



Natalia Jawiarczyk

Bioremediation of oil-rich wastewater: managing sewer Fats, Oils,
and Grease (FOG) deposits with energy uncoupler product.

School of Water, Energy and Environment
STREAM DC

Engineering Doctorate
Academic Year: 2023 - 2024

Supervisor: Prof. Bruce Jefferson
Associate Supervisor: Dr Yadira Bajon Fernandez
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This thesis is submitted in partial fulfilment of the requirements for
the degree of Engineering Doctorate

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- the thesis submitted has not been previously submitted to this university or any other.
- that all content, including primary and/or secondary data, is true to the best of my knowledge.
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Abstract

The disposal of Fats, Oils, and Grease (FOG) down drains in both residential and commercial settings results in the buildup of these substances within sewer systems. This accumulation can ultimately lead to blockages and subsequent sewer overflows, posing significant challenges for the water industry. The impacts include potential customer dissatisfaction, negative effects on business operations, and regulatory fines. Effectively managing FOG is a complex issue, and finding viable solutions is paramount. Solutions leading to an alleviation of this problem are of great value, nonetheless, no uniform approaches have been established so far, and the existing measures remain insufficient. FOG bioremediation is emerging as a promising alternative to traditional sewer cleaning methods, but effective, targeted implementation requires higher scientific understanding of FOG deposit formation and modes of action of biological products.

This research introduces a novel approach to understanding and addressing FOG deposit formation and treatment. It does so by tailoring these methods to the specific stages of FOG deposit development and utilizing an energy uncoupler product—specifically, a combination of yeast protein extract with surfactants.

To substantiate the effectiveness of this approach, comprehensive trials were conducted. These trials encompassed synthetic solutions to simulate deposit formation,

performed synthetic deposits, as well as real deposits collected from the UK's sewerage network. The results of the thesis question and provide an alternative to the currently accepted model of FOG deposit formation through saponification. Instead, the work proposes a two-stage model based on the initial starch-lipid complexation followed by growth through accumulation of fats, lipids, carbohydrates, proteins, and calcium. The study then assessed the uncoupler treatment's impact through two mechanisms of action, i.e., inhibition and rehabilitation, in terms of reducing deposit mass and removing organic fractions present in wastewater. The results provided compelling evidence for the advantageous use of metabolic uncouplers in minimizing FOG deposit formation within sewer systems. Finally, the economic assessment of using the metabolic uncoupler revealed its financial feasibility for both planned and unplanned sewer cleaning procedures through the reduced maintenance that occurred when using it.

Keywords:

Fatbergs, starch-lipid complexation, energy uncoupling, inhibition, rehabilitation, economic evaluation, biodosing

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Impact of COVID-19 Pandemic

This research was conducted during the global COVID-19 pandemic, which necessitated adjustments to the experimental methodology due to pandemic-related restrictions and reduced campus activity. The limited presence of students and staff on campus resulted in a lower discharge of fats into the wastewater system, affecting the availability of real wastewater with sufficient fat content. As a result, the planned pilot-scale trials in the experimental sewer loop, located at Cranfield Campus wastewater treatment plant and designed to utilise real wastewater, could not be completed as intended.

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List of Abbreviations

FOG	Fats, Oils and Grease
ADP	Adenosine Diphosphate
AI-2	Autoinducer-2
ASE	Accelerated Solvent Extraction
ATP	Adenosine Triphosphate
BA	Benzoic Acid
BSA	Bovine Serum Albumin
C/N ratio	Carbon/Nitrogen ratio
Ca	Calcium
CAS	Conventional Activated Sludge
CCCP	Carbonylcyanide m-chloromethoxy- phenylhydrazone
CCTV	Closed-circuit Television
COD	Chemical Oxygen Demand
CP	Chlorophenol
Cu	Copper
DOSS	Dioctyl Sulfosuccinate
ELSD	Evaporative Light Scattering Detector
EPS	Extracellular Polymeric Substances
EPSRC	Engineering and Physical Science Research Council
FA	Folic Acid
FCCP	Carbonylcyanide p-trifluoromethoxy- phenylhydrazone

FFA	Free Fatty Acid
FSE	Food Service Establishment
FTIR	Fourier Transform Infrared analysis
GI	Grease Interceptor
GT	Grease Trap
HEM	Hexane Extractable Material
HPLC	High Performance Liquid Chromatography
IC	Ion Chromatography
MA	Malonic Acid
MBR	Membrane Bioreactor
NEM	N- ethylmaleimide
NP	Nitrophenol
ODI	Outcome Delivery Incentive
Ofwat	Office for Water Services
PMF	Proton Motive Force
PPM	Part Per Million
PS	Pumping Station
QS	Quorum Sensing
SBR	Sequencing Batch Reactor
SLE	Sodium Lauryl Ether Sulphate
SMP	Soluble Microbial Products
SOUR	Specific Oxygen Uptake Rate

SS	Synthetic Sewage
TC	Total Carbon
TCS	Tertrachlorosalicylanilide
TGD	Triglyceride
THPS	Tetrakis (hydroxymethyl) phosphonium sulfate
TN	Total Nitrogen
TOC	Total Organic Carbon
TS	Total Solids
TSS	Total Suspended Solids
UCO	Used Cooking Oil
UK	United Kingdom
USA	United States of America
VFA	Volatile Fatty Acids
VS	Volatile Solids
VSS	Volatile Suspended Solids
WaSC	Water and Sewerage Company
WWTP	Wastewater Treatment Plant
Zn	Zinc

1 Chapter 1 Introduction

1.1 Background

Fats, oils, and greases (FOG) encompass a range of lipids, such as free fatty acids (FFAs), triglycerides (TGDs), and lipid-soluble hydrocarbons. These substances find their way into sewer systems from a wide array of origins, ranging from by-products generated at food processing facilities and food service establishments (FSEs) to residential properties. The most apparent form of FOG is spent cooking oil (UCO), but it can also be found in salad dressings, sauces, gravies, and various dairy-based products such as milk, cream, and ice creams (Iasmin et al., 2016; Keener et al., 2008).

The discharges of FOG into sewers can lead to blockages, resulting in sewer flooding and pollution incidents. These events, in addition to being unpleasant, can cause significant property and environmental damage (He et al., 2013). Detrimental consequences of FOG extend beyond the sewer network. If not effectively captured beforehand, FOG can interfere with biological processes at wastewater treatment plant (WWTP) hindering the treatment efficiency and increasing operating costs (Arthur & Blank, 2013; He et al., 2017; Husain et al., 2014; Wallace et al., 2017). As such, FOG present a considerable challenge for water companies, with potential far-reaching impacts on customer satisfaction, business performance, and regulatory penalties, for example Outcome Delivery Incentives (ODIs) set within the water companies in England and Wales by Ofwat, i.e., the water services Regulation Authority.

Despite significant efforts, the challenge posed by FOG persists as one of prominent concerns for Water and Sewerage Companies (WaSC), both within the UK and on a global scale. In the USA, FOG-related blockages account for 50% of sewer blockages (He et al., 2017) while in the UK, it's as high as 70% (British Water, 2015). In the USA, approximately 75% of sewage systems are operating at just half their capacity due to the accumulation of FOG (Copeland & Myers, 2006). As a consequence, the financial toll is substantial, with estimated costs of \$1 billion in the USA (NACWA, 2017) and £100 million in the UK (Water

UK, 2018). It is evident that a thorough reevaluation of the best FOG management options is needed, including effective methods for controlling oil-rich wastewater discharges into sewers and rehabilitating existing blockages. The estimated volumes of annual FOG generated, captured, and potentially disposed down the drain in the UK are presented in Table 1-1.

Table 1-1 Estimated volumes of FOG generated, captured, and disposed down the drain in the UK. Adapted from Smith et al. (2013) using conversion ratio 1.5 (Internal survey).

	FSEs	Food manufacturers	Large hospitals	Domestic	Total	Captured	Disposed
Volume (million L/yr)	75-134	149-164	4.5	75-112	304-415	45-49	259-365

In order to alleviate the detrimental consequences arising from the accumulation of FOG in sewer systems, FOG recovery approaches can be implemented both at the source, targeting UCO and GI waste (He et al., 2017; Wallace et al., 2017) and by retrieving FOG deposits from sewers, pumping stations, and WWTP (Collin et al., 2020). However, this process results in the generation of a waste stream that necessitates appropriate management or disposal. Presently (Figure 1-1), the majority of recovered FOG from grease interceptor (GI) and sewer sources is being disposed of in landfills or at rendering plants, whereas collected UCO is in majority converted to biodiesel (He et al., 2017).

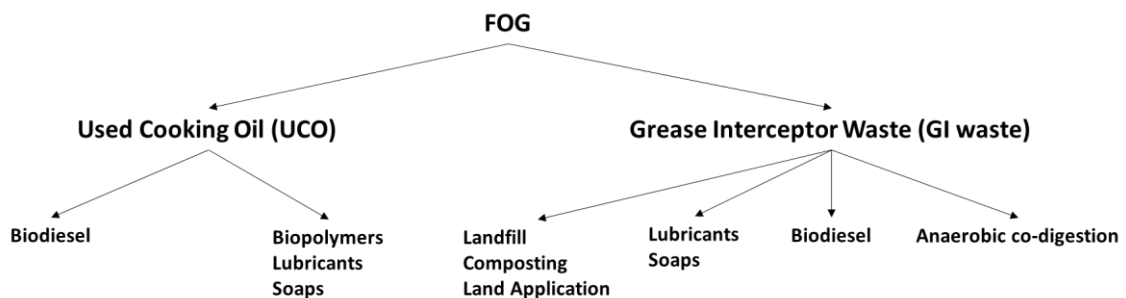


Figure 1-1 Current valorisation routes for recovered FOG.

The potential to recover FOG materials spans the entire sewer network, significantly impacting their physiochemical properties. This crucial consideration must guide the selection of appropriate recovery methods (Collin et al., 2020).

The development of processes to upcycle these waste streams holds immense value, primarily due to the substantial economic potential they represent. In Europe the recoverable biochemical products within wasted FOG, notably used cooking oil (UCO), are estimated to be worth around £86 million annually. Additionally, these materials possess a high energy-generating potential, estimated at 1000 GWh per year (Wallace et al., 2017). Collin et al., (2020) further estimated that the Thames Water Utility Catchment alone could yield 94,730 tonnes of recovered material each year, generating energy equivalent to 222 GWh annually, which could satisfy 19% of Thames' energy demand.

1.2 Current FOG Control Strategies

Water companies in the UK currently face limited options in addressing the uncontrolled discharges of FOG, leading to the prevalence of reactive, mechanical blockage rehabilitation. To prevent FOG from entering drains and sewers, the UK has implemented a series of legal mandates (as detailed in Table 1-2). However, these regulations frequently fall short of achieving their intended objectives, compounded by the infrequent exercise of rights by water companies in alignment with the regulatory frameworks (as outlined in Table 1-3).

Table 1-2 FOG and grease trap regulations in the UK (British Water, 2015).

Region	Rules and Policies for Handling FOG
UK Wide	UK Water Industry Act (WIA) 1991 S111(1). <i>'No person shall throw or empty any matter likely to injure the sewer or drain, to interfere with the free flow of its contents.'</i>
England and Wales	Building Regulations. Part H (Drainage and Waste Disposal) 2002. Point 2.21: Drainage serving kitchens in commercial hot food premises should be fitted with a grease separator complying with BS EN1825-1 and designed in accordance with EN 1825-2 or other effective means of grease removal. These regulations are generally only applied to new builds and refurbishments.
Scotland	Scottish Building Standards M2.6 and M2.7. Where a discharge into a drainage system contains oil, grease, or volatile substances, for example from a vehicle repair garage, there should be a facility for the separation and removal of such substances

Region	Rules and Policies for Handling FOG
Northern Ireland	Building Regulations (2012) relevant sections: Regulation 23 – fitness of materials and workmanship, Regulation 79 – drainage systems and Regulation 81 – underground foul drainage.

Table 1-3 Governance and Utility Interventions in the UK (Georges et al., 2017).

Regulation/Factor	Enacted by utilities in England and Wales
FOG classified as a trade waste	No
FSEs charged by utility to discharge FOG	No
FOG classified as a sewer pollutant under WIA (1991)	Yes
Authority for utility to prosecute under WIA (1991)	Yes
Authority for utility to enforce building regulations	No
Authority to enforce grease traps/interceptors servicing	No (although can advise)
Prosecuted commercial premises under WIA (1991)	Severn Trent Water, Southern Water, United Utilities
Engagement with FSEs to manage FOG	Southern Water, Severn Trent Water
Collecting and/or processing FOG	Thames Water

Table 1-4 provides the overview of the initiatives and measures undertaken within the UK to manage FOG. Achieving compliance involves the collection of used cooking oils and installation of grease interceptors at FSEs, designed to capture FOG materials from wastewater. While these measures help limit FOG discharges into sewers, long-term prevention largely relies on the behaviour and attitude of users. Consequently, numerous water companies have launched outreach and education campaigns to raise public awareness on the importance of proper FOG management. Furthermore, many countries have implemented

consent requirements for effluent as a viable alternative or a complementary measure to legislative mandates (Table 1-5).

Table 1-4 UK Initiatives for Controlling FOG Disposal in Sewage Networks (Georges et al., 2017).

Approach	Summary	Degree of implementation	Probability of success if implemented
Trade effluent permit	<p>WaCS classify grease trap waste as domestic waste, therefore commercial premises do not require trade effluent discharges permit.</p> <p>Industrial premises with trade effluent consent are allowed to discharge up to 300 mg/L of saponifiable material at a specific discharge rate.</p>	Low	Medium to High
Grease traps	<p>Companies are not taking responsibility for the following:</p> <ol style="list-style-type: none"> 1) Installing, monitoring, or servicing grease traps. 2) Getting involved in collecting, disposing, or treating FOG recovered from grease traps. 3) Recommending specific grease traps to their customers but may instead refer them to the Grease Contractors Association. <p>EU Standards relating to grease traps: 1) BS EN 1825-1 (2004)- design, performance and testing, quality control; 2) BS EN 1825-2 (2002)- size, installation, operation, and maintenance.</p>	Low (30% of FSEs in the UK)	High
Prosecution	<p>FSEs that repeatedly discharge FOG may receive a warning letter and face potential prosecution under Section 111 of the Water Industry Act.</p>	Low	Low to Medium

Approach	Summary	Degree of implementation	Probability of success if implemented
	Although water companies have the authority to prosecute repeat offenders under the Water Industry Act, most companies prefer not to exercise this option.		
Cost recovery	While not many companies opt for cost recharging, some do choose to recover the expenses related to clean-up and repairs from the responsible establishment.	Low	Medium
Reactive sewer maintenance	The most common approach to blockages and pollution incidents is a reactive maintenance, whereas mechanical maintenance of blockages is generally applied.	High	Low
Proactive sewer maintenance	Certain companies frequently utilize bio-dosing products in the areas with high densities of FSEs, high number of blockages (hotspots) and to sewers that are difficult to clean.	Low	Medium to High
Education	<p>Many companies opt for education campaigns as their approach to reducing FOG discharges. Some form dedicated teams to raise awareness of FOG management among both domestic and commercial customers. They also collaborate with Local Authority Environmental Health teams, conducting joint visits to FSEs to promote FOG management, which includes recommendations for:</p> <ol style="list-style-type: none"> 1) Installing grease traps. 2) Implementing best practices in the kitchens. 3) Encouraging UCO collection and appropriate disposal. 	Medium	Medium to High

Approach	Summary	Degree of implementation	Probability of success if implemented
Recovery and beneficial reuse	<p>There is a significant rise in interest in the recovery of FOG, including both UCO and brown grease (grease traps and sewer FOG).</p> <p>In general, third-party companies collect UCO from FSEs for biodiesel production.</p> <p>Certain WaSC are exploring the possibility of collecting UCO from local FSEs to use in their digesters, but only if regulations allow.</p> <p>In some cases, FOG removed from sewers is tinkered to WWTP and discharged into the digester.</p>	Low (brown grease)Medium (UCO)	Medium to High

Table 1-5 International Measures for Controlling FOG Release into Sanitary Sewers (Georges et al., Wallace et al., 2017).

Country	Discharge permit requirements	Legal requirements for Grease Trap (GT) installation	Additional comments
UK	x	x	In theory, commercial premises are required to obtain consent from the WaSC before discharging. However, in practice, the WaSC often does not enforce this requirement.
Australia	✓, defined as trade waste	✓, proof of maintenance is required	The installation of a grease trap requires formal consent.
Canada	✓, 100 mg/L	✓	Requirements vary across the country
Germany	x	✓	Strict rules on maintenance, fines up to €100,000 for noncompliance.
Ireland	✓, defined as trade waste	x	
Netherlands	x	✓	Regular inspections to ensure proper functioning and compliance of grease trap. A certificate of installation, along with a maintenance record, is essential for documentation and verification purposes.
New Zealand	✓, defined as trade waste	✓, proof of maintenance is required	The installation of a grease trap requires formal consent.
Portugal	x	✓	
Spain	x, 100 mg/L FOG limit on all discharges	x	Efforts are primarily focused on industrial discharges.

Country	Discharge permit requirements	Legal requirements for Grease Trap (GT) installation	Additional comments
Sweden	x	✓	Formal consent is necessary for the installation of a grease trap. The grease trap needs to be approved in accordance with the Swedish Standard (SS) 1825-2:2002 and manufactured in compliance with SS EN 1825-1.
USA	✓, 100 mg/L	✓, proof of maintenance is required	Requirements vary from state to state

1.3 Current model of FOG deposits formation

Knowledge regarding FOG deposits formation in sewers remains limited. In the past, FOG deposition was solely associated with fats cooling and solidifying on sewer walls due to the low temperature, and low hydraulic pressure in the network (Williams et al., 2012). The understanding has since evolved, and the current view indicates that primary reaction leading to deposit formation is fats saponification (He et al., 2011; Keener et al., 2008; Williams et al., 2012). Calcium has been identified as the predominant metal found in FOG deposits, while the major fatty acids present in these deposits are palmitic, oleic, stearic and linoleic (Benecke et al., 2017; He et al., 2011; Keener et al., 2008; Williams et al., 2012). The generation of free fatty acids is attributed to the combined effects of chemical and microbial hydrolysis of oil (Iasmin et al., 2014; Keener et al., 2008; Williams et al., 2012), while the presence of calcium is influenced by water hardness, concrete corrosion, and microbial reactions (He et al., 2011, 2013; Williams et al., 2012).

In general terms, saponification occurs when free fatty acids react with the positively charged metal ions such as calcium, sodium, or magnesium to form metal-based fatty acid salts (He et al., 2011). Keener et al., (2008) put forth an initial hypothesis that in alkaline conditions, calcium present in the wastewater

reacts with FFAs released from FOG's hydrolysis, to form soap. The author stated that FOG deposit formation in sewer pipes requires the simultaneous presence of four main components: calcium ions, FFAs, FOG and water and that the reaction of saponification begins at the FSEs discharge where the high pH condition needed for saponification is provided by the alkaline detergents, degreasers, and sanitizers regularly used for cleaning purposes. Similar conclusions were reached by He et al., (2011). Through a comparative study involving Fourier Transform Infrared (FTIR) analysis, they explored the structural resemblances between pure metallic salts, artificially created saponified solids in laboratory settings using Grease Interceptor (GI) effluent, and samples collected from the field. They suggested that, alongside the primary saponification, the buildup of surplus calcium and unreacted FFAs also plays a role in deposit formation as secondary mechanisms. The proceeding studies were predominantly designed to investigate calcium soaps formation under various experimental conditions (temperature, pH, FFA type) and have largely upheld previous statements, although have reported on greater heterogeneity of the field samples (He et al., 2013; lasmin et al., 2014; Williams et al., 2012). He et al., (2013) further confirmed that calcium excess, unreacted free fatty acids (FFAs) and debris from wastewater, are attracted to FOG saponified core, leading to intermittent deposit build up. In accordance to that, more recent work by Benecke et al. (2017) highlighted the role of FFAs aggregation in FOG deposit formation by revealing that FFAs rather than FFAs salts are the main species of fat found in the field deposits. Earlier studies were limited in this capability, as the analytical techniques employed for assessing FFA content were unable to differentiate between free and saponified FFAs. Moreover, Gross et al.(2017) demonstrated that the deposits could arise exclusively from FFAs, even in the absence of the calcium. This observation aligns with initial observations by Keener et al. (2008), who reported that a minor subset of analysed field samples exhibited minimal mineral and metal levels. Simultaneously, these samples also exhibited FFAs profiles resembling cooking oils, rather than being rich in palmitic, oleic and stearic acids predominant in other analysed FOG samples. Figure 1-2 depicts

how the understanding of FOG deposit formation has evolved and presents the current view on the process.

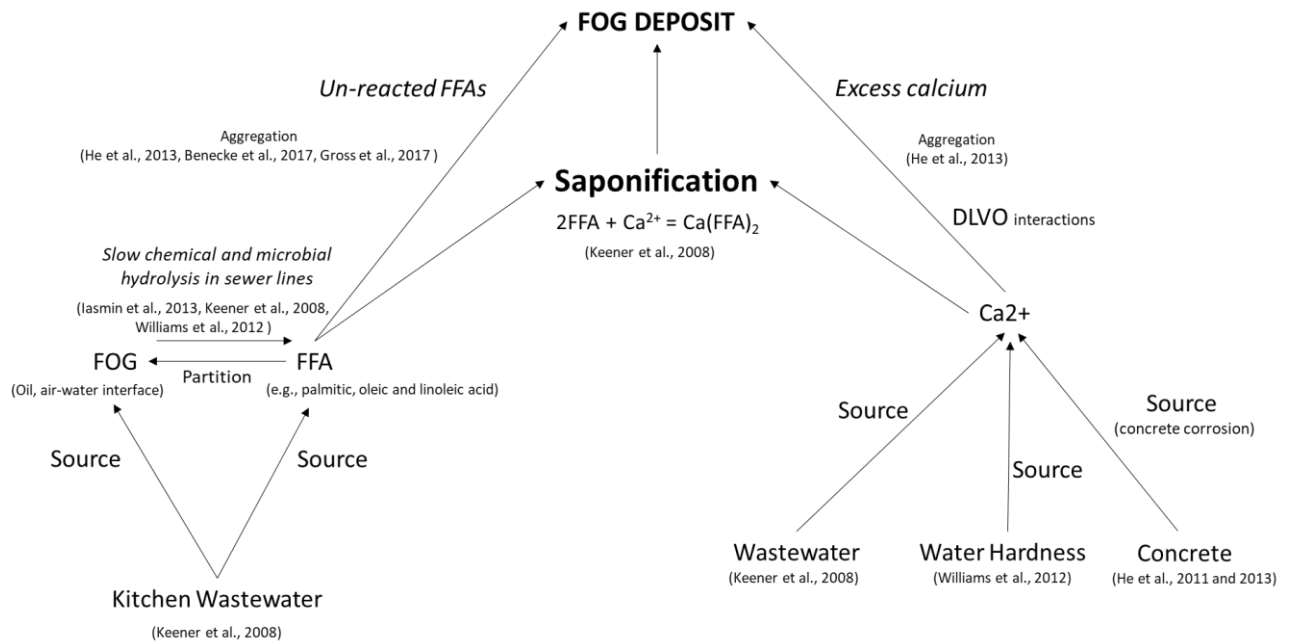


Figure 1-2 General understanding of FOG deposit formation in sewer pipelines. Adapted from He et al. (2013).

1.4 Flaws in the current FOG deposition model

To date, no study has provided a definitive replication of the intermittent step-by-step process of FOG formation in sewers. The reported studies have placed limited emphasis on generating FOG deposits using wastewater and have scarcely considered components beyond FOG, FFAs, and calcium (Dominic et al., 2013; He et al., 2011; Iasmin et al., 2014, 2016; Keener et al., 2008), yet conditions prevailing in the sewers are not consistent with those usually associated with saponification, except from dish-washer discharges (Gurd et al., 2019). Given the lack of an exhaustive assessment of the composition of FOG deposits generated in sewers, it is reasonable to assume that the presence of other chemical constituents leading to the generation of insoluble solids and contributing to FOG deposits is still possible. Consequently, questions persist as to what drives deposition.

The main source of organic matter in municipal wastewater are food-related discharges into sewers. The discharged wastewater is abundant in starch, protein, and FOG originating from food preparation, alongside alkaline and acidic chemicals used in cleaning practices (Ashley et al., 2015; Gurd et al., 2019). Notably, during food processing, specifically frying and cooking processes, frequent physical interactions between starch, proteins, and lipids occur (FFAs) (Kapusniak & Siemion, 2007; Liu et al., 2009; Wang et al., 2020), resulting in the formation of insoluble binary starch-FFA complexes (De Pilli et al., 2011) or ternary starch-FFA-protein complexes (Zhang & Hamaker, 2004). Indeed, this previously unconsidered phenomenon of physical interactions between wastewater compounds may hold significant implications for understanding and managing the formation of FOG deposits in the sewers. Further to that, recent studies have highlighted that conventional grease separators might be unable to efficiently remove around 67% of total FOG present in kitchen wastewater discharged from dishwashers and potwashing (Gurd et al., 2019; Sultana et al., 2022). The efficiency of physical separators is enhanced with larger droplet sizes with an effective cut off defined by some authors at around 150 μm (Barton, 2012) whereas on average 94% of FOG in dishwasher effluent and 76% of potwash sink comprises emulsified FOG droplets with diameters ranging from 20 to 45 μm . Considering their increased surface area and solubility, these oil droplets appear more suitable for microbial degradation than physical separation (Sultana et al., 2022), yet biological treatment of oil- rich wastewater is often a secondary measure.

The aforementioned factors underscore the significance of exploring alternative remediation approaches for effective management of FOG. To ensure effective and targeted strategies for FOG deposit management, it is essential to identify and understand the processes involved in deposit formation. This knowledge remains limited, leading to a variety of current practices, with numerous cases of unsuccessful implementation. A notable drawback in current mitigation approaches is the predominant focus on lipids' degradation. However, a more recent study by Otsuka et al. (2020) highlighted the significance of Total Suspended Solids (TSS) in FOG formation, while study by Brooksbank et al.

