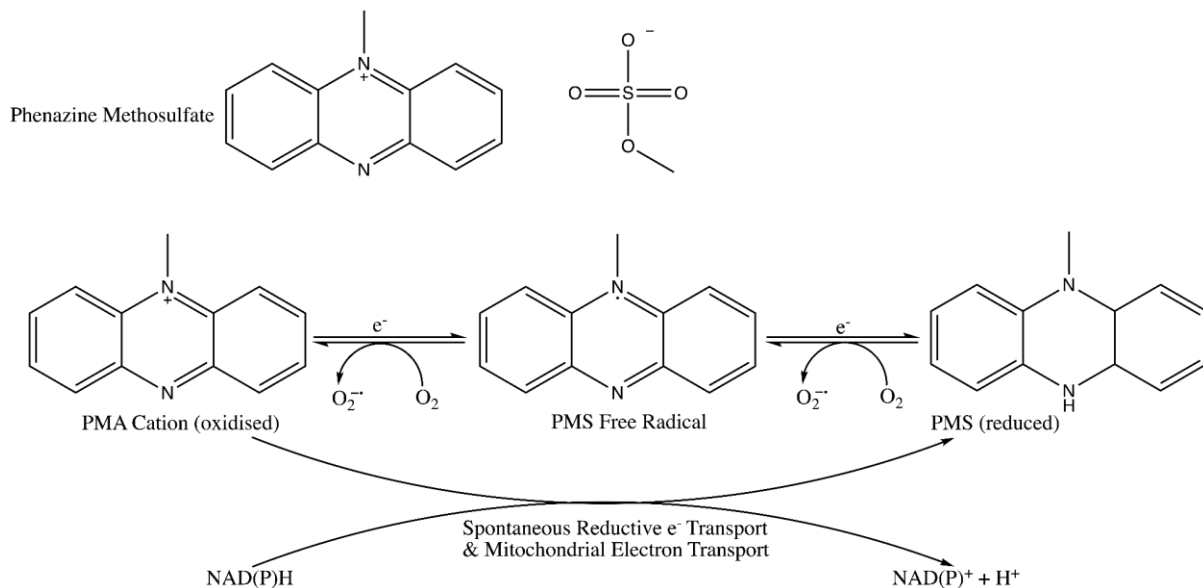


Fig.29 Diagram detailing PMS acting as electron acceptor, hence illustrating the role of PMS in ACAD assay^[31].

3.6.3 Extrapolating data to improve other parameters within ADM1 by utilising different inoculums in anaerobic digestion.

The techniques, procedures, and future studies established can support a wide range of future investigative efforts into anaerobic digestion. Robust data collection would be facilitated and applied to anaerobic digestion modelling, hence furthering understanding, and optimization of anaerobic digestion processes. An expansive variety of inoculum can be utilised to characterise anaerobic digestion performance; future studies with inoculation of longer chained, odd numbered, branched fatty acids, amino acids, monosaccharides, and complex composite particles can be explored.

3.7.0 Conclusion

The application of anaerobic digestion and production of biogas has far reaching implications in mitigating energy and waste management demands of a growing population. Traditionally AD has been widely associated with wastewater treatment, however AD can function as an energy producer. AD is therefore a crucial component of achieving a circular economy by turning waste streams into a viable energy source.

The preliminary results gathered suggest that while the methodologies were successful in gathering data, further improvements can be made to acquire more robust data sets. Anomalies observed in the preliminary data are only able to be disproven or confirmed following further repetition of experimentation.

Gas data indicates that butyric, hexanoic, and octanoic acid inoculated into starved anaerobic digester did have differing effects on gas production. 5 mM and 10 mM inoculation of digesters had shown increased gas production, while hexanoic and octanoic acid showed a lag-period for biogas production. Although fatty acid with chain lengths exceeding 4 carbons showed a lag period in gas production; biogas production did gradually increase and it's possible that beyond the 48 h of visualised gas data, when fatty acids are degraded into butyric acid the gas production profile would be similar to digesters inoculated with butyric acid. This indicates that this method was successful in generating data to investigate acetogenesis and the effects of fatty acid inoculation on biogas production in anaerobic digesters.

By staggering the inoculation of 3 digesters by 8 h, hourly time points could be taken for 24 h within 8 h. Data on digester characteristics such as chemical oxygen demand, volatile solids percentage, and fatty acid concentrations were analysed for the hourly samples taken over a 24 h time frame. A starved system was used to observe the effects of an inoculum; thereby excluding the effects of other organics which could be used by DMC; this demonstrated that a starved system was proficient in observing the systematic changes due to the inoculum introduced (in this case butyric, hexanoic, and octanoic acid).

The methods within this thesis were established to generate and provide robust datasets for modelling anaerobic digestion processes. By incorporating these methods, robust data acquisition on individual components (**Fig.3**) and their effect in AD will be generated.

Investigation into the individual components within AD allows for a systematic dissection of anaerobic digestion processes. Although the visualisation of data could be improved and further considered; the methodology deployed with the use of System-60 to gather the data was successful. As previously stated, the data used for the ADM1 were collated from multiple studies; the experimental possibilities and future applications of this methodology to generate data intended for modelling will thereby improve parametrisation and ground-truthing ADM1.

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