(2007) revealed that the genesis of FOG deposits within the sewers is fundamentally linked to the presence and actions of native microorganisms in oil-rich wastewater.

1.5 Bioremediation of FOG

The biological treatment of FOG is gaining traction as a promising alternative or complement to conventional sewer cleaning methods. This trend is attributed to a multitude of advantageous factors, including its relatively lower cost and energy requirements, ease of administration, and its long-term safety benefits for both humans and the environment (Lauprasert et al., 2017). While FOG deposits in sewers undergo a natural process of biodegradation, this degradation is significantly slower than the rate of FOG formation and accumulation. Nevertheless, this biodegradation can be enhanced by the addition of bioproducts, such as enzymes, live microorganisms, biosurfactants or biostimulant to sewers and grease traps. The degradative activities of microbial communities can be amplified either by enhancing the existing wastewater microorganisms, or by introducing new ones through a process known as bioaugmentation.

Several species of bacteria and fungi, tested as single cultures and mixed consortia, have shown capacity to enhance the breakdown of oil by producing external lipases. These enzymes work to break down triglycerides (TGDs) into free fatty acids (FFAs) and glycerol, making these components accessible for consumption by other microorganisms (He, 2012; Lauprasert et al., 2017). Using a combination of microorganisms (consortium) often results in more effective removal of lipids compared to using a single strain (Brooksbank et al., 2007; Lauprasert et al., 2017; Tzirita et al., 2019). Effective treatment of oil-rich wastewater was reported with the use of consortia comprising combinations of *Bacillus spp*, *Pseudomonas spp*, *Trichococcus*, *Staphylococcus epidermidis*, *Saccharomyces cerevisiae*, *Cellulomonas uda*, *Micrococcus sp.*, and *Thiobacillus novellus* (Anuforum, 2013; Brooksbank et al., 2007; Lauprasert et al., 2017; Tzirita, 2012). Although, there is a scarcity of studies evaluating the effectiveness of commercially available FOG-degrading supplements on large-

scale industrial trials documented in scientific literature, one notable example has been reported by Arthur and Blanc (2013). The practical application of bioremediation by Anglian Water at a challenging site resulted in a noteworthy 50% reduction in sewer blockage incidents, accompanied by substantial cost savings for the company. Notwithstanding that, the available data often shows considerable discrepancies in the efficiency of biological products, which raises doubts about the effectiveness of this approach.

1.6 Uncouplers for FOG remediation

Numerous studies have investigated the viability of employing chemical and biological uncouplers for TSS and biomass reduction. The majority of metabolic uncouplers are hydrophobic weak acids with a high degree of lipid solubility. Functioning as protonophores, these compounds have the ability to cyclically transport protons across the cellular membrane, bypassing their contribution to ATP synthesis (Jiang & Liu, 2010; Yang et al., 2003). In consequence, energy allocation for cell maintenance outweighs growth by restricting biomass anabolism, while allowing substrate catabolism to continue (Lin et al., 2022; Zheng et al., 2008). In essence, induced energy uncoupling means that lower biomass growth will occur for the same or higher substrate consumed (Lin et al., 2022; Yang et al., 2003; Ye & Li, 2005).

The initial attention in regards of using uncouplers has been on growth systems such as activated sludge. Nevertheless, the use of metabolic uncouplers in environmental research is steadily increasing annually, with the research focus expanding onto other microbially facilitated processes such as membrane biofouling (Xu & Liu, 2010), biofiltration (Gostomski & De Vela, 2018) and substrate bioremediation (Saini & Wood, 2008). The latest developments in identifying and comprehending the mechanisms behind metabolic uncoupling hold the potential to extend their applicability to other microbial-driven wastewater processes, including FOG bioremediation. The benefits of incorporating uncouplers include capability of treating complex organic pollutants, economic viability and ease of application (Guo et al., 2013).

1.7 Aims and objectives

The overall aim of this thesis is to establish how Fat, Oil and Grease (FOG) deposits form in sewer networks and how they can be effectively degraded (deposit) or inhibited (wastewater) with bio-additive products.

In pursuit of this aim, the thesis will establish a robust scientific foundation for evaluating alternative FOG management strategies based on deposit formation stage. Additionally, it seeks to offer actionable insights to water utilities for enhancing their existing bioremediation practices.

To deliver against the overall aim the following objectives were set:

Objective 1: To understand how FOG related deposits form in sewer networks.

Objective 2: To produce a state of the art critical review to investigate the mechanism of chemical uncoupling and its application in reducing biomass yield in biologically driven processes, with a potential focus on bioremediation of FOG deposits in sewers.

Objective 3: Establish the potential to manage FOG deposits through the dosing of an uncoupler.

1.8 Thesis structure

The steps involved in delivery of this work are reported in the Thesis visualisation diagram (Figure 1-3). This thesis is divided into chapters formatted as papers for publication. An outline of the objective(s) by each paper is shown below (Table 1-6). All papers were written by the primary author, Natalia Jawiarczyk and edited by Prof. Bruce Jefferson and Dr Yadira Bajon Fernandez. All experimental work was designed, co-ordinated and completed by Natalia Jawiarczyk at Cranfield University (UK) with contribution from MSc student Elvis Boampong to Chapter 3 as follows: scale-up experiments and mass balances; deposition compositional changes under inhibition and rehabilitation modes of action of the bioadditive, including analytical analysis of liquid and solid samples.

Volatile Fatty Acids (VFA) and Free Fatty Acids (FFA) analysis using high-performance liquid chromatography (HPLC) and ion analyses using ion chromatography (IC) were carried out by technician Monika Jodkowska, elemental analysis using Elemental Analyser were carried out by technicians Maria Biskupska and Richard Andrews, total carbon and nitrogen using TOC analyser were carried out by technician Rodrigo Leitao at Cranfield University (UK). Data interpretation was carried out by Natalia Jawiarczyk.

Chapter 2 is designed to explore FOG deposit formation. Knowledge regarding processes involved in sewer FOG deposits formation remains limited, and available reports lack or contain limited data regarding other compositional characteristics, i.e., carbohydrates and proteins levels, in analysed deposits or wastewater. This chapter was designed to review the existing mechanisms of FOG deposits formation and to gain more comprehensive view on FOG deposition. Work investigated the mechanisms of FOG deposit formation in synthetic sewage formula at 5 and 20°C with 500 mg/L of oil and 25-250 mg/L oleic and stearic acids addition. (Chapter 2, paper 1 – in preparation: Jawiarczyk, N., Bajon Fernandez Y., Villa R., Alibardi L. Mitchell., G. and Jefferson, B. Fats, Oils and Grease (FOG) deposit formation in sewers: Understanding the role of carbohydrates and other constituents in the water.)

Chapter 3 presents a critical literature review exploring the use of uncouplers for bioremediation of FOG deposits in sewers. Through their ability to induce metabolic changes, disrupt biofilm integrity, and challenge microbial diversity, uncouplers offer a potential pathway to mitigate FOG-related issues. Uncouplers have shown promising results in reducing biomass accumulation, inhibiting biofilm formation, and inducing biofilm detachment. The potential benefits of this approach include inhibiting the FOG formation, rehabilitation of existing deposits, and potentially enhancing downstream processes. (Chapter 3, paper 2 – in preparation: Jawiarczyk, N., Bajon Fernandez Y., Villa R., Alibardi L. Mitchell., G. and Jefferson, B. Understanding the potential for chemical uncouplers to aid the bioremediation of Fats, Oils and Grease (FOG) deposits in Sewers: Current Understanding of chemical uncouplers and future prospects.)

The outcome of chapter 2 and 3 informed the design of Chapter 4. Chapter 4 investigates the effectiveness of the commercial metabolic uncoupler (yeast protein extract) as a measure for effective inhibition of FOG formation in oil-rich synthetic sewage and rehabilitation of pre-deposited FOG under bioadditive range of 3-20 ppm. The chapter offers a novel approach to understand FOG deposit management by performing mass balances utilising controlled media and provides insights to changes occurring to deposits composition under uncoupler addition. (Chapter 4, paper 3 – in preparation: Jawiarczyk, N., Boampong E., Bajon Fernandez Y., Villa R., Alibardi L. Mitchell, G. and Jefferson, B. Inhibition and Rehabilitation of Fats, Oils, and Grease (FOG) deposits in sewers with an energy uncoupler with surfactants.)

The insights gained from the preceding chapters served as a foundation for conducting an economic analysis on uncouplers use for FOG management in sewers (Chapter 5). The costs and benefits of this approach are discussed. (Chapter 5, paper 4 – in preparation: Jawiarczyk, N., Bajon Fernandez Y., Villa R., Alibardi L. Mitchell., G. and Jefferson, B. Economic assessment of dosing an energy uncoupler with surfactants for Fats, Oils, and Grease (FOG) management in sewers.)

Chapter 6 provides a discussion to reflect on the overall outcomes of this thesis and how it relates to the wider context of FOG deposit formation and management using uncouplers.

Finally, Chapter 7 summarises the overall conclusions of this thesis and recommends future areas of focus to drive the development of bioremediation of FOG deposits in sewers.

Table 1-6 Thesis plan and status of paper submissions.

Chapter	Paper	Objective	Title	Journal	Status
2	1	1	Fats, Oils and Grease (FOG) deposit formation in sewers: Understanding the role of carbohydrates and other constituents in the water.	Water Research	In Preparation
3	2	2	Understanding the potential for chemical uncouplers to aid the bioremediation of Fats, Oils and Grease (FOG) deposits in Sewers: Current Understanding of chemical uncouplers and future prospects.	Environmental Technology Reviews	In Preparation
4	3	3	Inhibition and Rehabilitation of Fats, Oils, and Grease (FOG) deposits in sewers with an energy uncoupler with surfactants.	Water Research	In Preparation
5	4	3	Economic assessment of dosing an energy uncoupler with surfactants for Fats, Oils, and Grease (FOG) management in sewers.	Science of the Total Environment	In Preparation
6	--	1, 2, 3	Implication of the work – Practical guide to effective sewer Fats, Oils and Grease (FOG) management with biologically based energy uncoupler solutions.	--	--
7	--	--	Conclusions and Future Work	--	--

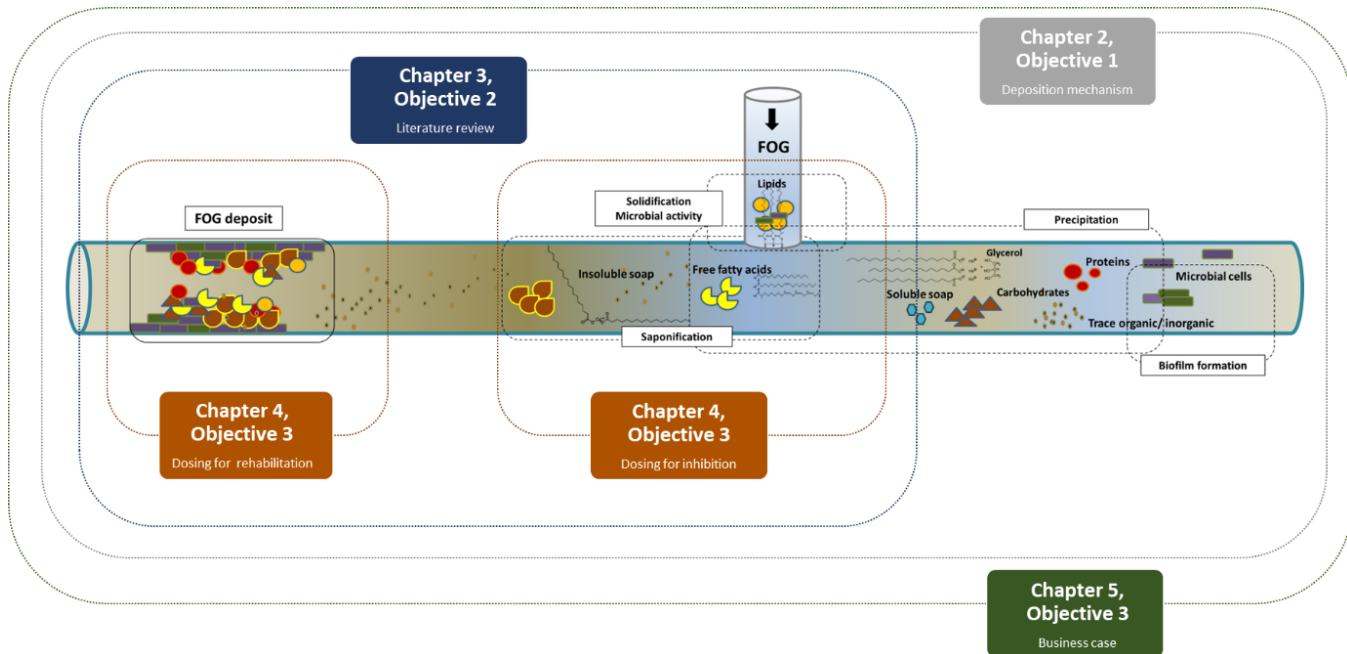


Figure 1-3 Visualisation of Thesis structure.

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2 Chapter 2 Fats, Oils and Grease (FOG) deposit formation in sewers. Understanding the role of carbohydrates and other constituents in the water

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Abstract

The study investigated the mechanisms of FOG deposit formation in synthetic sewage at 5 and 20°C with 500 mg/L of oil and 25-250 mg/L oleic and stearic acids addition as well as analysis of field deposits. Deposits formed in synthetic sewage displayed compositional characteristics comparable to field FOG samples, however despite the generally hold view, analysis of field and synthetic FOG revealed that saponification is not the leading process in deposit formation. Knowledge regarding processes involved in sewer FOG deposits formation remains limited, and available reports lack or contain limited data regarding other compositional characteristics, i.e., carbohydrates and proteins levels, in analysed deposits or wastewater and this research responds to this knowledge gap. Results of this research suggest that the composition of FOGs is dependent on chemistry of wastewater and external conditions in which they form, with only limited influence of temperature at investigated range. Overall results shows that synthetic sewage is a good matrix to investigate deposit formation, and that FOG formation can be successfully replicated under controlled lab conditions using synthetic wastewater. In general, the results of the research question and provide an alternative to the currently accepted model of FOG deposit formation through saponification. Instead, the work proposes a two-stage model based on the initial starch-lipid complexation followed by growth through accumulation of fats, lipids, carbohydrates, proteins and calcium.

Keyword: Fat, oil and grease, FOG deposits, deposition mechanism, starch, binary starch complexes, tertiary starch complexes, FFAs

2.1 Introduction

Waste fats, oils and greases (FOG) discharged down the drain from residential areas and food service establishments (FSEs) deposit and accumulate in sewers, typically between 50 and 200 meters from the point of discharge (Keener et al., 2008). This highly adhesive, congealed material adheres to sewer walls mainly

above the water levels (Iasmin et al., 2014) and attracts debris and non-flushable items, i.e. wet wipes and sanitary products, from wastewater, cementing them together to form much harder masses called fatbergs (Arthur & Blanc, 2013; Wallace et al., 2017). Build-up of FOG deposits have damaging consequences to the system, ranging from local domestic pipes blockages to disruption of the sewers operation through network blockages and associated sewer overflows, and in the worst case, properties flooding.

To prevent FOG from entering the drains and sewers numerous countries have introduced consent requirement for effluent from non-domestic discharges as alternative or supplement to legislation. Compliance can be achieved through collection of used cooking oils and installation of grease interceptors at FSEs designed to capture FOG materials from wastewater. Outreach and education campaigns have also been implemented by many water companies to raise public awareness about the detrimental consequences of incorrect FOG disposal. However, current approaches remain insufficient, and FOG-related problems re-occur with increasing frequency. Ultimately, if not prevented, reactive, mechanical rehabilitation of blockages is required which is time consuming and costly. Recent estimates suggest that FOG related sewer blockages account for around 50% of all sewer blockages in USA (Keener et al., 2008) and up to 70% (British Water, 2015) in the UK, translating to an annual maintenance and rehabilitation cost of \$1 billion (NACWA, 2017) and £100 million (Water UK, 2018) in the USA and UK respectively.

Although FSEs are often referred as primary polluter, domestic wastewater is also an important contributor to FOG contamination (Nieuwenhuis et al., 2018). Both contain FOG, carbohydrates and proteins with different amounts of fibres (predominately from domestic wastewater) and alkaline and acidic chemicals used in cleaning practices (predominately from FSEs) (Huang et al., 2010; Raunkjer et al 1994; Sophonsiri & Morgenroth, 2004; Gurd et al., 2019). In addition, food processing, i.e., cooking and frying, can trigger a series of changes and interactions resulting in the formation of binary starch-lipid or ternary starch-lipid-protein complexes (Liu et al., 2009; Wang et al., 2020). The longer the chain

and higher saturation degree the more stable the complexes that formed are (Wang et al., 2020; Zhang et al., 2010).

The commonly hold view on FOG deposit formation is that it is mainly driven by two overlapping reactions (Figure 2-1): 1) fat solidification, due to temperature and hydraulic pressure drop in sewers (Williams et al., 2012), and to a greater extent 2) saponification, the chemical reaction of animal and/or plant lipids with calcium ions to form soaps (He et al., 2011; Keener et al., 2008; Williams et al., 2012). The lipids first undergo a hydrolysis reaction to form free fatty acids (FFAs) which react with calcium in the wastewater under alkaline conditions (Keener et al., 2008). This saponified core then attracts calcium excess, unreacted fatty acids and debris from wastewater toward itself leading to deposit build up (He et al., 2013). During saponification, two moles of FFAs react with one mole of calcium to produce calcium- based metallic salts. Hydrolysis of triglycerides to FFAs follows a one-step fourth order irreversible reaction proportional to the presence of un-reacted triglycerides and the water in the system. FFAs are then consumed following third order reaction kinetics (Iasmin et al., 2016). Once 25-30% of the stoichiometry of resultant soap has been formed, the process becomes autocatalytic.

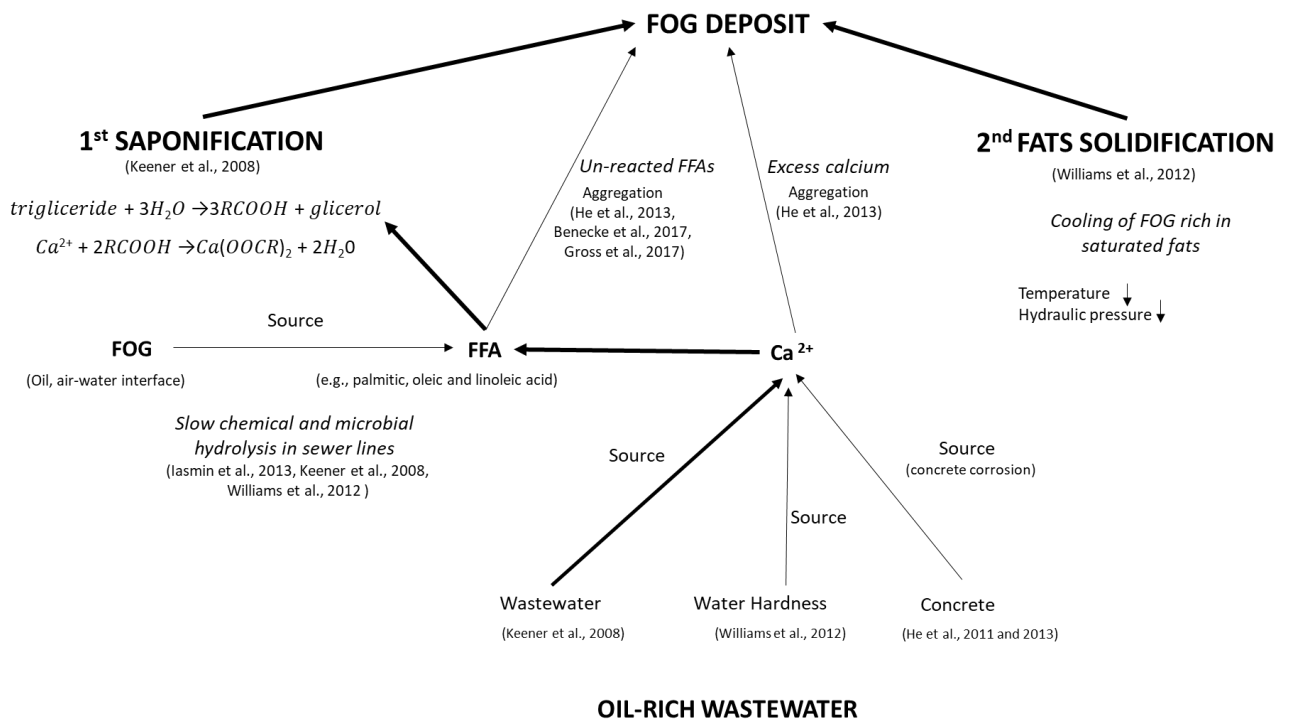


Figure 2-1 Current understanding of Fats, Oils and Grease (FOG) deposition mechanism in sewer.

The importance of saponification was initially hypothesised based on high concentrations of fats and calcium measured in field samples and observations on physical properties, and visual characteristics of analysed deposits (Keener et al., 2008). Support was provided by Fourier Transform Infrared (FTIR) analysis comparing pure metallic salts, laboratory induced saponified solids from GI effluent and field samples (He et al., 2011). Subsequent studies have supported such observation whilst reporting greater heterogeneity (He et al., 2013; Iasmin et al., 2014; Williams et al., 2012).

The authors also suggested that excess calcium and unreacted FFAs aggregation are the secondary mechanisms in deposit formation (He et al., 2013). In part these observations were driven by the analytic techniques which were not able to distinguish between free and saponified FFAs (Gross et al., 2017). Consequently, the concept has been refined to focus on FFAs aggregation rather than just the FFA salts and can occur without the presence of metals (Benecke et al., 2017; Gross et al., 2017). The predominate FFAs

found in FOG deposits are palmitic and oleic acids followed by stearic and linoleic acids (Benecke et al., 2017; He et al., 2011; Keener et al., 2008; Williams et al., 2012) offering both saturated and unsaturated FFAs.

The required alkaline conditions for saponification (i.e. pH 10.1-11.4) (Dunn, 2010) are not commonly found in sewers with the exception of dish-washer discharges (Gurd et al., 2019). Accordingly, some authors suggest that the initial saponified deposit forms in these alkaline niches and then grow within the more general sewer conditions (He et al., 2013; lasmin et al., 2014; Keener et al., 2008). Ultimately, there remain questions as to what drive deposition. Previous approaches to resolve this have focussed on analysing FOG deposits although not all critical constituents have been fully identified (Benecke et al., 2017; Collin et al., 2020; Gross et al., 2017; He et al., 2011; Keener et al., 2008; Nieuwenhuis et al., 2018; Williams et al., 2012). The majority of the work has been conducted on real wastewater sources with challenges around consistency and comparability of findings (Benecke et al., 2017; Ducoste et al., 2008; Williams et al., 2012). Other groups have tried to resolve this by using synthetic wastewater but limit their constituents to one or two components negating potential synergistic interactions (He et al., 2011; lasmin et al., 2014; Keener et al., 2008). Importantly, the conducted studies neglect the role of indigenous microflora on the rate of deposit formation, although previous work has indicated its potential significance (Brooksbank et al., 2007)

The current research aims to resolve such ambiguities through working with both real and synthetic systems in terms of deposit analysis and conducting controlled deposition trials with a synthetic recipe containing all major components in order to test the current reported mechanisms and control parameters. The work is based on the hypothesis that FOG deposits on sewer pipe wall occurs through a two-stage process initiated by co-precipitation of starch and FFAs released from triglycerides through microbial activity, followed by subsequent growth by saponified and unreacted fats and protein accumulation and compositional transformations caused by deposit ageing.

2.2 Materials and Methods

All reagents used were purchased from Fisher Scientific (UK) unless otherwise specified.

2.2.1 Field samples

FOG samples used in this study were collected from various locations on the UK sewage network. Samples were sourced from pumping stations (pumping station 1-2), sewers (sewer 1-2) and wastewater treatment plants (WWTP 1-4). Pumping station and sewer samples were manually excavated during the clean-up of the locations. Wastewater treatment plant samples were collected by Severn Trent Water operatives, who identified them as fat related. WWTP samples were composed of floating scum accumulating on grit removal and primary tank walls (WWTP1 and WWTP 2, respectively), and grease balls accumulating at the primary tank wall and pipe from the sludge thickener to the holding tanks (WWTP 3 and WWTP4, respectively). Sewers samples were provided by Thames Water. All samples were stored at 4°C.

2.2.2 Synthetic Sewage

Synthetic sewage (SS) was prepared using a recipe adapted from OECD guidelines, Test No. 303 (OECD, 2001) using tap water and addition of raw wastewater at 2 mL wastewater/1L SS to introduce indigenous microorganisms. The formula contained starch (100 mg/l), peptone (160 mg/L), meat extract (110 mg/L), urea (30 mg/L), sodium chloride (7 mg/L), potassium dihydrogen phosphate (28 mg/L), calcium chloride (4 mg/L) and magnesium sulphate (2 mg/L). Finally, synthetic sewage was spiked with vegetable oil (Co-op, Cranfield, UK) (500 mg/L) to replicate oil-rich wastewater environment (Table 2-1).

Table 2-1 Characteristics of synthetic sewage according to standard laboratory methodology used for the study.

Compound	Concentration (mg/L)
Total Suspended Solids (TSS)	157±2
pH	7.1
Temperature (°C)	20
Chemical oxygen demand (COD)	1361±166
Lipids (HEM)	516±44
Carbohydrates	130±4
Proteins	140±14
Carbon/Nitrogen (C/N) ratio w/out lipids	2.5
Carbon/Nitrogen (C/N) ratio with lipids	6.2

2.2.3 Synthetic FOG samples

2.2.3.1 Deposit ageing

To explore the leading reactions in deposit formation and how deposits transform over time synthetic, triplicate FOG samples were formed in 8 L batch reactors using the SS recipe spiked with 500 mg/L vegetable oil. This volume was chosen to ensure that a sufficient quantity of synthetic FOG was generated for subsequent analysis of its composition, including the quantification of lipid, protein, and carbohydrate fractions, as well as further differentiation of fat components such as free fatty acids, saponified fats, and unsaponified triglycerides. Deposits were formed at room temperature, during 7-180 days periods, under constant stirring (Overhead stirrer, Heidolph, Schwabach, Germany) at 80 rpm. At the end of each experiment, solid and liquid samples were recovered by centrifugation at 4000 G, 5 minutes, 20°C (ThermoScientific Sorvall Legend RT+, Massachusetts, USA).

2.2.3.2 Deposit formation investigation

To understand how the characteristics of wastewater and external conditions influenced the deposit formation, triplicate samples were prepared in 250 mL flasks filled with 50 mL of synthetic wastewater. This volume was selected as it was sufficient to allow the formation of visible precipitates while also providing enough material to be retained on a filter and weighed for meaningful and reliable results. For each set of experiments, control tests were run in parallel. Flasks were kept under constant mixing under aerobic conditions, using an orbital shaker at a rotation speed of 80 rpm (Stuart Digital Shaker, Cole-Parmer, St. Neots, UK). After completion of trials FOG precipitates (15 min) and FOG deposits (days) were harvested by vacuum filtration (Whatman GF/F glass 0.7 μm retention filter, Kent, UK) to measure the dry weight of material retained by the filter (EPA, 1684).

2.2.3.2.1 Temperature and length of the trials

To investigate how the temperature influence the FOG formation, trials were performed in synthetic sewage spiked with 500 mg/L vegetable oil, 50 mg/L of oleic acid (99%, Alfa AesarTM) or 50 mg/L of stearic acid (97%, ACROS OrganicsTM), under 5°C and 20°C. Deposits were harvested on day 0, 2, 7 and 9.

2.2.3.2.2 Initial concentration of FFAs

Trials were conducted with additions of oleic, stearic, or oleic and stearic acids (50:50, w/w) across a range of concentrations between 25-250 mg/L. Deposits were formed for 2 days at 5°C and 20°C in synthetic sewage spiked with 500 mg/L vegetable oil

2.2.3.2.3 Significance of individual components of wastewater

2.2.3.2.3.1 Formula built up

The individual components within the recipe were trialled progressively by adding an additional component from trial to trial (Table 2-2). Preliminary experiments showed this sequence ensured consistent results, with no effect of the additive order on the final outcomes. The trials were conducted with either 25 and 50 mg/L

oleic and stearic acid and ran for 15 minutes (precipitates), or, 500 mg/L vegetable oil for 2 days at 20°C (deposits).

Table 2-2 Built up of synthetic sewage formula.

Solution	Composition
Control	Water
1	Water, starch
2.	Water, starch, peptone
3	Water, starch, peptone, meat extract
4	Water, starch, peptone, meat extract, urea
5	Water, starch, peptone, meat extract, urea, NaCl
6	Water, starch, peptone, meat extract, urea, NaCl, CaCl ₂ .
7	Water, starch, peptone, meat extract, urea, NaCl, CaCl ₂ , HK ₂ O ₄ P,
8	Water, starch, peptone, meat extract, urea, NaCl, CaCl ₂ , HK ₂ O ₄ P, MgSO ₄

2.2.3.2.3.2 Thermal deactivation of main compounds of synthetic sewage.

Each component was independently thermally deactivated at 121°C, for 15 min (Priorclave Compact C60 Autoclave, 4590, London, UK). Solutions were then left to cool down and synthetic sewage formula was built up by addition of remaining compounds. The trials were conducted with either 25 and 50 mg/L oleic and stearic acid and run for 15 minutes (precipitates), or, 500 mg/L vegetable oil for 2 days at 20°C (deposits).

2.2.3.2.3.3 Role of Microorganisms

To investigate the significance and the role of microorganisms in deposit formation, synthetic sewage, and glassware were autoclaved at 121°C, for 15 min (Priorclave Compact C60 Autoclave, 4590, London, UK). Oil and FFAs were filter sterilized (Sartorius 0.2 µm retention syringe filter, Göttingen, Germany). The trials were conducted with either 25 and 50 mg/L oleic and stearic acid and ran for 15 minutes (precipitates), or, 500 mg/L vegetable oil for 2 days at 20°C (deposits).

2.2.3.2.3.4 Free Fatty acids and Starch interactions

To identify the significance of the initial starch and free fatty acids concentrations on the first stage of deposit formation, co-precipitation of compounds was induced in synthetic sewage solutions containing various molar ratios of starch vs. free fatty acids: 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 10.

2.2.4 Analytical methods

All samples were stored at 4°C prior each analysis to minimise microbial activity.

To assure the homogeneity of solid samples, synthetic FOG and field samples were subjected to pre-treatment comprised of moisture removal and grinding prior to compositional analysis. Samples were frozen at -80°C for two days and subsequently freeze-dried using Christ Alpha 1-2 LD (Osterode am Harz, Germany) until arriving to a stable weight. To produce fine samples, dry deposits were then grinded using pestle and mortar for 5 min.

2.2.4.1 Solid samples compositional analysis

Total solids (TS), volatile solids (VS) and ash content were determined according to standard methods EPA, 1684 (EPA, 2001). Characterization of the organic fractions of the deposits included lipids, carbohydrates, proteins, and fibre content determination. Total lipid content was established using the reported ThermoScientific Accelerated Solvent Extraction method, Application Note 334 (ThermoFisher, 2012) with hexane as a solvent, on a Dionex ASE 200 (Dionex, California, USA), and determined by gravimetric analysis. Proteins were determined following the Dumas method BS EN 13652-2:2001 (British Standard Institution, 2001) where total nitrogen content was measured using the Elemental Vario EL cube analyser (Elementar, Stockport, UK). The content of proteins was quantified by multiplying the total nitrogen content by the conversion factor of 6.25 (Gurd et al., 2019). Fibre content was established as dried and ashed residue from neutral detergent digestion of the sample under controlled conditions which include cellulose, hemicellulose and lignin as the major components following AOAC Method 973.18 (AOAC, 1990). Carbohydrates were estimated as the remaining fraction after lipids, fibre, proteins and ash deduction from dry sample

weight. Analysis of saponified and unsaponified fractions of total lipid content were established by sample fractionation with dichloromethane (Benecke et al., 2017).

2.2.4.2 Liquid samples compositional analysis

Liquid samples taken prior and after deposit formation were analysed for their composition. Total suspended solids (TSS) and Volatile Suspended Solids (VSS) were determined following standard methods APHA, 2540D (APHA, 1999). pH was measured with laboratory pH meter (Jenway 3540, Cole-Parmer, St. Neots, UK). Chemical Oxygen Demand (COD) was determined photometrically using spectroquant cell test kits with a Nova 60 A Spectroquant photometer (Spectroquant® NOVA 60, Merck KGaA, Darmstadt, Germany). Ions including chloride, nitrite, nitrate, bromide, lithium, sodium, ammonium, potassium, magnesium, calcium, phosphate and sulphate were measured using the Thermo Scientific Ion Chromatography system ICS900 (Dionex, California, USA) with an IonPac™ CS12A column and guard. A 20mM methanesulphonic acid was used as eluent and 100mM tetrabutylammonium hydroxide as regenerant. An injection volume of 20 µl with a 1ml/min flow and ambient temperature was used for the analysis following Standard Test Methodology, D6919-09 (ASTM, 2009). Ion concentrations were calculated via Chameleon software using standard calibration curves. The total carbon and nitrogen (TC-TN) were measured using Shimadzu TOC-V analyser (Shimadzu, 2007).

The compositional characterisation of liquid samples included measurements of oil, carbohydrates, and protein levels. The oil content of the samples was quantified as hexane extractable material (HEM) using the centrifugation method (Barton, 2012). Carbohydrates content was established by phenol-sulphuric colorimetric method (Dubois et al., 1956). A standard calibration was prepared using glucose. Protein concentrations were measured using the Thermo Scientific™ Commassie Protein Assay Kit. A standard calibration was prepared using bovine serum albumin (BSA). In addition to that, proteins were estimated from the difference between TN, NH₄-N, NO₃-N and NO₂-N multiplied by a standard nitrogen conversion factor of 6.25. During colorimetric tests,

absorbance was measured using Tecan Infinite 200 PRO multifunctional microplate reader (Tecan, Männedorf, Switzerland).

2.2.4.3 Triglycerides and Free Fatty Acids analysis

Hexane extracts of solid and liquid samples were analysed for triglycerides (TGDs) and free fatty acids (FFAs) content using high- performance liquid chromatography with evaporative light scattering detector (HPLC-ELSD). Dry lipid samples were reconstituted in HPLC-grade chloroform-methanol (50:50%, v/v) solvent mixture, filtered through 0.45µm retention membrane filter (Whatman, Kent, UK) and stored at -20°C protected from light.

5 mg/mL stock solutions of trioleate and tripalmitate (TGDs), arachidonic, cis-4,7,10,13,16,19-docosahexaenoic, elaidic, erucic, linoleic, linolenic, nervonic, oleic, palmitoleic, petroselinic, arachidic, behenic, decanoic, dodecanoic, hexanoic, lignoceric, myristic, octanoic, palmitic and stearic acid (FFAs) were prepared in chloroform-methanol (50:50%, v/v), and glycerol in MiliQ water. Working standard solutions in range 5-500 mg/L (TGDs), 5-1000 mg/L (FFAs), 5-500 mg/L glycerol were prepared by serial dilution from the respective stock solution. Mixture of standards was prepared by mixing equal concentrations of triglycerides (TGDs) standards and free fatty acids (FFAs) standards. The chromatographic system included an Agilent 1260 Infinity module equipped with an autosampler (G132913 – 1260 ALS), evaporative light scattering detector (ELSD, G4218A), Quaternary Pump (G1311C) and thermostat (G1330B). The separation of FFAs was performed on a Phenomenex Luna 3µ 150x2.1mm analytical column, while separation of triglycerides on Phenomenex Kinetex 250x4.6 mm 5µ. The signal was acquired and processed with ChemStation software. The composition of the solvents and running program for the HPLC-ELSD method are summarized in Table 2-3 (FFAs) and Table 2-4 (TGDs). The method for TGDs was adapted from AOCS Official Method, Ce 5b-89 (AOCS, 2017) and for FFAs from Phenomenex Luna Applications: ID 1258. Resolution of FFAs was achieved within total run time of 30 minutes using the flow rate of 0.3 mL/min at gradient conditions while the resolution of TGDs within total run of 25 minutes at isocratic conditions using the flow rate of 1.0 mL/min. The columns

temperature was 25°C. The optimized ELSD detector conditions were temperature of 40°C and gain 8. Scientific grade nitrogen gas was used as nebulizing gas. The volume of standards and samples injected was 10 µL. The identification of each compound was performed by comparing the retention time with the peak of corresponding standard.

Table 2-3 Solvent Program Gradient for Free Fatty Acids (FFAs).

Time (min)	Mobile phases	
	A ^a (%)	B ^b (%)
0	20	80
15	20	80
20	0	100
31	20	80
40	20	80

^aWater, ^bAcetonitrile

Table 2-4 Isocratic Solvent Program for Triglycerides (TGDs).

Time (min)	Mobile phases	
	A ^a (%)	B ^b (%)
0	50	50
25	50	50

^aAcetonitrile, ^bIsopropanol

2.2.5 Statistical Analysis

All tests were conducted in triplicates and reported as the mean of observation. Standard deviation was applied to determine variation in data.

2.3 Results

2.3.1 Understanding the significance of saponification in field and synthetic deposits

The total fat content reported across the collected samples revealed a general trend for decreasing total fat content in relation to the distance away from the source point (Table 2-5). To illustrate, the average total fat content decreased from a high of 76.9% taken from the domestic samples to 69.6% at the pumping stations, 64% in the sewers and 44.4% at the sewage treatment works. Interestingly, saponified fat represented a relatively small proportion of the total at between 3.5 and 24.7%, apart from fat balls collected at primary tank that had saponified fats content of 68%. The total fat was predominately comprised of free fatty acids (FFAs) which constituted between 68% and 100% of the total fat. Further, no link could be established between relative position of the sampling, total fat content and the levels of saponification, indicating the levels were not kinetically limited. Interestingly, high levels of carbohydrates were measured in FOG samples which varied between 0.4 and 43.7% (Table 2-5). The richest in carbohydrates were deposits collected from sewage works with an average of 21.7%, followed by domestic at 14.7% and pumping stations at 9.6%. The lowest content, 5.6% was detected in sewer samples. The presence of carbohydrates measured in the majority of field deposits suggest that carbohydrates from wastewater are transferred into the deposits. In contrast, the average amounts of proteins detected in samples (expressed as TN) were relatively low and showed the general trend for increasing in relation to the distance away from the source point, i.e., domestic 0.6%, pumping station 1.8%, sewer 5.6% and WWTP 11.1% (Table 2-5). Low concentrations of proteins in the samples suggest minor to moderate contribution to deposition.

Deposits formed in synthetic sewage showed variability associated with its relative age, equivalent to the location of the field deposit. For instance, the total fat content of the synthetic trials was between 0.9-51.3% and compared to the average values of 69.9% for domestic samples and 44.4% for sewage works samples (Table 2-5). Further, the proportion of FFAs and saponified solids were

0-57.5% and 42.5-100% of the TF for the synthetic and 32-98% and 2-68% of the TF for the field samples (Figure 2-2 and Figure 2-4).

Table 2-5 Compositional profiles of field and synthetic FOG samples.

Sample	Moisture	Ash	Total (TF)	fat	FFAs	Saponified	Total nitrogen	Total carbohydrates	References
	%wt.	%DS	%DS	% of TF	% of TF	%DS	%DS		
FOG field samples									
Sewer1	29.3 (2.20)	0.9 (0.5)	89.8 (2.6)		79.2	20.8	0.2 (0.0)	6.6 (1.1)	This research
Sewer2	9.9 (2.4)	5.1 (1.4)	87.1 (10.1)		96.5	3.5	0.2 (0.0)	5.2 (4.7)	This research
Sewer	30.0 (2.9)	1.5 (0.9)	93.1 (9.2)				0.1 (0.0)	5.0 (8.7)	(Collin et al., 2020)
Sewer			79.1		100				(Gross et al., 2017)
Sewer1-3			26.1-48.9		91.2- 97.0				(He et al., 2011)
Sewer 1-30	21.5-87.1	1.7-6.5	0.0-54.8		100 ¹				(Williams et al., 2012)

Sample	Moisture	Ash	Total (TF)	fat	FFAs	Saponified	Total nitrogen	Total carbohydrates	References
	%wt.	%DS	%DS	% of TF	% of TF	%DS	%DS		
Sewer 1-27	53.9 (21.0)		85.5 (29.8)		88				(Keener et al., 2008)
Sewer			25-45		53-70		30-47		(Iasmin et al., 2014)
Sewer 1 -3		5.4-6.4	76.2-93.0		61.1-79.0		21.0- 37.9	0.81-2.80	(Benecke et al., 2017)
Sewer average	38.6 (30.7)	3.1 (2.5)	64 (30.7)		82.5 (15.6)		26.3 (13.9)	0.9 (1.0)	5.6 (0.9)
Domestic	1.2 (0.1)	0.0	84.5 (5.3)				0.1 (0.0)	14.7 (5.1)	(Collin et al., 2020)
Domestic- Lateral house connections	52.5-80.8		32-110		43-101				(Nieuwenhuis et al., 2018)

Sample	Moisture	Ash	Total (TF)	fat	FFAs	Saponified	Total nitrogen	Total carbohydrates	References
	%wt.	%DS	%DS	% of TF	% of TF	%DS	%DS		
Domestic-Kitchen drains	75-90.2		9-22		47-95				(Nieuwenhuis et al., 2018)
Domestic average	66.0		72.6		71.6				
FSE	14.8	0.0	100.0 (0.4)				0.1	0.0	(Collin et al., 2020)
Grease removal unit	(11.7)						(0.0)		
PS1	24.9	0.5 (0.2)	88.0 (1.3)	95.9	4.1		0.02	11.3	This research
Pumping station	(3.1)						(0.0)	(0.6)	
PS2	33.0	1.3 (0.02)	80.2 (17.3)	97.7	2.3		0.18	17.2	This research
Pumping station	(0.7)						(0.0)	(8.6)	
PS	46.1	3.5 (0.1)	93.1				0.6	0.4	(Collin et al., 2020)
Pumping station	(2.3)		(4.5)				(0.1)	(0.7)	
PS 1-3			78.5-91.4	100.0					(Gross et al., 2017)
Pumping station									

Sample	Moisture	Ash	Total (TF)	fat	FFAs	Saponified	Total nitrogen	Total carbohydrates	References
	%wt.	%DS	%DS	% of TF	% of TF	%DS	%DS		
PS 1-30	31.0-71.4	0.9-1.7	0.3-56.0		100.0				(Williams et al., 2012)
Pumping station									
PS average	41.3	1.6	69.6		98.4	3.2	0.3	9.6	
	(18.5)	(1.2)	(33.0)		(2.0)		(0.3)	(8.5)	
WWTP-1	98.6	0.2 (0.0)	32.0 (1.8)		95.6	4.4	2.7	36.1	This research
Grit removal- floating scum	(0.1)						(0.1)	(1.0)	
WWTP			88.8		100.0				(Gross et al., 2017)
Headworks									
WWTP	91.1	4.8 (3.9)	13.7				1.5	43.7	(Collin et al., 2020)
Inlet- floating scum	(1.5)		(2.4)				(0.3)	(8.8)	
WWTP-2	93.1	0.9 (0.0)	9.8		75.3	24.7	2.5	16.5	This research
Primary tank- floating scum	(0.1)		(4.3)				(0.1)	(1.9)	
WWTP-3	95.0	7.0	83.0		32	68	0.6	6.0	This research

Sample	Moisture	Ash	Total (TF)	fat	FFAs	Saponified	Total nitrogen	Total carbohydrates	References
	%wt.	%DS	%DS		% of TF	% of TF	%DS	%DS	
Primary tank	(1.0)	(4.0)	(4.0)				(0.1)	(2.0)	
Fat balls									
WWTP -1-	47.2	5.0 (0.8)	94.5				0.6	0.0	(Collin et al., 2020)
Inlet- fat balls	(10.9)		(3.3)				(0.0)		
WWTP	14.9-94.7	1.1-7.4	0.0-45.6		100.0				(Williams et al., 2012)
Fat balls									
WWTP-4	62.3	4.1 (0.2)	31.8 (4.4)	91.0	9.0		2.0	27.8	This research
Pipe to sludge thickener-solid	(0.4)						(0.1)	(2.0)	
WWTP average	74.6	3.8	44.4	88.3	17.5		1.7	21.7	
	(30.4)	(2.8)	(36.0)	(13.5)	(13.0)		(0.9)	(17.2)	
Sewage sludge	90.1	22.7	11.2				4.91	12.4	(Collin et al., 2020)
	(0.03)	(0.6)	(1.3)				(0.2)	(5.5)	

Sample	Moisture	Ash	Total (TF)	fat	FFAs	Saponified	Total nitrogen	Total carbohydrates	References
	%wt.	%DS	%DS		% of TF	% of TF	%DS	%DS	
Laboratory induced FOG deposits									
Synthetic sewage	78.2	2.4	21.3		19.3	80.7	9.2	18.7	This research
FOG1, day 7	(2.5)	(0.6)	(3.4)				(0.4)	(2.6)	
Synthetic sewage	78.5	2.1	51.3		51.9	48.1	4.6	17.7	This research
FOG2, day 14	(1.5)	(0.1)	(14.2)				(2.0)	(1.9)	
Synthetic sewage	77.8 (3.3)	2.6 (0.8)	43.5 (3.7)		57.5	42.5	4.0	28.7	This research
FOG3, day 28							(0.4)	(2.1)	
Synthetic sewage	75.0	2.7	18.1		14.4	85.6	4.3	52.2	This research
FOG4, day 90	(3.0)	(0.7)	(4.2)				(0.7)	(1.5)	
Synthetic sewage	70.4	0.4	0.9		0	100	3.5	77.8	This research
FOG5, day 180	(1.4)	(0.2)	(0.6)				(0.3)	(1.2)	
Synthetic sewage FOG average	76.0	2.0	27.0		28.6	71.4	5.1	39.0	
	(3.4)	(1.0)	(20.3)				(2.3)	(25.7)	

Sample	Moisture	Ash	Total fat	FFAs	Saponified	Total nitrogen	Total carbohydrates	References
	%wt.	%DS	%DS	% of TF	% of TF	%DS	%DS	
GI effluent 1-3			18.0-34.4	67.1-89.4				(He et al., 2011)
Distilled water	0.45-13.39		80.4-98.9	100.0				(Gross et al., 2017)

FSE- Food Service Establishment; PS- Pumping Station; WWTP- Wastewater Treatment Plant.

Total fat, ash and carbohydrate content are represented on sample dry weight. Free fatty acid content is shown as percentage of total fat. All experiments were performed in triplicate, and the data presented in Table 2-5 are three sample average, standard deviation is given in brackets.

Although, it is generally accepted that the presence of a strong oxidizing agent is required to initiate saponification, several authors also reported saponification under neutral pH (Dominic et al., 2013; He et al., 2011; lasmin et al., 2014; lasmin et al., 2016). This is illustrated in the current study with saponified levels of 18-25% at pH 7.1 and room temperature and a previous study at 22°C and pH 7 where saponified levels were reported to be 21.4-42.9% (lasmin et al., 2014). Further, saponification was observed to occur in the early phases of the synthetic experiments while accumulation of un-saponified fats occurred progressively through the experiments (Figure 2-3). To illustrate, saponified fats represent 80.7% on day 7, 48.1% on day 14, 42.5% on day 28, 85.6% on day 90 and 100% on day 180. The increase in the saponified fraction as time extended reflects degradation of un-saponified lipids rather than further generation of saponified material with lipid removal levels of 94±1% and 99% by day 90 and 180, respectively. Interestingly, the saponification ratio did not exceed 25% of total deposit despite a sufficient concentration of calcium and fat available in the synthetic sewage for it to progress. Instead, increasing levels of fats were incorporated through aggregation of unreacted fat, i.e., FFAs and triglycerides. A similar pattern of results was obtained by He et al.(2011), who reported limited saponification and aggregation of unreacted FFAs and excess calcium as a means of deposit mass growth. In the current trials only 14% of the available calcium was utilised within the first 90 days of the synthetic trials. Additional calcium accumulation into the deposit occurred by 180 days consistent with others who have reported accumulation of calcium within deposit not associated with saponified fats (Keener et al., 2008). Similarly, previous research has shown that increasing concentrations of calcium added to GI effluent (75-750 mg/L) led to increased levels of calcium incorporated into deposits, but the change was not accompanied by increase of the total fat content in deposit (He et al., 2011).

Deposit ageing also impacted the carbohydrate and protein levels with overall reduction across the 180 days of 66±1% and 53±1% for proteins and carbohydrates compared to 99±0% for lipids (Figure 2-3). The aged deposit sample (180 days) characteristics were similar to deposit described by Gross et

al. (2017) and Keener et al. (2008) who reported samples with negligible content of lipids and high levels of calcium. Furthermore, it has been observed that the physicochemical characteristics of synthetic deposits changed with age. Fresher deposits (7-28 days) formed sticky, large yellowish aggregates, whilst aged samples (90-180 days) appeared as greyish dispersed small flocs. A similar range of characteristics in field samples were reported by previous authors (Benecke et al., 2017; Collin et al., 2020; Gross et al., 2017; Keener et al., 2008).

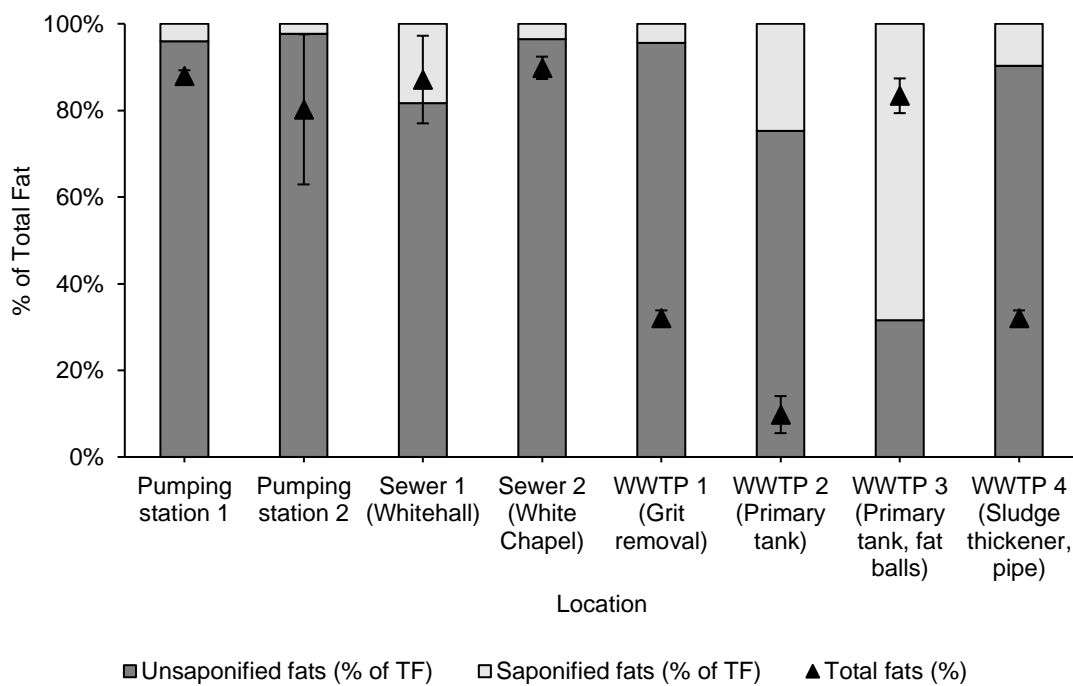


Figure 2-2 Fat profiles of field samples. Saponified vs. unsaponified solids

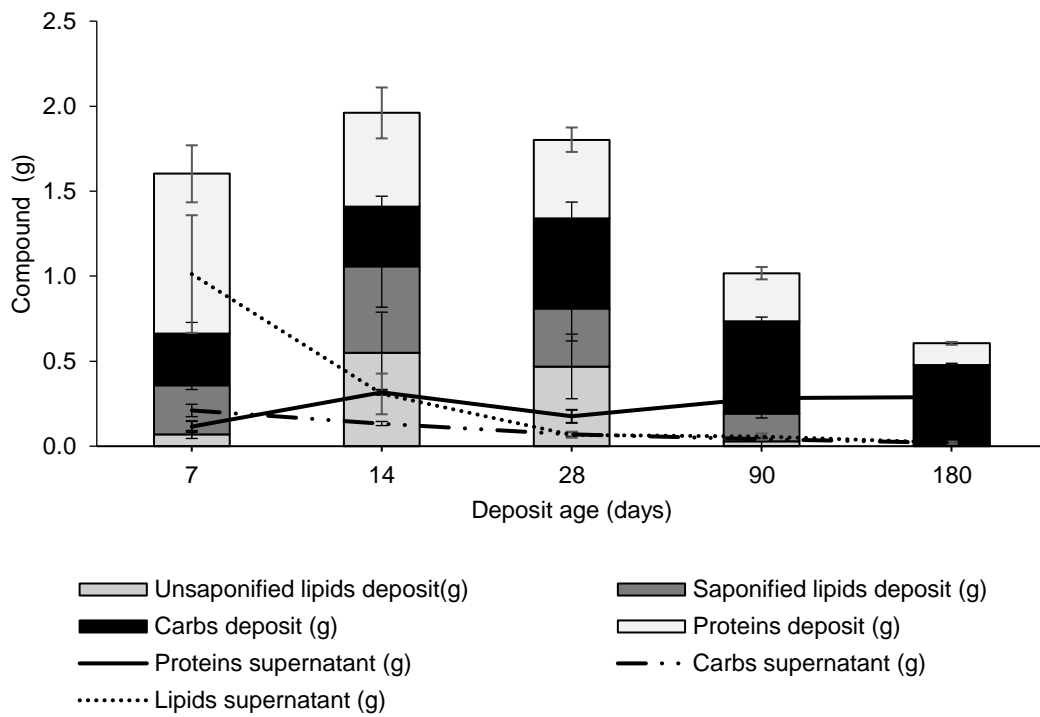


Figure 2-3 Mass balance and composition of synthetic FOG. Sum of saponified and unsaponified fats accounts for total lipid content in deposit samples. Error bars indicate the standard deviation of the experimental results.

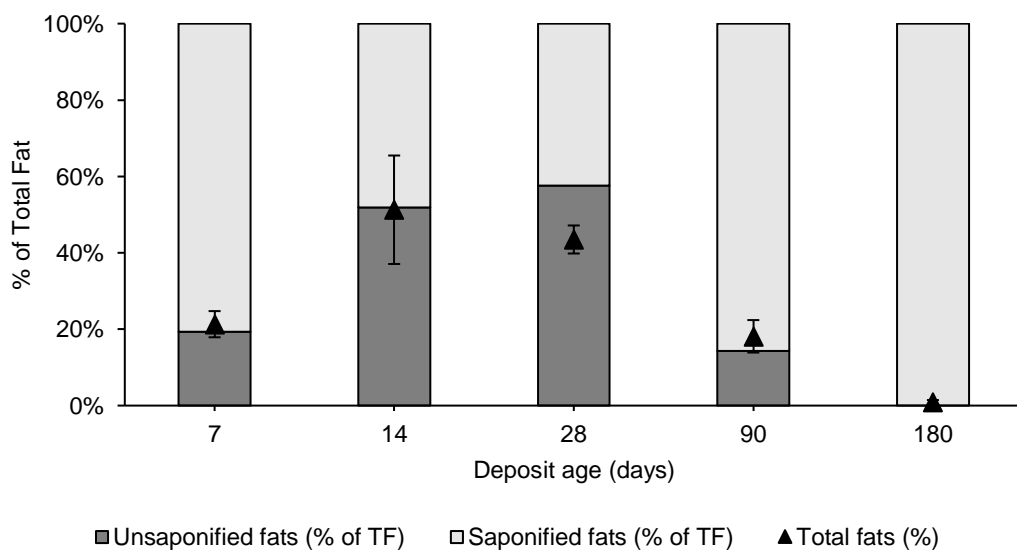


Figure 2-4 Fat profile of synthetic FOG versus the age. Error bars indicate the standard deviation of the experimental results.

2.3.2 Stages of deposit formation. Significance of temperature and initial FFAs presence and concentrations on FOG deposit formation process

Comparison of deposit formation at 5 and 20°C with stearic and oleic acid revealed no observable impact of temperature (Figure 2-5). To illustrate, in the case of stearic acid after 9 days the mass of deposit was 55.9 ± 11.9 mg at 5°C and 60.2 ± 15 mg at 20°C, whilst for oleic acid, the mass of deposit was 70.4 ± 15.1 mg at 5°C and 42.8 ± 15.6 mg at 20°C after 9 days. Alteration of the initial FFA concentration did not lead to significant differences in the observed amounts of deposits formed for both FFAs (Figure 2-6). To illustrate, the normalised deposit mass decreased from 18.1 ± 3.8 mg/mg when 25 mg/L of FFA was added to 3.0 ± 0.7 mg/mg when 250 mg/L of FFA was added, equivalent to an actual mass formed of 22.6 ± 4.8 mg with 25 mg/L of FFA and 37.8 ± 8.7 mg when 250 mg/L of FFA was added (Figure 2-6). Further, the calcium concentration in the water was 47.7 mg/L which exceeded the stoichiometric requirement for even the highest FFA dose, suggesting that saponification of FFA was not the principle driving reaction. Measurement of the ions in the water revealed reductions to calcium, phosphate, and sulphate by 23.7%, 39.8% and 15.5% respectively. No change was observed with regards to any other ions monitored which included chloride, nitrite, bromide, lithium, sodium, ammonium, potassium and magnesium. Interestingly no noticeable difference was again observed when operating at either 5°C or 20°C indicating that seasonal temperature changes in sewers were unlikely to impact deposition rates.

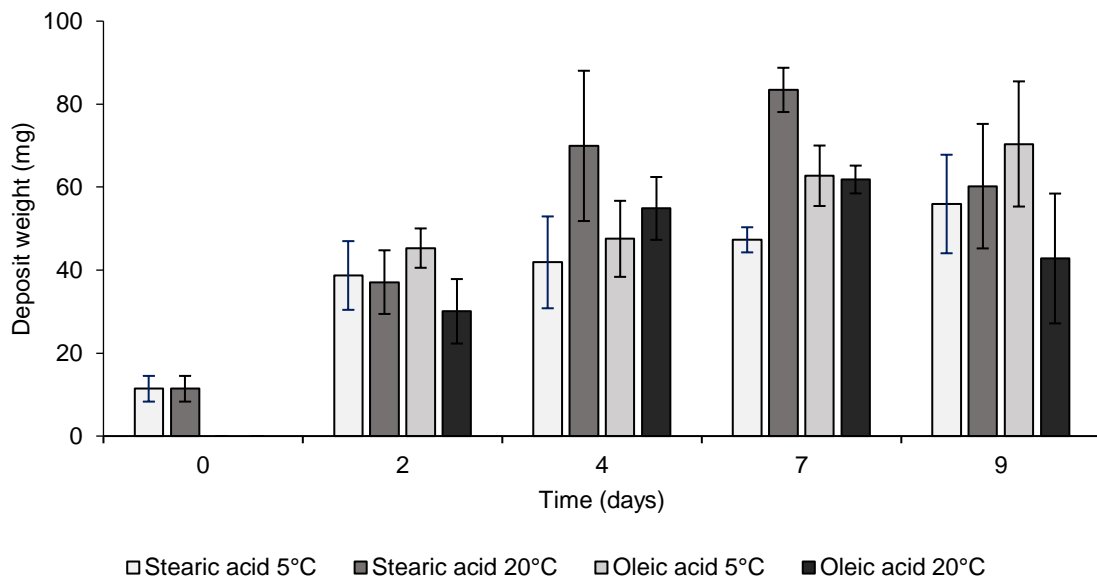


Figure 2-5 Formation of FOG precipitates during batch tests with different free fatty acid (FFA) types added (50 mg/L) expressed as deposit mass. Error bars indicate the standard deviation of the experimental results.

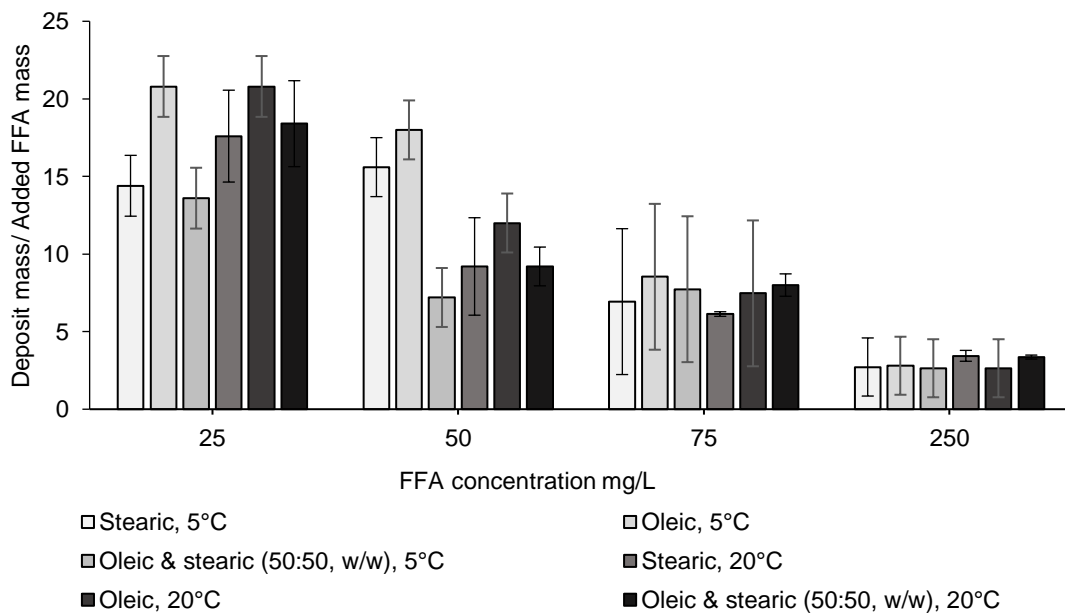


Figure 2-6 Formation of FOG deposits during batch tests (2 days) with different free fatty acid (FFA) concentrations and types added expressed as normalized deposit mass. Error bars indicate the standard deviation of the experimental results.

2.3.3 Wastewater components influencing 1st stage of deposit formation – precipitation

Deposit formation was further investigated by decoupling the different components within the synthetic wastewater. A series of trials were conducted where the recipe was progressively built up and the resultant deposit mass measured (Figure 2-7). In the case of the experiments where 25 mg/L of FFA were included, the precipitate mass increased from 1 ± 0.5 mg in just water to 2.8 ± 0.9 mg when starch was also added. No further measurable change in precipitate mass was observed due to the addition of peptone, meat extract or urea, indicating that starch interactions with FFAs are a key driving reaction. A similar observation was made in the case of adding 50 mg/L of FFA with the overall deposit mass increasing congruently with the added mass of FFA. Overall, the deposit mass was similar when using all the components (synthetic sewage) for the 50 mg/L FFA case but showed an increase compared to the component trials for the 25 mg/L FFA case.

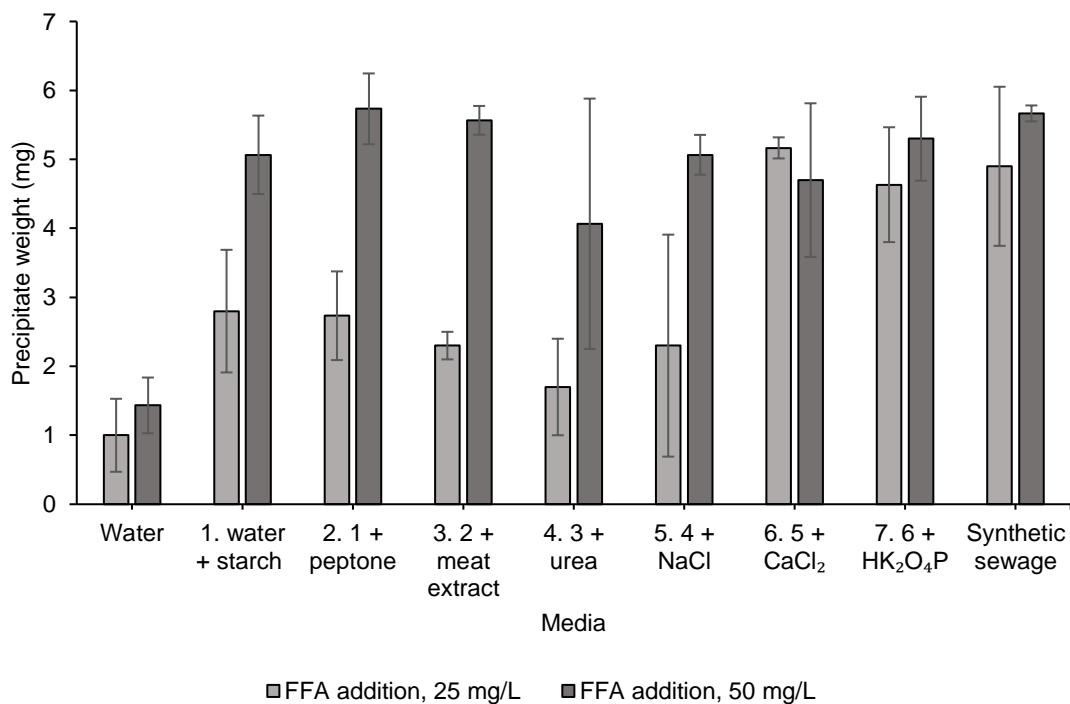


Figure 2-7 Average weight of FOG precipitates formed during batch tests using formulas with increasing number of individual synthetic sewage macro compounds. Error bars indicate the standard deviation of the experimental results.

Thermal pre-treatment of the different individual components of synthetic sewage (Figure 2-8) revealed no change in the deposit mass apart from when the carbohydrates or the overall recipe were pre-treated. To illustrate, the precipitate mass decreased on average by 55% when the carbohydrates were pre-treated compared to control. Importantly, sterilization of either tap or real wastewater had no influence on the precipitate mass indicating that the process was driven by an abiotic carbohydrate reaction.

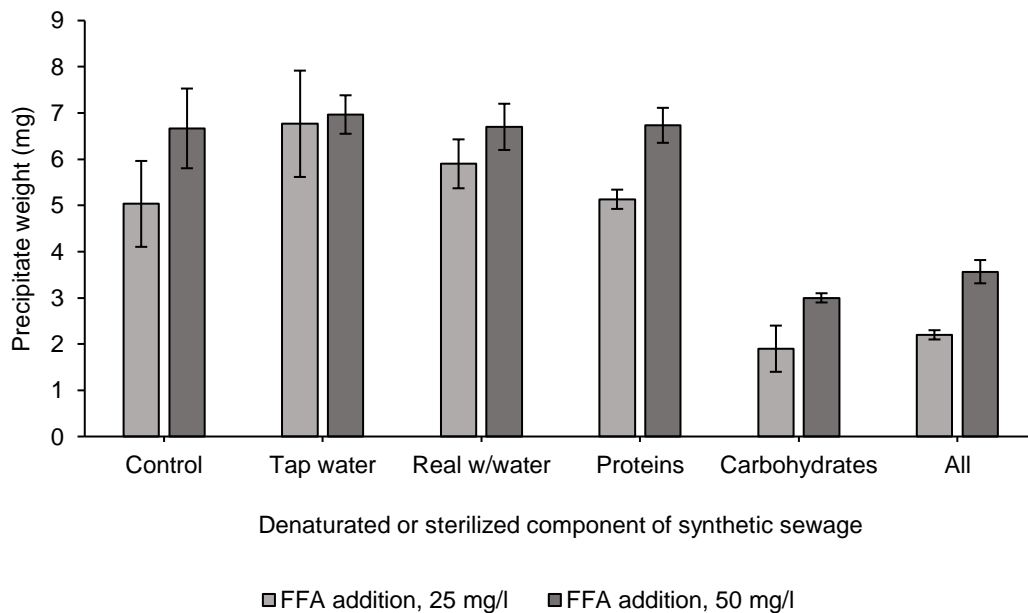


Figure 2-8 Average weight of FOG precipitates formed during batch tests using formulas with deactivated synthetic sewage main compounds. Error bars indicate the standard deviation of the experimental results.

2.3.3.1 Free Fatty Acids and Starch interactions

To identify the significance of the initial starch and free fatty acids concentrations on nucleus formation, co-precipitation was induced in solutions containing various molar ratios of starch vs. free fatty acids. In the case of a constant starch concentration of 0.036 g and an increasing concentration of oleic acid, the mass of precipitate remained constant at between 0.03 ± 0.01 g and 0.05 ± 0.01 g across the full range of mole ratios (Figure 2-9). A similar pattern was observed for steric acid up to a mole ratio of 1.75 with a mass of 0.07 ± 0.01 g was formed. However, at the maximum mole ratio of 10:1, more deposit was formed generating a mass

of 0.11 ± 0.02 g. In contrast, when the FFA concentration was kept constant and the starch concentration adjusted, a linear increase in deposit mass was observed as a function of mole ratio ($R^2 = 0.99$). To demonstrate, in the case of oleic acid, the mass of deposit formed went from 0.05 ± 0.01 g at a mole ratio of 0.25 to 0.58 ± 0.01 g at a mole ratio of 10:1. The finding indicate that the carbohydrate concentration controls the level of initial precipitate formation consistent with general finding concerning amylose (starch)-lipid complexation where three amylose helical turns are required to complex with one fatty acid molecule (Liu et al., 2009; Shah et al., 2011; Zhang et al., 2010). This equates to a molar ratio in the current study of 1:68 and exceeds the maximum ratio tested of 1:10 which indicates why the data remained on the same linear trend (Zhang et al., 2010) and suggests the overall importance of managing the mole ratio through controlling dissolved carbohydrate concentration to limit initial precipitation.

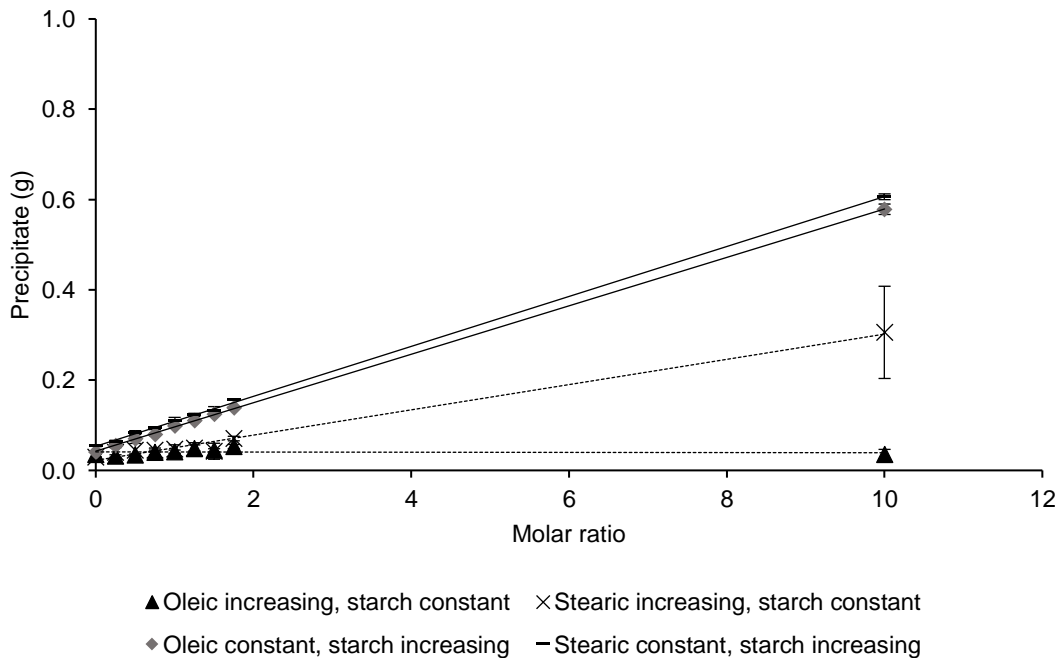


Figure 2-9 Average weight of starch-FFAs precipitates formed during batch tests using formulas with various molar ratios of starch vs. free fatty acids. Error bars indicate the standard deviation of the experimental results.

2.3.4 Understanding deposit formation beyond the initial precipitation (1st stage). Wastewater components promoting deposit growth (2nd stage) through aggregation on pre-formed core, and the role of the microorganisms in FOG deposit formation

The previous trials on the role of individual components, which also included 500 mg/L of oil (25 mg) and thermal impacts were extended to prolonged trials of 2 days to establish the impact on both initial nucleation and then subsequent growth (Figure 2-10). No deposits were formed in the control flasks where only oil and water were used confirming the need for initial nucleation before oil can accumulate into the deposit. In all remaining flasks, a deposit mass of 25.7 ± 3.8 mg was formed with no observable difference between the flasks. This included flasks where there was no initial FFA added and reflected microbial breakdown of the oil by the native microbial community. This was confirmed through pre-sterilization of the water sources which resulted in a reduction of oil uptake from $71.1 \pm 6.6\%$ in control to $36.9 \pm 20\%$ for tap water and $28.9 \pm 14\%$ for wastewater respectively. Further, under completely sterile conditions (both glassware and solutions were sterilised), no oil removal was observed, and the measured deposit mass was equal to initial starch addition (4.7 ± 2.8 mg) (Figure 2-11). Further, thermal pre-treatment of both starch and protein resulted in a reduction in the oil uptake into the deposit reducing the uptake from $71.1 \pm 6.6\%$ in the control to $64.9 \pm 2.0\%$ with inactivation of the proteins and $46.7 \pm 24.1\%$ with inactivation of carbohydrates.

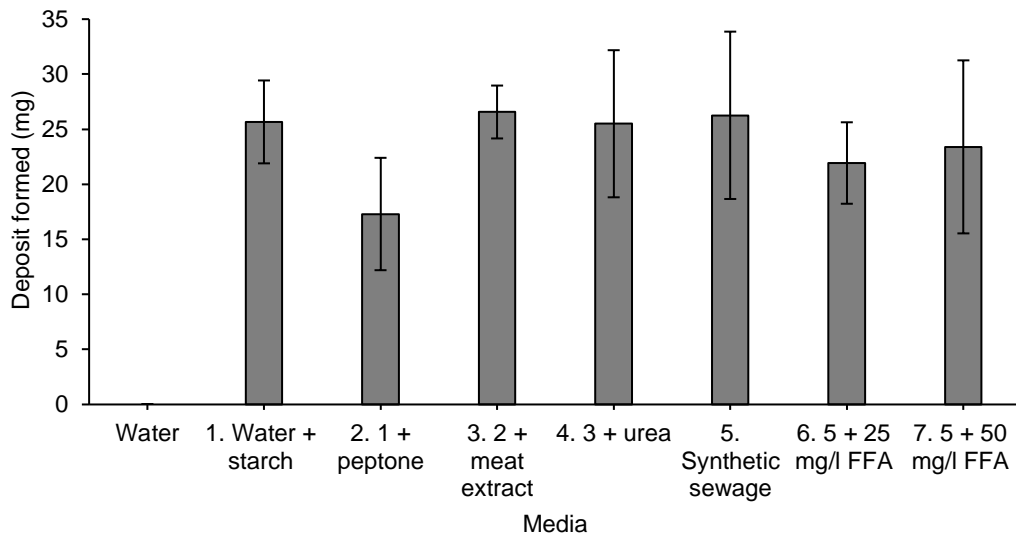


Figure 2-10 Average weight of FOG deposits formed during batch tests using formulas with increasing number of individual synthetic sewage macro compounds enriched with 500 mg/l oil and real wastewater. Error bars indicate the standard deviation of the experimental.

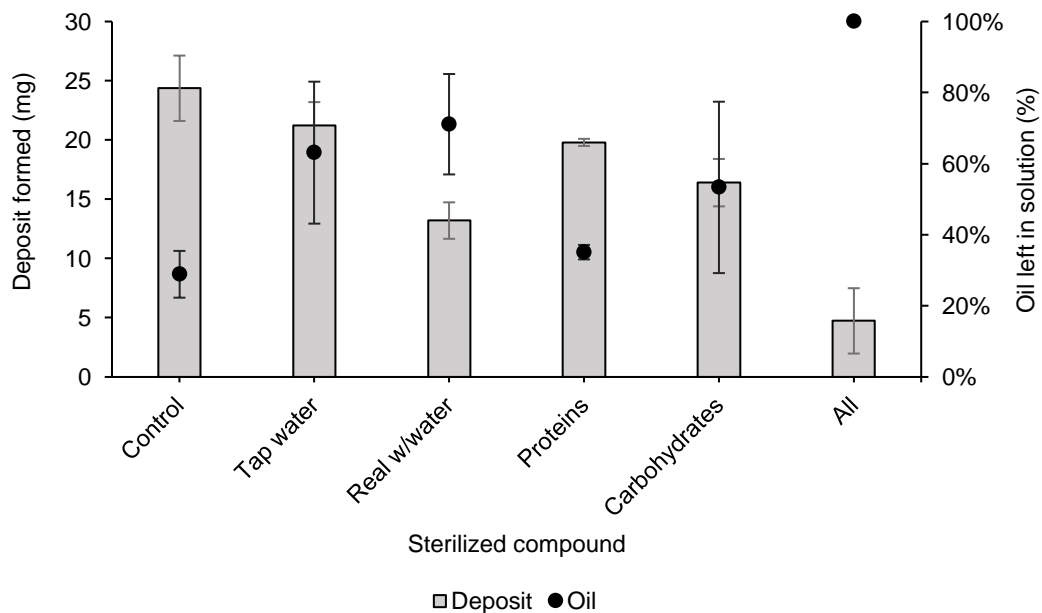


Figure 2-11 Significance of main groups of synthetic sewage and role of microorganisms in FOG deposit formation. Average weight of FOG deposits formed during batch tests using formulas with deactivated synthetic sewage main compounds added with 500 mg/l oil. Error bars indicate the standard deviation of the experimental results.

2.4 Discussion

Overall, the results provide evidence that FOG deposition is driven by the interactions between proteins, carbohydrates, and FFAs and is mediated by microbial activities leading to release of FFAs from available oil. Accumulation of oil into deposits is driven post formation and is not part of the deposition process. In contrast to the generally held view, the results of this research suggest that the process of saponification is not the leading attributer in FOG deposit formation. Such suggestions are supported by the conditions known to favour saponification pathways such as higher temperatures (40-65°C) and pHs (10.1-11.4) (Dunn, 2010) than are normally observed in sewers, i.e. 5-20°C, pH 6-7 (He et al., 2011; Keener et al., 2008). The only exception is the discharge of dishwasher wastewater where the exiting water characteristics are typically 4-57°C and pH of 10.6-11.8 (Gurd et al., 2019). The relative significance has previously been demonstrated by lasmin et al. (2016) who reported saponified fats formation levels of 21.4%, 60.1% and 71.9% at pH 7, 10 and 14 at 22°C respectively, in comparison to 87.8%, 87.3% and 41.7% at pH 7, 10 and 14 at 45°C. Although saponification under neutral pH is achievable (Dominic et al., 2013; He et al., 2011; lasmin et al., 2014; lasmin et al., 2016) it results in much lower yields than when performed in presence of strong oxidizing agent (high pH) as seen in the current work.

Based on the observed results in the current study an alternative model of deposition is proposed, featuring a 2-step process (Figure 2-12) (1) initial precipitation driven by complexation of starch with FFAs (nucleation) and (2) subsequent growth caused by accumulation of fats, proteins, calcium and other detritus.

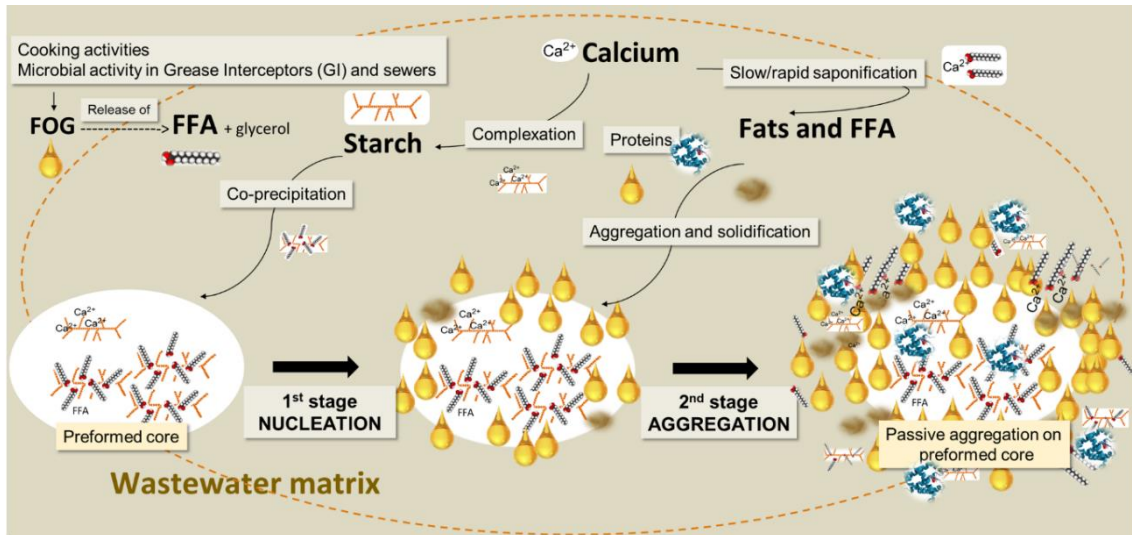


Figure 2-12 Novel model of FOG deposit formation in sewers.

Investigation into the critical components of wastewater revealed that the process of deposit formation starts with the formation of a nucleus, which is predominately driven by co-precipitation of starch and free fatty acids. Support for this idea is provided from the associated field of Food Science and Engineering. Starch is built from two major components; relatively linear amylose and highly branched amylopectin (Ai et al., 2013) with lipid interactions mostly associated with the helical structure of amylose (De Pilli et al., 2011; Liu et al., 2009). Importantly, only FFAs, monoglycerides and alcohols can form complexes with starch, while both diglycerides and triglycerides are incompatible with amylose pockets (Ai et al., 2013). The importance of FFA characteristics relates to the yield and stability of the complexes (Akuzawa et al., 1995; Wang et al., 2019; Zhang et al., 2010). Longer chains produce more stable complexes but generate a lower yield and the more unsaturated the FFA is, the more it will complex but the crystallinity and stability of complexes decrease (Shah et al., 2011). In a related study comparing oleic, palmitic, stearic, and linoleic acids at high concentrations of 20 g/L (compared to a maximum of 50 mg/L used in the current work), an order of complexation was seen in decreasing levels of complexation as oleic, stearic, palmitic and linoleic acids (Akuzawa, et al., 1995). All four FFAs have been reported in FOG samples but do not match the profiles found in the original cooking oils used. This has previously been attributed to biological transformation, i.e. oleic to palmitic through beta-oxidation (Iasmin et al., 2014)

but could also be influenced by preferential interactions between starch and the different FFAs. In the current work, no real differences were observed between the saturated (stearic) and the unsaturated (oleic) FFAs. This most likely represents the temperature and concentrations used which are below optimum and this likely masks potential differences. Importantly they were within ranges typically observed in sewers suggesting that potential difference due to changes in FFA character are unlikely to be significant in sewers.

During the second stage of FOG deposit formation, proteins, oils, FFAs, saponified FFAs and calcium aggregate onto the pre-formed core built through the initial amylose-lipid complexation pathway. In particular, the exposed negatively charged carboxylic groups from the starch molecules (both amylose and amylopectin components) bind to the other material through a combination of calcium bridging, hydrophobic interactions, and electrical interactions (Wang et al., 2020). Proteins also act as bridging agents but through interactions with FFA and do not directly interact with starch. The reaction is also less favourable compared to the amylose-lipid complex which is why only relatively small amounts of proteins are normally found within deposits (Zhang et al., 2010; Shah et al., 2011). The characteristics and reactions associated to the ternary complexes are still uncertain but are thought to form through a reversible process whereby over times they can be replaced with additional FFAs and transformed to the more stable binary complex (Zhang et al., 2010; Liu et al., 2009). Accordingly, the compositional make-up of the deposit can change with time, replacing proteins with additional FFAs and carbohydrates. Both amylose-lipid and amylose-lipid-protein complexes are readily formed as insoluble precipitates in aqueous media at neutral pH (Wang et al., 2020) supporting the view of their importance over that of saponification. Further, the large excesses of calcium commonly observed in FOG deposits (Benecke et al., 2017; He et al., 2011; Keener et al., 2008; Williams et al., 2012) reflect calcium bridging with starch or FFAs such that calcium competes with FFAs for available amylose binding sites. This is reflected in the experimental data where the most profound reduction in calcium concentration in synthetic sewage was observed for deposits with the highest carbohydrates concentrations and negligible lipid content (180 days).

However, FFAs are known to also self-associate when in sufficient concentration (Wang et al., 2020). Consequently, large concentrations of FFA can accumulate beyond levels defined by the available calcium concentration.

Overall, the myriad of possible bindings pathways and the fact that compositional changes as a function of time means that high variability in deposit composition is to be expected and reflects variations in the input of the different components and the background water characterisation (as observed in Table 2-5). For instance, the reported protein, carbohydrates, and lipids content in wastewater as fraction of tCOD can vary between 5.8-38%, 6-35.8% and 6.6-82% respectively (Heukelekian & Balmat, 1959; Huang et al., 2010; Sophonsiri & Morgenroth, 2004). Further, the most common locations for deposit formation are near high concentrations of FSEs whose effluent is known to be particularly high in carbohydrates and fats as well as alkaline and acidic chemicals from the cleaning activities (Gurd et al., 2019).

2.5 Conclusions

The results of this research challenge the generally accepted model of FOG deposit formation, which posits that saponification is a leading reaction in FOG deposit formation. Based on the observed results in the current study a novel model of deposition is proposed, featuring 2-step process: (1) initial precipitation driven by complexation of starch and FFAs followed by (2) subsequent growth caused by fats, protein and calcium accumulation. The new model brings to light flaws in current FOG mitigation practices focused solely on elimination of fats, neglecting the role of other significant wastewater components like starch. The initial starch concentration in wastewater has been identified as directly correlated to the amount of the FFAs-starch complexes formed during the nucleation stage of FOG deposit formation, as well as factor drawing excess calcium and protein towards deposit core.

The novel, two-stage deposition model presented here indicates that current deposit management practices are overly focussed on lipids elimination which is difficult to deliver. Whereas the model highlights the potential to tailor mitigation practices to target the different stages of deposit formation, i.e., nucleation or

deposit growth. In either case, inhibition of deposit formation can occur through blocking the available pathways or altering the make-up of the different components through dosing chemical/biological agents into the flow. Reimagine such practices through a more evolved understanding of deposit formation will increase the efficacy of the treatment and significantly reduce the risk of sewer blockages. This can be delivered using controlled research with synthetic solutions which are shown to successfully replicated field deposits providing a robust matrix for currently undeveloped laboratory-based testing procedures beyond induced saponification trials.

2.6 Acknowledgements

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3 Chapter 3 Understanding the potential for chemical uncouplers to aid the bioremediation of Fats, Oils, and Grease (FOG) deposits in Sewers. Current understanding of chemical uncouplers and future prospects.

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Abstract

This review explores the use of chemical uncouplers for bioremediation of FOG deposit in sewers. Recent advancements in understanding of deposit formation and pivotal role played by the indigenous wastewater bacteria have shed the light on the potential benefits of employing uncouplers in this context. Through their ability to induce metabolic changes, disrupt biofilm integrity, and challenge microbial diversity, uncouplers offer a potential pathway to mitigate FOG-related issues. Uncouplers have shown promising results in reducing biomass accumulation, inhibiting biofilm formation, and inducing biofilm detachment. The potential benefits of this approach include inhibiting the FOG formation,

rehabilitation of existing deposits, and potentially enhancing downstream processes.

Ultimately this review suggests that future research should focus on developing and investigating environmentally friendly, non-toxic uncouplers that can be safely applied in sewer systems without adverse effects on in sewer processes, and the environment. Research should also aim to quantify the added value generated by the use of the uncouplers in terms of improved effluent quality and reduced maintenance costs. In so doing it is predicted that uncouplers can play a significant role in FOG deposits management practices.

Keyword: chemical uncoupler, biomass control, metabolic uncoupling, FOG deposits management

3.1 Introduction

Biological wastewater treatment converts dissolved and suspended organic pollutants in sewage into biomass (sludge), residual dissolved components (unused and breakdown products) and gases (CO₂, CH₄, N₂ and SO₂). Across the years the global focus on wastewater purification has led to a significant increase in excess sludge generation, which raises environmental concerns. The conventional activated sludge (CAS) process is the predominant biological treatment method for domestic and industrial wastewater, yielding on average 0.3-0.5 grams of sludge (TSS) per gram of chemical oxygen demand (COD) removed (Han et al., 2017; Morello et al., 2022). Countries with more intensive treatment tend to also have higher per capita sludge production rates with countries such as Germany, the UK, Spain, Italy, and France reporting the highest rates with an average specific sludge production of 26.1 kg DS/person year (Eurostat, 2021). Conventional sludge disposal methods, like landfilling, are unsustainable, while agricultural reuse faces restrictions due to regulations (Guo et al., 2020; Wei et al., 2003).

Managing and disposing of excess waste activated sludge have become major challenges in biological sewage treatment due to the associated environmental burdens and high costs. For instance, the handling and disposal of sewage

sludge can contribute to 25- 65% of the overall operating costs of the plant (Guo et al., 2013; Hassani et al., 2011; Low et al., 2000; Wei et al., 2003; Yang et al., 2003). Moreover, excessive biomass accumulation in biological wastewater treatment systems like activated sludge, biofilters, trickling filters and membrane bioreactors poses challenges such as increased energy costs and system failure risks (Gostomski & De Vela, 2018). Therefore, it becomes crucial to reduce the yield of biomass where undesired biofilm formation can occur.

CAS systems can manage solids production through mechanical, biological, and chemical approaches (Morello et al., 2022). Reduction approaches can be categorized into two main trends: post-treatment methods and in-situ reduction. Compared to post treatments, in-situ excess sludge reduction has prominent advantages, i.e., minimizing sludge yield directly within the sewage treatment process and avoiding operational complexities, high expenses, and energy consumption (Li & Tabassum, 2022; Tian et al., 2013). In-situ sludge reduction can be achieved by 1) Lysis-cryptic growth induced by chemical treatment like ozonation or chlorination; or physical treatment like ultrasound, alkaline and thermal treatment; 2) worms' predation by two-stage sludge predation systems and Oligochaeta addition, 3) uncoupling metabolism by oxic-settling-anaerobic (OSA) process, repeatedly coupling of aerobes/anaerobes treatment, extended aeration and chemical uncouplers (Lin et al., 2022a). Energy uncoupling and spilling, as well as biological predation, enhance energy dissipation in microbial individuals and the food network, resulting in reduced energy utilization for cell synthesis (Feng et al., 2020a). The aforementioned strategies can be classified into three primary categories based on the principle they affect: thermodynamics, substance metabolism and electron transfer (Feng et al., 2020a). Among these, dosing metabolic uncouplers stands out as a promising method due to its convenience, simplicity and high efficiency (Aragón et al., 2009; Fang et al., 2015, 2020). The advantages of adding an uncoupler include 1) capability of treating complex components or high strength organic pollutants, 2) economic efficiency and environmental friendliness (biological and non-toxic chemical uncouplers), 3) no significant change in the configuration of the biological processes (Guo et al., 2013). Several studies have explored the potential of using

uncouplers in biomass reduction applications, operating on the principle that energy allocation for cell maintenance outweighs growth by restricting biomass anabolism, while allowing substrate catabolism to continue (Lin et al., 2022b; Zheng et al., 2008). Over the past decade, around 20 metabolic uncouplers have been tested in wastewater treatment systems, demonstrating their ability to impact biomass yield. The use of metabolic uncouplers in environmental research is growing annually and although the primary emphasis has been on growth systems like activated sludge, it has been suggested that uncouplers can mediate other microbial applications as membrane biofouling (Xu & Liu, 2011), biofiltration (Gostomski & De Vela, 2018) and substrate bioremediation (Saini & Wood, 2008). The emergence of these applications offers opportunity to expand research efforts and evaluate the potential of uncouplers in addressing other microbially facilitated wastewater processes, such as the deposition of Fat, Oil, and Grease (FOG) in sewer systems.

The accumulation of FOG deposits in sewer networks can lead to blockages, overflows, and property flooding, causing significant financial and reputational damage to water companies (He et al., 2017; Wallace et al., 2017). Current FOG management practices primarily rely on costly mechanical maintenance, which poses risks to public health and the environment. Recent studies highlight the role of total suspended solids (TSS) in FOG formation (Otsuka et al., 2020) and emphasize the interactions between fatty acids (FFAs), carbohydrates, proteins, and indigenous wastewater bacteria in the deposition process (Brooksbank et al., 2007). Considering these findings, the use of uncouplers holds potential to facilitate FOG management.

Although energy uncoupling metabolism can be induced by several factors, such as excess energy sources, inhibitory compounds, nutrient limitation, exposure to cyclic changes in ATP, and abnormal temperatures, this review article aims to investigate the mechanism of chemical uncoupling and its application in reducing biomass yield in biologically driven processes, with a potential focus on bioremediation of FOG deposits in sewers.

3.2 Uncoupling mechanism

Microbial metabolism is a sum of catabolism and anabolism, essential for energy generation and utilization in living cells (Figure 3-1). In biological systems, microorganisms engage in a series of biochemical reactions with a terminal electron acceptor to fully oxidize and decompose a portion of available substrates to generate the energy they need (catabolism). In addition, the process of cell synthesis (anabolisms) relies on these reactions for energy influx to convert a carbon and nitrogen sources in the environment into cells' biomass (González-Cabaleiro et al., 2015; Morello et al., 2022; Ye & Li, 2005). The forementioned metabolic pathways comprise of the electron transport chain and oxidative phosphorylation, tightly linked by the proton force, resulting in production and regeneration of adenosine triphosphate (ATP). Synthesized through these two interrelated reactions in which electrons are transported through the electron transport system from an electron donor (substrate) to a final electron acceptor (Xu & Liu, 2010), ATP serves as the universal energy currency in biological systems (Gostomski & De Vela, 2018). Under normal circumstances, a portion of this energy is allocated to cell maintenance, while the remaining energy is utilized to fuel growth through anabolic reactions (Aragón et al., 2009). When energy is needed for anabolic processes, it is released through the hydrolysis of the high-energy bond in ATP, yielding 8 kcal per mole (González-Cabaleiro et al., 2015). To ensure efficient energy transfer between two processes, bacterial anabolism is coupled to substrate catabolism by rate-limiting respiration (Wei et al., 2003). Uncoupled metabolism in microorganisms occurs when the regulatory control between catabolism and anabolism is disrupted, and the biosynthetic processes become rate limiting, resulting in a reduction of ATP synthesis. The presence of an uncoupler allows for the continuation of electron transport, but it inhibits oxidative phosphorylation. In consequence, the oxidation of substrate occurs with less energy available for formation of new biomass (sludge), because phosphorylation of ADP to ATP has been decreased (Ye and Li, 2005). When ATP synthesis becomes less efficient, more substrate oxidation per ATP molecule production is required, effectively increasing the maintenance coefficient (Low et al., 2000). Diversion of excess energy away from biomass

production towards non-growth-associated reactions, like maintenance or heat, reduces biomass yield (Aragón et al., 2009; Detchanamurthy & Gostomski, 2012; Li et al., 2012; Low & Chase, 1998).

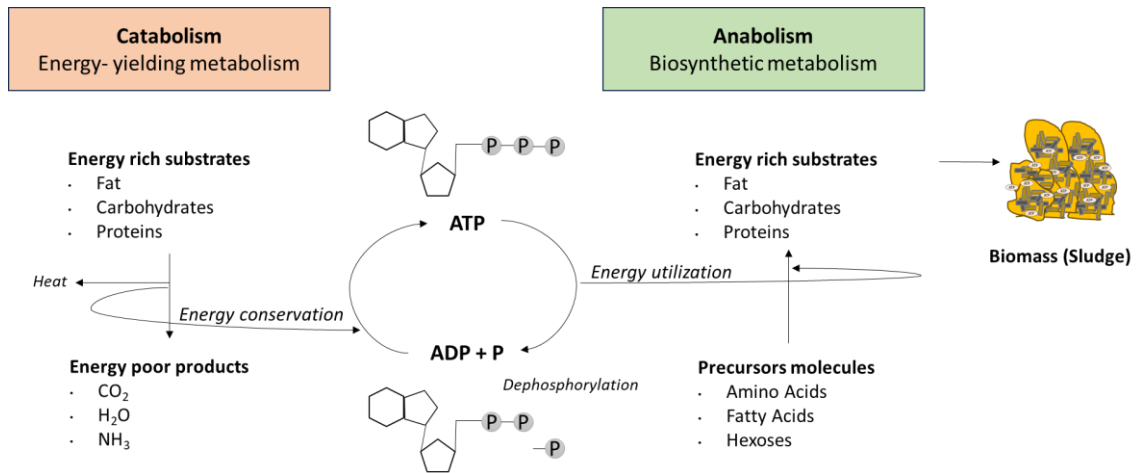


Figure 3-1 Energy generation and utilization in microbial cells.

3.3 Uncoupling engineering

Regulation of the factors that influence substrate allocation and energy conversion represent potential mechanisms to reduce biomass production in microbial metabolism (Feng et al., 2020a). From the engineering perspective, the concept of induced energy uncoupling means that the process has been modified in the way that led to the lower biomass growth for the same amount of substrate consumed (Lin et al., 2022a; Yang et al., 2003; Ye & Li, 2005). Induction of overconsumption of substrate with limited energy supply to anabolism however necessitates avoidance of accumulation of surplus intracellular energy and metabolites (Low & Chase, 1999). This can be achieved by deletion of oxidative phosphorylation sites, branching of respiratory chain, shifting metabolic pathways, dissipation of membrane potential, ATP hydrolysis and futile cycles (González-Cabaleiro et al., 2015; Podella et al., 2009; Ye and Li, 2005).

Uncoupling can occur through three mechanisms: (1) Protein-independent uncoupling, which is induced by metabolic uncouplers (2) Protein-mediated uncoupling, where certain proteins increase the H⁺ conductance of the inner membrane and (3) Proton leakage across the inner membrane, where protons

return across the membrane via routes other than the ATP synthase, all of which diminish the protonmotive force required for ATP production (Gostomski & De Vela, 2018).

Among these, the use of protein-independent metabolic uncouplers has proven to be a highly efficient approach. Under normal circumstances, during electron transfer across the cell membrane, there is a division of protons and electrons, leading to an acidification of the external surface and an alkaline environment within the cytoplasm (Figure 3-2). This pH gradient and electrochemical potential (proton motive force) energize the membrane enabling it to drive ATP synthesis. To avoid short-circuiting during electron transfer through the cell membrane, the membrane must remain closed and possess a high resistance or permeability to protons. This tight association is a target for metabolic uncouplers. Metabolic uncouplers are typically hydrophobic weak acids with substantial lipid solubility, able to carry protons across the cellular membrane in their protonated form, dissociate, and move back across the membrane in a cyclical manner, uncoupling at a ratio of 1 molecule per 20 respiratory chains (Jiang & Liu, 2010; Yang et al., 2003). These substances are called protonophores, and they possess ability to shift protons at rates significantly surpassing the speed of the proton pump of the respiration chain. Due to their nature, i.e., positive charge and hydrophilic properties, protons would not be able to cross the membrane without a channel or transporter. Protonophores diffuse passively through membranes, simultaneously facilitating proton transfer. In consequence, they dissipate the transmembrane proton gradient, i.e., the driving force of phosphorylation. Upon entering the membrane matrix, the higher pH environment leads to deprotonation of the uncoupler. Consequently, the uncoupler acts by transporting hydrogen ions back into the matrix, circumventing the proton channel, and effectively inhibiting ATP synthesis (Xu & Liu, 2010) (Figure 3-2). During the uncoupling, the respiratory chain and ATP synthase activity remain unaffected, but the ATP production is lowered due to the loss of proton motive force. Simultaneously, freed from phosphorylation electron flow (nutrients oxidation) accelerates.

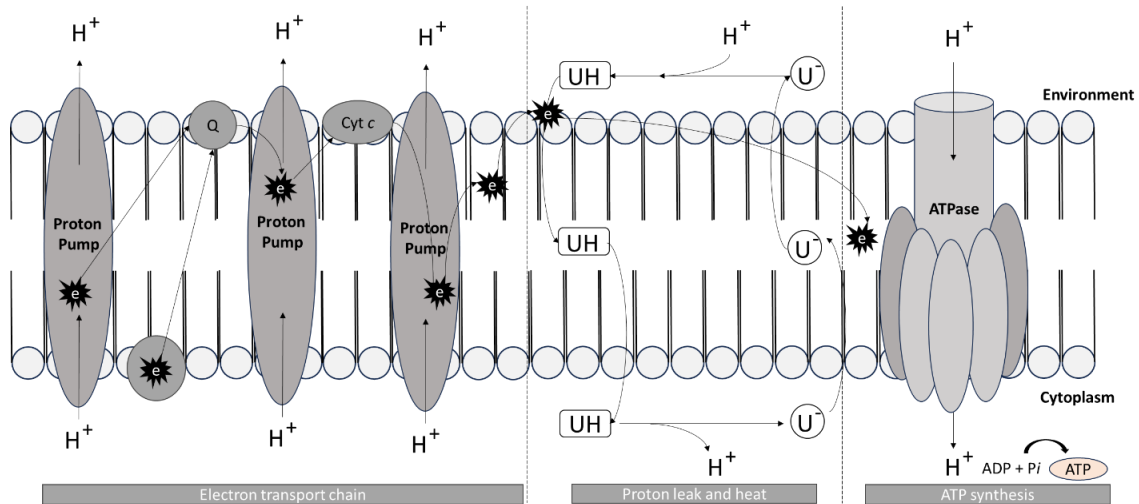


Figure 3-2 Simplified illustration of electron transport system, oxidative phosphorylation and proton leak induced by addition of the uncoupler (protonophore). UH-protonated uncoupler, U- unprotonated uncoupler.

Extensive number of compounds are known to be protonophores. These compounds encompass oxidants, detergents, heavy metals (Aragón et al., 2009), ammonia, fatty acids, plants thionins and coumarins, animal defensins, and chemicals as phenols, benzimidazoles, salicylic acids, and aromatic amines (Detchanamurthy & Gostomski, 2012; Gostomski & De Vela, 2018). The widely described chemical uncouplers (Table 3-1) in the literature include 2,4-dinitrophenol (2,4- dNP), 2,4-dichlorophenol (2,4- dCP), para-nitrophenol (pNP), pentachlorophenol (pCP), m-chlorophenol (mCP), o-nitrophenol (oNP), o-chlorophenol (oCP), N- ethylmaleimide (NEM), 3,3',4',5-tetrachlorosalicylanilide (TCS), 2,4,5-trichlorophenol (TCP), carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP), carbonylcyanide m-chloromethoxy- phenylhydrazone (CCCP), cresol and aminophenol (Aragón et al., 2009; Detchanamurthy & Gostomski, 2012; Wang et al., 2013; Yang et al., 2003).

3.4 Possible mechanism of action

Biomass control using uncouplers can be divided according to their mechanisms, including: 1) true metabolic uncoupling, 2) maintenance metabolism i.e., diversion of energy to non-growth processes, 3) reduction of biofilm integrity and

stability, and 4) alteration of the microbial ecology within the system (Gostomski & De Vela, 2018) (Table 3-1).

The effectiveness of uncoupler compounds in controlling biomass generation is determined by the percentage of biomass reduction compared to control without the uncoupler, which varies based on their concentration. Each type of uncoupler has an effective concentration that strikes a balance between achieving the highest biomass reduction and minimizing negative impacts on effluent quality. The compiled available data (Figure 3-3) reveals that the uncouplers are effective even at very low concentrations of between 2.7 mg/L and 100 mg/L for maintenance metabolism; 0.025 mg/L and 110 mg/L for true metabolic uncoupling and between 0.1 mg/L and 30 mg/L for undermining biofilm stability. Furthermore, the results also indicate that beyond a certain threshold, increasing the dosage fails to yield any additional benefits. On the contrary, increased doses can inhibit microbial activity leading to sludge properties and effluent quality deterioration, while at very high concentrations causing overall system collapse. According to Liu (2000), the true strength of a chemical uncoupler is more accurately represented by the ratio of the initial uncoupler concentration to the initial biomass concentration, as opposed to considering the uncoupler's initial concentration alone.

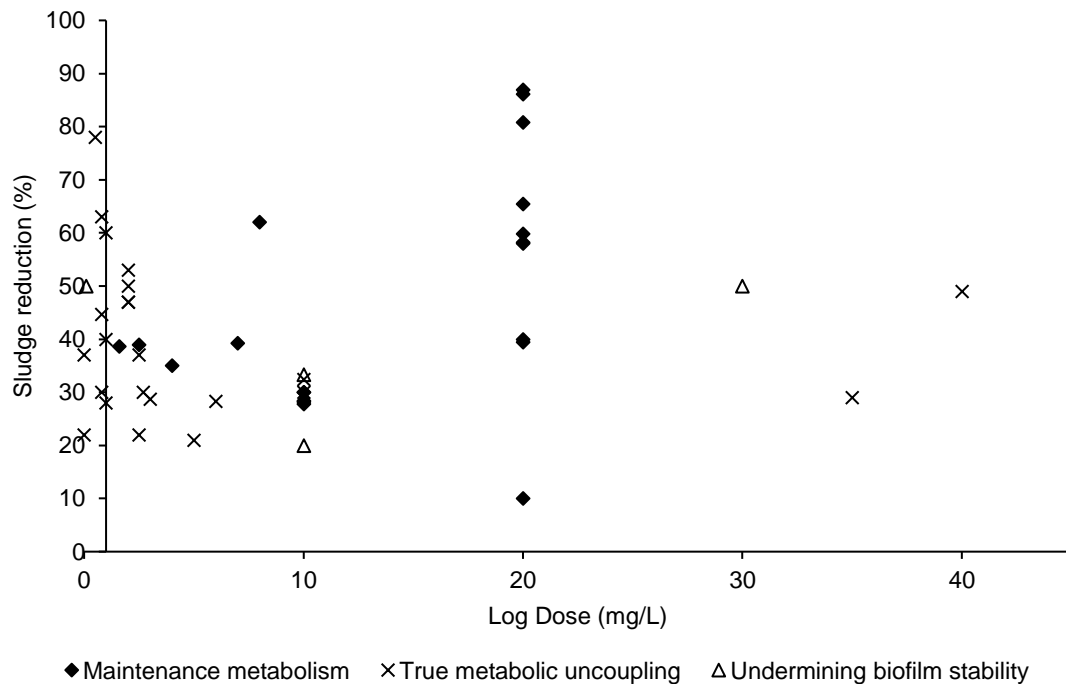


Figure 3-3 Sludge reduction efficiency and dosage requirements across metabolic uncoupling mechanisms.

Some of the most frequently reported disadvantages associated with the dosing of chemical uncouplers are reduced COD and nitrogen removal, and worsened properties of the produced sludge, i.e., settling and dewatering (Aragón et al., 2009; Morello et al., 2022). The reduced nitrogen removal is attributed to uncouplers restraining the activity of nitrifying bacteria (NOB) (Fang et al., 2020; Ferrer-Polonio et al., 2019a; Low et al., 2000), while the lowered COD removal has been associated with lower biomass counts (Qiong et al., 2013; Zheng et al., 2008). The worsened sludge settleability is linked to changes in the secretion of soluble microbial products (SMP) and extracellular polymeric substances (EPS), changes in population dynamics (Li & Tabassum, 2022; Low et al., 2000; Zheng et al., 2009) and uncoupler induced cell lysis (Fang et al., 2020; Wang et al., 2022).

The detrimental effects of uncoupler dosing are particularly pronounced for uncouplers inducing maintenance metabolism. The addition of uncouplers such as 2,4-dCP (Han et al., 2017; Tian et al., 2013), TCS and THPS (Li et al., 2016)

have been shown to reduce substrate removal, most profoundly for nitrogen compounds (7.7-10.3%) along with deteriorated sludge settleability. The majority of studies examining chemical uncouplers comprising phenolic ring such as pNP, mCP, mNP, oNP, dNP and pCP, which induced changes in population dynamics, have consistently demonstrated that the addition of these specific uncouplers results in significant inhibition of COD removal (6-30%) and sludge settleability deterioration. The detrimental effect is positively correlated to increasing uncoupler dosage (Li & Tabassum, 2022; Low et al., 2000; Yang et al., 2003; Zheng, et al., 2009). Yang et al. (2003) suggested that the decreased degree of COD removal efficiency is affected by combined effect of species shift, reduced biomass production and potential inhibition induced by the uncoupler. Notwithstanding that, it is important to note that deteriorated effluent quality is not always correlated with reduced sludge quality; nor does a decreased nitrogen assimilation necessary result in decreased COD removal. For instance, Tian et al. (2013) reported strong inhibitory effect of 2,6-dCP on COD removal and nitrification performance, but no significant effect on sludge properties. Fang et al. (2015 and 2020) reported significant inhibition of ammonia removal upon addition of pCP, oCP, and oNP, while no noticeable effect on COD removal or sludge settleability was observed. On the contrary, Zuriaga-Agustí et al. (2016) have shown that the biological process performance of the system was not affected by addition of pNP, recording high COD removal of 96%.

Research concerning reduction of biofilm stability have shown that the use of uncoupler such as TCS (Ding et al., 2019; Jiang & Liu, 2010) and 4-NP (Qiong et al., 2013) can hinder COD and TN removal by 5-8%. There is limited information available regarding the influence on sludge properties in this particular metabolic uncoupler mechanism group, but the existing evidence show no (Lin et al., 2022a) to moderate (Jiang & Liu, 2010) influence on sludge settleability, while the improved sludge dewaterability upon addition of TCS was reported by Lin et al. (2022b).

Notably, numerous studies have provided substantial evidence for the effectiveness of true metabolic uncoupling in reducing biomass accumulation,

with the majority of findings indicating no or minimal impact on pollutant removal efficiency (Chen et al., 2002; Chen et al., 2008; Ferrer-Polonio et al., 2019a; Zuriaga-Agustí et al., 2016) and sludge settleability (Chen et al., 2008; Ye & Li, 2005; Zheng et al., 2008) with exception of a study that reported a 44% reduction in TN removal upon the addition of 2,4-dNP (Li & Tabassum, 2022). Additionally, in most of the documented cases the application of true metabolic uncouplers did not result in significant changes in the biomass community structure (Lin et al., 2010; Zheng et al., 2008). However, in instances where changes were reported, they had a greater impact on higher organisms, such as protozoa (Chen et al., 2008; Ye & Li, 2005).

Table 3-1 Uncouplers and their mechanisms of action.

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
2,4- dichlorophenol (2,4- dCP)	40 mg/h; Continuous	Activated sludge	Maintenance metabolism		Continuous, A2O (anaerobic- anoxic-oxic) laboratory bench scale, 85d, SRT=10d, MLSS=2000- 4000 mg/L, activated sludge, 20-28°C, pH 7.56-8.43	43.4	Decreased by 3.2%	Decreased by 8.6%	Slightly affected sludge settleability	(Li et al., 2016)
2,4- dichlorophenol (2,4- dCP)	10 mg/L; Continuous	Activated sludge	Maintenance metabolism		SBR (sequencing batch reactor), 85d, MLSS=2000- 4000 mg/L, real wastewater, 20- 28°C	27.8	Decreased by 3.8%	Decreased by 10.3%	Deteriorated sludge settleability Changes in EPS composition; increased PS content	(Han et al., 2017)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
2,4- dichlorophenol (2,4- dCP)	10 mg/L; Continuous	Activated sludge	Maintenance metabolism		A2O (anaerobic- anoxic-oxic), 85d, MLSS=2000- 4000 mg/L, real wastewater, 20- 28°C	28.2	Decreased by 6%	Decreased by 8.6%	Deteriorated sludge settleability Changes in EPS composition; increased PS content of EPS.	(Han et al., 2017)
2,6- dichlorophenol (2,6- dCP)	20 mg/L; 3 times per day	Activated sludge	Maintenance metabolism		Continuous, DO=5 mg/L, MLSS= 2000 mg/L, synthetic sewage, CAS seed, 25°C, pH 7	40	Decreased by 20%		Strong inhibitory effect on nitrification performance	(Tian et al., 2013)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
2,4-dinitrophenol (2,4- dNP)	49-140 mg/L (0.26-0.76 mM); Once	Impact of chemical stress on microbiological metabolism under sub- lethal concentrations of a chemical stressor.	Maintenance metabolism		<i>Pseudomonas aeruginosa</i> isolated from activated sludge SBR consortium	Decreased (not provided)	Unaffected		Uncoupler induced the heat shock response in the microbiome	(Ray & Peters, 2008)
2,4-dinitrophenol (2,4- dNP)	8 mg/L (3-12 mg/L)	Oxic-settling- anaerobic biomass	Maintenance metabolism	Energy spilling	Continuous, HRT= 6h, STR=2d MLSS= 50-200 mg/L, synthetic sewage, CAS seed, 22°C, pH 7.2	62			The reduced degree of observed growth yield (Yobs) was closely related to the initial biomass concentration. Energy spilling induced by futile cycles.	(Liu, 2000)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
3,3',4',5- tetrachlorosalicylanilide (TCS)	1.6 mg/h; Continuous	Activated sludge	Maintenance metabolism		Continuous, A2O (anaerobic- anoxic-oxic) laboratory bench 85d, SRT=10d, MLSS=2000- 4000 mg/L, activated sludge 20-28°C, pH 7.56-8.43	38.6	Decreased by 5%	Decreased by 7.7%	Slightly affected sludge settleability	(Li et al., 2016)
3,3',4',5- tetrachlorosalicylanilide (TCS)	2.5 mg/L (1- 10 mg/L); Once	Activated sludge	Maintenance metabolism	Lysis-cryptic growth	Batch, MLSS= 2000mg/L, DO= 2 mg/L, synthetic sewage, CAS seed, 25°C	39	Severely inhibited at high dosage (>7.5mg/L)		The reduced degree of observed growth yield (Yobs) was closely related to the uncoupler dose. Increased EPS and SMP production.	(Li et al., 2012)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
Copper (Cu)	5 (5-15 mg/L)	Activated sludge	Maintenance metabolism		Batch, short- term test study, MLSS= 2000- 2500 mg/L, synthetic wastewater, CAS seed, 20°C	No effect	Inhibited		Copper inhibited both anabolic and catabolic reactions. No effect on sludge growth yield	(Aragón et al., 2009)
m- chlorophenol (mCP)	20 mg (1-20 mg/L)	Activated sludge	Maintenance metabolism	Changes in population dynamics	Batch, MLSS= 2000 mg/L, COD= 400 mg/L, synthetic wastewater, CAS seed, 25°C, pH 7	86.9	Decreased by 13.5%		Dissociation of energy metabolism was proportionally related to the concentration of the uncoupler.	(Yang et al., 2003)
p- chlorophenol (pCP)	20 mg (1-20 mg/L)	Activated sludge	Maintenance metabolism	Changes in population dynamics	Batch, MLSS= 2000 mg/L, COD= 400 mg/L, synthetic wastewater, CAS seed, 25°C, pH 7	58	Decreased by 8.9%		Dissociation of energy metabolism was proportionally related to the concentration of the uncoupler.	(Yang et al., 2003)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
p- chlorophenol (pCP)	20 mg (1-20 mg/L)	Activated sludge	Maintenance metabolism		Batch, SBR, HRT=12h, SRT=15d, DO=4 mg/L, MLSS= 2500 mg/L, synthetic wastewater, CAS seed, 20- 22°C	58.2	Unaffected		Strong inhibitory effect on ammonia removal. Increased EPS production; increased PN content of EPS.	(Fang et al., 2015)
o-chlorophenol (oCP)	20 mg/L (5-20 mg/L)	Activated sludge	Maintenance metabolism		Batch, SBR, HRT=12h, SRT=15d, DO=4 mg/L, MLSS= 2500 mg/L, synthetic wastewater, CAS seed, 20- 22°C	59.8	Unaffected		Strong inhibitory effect on ammonia removal. Increased EPS production; increased PN content of EPS.	(Fang et al., 2015)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
o-chlorophenol (oCP)	10 mg/L; Daily	Activated sludge	Maintenance metabolism	Lysis-cryptic growth Changes in population dynamics	Anaerobic-oxic process, SRT=91 d, synthetic wastewater, CAS seed.	28.4	Decreased by 8.4%	Decreased (not provided)	Permanent loss of bacterial richness and diversity. Reduced nitrogen and phosphorus removal. Increased production of SMP.	(Wang et al., 2022)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
o-chlorophenol (oCP)	20 mg/L (5-20 mg/L)	Activated sludge	Maintenance metabolism	Lysis-cryptic growth Changes in population dynamics	Long-term operated SBR, DO=6 mg/L, MLSS= 3000 mg/L, 1 month adaptation; HRT=12h, SRT=15d, synthetic wastewater, CAS seed, 25°C	39.5	Decreased by 13.82%	Decreased by 8.67%	Decreased production of SMP. Increase production of EPS and PHA. Inhibition of nitrate-oxidizing bacteria (NOB).	(Fang et al., 2020)
Pentachlorophenol (pCP)	15-38 mg/L; Once	Impact of chemical stress on microbiological metabolism under sub- lethal concentrations of a chemical stressor.	Maintenance metabolism		<i>Pseudomonas aeruginosa</i> isolated from activated sludge SBR consortium	Decreased (not provided)	Unaffected		Uncoupler induced the expression of stress proteins.	(Ray & Peters, 2008)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
Para-nitrophenol (pNP)	100 mg/L (40- 120 mg/L); Continuous	Activated sludge	Maintenance metabolism	Energy spilling	Continuous mono-culture of <i>P.putida</i> , synthetic wastewater, 30°C, pH 6.2-7.7	62-77	Unaffected		<i>P.putida</i> is major constituent of microbial population in CAS	(Low & Chase, 1998)
Para-nitrophenol (pNP)	100 mg/L; Continuous	Activated sludge	Maintenance metabolism	Changes in population dynamics.	Continuous, bench scale system, MLSS= 0.71 g/L, synthetic wastewater, CAS seed, 20°C, pH 7.7	49	Decreased by 25%		Decrease in protozoa and proliferation of filamentous bacteria. 3.3-fold increase in specific substrate uptake rate. Deteriorated sludge settleability.	(Low et al., 2000)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
Para-nitrophenol (pNP)	20 and 25 mg/L; Daily	Activated sludge	Maintenance metabolism		SBR, MLSS=2500 mg/L, HRT= 0.67d, synthetic wastewater, CAS seed, 20- 22°C	10	Unaffected		Negligible toxic impact on activated sludge.	(Zuriaga-Agustí et al., 2016)
m- nitrophenol	20 mg/L (1-20 mg/L)	Activated sludge	Maintenance metabolism	Changes in population dynamics	Batch, MLSS= 2000 mg/L, COD= 400 mg/L, synthetic wastewater, CAS seed, 25°C, pH 7	65.5	Decreased by 13.2%		Dissociation of energy metabolism was proportionally related to the concentration of uncoupler. Increase in substrate uptake rate.	(Yang et al., 2003)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
o- nitrophenol	20 mg/L (1-20 mg/L)	Activated sludge	Maintenance metabolism	Changes in population dynamics	Batch, MLSS= 2000 mg/L, COD= 400 mg/L, synthetic wastewater, CAS seed, 25°C, pH 7	86.1	Decreased by 26%		Dissociation of energy metabolism was proportionally related to the concentration of uncoupler. Increase in substrate uptake rate.	(Yang et al., 2003)
o- nitrophenol	20 mg/L (5-20 mg/L)	Activated sludge	Maintenance metabolism		Batch, SBR, HRT=12h, SRT=15d, DO=4 mg/L, MLSS= 2500 mg/L, synthetic wastewater, CAS seed, 20- 22°C	80.8	Unaffected		Strong inhibitory effect on ammonia removal. Increased EPS production; increased PN content of EPS.	(Fang et al., 2015)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
N- ethylmaleimide (NEM)	2.7 mg/L; Once	Impact of chemical stress on microbiological metabolism under sub- lethal concentrations of a chemical stresser.	Maintenance metabolism	Lag in biological activity	<i>Pseudomonas aeruginosa</i> isolated from activated sludge SBR consortium	Decreased (not provided)	Significantly decreased		Uncoupler induced oxidative stress.	(Ray & Peters, 2008)
Tetrakis (hydroxymethyl) phosphonium sulfate (THPS)	7 mg/h; Continuous	Activated sludge	Maintenance metabolism	Lysis-cryptic growth	Continuous, A2O (anaerobic- anoxic-oxic) laboratory bench 85d, SRT=10d, MLSS=2000- 4000 mg/L, activated sludge 20-28°C, pH 7.56-8.43	39.3	Decreased by 3.8%	Decreased by 8.7%	Slightly affected sludge settleability	(Li et al., 2016)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
Zinc (Zn)	10 mg/L (0.5- 15 mg/L)	Oxic-settling- anaerobic	Maintenance metabolism	Energy spilling	Continuous, HRT= 6h, STR=2d, MLSS= 50-200 mg/L, synthetic sewage, CAS seed, 22°C, pH 7.2	30				(Liu, 2000)
Zinc (Zn)	10 mg/L (5-15 mg/L); Once	Activated sludge	Maintenance metabolism	Energy spilling	Batch, short- term test study, MLSS= 2000- 2500 mg/L, synthetic wastewater, CAS seed	30	Decreased by 7%		Energy spilling above 5 mg/L. Slight decrease in SOUR.	(Aragón et al., 2009)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
2,4- dichlorophenol (2,4- dCP)	3 and 4 mg/L (1-8 mg/L); Continuous	Minimization of sludge production in MBR	True metabolic uncoupling		Batch, DO=5 mg/L, MLVSS= 100 mg/L, SRT= 45d, synthetic wastewater, CAS seed, 20- 22°C,	28.7 (3 mg/L) 35 (4 mg/L)	Slightly decreased		Slightly reduced sludge settleability. Significant improvement in biomass bioactivity.	(Lin et al., 2010)
2,4-dinitrophenol (2,4- dNP)	35 mg/L; Continuous	Activated sludge	True metabolic uncoupling		Continuous, MLSS= 2.5 g/L, SRT=1.5d, HRT=5.5h, sewage as substrate, 20°C, pH 7	29	Decreased by 3.7%		Dissociation of energy metabolism was proportionally related to the concentration of uncoupler. Increase in SOUR.	(Mayhew et al., 1998)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
2,4-dinitrophenol (2,4-dNP)	5 mg/L	Activated sludge	True metabolic uncoupling	Changes in population dynamics	SBR, DO=2 mg/L in the aeration period, MLVSS=2000 mg/L, SRT=10- 30d, operating time=220d, synthetic wastewater, seeding sludge from oxidation ditch, 20-22°C	21	Unaffected		Uncoupler protozoan inclined to grow in disperse. Enhanced sludge bioactivity.	(Chen et al., 2008)
2,4-dinitrophenol (2,4-dNP)	40 mg/L (20- 40 mg/L); Once	Activated sludge	True metabolic uncoupling		Batch, short- term test study, MLSS= 2000- 2500 mg/L, synthetic wastewater, CAS seed, 20°C	49	Decreased (not provided)		20% SOUR increase Detrimental effect on COD removal increased with dose.	(Aragón et al., 2009)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
2,4- dinitrophenol and diatome (carrier) (2,4- dNP)	6 mg/L	Activated sludge	True metabolic uncoupling	Changes in population dynamics	Continuous, synthetic sewage, CAS, 25°C, pH 7	28.28	Decreased by 7-30%	Decreased by 44%	Increased microbial activity at low dosage. Effluent quality and sludge settleability deteriorated with increasing dose of uncoupler.	(Li & Tabassum, 2022)
2,4,5-trichlorophenol (TCP)	2-2.5 mg/L; Continuous	Activated sludge	True metabolic uncoupling		Continuous, VSS/TSS=0.83, SRT=5.0d, HRT=3.5h, 21°C, pH 7	50				(Strand et al., 1999)
2,4,6-trichlorophenol (2,4,6-TCP)	2 mg/L (0.01 mM)	Activated sludge	True metabolic uncoupling		SBR	47	Unaffected			(Verduyn et al., 1992)
2,4,6-trichlorophenol (2,4,6-TCP)	236 mg/L (1.2 mM)	Activated sludge	True metabolic uncoupling			67				(Tao et al., 2010)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
2,4,6-trichlorophenol (2,4,6-TCP)	2 mg/L; Continuous	Activated sludge	True metabolic uncoupling		Continuous, SBR, 70 d, DO= 4 mg/L, MLSS= 2000 mg/L, municipal wastewater, CAS seed, 20- 22°C	47	Decreased by 8%			(Zheng et al., 2008)
3,3',4',5- tetrachlorosalicylanilide (TCS)	1 mg/L (0.8-1 mg/L); Once per day	Activated sludge	True metabolic uncoupling		Continuous, MLSS=2000 mg/L, SRT=7d, HRT=8h, synthetic wastewater, CAS seed, 20°C, pH 7	40	Nearly unaffected		Increase of active bacteria. 50% increase in SOUR	(Chen et al., 2002)
3,3',4',5- tetrachlorosalicylanilide (TCS)	0.5 mg/L	<i>Escherichia coli</i> biomass yield reduction	True metabolic uncoupling			78	Nearly unaffected			(MacLeod et al., 1988)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
3,3',4',5- tetrachlorosalicylanilide (TCS)	0.8 mg/L (0.2- 0.8 mg/L); Once	Activated sludge	True metabolic uncoupling		Batch, short- term test study, MLSS= 2000- 2500 mg/L, synthetic wastewater, CAS seed, 20°C	30	Increased by 13%		Increase in microbial activity. 7.5% increase in SOUR	(Aragón et al., 2009)
3,3',4',5- tetrachlorosalicylanilide (TCS)	1% gTCS/gMLSS (1-20%); 10 days intervals	Activated sludge	True metabolic uncoupling		Side-stream activated sludge pilot reactor (CAS), 8 months, synthetic wastewater, CAS seed, 20- 22°C	80% per day	Slightly reduced			(Hassani et al., 2011)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
3,3',4',5- tetrachlorosalicylanilide (TCS)	2.7 mg/L; Daily	Activated sludge	True metabolic uncoupling	Changes in population dynamics	Continuous, lab bench, mixed activated sludge process, MLSS= 3000 mg/L, DO=6 mg/L, 60 days, synthetic wastewater, CAS seed, 25°C	30	Unaffected		Alteration in protozoa population.	(Ye & Li, 2005)
3,3',4',5- tetrachlorosalicylanilide (TCS)	2 mg/L (0.5- 2mg/L); Once	Control of the biomass growth of a pure culture.	True metabolic uncoupling		<i>Shewanella</i> <i>oneidensis</i> MR- 1, 20 mM pyruvate minimal media	53			Increase in average protein content of the bacterial cells. Significant drop in cells count.	(Saini & Wood, 2008)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
3,3',4',5- tetrachlorosalicylanilide (TCS)	1 mg/L	Activated sludge	True metabolic uncoupling		Continuous, DO= 2 and 9 mg/ L, two F/M ratio (0.18 and 0.35 g COD/ g MLVSS*d), HTR=1d, 42 d, municipal wastewater, 20°C, pH 7.7	28 (low F/M) 60 (high F/M)	80-85%		Increase in EPS production. Sludge reduction was enhanced by the increase of the F/M ratio. Increased aerobic volume inside the flocks.	(Ferrer-Polonio et al., 2017)
3,3',4',5- tetrachlorosalicylanilide (TCS)	0.8 mg/L	Activated sludge	True metabolic uncoupling	Lysis-cryptic growth	SBR, MLSS= 2500 mg/L, HRT=16h, synthetic wastewater, CAS seed, pH 7.9	63	Unaffected		Strong inhibitory effect on ammonia removal. Bacteria diversity diminution. 69% reduction in ATP production.	(Ferrer-Polonio et al., 2019a)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
Benzoic acid (BA)	1.2 mg/L (10 mM)	Metabolic flux reduction in yeast	True metabolic uncoupling			Decreased (not provided)				(Verduyn et al., 1992)
LODORed	110 mg/L; Daily	Activated sludge	True metabolic uncoupling		SBR, MLSS=2500 mg/L, HRT= 0.67d, synthetic wastewater, CAS seed, 20- 22°C	26.2	Unaffected		No impact on microbial respiration. Improved settleability of sludge.	(Zuriaga-Agustí et al., 2016)
Malonic acid (MA)	10 mg/L; Continuous	Activated sludge	True metabolic uncoupling	Changes in population dynamics	Continuous, SBR, 25 d, DO= 4 mg/L, MLSS= 2000 mg/L, municipal wastewater, CAS seed, 20- 22°C	30	Decreased by 6%		Significantly deteriorated sludge settleability and structure.	(Zheng et al., 2009)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
Folic acid (FA)	0.8 mg/L (0.8- 1.6 mg/L); Once per day	Activated sludge	True metabolic uncoupling		Batch SBR, MLSS= 2500 mg/L, synthetic wastewater, CAS seed, 21°C	44.7	Unaffected		Increased endogenous respiration. Increased SMP production.	(Ferrer-Polonio et al., 2019b)
Carbonylcyanide m- chloromethoxy- Phenylhydrazone (CCCP)	0.0025 mg/L; Once	Metabolic flux reduction in yeast	True metabolic uncoupling			37				(Slonczewski et al., 2009)
Carbonylcyanide m- chloromethoxy- Phenylhydrazone (CCCP)	2.5 mg/L; Once	<i>Escherichia coli</i> biomass yield reduction	True metabolic uncoupling			22				(MacLeod et al., 1988)
Carbonylcyanide p- trifluoromethoxy- phenylhydrazone (FCCP)	0.0025 mg/L; Once	Metabolic flux reduction in yeast	True metabolic uncoupling			22				(Slonczewski et al., 2009)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
Carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP)	2.5 mg/L; Once	<i>Escherichia coli</i> biomass yield reduction	True metabolic uncoupling			37				(MacLeod et al., 1988)
Dicoumarin with tourmaline (carrier)	10 mg/L (10- 40 mg/L); Once per day	Activated sludge	True metabolic uncoupling		SBR, MLSS= 2000 mg/L, real wastewater, 15- 35°C, pH 6.5-8.5	32.4	Unaffected			(Li & Tabassum, 2020)
2,4-dinitrophenol (2,4-dNP)	45 mg/g VSS (15-45 mg/gVSS)	Effects of dNP addition on membrane biofouling.	Undermining biofilm stability - changes in EPS and SMP production	Restrained ATP production	Batch, activated sludge, 25°C	37.5			High inhibition of biofilm's EPS production.	(Ding et al., 2019)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
2,6- dichlorophenol (2,6- dCP)	10 mg/L (10-40 mg/L)	Activated sludge	Undermining biofilm stability - changes in EPS and SMP production	Changes in population dynamic.	SBR, DO=4 mg/L, MLSS= 3000 mg/L, SRT=15d, synthetic wastewater, raw sludge, 25°C, pH 7	33.3			Weakened interactions between microorganisms and environment. Reduced SMP production. Dispersed flocks, but no effect on settleability.	(Lin et al., 2022a)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
3,3',4',5- tetrachlorosalicylanilide (TCS)	4 mg/L (1-4 mg/L); Once	Effect of uncoupled metabolism on aerobic granulation.	Undermining biofilm stability - changes in EPS and SMP production	Restrained ATP production	Batch SBR, 30d, MLSS= 2000 mg/L, synthetic wastewater, activated sludge flocs from WWTP, 20-22°C		Decreased by 8%		4-fold deteriorated stability and integrity of precultivated granules. Inhibited formation of new granules. 75.2% reduction in ATP synthesis. Reduced EPS production; lower PN	(Jiang & Liu, 2010)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
3,3',4',5- tetrachlorosalicylanilide (TCS)	0.1 mg/L; Continuous	Inhibition of biofilm and biofouling formation in membrane bioreactor (MBR) systems	Undermining biofilm stability - changes in EPS and SMP production		<i>Bacillus subtilis</i> in different development stages, incubation on membrane for 2 and 24 h				Significant reduction in the initial bacterial attachment stage and subsequent biofilm development. Inhibited motility in semisolid medium. Reduced EPS production.	(Feng, et al., 2020b)
3,3',4',5- tetrachlorosalicylanilide (TCS)	0.12 g/ g VSS (0.03-0.12g/ g VSS); Once	Activated sludge	Undermining biofilm stability - changes in EPS and SMP production	Restrained ATP production	Batch, MLSS= 8000 mg/L, DO=0.4 mg/L, secondary sludge from WwTP, 24 hrs mixing, 25°C	51.1			60.8% reduction in ATP synthesis. Significant loss of microbial diversity and richness. Reduced EPS production (43%)	(Lin et al., 2022b)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
3,3',4',5- tetrachlorosalicylanilide (TCS)	30 mg/L (30-120 mg/L); Once	Ultrafiltration process (sludge water recovery)	Undermining biofilm stability - changes in EPS and SMP production	Restrained ATP production	MLSS= 3000 mg/L, 7 days, activated sludge from secondary tank, 20°C	50	Slightly reduced	Slightly reduced	Reduced EPS production. Increase in SMP production. Flocks' fragmentation.	(Ding et al., 2020)
4- nitrophenol (4-NP)	3 mg/L	Activated sludge, membrane bioreactor	Undermining biofilm stability - changes in EPS and SMP production		Bench scale submerged MBR, DO=4mg/L, SRT=45 days, MLSS=1000 mg/L, HRT=4- 5.5h, synthetic wastewater, CAS seed, 20- 22°C, pH 6	62	Decreased by 5%		Reduced EPS and SMP production Shift to greater particle size range.	(Qiong et al., 2013)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
2,4-dinitrophenol (2,4-dNP)	10 mg/L; Once	Microbial attachment to solid surfaces.	Undermining biofilm integrity - suppression of quorum signalling molecules	Restrained ATP production	Batch, synthetic wastewater, CAS seed, 25°C				68-82.8% reduction in attachment to solid surface 57.4% reduction in AI-2 secretion 2.5-fold reduction in ATP production	(Xu & Liu, 2010)
2,4-dinitrophenol (2,4-dNP)	10 mg/L; Once	Influence on granulation, biofilm attachment and detachment.	Undermining biofilm integrity - suppression of quorum signalling molecules	Restrained ATP production	Batch, synthetic wastewater, CAS seed, 25°C				20% reduction in membrane biofouling. Attachment to membrane reduced by 46%.	(Xu & Liu, 2011)

Uncoupler	Dose/ Range Addition	Application	Primary mechanism	Secondary mechanisms	Test conditions	Sludge reduction (%)	COD	TN	Main observations	References
3,3',4',5- tetrachlorosalicylanilide (TCS)	0.1 mg/L; Continuous	Biofouling inhibition in membrane bioreactor (MBR)	Undermining biofilm integrity - suppression of quorum signalling molecules		Continuous, lab- scale parallel MBR systems, 100 d, HRT=8h, SRT=40d, MLSS= 9000 mg/L, synthetic sewage, CAS seed, 20-25°C, pH 5.5-7.5	50	Unaffected		Significantly inhibited secretion of AI-2 and C8-HSL (N- octanoyl-DL- homoserine lactone). Reduced EPS production.	(Feng et al., 2020a)
Carbonylcyanide m- chloromethoxy- Phenylhydrazone (CCCP)	1 mg/L; Once	Inhibition of biofilm formation	Undermining biofilm integrity - suppression of quorum signalling molecules		<i>Salmonella</i> <i>Typhimurium</i> ATCC 14028S (L828) was used as a control strain throughout.				Significantly inhibited biofilm formation	(Baugh et al., 2012)

3.4.1 True metabolic uncoupling

True metabolic uncoupling is characterized by meeting two specific criteria: (1) inducing a decrease in the growth yield of microorganisms without significantly compromising the overall efficiency of pollutant removal, (2) sustaining or increasing the respiration rate of the microbial culture (Aragón et al., 2009). Furthermore, genuine uncoupling ability is demonstrated by resumption of the sludge production rate to an increasing trend after the cessation of continuous uncoupler addition (Figure 3-4).

The specific uptake rate of biomass determines its catalytic efficiency in removing substrates. True metabolic uncouplers have been shown to significantly increase this rate by enhancing the oxidation of catabolic intermediates (Ferrer-Polonio et al., 2019; Ferrer-Polonio et al., 2019b; Low et al., 2000). Several authors have reported that the addition of these uncouplers led to a non-growth-associated increase in specific oxygen uptake rate (SOUR). For instance, Aragón et al. (2009) observed a 7.5% increase in SOUR and a 13.5% increase in COD removal with the addition of TCS, and 20% SOUR increase upon addition of 2,4- dNP to a batch activated sludge system fed with synthetic wastewater. Additionally, Chen et al. (2002) reported 50% increase in oxygen consumption with TCS to a continuous activated sludge system fed with synthetic wastewater. This overconsumption of oxygen implies a high-level dissipation of energy for metabolic regulation confirming that the addition of the forementioned uncouplers stimulates the respiratory activity of microorganisms. In accordance with that Chen et al. (2002) and Aragón et al. (2009) reported an increase in the percentage of active bacteria over the total microbial population of around 50% upon addition of TCS. These findings are in line with the observations of Ferrer-Polonio et al. (2017, 2019a) who reported enhanced reactor performance due to increased microbial hydrolytic enzymatic activities, accompanied by an average 60% reduction in biomass growth and a 69% decrease in ATP synthesis upon the addition of low concentrations of TCS to a sequencing batch reactor (SBR) fed with synthetic wastewater. Further to that, Lin et al. (2010) reported a significant improvement in the bioactivity of the biomass with 2,4- dCP, while

Zheng et al. (2008) observed slightly enhanced catabolism capability of the sludge with the addition of 2,4,6-TCP, all in line with true metabolic uncoupling.

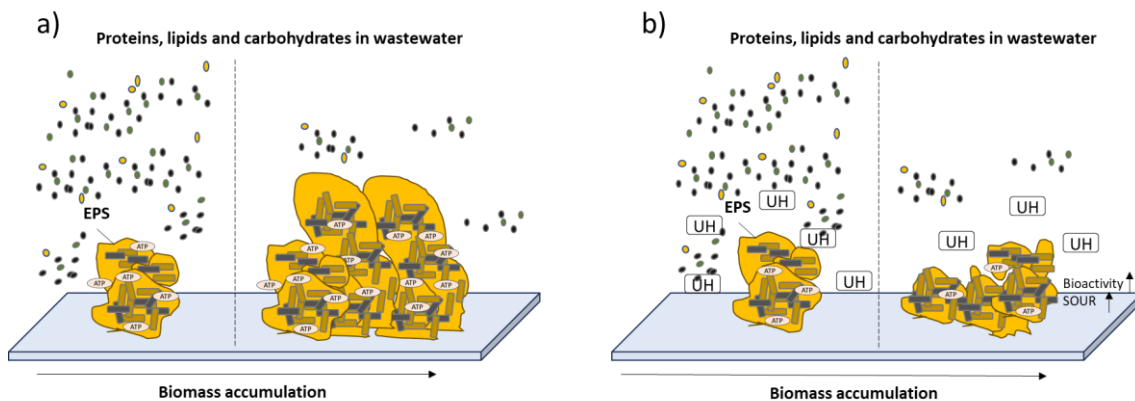


Figure 3-4 True metabolic uncoupling mechanism of action: a) untreated system state, b) alterations upon uncoupler addition.

3.4.2 Maintenance metabolism

Uncouplers have been found to elevate the demand for maintenance energy by redirecting energy towards non-growth processes (Gostomski & De Vela, 2018; Low & Chase, 1999). Microbial catabolism and anabolism are linked through rate limiting respiration. However, in cases where this control is eliminated, biosynthetic processes become the rate limiting factor. In such cases, where there is an excess of free energy, bacterial cells need to redirect this energy away from biomass production. To achieve this, they employ various strategies such as dissipating the energy as heat through ATP systems and futile cycles (Aragón et al., 2009; Liu, 2000), activating alternative metabolic pathways that bypass energy-conserving reactions (Wang et al., 2022), and accumulating polymerized products in storage form or secreting them as a waste (Fang et al., 2015; Han et al., 2017; Li et al., 2016; Li et al., 2012) (Figure 3-5). These mechanisms enable cells to effectively manage and utilize the surplus energy (Low & Chase, 1999). Ray and Peters (2008) suggested that under the forementioned conditions, a portion of the energy is used in stress management rather than growth, while Liu (2000) and Aragón et al. (2009) suggested that energy spilling might serve as defence mechanism in challenging environmental conditions.

One notable stress response observed in the presence of metabolic uncouplers is an increase in the production and alteration of the composition of extracellular polymer substances (EPS), believed to be a protection mechanism against the Proton Motive Force (PMF)-dissipating effect of the uncoupler (Fang et al., 2020). Fang et al. (2015) reported that the addition of pCP, oCP and oNP influenced not only the quantity, but also the composition of EPS. All three metabolic uncouplers increased the quantity of EPS produced, while the EPS was significantly richer in proteins (PN) than carbohydrates (PS). These results are consistent with those reported by Li et al. (2012) who found that EPS production was promoted in the presence of TCS and Han et al. (2017) reporting increased generation of protein content (PN) of EPS in d-CP existent environment. Nevertheless, the upregulation of EPS production is not a direct strategy aimed at reducing sludge formation; rather, it represents a maintenance response that diverts energy away from growth processes. Reported substantial increases in protein content, coupled with a minimal increase in polysaccharide content, indicates a significant shift in the energy utilization strategy of microorganisms. Protein synthesis requires approximately three times more energy than synthesis of polysaccharides; 36.4 mmol ATP/gram versus 12.6 mmol ATP/ g respectively. Consequently, this diversion of energy allocation constrains the availability of energy for microbial growth (Fang et al., 2020; Gostomski & De Vela, 2018).

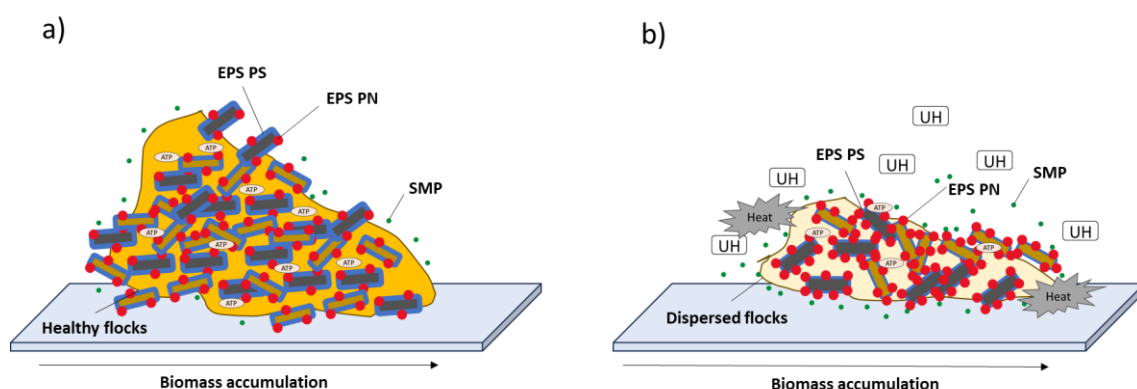


Figure 3-5 Maintenance metabolism mechanism of action: a) untreated system state, b) alterations upon uncoupler addition.

3.4.3 Undermining biofilm integrity, ATP driven destabilisation of biofilm

Bacteria exhibit clear preference for forming biofilms, benefiting from the cooperative advantages and multicellular lifestyle such communities offer (Feng et al., 2020b). Biofilms are intricate and diverse communities of bacteria that exhibit a well-organized three-dimensional structure enveloped in a self-produced polymeric matrix that attach to surfaces, interfaces, or each other (Ding et al., 2019; Xu & Liu, 2010). Bacteria cells living in biofilms are physiologically and behaviourally integrated, which provides them with higher resistance and adaptivity to periods of environmental stress (Li et al., 2012; Xu & Liu, 2010).

The growth and development of biofilms rely on three factors: (1) the presence of microorganisms, (2) production of extracellular polymeric substances (EPS), and (3) a surface for attachment. Since the biofilm itself is composed of a community of microorganisms that are brought together through attractive forces and the production of extracellular polymeric substances, the physical, chemical, and biological processes involved in bacterial adhesion, biofilm establishment, and biofilm maturation play a crucial role in determining the adhesive and cohesive properties of the biofilm (Feng et al., 2020b; Lin et al., 2010; Xu & Liu, 2011). The viscoelasticity of biofilms, which determines their behaviour under shear stress, results from the combined interactions of EPS and bacteria through quorum sensing, whereas the soluble microbial products (SMP) and biopolymers play a crucial role in the initial adhesion of microorganisms to surfaces facilitating subsequent attachment of EPS and sludge flocs (Ding et al., 2019; Wang et al., 2013). In consequence, the overall stability of the biofilm is achieved by the production of EPS and SMP (Ding et al., 2019; Qiong et al., 2013), bacterial communication (Baugh et al., 2012; Feng, et al., 2020b; Xu & Liu, 2010, 2011), and, to some extent, the hydrophobicity of the cell surface (Lin et al., 2022b) (Figure 3-6).

Energy metabolism plays a pivotal role in initiating microbial attachment to, and detachment from solid surfaces. Bacterial motility is especially significant during initial phase of attachment (Feng et al., 2020b; Xu & Liu, 2010). When

approaching substratum surface, microorganisms rely on localized proton motive force (PMF) and ATP as the driving force to facilitate the adhesion (Xu & Liu, 2011). Metabolic uncouplers are recognized for their capacity to dissipate the PMF and to suppress ATP synthesis. Studies have indicated that by interfering ATP production metabolic uncouplers can: 1) reduce bacteria motility (Feng et al., 2020b) 2) inhibit the attachment of microorganisms, 3) promote the detachment of biofilms at different development stages, 4) suppress the secretion of extracellular polymeric substances (EPS), and 5) suppress the secretion of autoinducer-2 (AI-2), a signalling molecule that plays a role in intercellular communication during biofilm formation and bacteria motility (Feng et al., 2020b; Li et al., 2012; Qiong et al., 2013). To date, chemical uncouplers have been successfully implemented to regulate biofilm development and induce the detachment of mature biofilms in activated sludge suspensions (Ding et al., 2020; Lin et al., 2022a; Lin et al., 2022b; Qiong et al., 2013) and membrane biofouling (Feng et al., 2020b).

3.4.3.1 Changes in EPS and SMP production

Extracellular polymeric substances (EPS) have been identified as key components responsible for the matrix structure and stability of aerobic granules and biofilms (Jiang & Liu, 2010). EPS play a vital role in the formation, maintenance, and protection of microbial aggregates by occupying the intracellular space. This solid and adhesive material, produced by microorganisms, constitutes a significant portion of the total organic carbon in activated sludge, ranging from 50% to 90%. It is primarily composed of proteins (PN) and polysaccharides (PS), which make up the majority of EPS (75-89%), along with nucleic acids and heteropolymers (Gostomski & De Vela, 2018; Li et al., 2012). SMPs are small, water-soluble molecules such as proteins, carbohydrates, enzymes, and secondary metabolites, released by microorganisms to surrounding environment during their metabolic activities. SMPs play a significant role in microbial interactions, biofilm formation, and overall functioning of microbial communities (Ding et al., 2019; Lin et al., 2022b).

A growing body of evidence supports the notion for the existence of intricate relationship between microbial aggregation, granulation, and energy metabolism. Studies have demonstrated that inhibiting energy-generating functions can significantly impact granules' stability and hinder microbial competences for granulation. For instance, the addition of TCS (Feng et al., 2020b; Jiang & Liu, 2010) and CCCP (Baugh et al., 2012) has been found to inhibit biofilm formation and to induce disintegration of mature granules.

Apart of their effect on sludge yield, metabolic uncouplers also exert a profound influence on the formation of microbial products such as EPS and SMPs. Studies have revealed that the addition of uncouplers like TCS (Ding et al., 2020; Feng et al., 2020a, 2020b; Jiang & Liu, 2010; Lin et al., 2022b), 4-NP (Qiong et al., 2013) and 2,4-dNP (Ding et al., 2019) leads to an increase in the PN content of EPS, with a less pronounced effect on PS content. Simultaneously, the overall EPS content is substantially reduced. This reduction in EPS production is directly linked to a significant decrease, up to 75%, in ATP production (Ding et al., 2019, 2020; Jiang & Liu, 2010; Lin et al., 2022b)

Furthermore, the impact of uncouplers extends to the formation of SMPs. Several studies have reported the effects of specific uncouplers on SMP production. For instance, the addition of o-CP (Fang et al., 2020), 2,6-dCP (Lin et al., 2022a) and 4-NP (Qiong et al., 2013) has been associated with reduction in overall SMP production, equally affecting the concentrations of proteins and polysaccharides. The decrease contributed to the formation of more dispersed flocs and weakened interactions between microorganisms and their environment. Conversely, the addition of TCS (Li et al., 2012) and 2,4-dNP (Ding et al., 2019) has been observed to increase the levels of both polysaccharide and protein SMPs. These findings reveal that different uncouplers will have distinct effects on the EPS and SMPs formation, leading to varying impacts on the characteristics and behaviours of microbial communities.

3.4.3.2 Suppression of Quorum Signalling molecules

Bacteria utilize a communication system called quorum sensing (QS) to regulate various functional genes, such as those involved in competence and biofilm

formation (Feng et al., 2020a; Xu & Liu, 2011). This system relies on the synthesis, emission, and detection of specific chemical molecules known as QS signal molecules. Autoinducers, a group of QS signal molecules, play a critical role in coordinating collective behaviours within bacterial communities, for both Gram-negative and Gram-positive bacteria. Among these, autoinducer-2 (AI-2) has been identified as a key mediator of interspecies communication during biofilm formation, whereas the synthesis of AI-2 is energy-dependent (Xu & Liu, 2011). While the understanding of the impact of energy metabolism on microbial attachment and detachment is still limited, the control of cellular communication pathways using uncouplers offers a viable option for control of biofilm formation.

Studies have shown that the attachment of the microorganisms to the solid surfaces is positively linked to AI-2 concentration in microbial cells. This indicates that bioenergetically active state of suspended microorganisms will promote microbial attachment to solid surfaces. It has been hypothesised that to overcome the barriers of adhesion between surfaces, microorganisms require certain amount of ATP, relying on localized PMF to serve as driving force for the adhesion (Xu & Liu, 2010). The lowered secretion of AI-2 induced by inhibited ATP production has been associated with the addition of 2,4-dNP (Xu & Liu, 2010), TCS (Feng et al., 2020a) and CCCP (Baugh et al., 2012). For instance, the addition of 2,4-dNP showed 2.5-fold reduction in net synthesis of cellular ATP leading to 57.4% reduction in AI-2 secretion, whereas the TCS showed to significantly reduce the secretion of AI-2 and C8-HSL (N-octanoyl-DL-homoserine lactone).

On the other hand, it is reasonable to hypothesise that the energy dissipation caused by uncouplers can ultimately destabilize the existing biofilm. The inadequate energy availability to maintain biofilm stability can lead to the disintegration of the biofilm structure and the subsequent release of individual cells from the surface. The addition of 2,4-dNP have been proven to not only suppress biofilm attachment, but also to enhance biofilm detachment from solid surfaces (Xu & Liu, 2011). The authors observed that the significantly impaired ATP and AI-2 production induced detachment of the biofilm from the membrane

surface, whereas the measure was more effective in disturbing old biofilms than young ones.

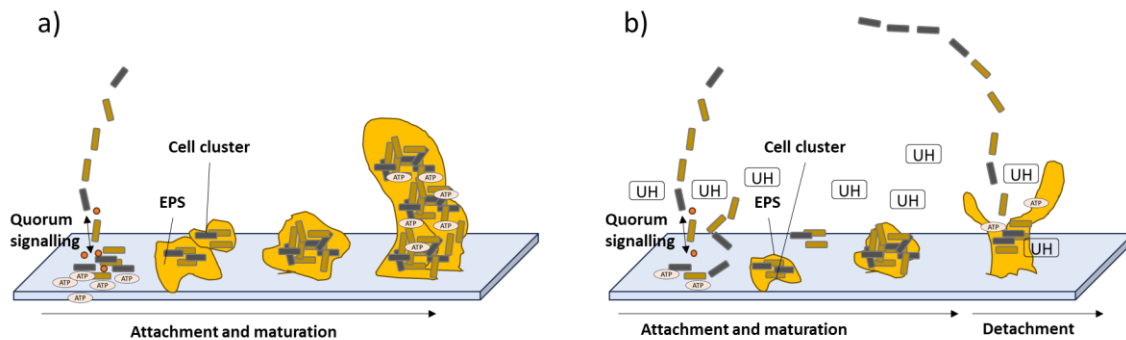


Figure 3-6 Undermining biofilm stability mechanism of action: a) untreated system state, b) alterations upon uncoupler addition.

3.4.4 Challenging the microbial diversity

The biomass encompasses a rich and interconnected microbial population. This diverse community consists of both individual cells and clusters forming flocs and biofilms, where cells undergo life cycles and reproduction. This system fosters a wide array of intricate interactions, characterized by symbiotic, cooperative, aggressive, and competitive behaviours. These interactions contribute to the dynamic and constantly evolving nature of biomass population. Studies on mixed cultures have revealed that the introduction of uncouplers can induce changes in population morphology and dynamics, leading to the demise of certain species and the proliferation of the others (Ferrer-Polonio et al., 2019a). This shift in microbial composition often favours the microorganisms that have fully adapted to the presence of uncouplers (Lin et al., 2022a). The microbial shift typically occurs after an extended exposure period and tends to alleviate the initial inhibitory effect of uncouplers on nutrient removal efficiency (Gostomski & De Vela, 2018; Low & Chase, 1999). Activated sludge is an artificial ecosystem serving as favourable habitat not only for bacteria, but also for a diverse range of higher organisms such as protozoa and metazoa predated on bacteria.

Interestingly, the induced variation in the microbial population is often a secondary mechanism to primary occurring maintenance metabolism (Low et

al., 2000; Yang et al., 2003), true metabolic uncoupling (Chen et al., 2008; Li & Tabassum, 2022; Ye & Li, 2005; Zheng et al., 2009) or undermining biofilm stability (Lin et al., 2022a; 2022b). This implies that in these cases the observed reduction in sludge production is attributed to a combination of altered microbial dynamics and metabolic processes. Uncouplers such as mCP, mNP, oNP, pCP (Yang et al., 2003), dNP (Li & Tabassum, 2022), 2,6-dCP (Lin et al., 2022a), TCS (Ferrer-Polonio et al., 2019b; Lin et al., 2022b), pNP (Low et al., 2000), malonic acid (Y. Zheng et al., 2009) and oCP (Fang et al., 2020) have been reported to cause significant losses in the relative abundance, richness, and diversity of microbial population. For instance, 2,6-dCP reduced the number of active and respiring bacteria within eleven bacteria phyla, while increasing the abundance in five others in a sequencing batch reactor (SBR) fed with synthetic wastewater and inoculated with raw sludge (Lin et al., 2022a). The addition of TCS has reportedly led to 60% reduction in *Chloriflexi spp.* and 31% reduction in *Nitrospirota spp.* (Lin et al., 2022b), while the dosing of pNP (Low et al., 2000) and malonic acid (Zheng et al., 2009) has been reported to lead to proliferation of filamentous bacteria and disappearance of protozoa.

3.5 Discussion

Biomass reduction realised through a combination of true metabolic uncoupling, maintenance metabolism, undermining biofilm integrity and/or shift in microbial diversity, provides a potential alternative to traditional sewer FOG bioremediation practices. Recent advancement in the identification and understanding of mechanisms underlying metabolic uncoupling support the notion that this approach holds a significant promise in controlling biomass growth across various microbial processes. Successful applications of uncouplers have been observed in reducing excess sludge production and biofouling in various sewage treatment systems including sequencing batch reactors (SBR), membrane bioreactors (MBR) and full scale conventional activated sludge systems (CAS). Consequently, consideration of the use of uncouplers can be reasonably extended to any fixed film processes to treat aqueous pollutants, including minimalization of FOG deposit formation and accumulation in sewers.

Notwithstanding that, further research is crucial to substantiate this hypothesis and gain a deeper comprehension of the potential advantages that uncouplers could possibly provide beyond the immediate dosing point, as adopting and replicating the enhanced performance of biomass catalyst could enable existing facilities to effectively handle higher pollutant loads or accommodate reduced reactor volumes in the new installations.

FOG mitigation potential based on the uncoupler mechanism of action.

Wastewater is a complex mixture of components that undergo degradation through microbial metabolism. FOG deposit formation is influenced by biological, chemical and physical phenomena associated with nutrients removal and molecules interactions therefore these shall be considered in conjunction with strategies aiming for deposit minimalization.

1) *True metabolic uncoupling:*

Recent development in understanding how FOG deposits form and accumulate in sewers revealed that the deposit formation is inherently associated with the presence of not only lipids, but also proteins and carbohydrates (Jawiarczyk et al., 2023, Chapter 2), and that the total suspended solids are indispensable in FOG deposits formation (Otsuka et al., 2020). By increasing the specific uptake rate of pollutants through the enhancement of oxidation in catabolic intermediates, uncouplers can significantly improve the bioactivity of the biomass. This, expressed through increased hydrolytic enzymatic activities, can enable a more efficient and rapid elimination of fats, carbohydrates, and proteins from wastewater. Consequently, the opportunity for their physical and chemical interactions leading to deposit formation and growth can be minimized.

2) *Metabolism maintenance:*

It has been reported that EPS facilitate the adsorption of pollutants present in wastewater. They contain functional groups that serve as binding sites for various compounds, whereas the electrostatic interactions and complexing bonding are responsible for binding smaller molecules, while hydrophobic interactions play a major role in binding larger molecules. Although the further research is needed

to fully comprehend the intricate processes involved in pollutants binding to the biofilm, it is reasonable to assume that these interactions might contribute to FOG accumulation. One of the notable stress responses induced by metabolism maintenance inducing uncouplers is alteration in EPS production and composition, both potentially holding an opportunity to minimize pollutant binding to the biofilm.

3) *Undermining biofilm integrity:*

FOG deposits undesirably accumulate at the interface between solid and liquid phases, and are likely facilitated by deposition, growth, and metabolism of bacteria in biofilm attached to sewer walls and in flocks. While complete inhibition of biofilm formation is unattainable due to its spontaneous occurrence in nature, uncouplers hold promise in terms of process control by specifically disrupting microbial metabolism involved in the biofilm formation process, as well as its attachment and detachment. Uncouplers have been shown to affect the integrity of biofilms through various mechanisms, including changes in EPS, and SMP production, quorum signalling molecules, and cell hydrophobicity. Moreover, the dosing of uncouplers can potentially soften pre-deposited FOG deposits, promoting their disintegration. It has been reported that the uncouplers are more effective in disruption of mature biofilm, therefore offering attractive option for deposits rehabilitation.

4) *Challenging the microbial diversity:*

There is undoubtful correlation between indigenous wastewater bacteria and FOG deposit formation (Brooksbank et al., 2007). Deposit formation is likely facilitated by the deposition, growth, and metabolism of the bacteria within biofilms attached to sewer walls and suspended flocks. Studies on mixed cultures have revealed that the introduction of uncouplers can induce changes in population morphology and dynamics, leading to the demise of certain species and the proliferation of others. This suggests that some uncouplers may have the potential to alter the balance of the microbial community, potentially diminishing the presence of bacteria responsible for FOG deposit formation. Notwithstanding

that, understanding the relationship between indigenous wastewater bacteria and FOG is crucial for further developments in effective mitigation measures.

3.6 Considerations

Despite of promising results in reducing excess sludge in laboratory-scale experiments, a practical application of uncouplers entails inherent limitations and challenges. For instance, introducing stress to the existing microbial ecosystem can cause population shift that can decrease sludge reduction capability due to microbial acclimatization to stressor and/or adversely affect biomass settling characteristics. Moreover, chemical stress can deteriorate the effluent quality by reducing nitrogen assimilation into biomass, in consequence increasing the demand for downstream nitrification and denitrification processes (Low & Chase, 1999; Ray & Peters, 2008). Further to this, reduced sludge production is often associated with lowered COD removal efficiency. Finally, several metabolic uncouplers including 2,4-dNP; 2,4-dCP and p-nitrophenol proved to be xenobiotic and even toxic to the microorganisms and environment (Chen et al., 2002). Encouragingly, the majority of current studies indicate that many uncouplers can effectively reduce sludge production without significant disruption to system performance.

3.7 Final remarks

While the dosing of additives to the system without significant system modifications and at a comparatively low cost can accelerate the adoption of the measure for FOG remediation once its efficacy is demonstrated by comprehensive research, the commercial implementation of the uncoupling metabolism approach hinges on the availability of inexpensive and non-toxic uncouplers. This will become increasingly important in positioning uncouplers as part of FOG remediation practices, which is expected to increasingly shape future applications considerations. Ultimately this will enable potential for such practices to be considered in places where traditional sewer cleaning methods and FOG capture through physical separators are either not practical, desirable, or sufficient. This includes domestic outflows and commercial dishwashers'

discharges. Furthermore, in the context of full-scale application when selecting an uncoupler and optimizing its dosage, due consideration should be given to achieving a careful balance between sludge reduction and system performance, as well as the dosing influence on processes downstream of dosing point.

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4 Chapter 4 Inhibition and Rehabilitation of Fats, Oils, and Grease (FOG) deposits in sewers with an energy uncoupler with surfactants.

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Abstract

The study investigated the effectiveness of a product based on a mixture of yeast protein extract (uncoupler) and surfactants for effective inhibition of FOG formation in oil-rich synthetic sewage and rehabilitation of pre-deposited FOG. Trials were conducted with a combination of synthetic solutions for deposition formation, preformed synthetic deposits and real deposits taken from the UKs

sewerage network. Trials were conducted with the product across a dose range of 3-20 ppm and the impact was measured in terms of deposit mass reduction and the removal of lipids, carbohydrate, proteins, nutrients, VFAs and FFAs. Effective FOG degradation was revealed to be strongly influenced by the other constituents with reduction in FOG degradation associated with high C:N ratios or the presence of available carbohydrates. This emphasised the need to monitor all components when assessing the ability to degrade FOGs. The results of the study showed high potential for implementing the product in FOG bioremediation practices, offering improved TSS and nutrients removal, without associated biomass generation. The findings raise the possibility of employing yeast proteins extract to achieve minimalization of FOG deposit formation in sewers, requiring only addition of a series of low doses of uncoupler to sewers, providing low-price and non-toxic alternative to traditional sewer cleaning method.

Keywords: FOG bioremediation, uncoupler, carbon/nitrogen ratio

4.1 Introduction

Fats, oils and greases (FOG) deposition and accumulation in sewer lines can lead to sewer blockages and bursts resulting in raw sewage spilling into water bodies or flooding events. Current estimates in the UK and USA indicate that between 50-70% of such events are caused by FOG accumulation (Keener et al., 2008; British Water, 2015) with the associated cost of rehabilitation and maintenance in the UK and USA reported to be £100 million (Water UK, 2018) and \$25 billion (Marie et al., 2017) respectively. Rehabilitation involves mechanical removal using either jetting or manual excavation with power tools and shovels, followed by landfill disposal. Accordingly, mitigation measures are being employed to reduce FOG inputs into sewers such as grease traps, education and best practice campaigns, complimented with appropriate regulations and legislations. Despite implementing such measures, problems related to sewer blockages caused by FOG persist and are, in fact, becoming more frequent. As a result, there is a need for supplementary interventions.

The most common alternative involves dosing bio-products into FSE wastewater or the sewer, to enhance biological degradation pathways (Lauprasert et al.,

2017). These include enzymes, live microorganisms (bioaugmentation), biosurfactants or bio-nutrients and have gained popularity as they are relatively low cost, easy to implement, require low energy inputs and are safe for both humans and the environment. To illustrate, implementation of bioremediation by Anglian Water at one of their problematic sites led to 50% reduction in sewer blockages events, and considerable cost savings for the company (Arthur & Blanc, 2013). Even in the absence of bio-product dosing, deposited FOG is degraded by the native microbial community. However, in problem sites, the rate of deposition far exceeds the rate of degradation due to minimal hydrolysis of triglycerides, and so bio-products are added to enhance the latter. The applied organisms/products are selected based on their ability to produce extracellular lipases, i.e., enzymes that degrade triglycerides into free fatty acids and glycerol, making them accessible for microbial consumption (He, 2011; Lauprasert et al., 2017). Previously reported trials that demonstrated the potential include dosing *Bacillus coagulans*, *Bacillus simplex* and *Trichococcus* (diary effluent, 88% removal), *Bacillus spp.* and *Pseudomonas spp* (80% removal) (Tzirita, 2012), *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* (Lauprasert et al., 2017), and a consortium of *Bacillus spp.*, *Lactobacillus sporogenes*, *Saccharomyces cerevisiae*, *Pseudomonas spp.*, *Cellulomonas uda*, *Micrococcus sp.*, and *Thiobacillus novellus* (37-62% removal) (Brooksbank et al., 2007). However, there is a limited number of reported trials under true environmental conditions and at industrial scale. Moreover, the existing data reveals significant variation in product efficiency, sufficient to question the overall effectiveness of the approach (Brooksbank et al., 2007).

Another weakness with the current mitigation practices is their almost sole focus on management of lipid degradation brought about, in part, through a belief that saponification of available lipids is the predominate pathway for deposition. Previous work by the authors (Jawiarczyk et al., 2023; Chapter 2) challenges this notion and proposes an alternative two stage model of deposition based on initial formation through precipitation of carbohydrate-lipid complexes, followed by accumulation of fats, other lipids, carbohydrates, proteins, and other debris. This aligns to other work that has emphasised the important role of TSS in FOG

formation (Otsuka et al., 2020) and the link between wastewater bacteria consuming available FFAs and the formation of solids (Brooksbank et al., 2007). These findings suggest a link between wastewater bacteria and deposit formation, therefore efforts to improve bioremediation should either adjust the activity of overall microbial community using bioaugmentation or reduce the net yield of solids production. A number of authors have suggested that native populations are better suited for the purpose as they are 'pre-activated' populations in a variety of physiological states associated to the specific local conditions (Rani et al., 2007; Loperena et al., 2006).

Management of yield requires alteration of the balance of the metabolic biochemical reactions associated with catabolism and anabolism. Catabolism is the energy-generation component of metabolism, that converts substrates into lower energy products as adenosine triphosphate- ATP. Whereas anabolism refers to processes that convert a carbon source and a nitrogen source into cells' biomass using energy derived for ATP hydrolysis (González-Cabaleiro et al., 2015; Ye & Li, 2005a). Catabolism and anabolism are linked by the energy exchange through the production and consumption of ATP (González-Cabaleiro et al., 2015). Chemicals can be added to uncouple these pathways increasing the discrepancy in the energy level between catabolism and anabolism (Ye & Li, 2005). From the engineering standpoint, the concept of energy uncoupling means that the modified metabolic pathways lead to overconsumption of substrate required for maintenance of the cells, while the energy supply to anabolisms becomes limited. Consequently, the oxidation of substrate occurs with less energy available for formation of excess biomass (sludge) because phosphorylation of ADP to ATP has been decreased (Ye & Li, 2005). Metabolic uncoupler addition is attracting considerable research interest due to its relatively low cost, high efficiency, and convenience as a measure, as it does not require any modifications to existing wastewater processes or installation of expensive facilities. Many standard protonophore uncouplers are hydrophobic weak acids such as 2,4-dinitrophenol (DNP) and lauric acid (Hiraishi & Kawagishi, 2002; Podella et al., 2009).

Similar impacts have been reported when using a mixture of heat-shock yeast proteins and surfactants that offer a more environmentally friendly chemical to dose (Podella et al., 2009). For example, a protein-rich, cell free supernatant from the heat-shocked yeast *Saccharomyces cerevisiae* combined with certain synthetic surfactants has been reported to affect the growth rate of indigenous microflora in order to synergistically enhance microbial decontamination of wastewater and decrease biomass growth (Podella et al., 2009). Commercial application of the product has been reported to improve water decontamination, reduce interfacial tensions in water/hydrocarbon and water/oil systems, act as degreasers in sewers and cleaners for petroleum oil spillages, inhibit biofilm formation in reverse osmosis membranes, treat odours, as well as minimize excess biomass production in an activated sludge process (Baldrige & Podella, 2005; Podella et al., 2009). The latter reflects previous focus on the use of uncouplers as a means to reduce yield in activated sludge systems (Ye and Li, 2005).

To date there is a paucity of available information on the potential for utilising uncouplers for management of FOG deposits in sewers. Given that reduction in TSS should reduce FOG deposition, it seems prudent to explore the potential of uncouplers to stimulate indigenous wastewater microbiota combined with TSS reduction in oil-rich wastewater. To this end, the current research responds to this knowledge gap by assessing the efficacy of a commercially available liquid mixture of a protein-rich, cell-free supernatant (uncoupler) towards inhibition or rehabilitation of FOG deposits.

4.2 Materials and Methods

All reagents, unless otherwise specified, were purchased from Fisher Scientific (UK).

4.2.1 Energy uncoupler with surfactants

The uncoupler used is a commercially available liquid mixture of a protein-rich, cell-free supernatant obtained from a heat-shocked yeast, and proprietary

surface-active agents (Table 4-1 and Table 4-2). This uncoupler was selected as it is the only commercially available uncoupler designed for FOG bioremediation.

Table 4-1 Commercial product composition %vol (Podella et al., 2009)

	Protein Ferment	Propylene glycol	Etox. Alcohol 6EO	SLE (60%)	DOSS (75%)	Hexylene glycol
Yeast ferment	76.67%	21.09%	0%	0%	0%	0%
Product	54.37%	14.76%	22.44%	7.48%	0%	0%

Etox. Alcohol- Ethoxylated Alcohol; SLE- Sodium Lauryl Ether Sulphate; DOSS- Dioctyl Sulfosuccinate.

Table 4-2 Product properties (Advanced BioCatalytics, 2023).

Property	Value
pH value	4.5 - 5.5
Dilution pH	4.0 - 5.0
Specific Gravity (25°C)	1.02 - 1.05
Density	1.02 - 1.05 g/cm ³
Flash Point	Min 93.3
Percent Volatile (15 min, 105°C)	73 - 75
Boiling Point	100°C
Freezing Point	0°C
Colour	Amber
Shelf life	2 years

4.2.2 Synthetic sewage

The efficiency and modes of action of the uncoupler solution were investigated in synthetic sewage solution (SS) enriched with 500 mg/L vegetable oil (Co-op, Cranfield, UK). The SS recipe was adapted from OECD guidelines Test No. 303 (OECD, 2001) and contained starch (100 mg/L), peptone (160 mg/L), meat extract (110 mg/L), urea (30 mg/L), sodium chloride (7 mg/L), potassium

dihydrogen phosphate (28 mg/L), calcium chloride (4 mg/L) and magnesium sulphate (2 mg/L). Indigenous microbiota were added through addition of 2 mL of raw wastewater per L of solution. The characteristics of synthetic sewage, according to standard laboratory methodology used for this study, were: Total Suspended Solids (TSS) 157 ± 2 mg/L, Chemical Oxygen Demand (COD) 1361 ± 166 mg/L, lipids (HEM) 516 ± 44 mg/L, carbohydrates 130 ± 4 mg/L, proteins 140 ± 14 mg/L, Carbon/Nitrogen ratio (C/N) with lipids 6.2, pH 7.1, and temperature 20°C.

4.2.3 Field samples

Fat, oil, and grease (FOG) samples used in this study were sourced from sewer networks across the UK. Samples were provided by courtesy of Severn Trent Water. Samples were manually excavated during the clean-up of the location. All samples were stored at 4°C prior the analysis.

4.2.4 Uncoupler trials

To determine the ability to prevent FOG deposit formation and to degrade FOG blockages, two modes of action of the product were investigated: inhibition- addition of the uncoupler to oil-rich wastewater to prevent deposit formation and, rehabilitation- addition of the uncoupler to degrade pre-deposited FOG deposits.

4.2.4.1 Laboratory bench scale experiments

Batch experiments, in triplicates, were run in 250 ml flasks filled with 50 mL of SS. For each set of the experiments, control tests were run in parallel. Flasks were kept under constant mixing, at aerobic conditions and room temperature, using an orbital shaker at a rotation speed of 80 rpm (Stuart Digital Shaker, Cole-Parmer, St. Neots, UK).

4.2.4.1.1 Evaluation of uncoupler mode of action

To evaluate product potential mode of action of the uncoupler, trials were conducted at the producer's recommended dose of 15 ppm, acting on oil-rich wastewater and pre-deposited FOG. For inhibition trials, uncoupler was added at the start of the experiment. For rehabilitation purpose, deposits were left to form

in synthetic sewage for 2 days, i.e., time required for visible deposits to appear in the flasks. On the completion of day 2, uncoupler at 15 ppm was added to the mixtures. Sacrificial samples were collected on day 2, 7, 11, 15 and 39. FOG deposits were collected by vacuum filtration (Whatman GF/F glass 0.7 μm retention filter) and synthetic FOG formation (inhibition), and degradation (rehabilitation) were assessed by evaluation of dry weight of material retained by the filter (Method EPA, 1684) (EPA, 2001).

4.2.4.1.2 Optimal uncoupler dose assessment for inhibition and rehabilitation of FOG

To evaluate bioadditive optimal dose for inhibition and rehabilitation of FOG, doses based on recommendation from Podella et al. (2009) of 3, 6, 10, 12, 15 and 20 ppm were applied in seven day duration trials. For inhibition, uncoupler was added at the start of the trials. For rehabilitation, deposits were left to form for 7 days, and on the completion of day 7, uncoupler was added to pre-deposited FOG. Rehabilitation of pre-deposited FOG was then allowed for 7 days. On the completion of the trials, FOG deposits were collected by vacuum filtration (Whatman GF/F glass 0.7 μm retention filter, Kent, UK) and synthetic FOG formation (inhibition), and degradation (rehabilitation) were assessed by evaluation of dry weight of material retained by the filter (Method EPA, 1684) (EPA, 2001). In addition to deposit mass evaluation, compositional analysis of supernatant was performed.

4.2.4.2 Scale-up experiments and mass balances

Batch experiments, in triplicates, were run in 10 L reactors filled with 8 L of media. For each set of the experiments, control tests were run in parallel. Reactors were kept under constant stirring (Overhead stirrer, Heidolph, Schwabach, Germany) at 80 rpm, at room temperature and aerobic conditions. Post trials completion, solid and liquid samples were recovered by centrifugation at 4000 G, 5 min, 20°C (ThermoScientific Sorvall Legend RT+, Massachusetts, USA) and used for compositional analysis and subsequent mass balance calculations. All samples were kept at 4°C prior analysis.

4.2.4.2.1 Deposit compositional changes under inhibition and rehabilitation mode of action of the uncoupler

To understand compositional changes of FOG deposit associated with uncoupler mode of action and product dose, batch experiments were performed in SS employing three uncoupler dosage of 3, 12 and 20 ppm. For inhibition, uncoupler was added at the start of the experiment. For rehabilitation, deposits were left to form for 7 days, and on the completion of day 7, uncoupler was added to pre-deposited FOG. Rehabilitation of pre-deposited FOG was then allowed for 7 days. After completion of the trials, FOG deposits were recovered by centrifugation. It is important to note, that by the default, the bacteria residence time in synthetic wastewater during inhibition and rehabilitation trials was longer for rehabilitation. Rehabilitation had 7-day incubation period, to allow sufficient deposit formation available for subsequent degradation, while for inhibition, bacteria and bioadditive were added simultaneously to the system. Initial microbial count therefore upon addition in rehabilitation was higher than for inhibition. Nevertheless, the variable microbial count has been accounted for by the control, incubated under same conditions for 7-days.

4.2.4.2.2 Rehabilitation of aged and real deposits

To investigate the influence of deposit composition, associated with its age, on its biodegradability, rehabilitation of pre-deposited FOG was attempted using deposits formed for 7, 14, 28, 90 and 180 days in synthetic sewage, in comparison to real sewer FOG deposit (age unknown). Freeze-dried deposits were re-suspended in tap water comprising TSS concentration of 400 ± 50 mg/L to match control TSS concentration on day 7 from the previous trials with synthetic sewage and spiked with 2 mL of raw wastewater per 1 litre of sample. Uncoupler dosed at 20 ppm was added to the solution and degradation of deposits was carried for 7 days. After completion of the trials, FOG deposits were recovered by centrifugation at 4000 G, 5 min, 20°C (ThermoScientific Sorvall Legend RT+, Massachusetts, USA).

4.2.5 Analytical methods

Solid samples were subjected to pre-treatment comprising of moisture removal and grinding, to assure samples homogeneity prior to compositional analysis. Samples were freeze-dried until a stable weight was achieved (Christ Alpha 1-2 LD, Osterode am Harz, Germany), and subsequently ground for 5 min using pestle and mortar.

4.2.5.1 Solid samples compositional analysis

Total solids (TS), volatile solids (VS) and ash content were determined according to standard methods (Method EPA, 1684) (EPA, 2001). Characterisation of deposits comprised of total lipids, carbohydrates and protein content evaluation and fractionation of lipids into saponified and un-saponified fats. Total lipid content was determined gravimetrically on an Accelerated Solvent Extractor (Dionex ASE 200, Dionex, California, USA) following Application Note 334 (ThermoFisher, 2012) using hexane as solvent. Saponified and un-saponified fats evaluation was performed by dichloromethane fractionation following Benecke et al.(2017). Protein content was established by the Dumas method (BS EN 13652-2:2001) (British Standard Institution, 2001). Total nitrogen was measured on Elemental Vario EL cube analyser, and multiplied by conversion factor of 6.25 (Gurd et al., 2020) to calculate total protein content of the sample. Carbohydrates were estimated by deducting total lipid, protein, and ash content from the sample.

4.2.5.2 Liquid samples compositional analysis

Total suspended solids (TSS) and Volatile Suspended Solids (VSS) were determined following standard methods (APHA, 2540D) (APHA, 1999). Chemical Oxygen Demand (COD) was measured photometrically using spectroquant cell test kits with a Nova 60 A Spectroquant photometer (Spectroquant® NOVA 60, Merck KGaA, Darmstadt, Germany) where filtered samples -COD_f, centrifuged samples- COD_c. The organic fractions characterization included measurements of oil, protein, and carbohydrates content. Oil concentration was quantified as hexane extractable material (HEM) following the centrifugation method of (Barton, 2012). Protein concentration was established using Thermo Scientific™

Commasie Protein Assay Kit, where bovine serum albumin (BSA) was used as standard for calibration curve. In addition to that, proteins were estimated from the difference between TN, NH₄-N, NO₃-N and NO₂-N multiplied by a standard nitrogen conversion factor of 6.25. Carbohydrates were measured following phenol-sulphuric colorimetric method (Dubois et al., 1956), using glucose as standard. For colorimetric tests, absorbance was measured using Tecan Infinite 200 PRO multifunctional microplate reader (Tecan, Männedorf, Switzerland). The ions in synthetic sewage: chloride, nitrite, nitrate, bromide, lithium, sodium, ammonium, potassium, magnesium, calcium, phosphate and sulphate, were measured on Thermo Scientific Ion Chromatography system ICS900 (Dionex, California, USA) with an IonPac™ CS12A column and guard A 20mM, using methanesulphonic acid as eluent and 100mM tetrabutylammonium hydroxide as regenerant. An injection volume of 20 µl with a 1mL/min flow and ambient temperature was used for the analysis (Standard Test Methodology, D6919-09) (ASTM, 2009). Ion concentrations were calculated via Chameleon software using standard calibration curves. The total carbon and nitrogen (TC-TN) were evaluated on Shimadzu TOC-V analyser (TOC-V Series, User Manual) (Shimadzu, 2007). pH of solutions was measured with laboratory pH meter (Jenway 3540, Cole-Parmer, St. Neots, UK).

4.2.5.3 Triglycerides and Free Fatty Acids analysis

Triglycerides (TGDs) and free fatty acids (FFAs) content of hexane extractable material (HEM) from liquid samples were evaluated using high- performance liquid chromatography with evaporative light scattering detector (HPLC-ELSD). Dry lipid extracts were reconstituted in HPLC-grade chloroform-methanol (50:50%, v/v) solvent mixture and filtered through 0.45 µm retention membrane filter (Whatman, Kent, UK) prior to analysis. All samples and standards were protected from light and stored at -20°C.

Working standard solutions in the range 5-500 mg/L (TGDs), 5-1000 mg/L (FFAs), 5-500 mg/L glycerol were prepared by serial dilution from the respective 5 mg/mL stock solutions. TGDs and FFAs were prepared in chloroform-methanol (50:50%, v/v) and glycerol in MiliQ water. A mixture of standards was prepared

by mixing equal concentrations of triglycerides (TGDs) standards and free fatty acids (FFAs) standards respectively. The standards used for TGDs were trioleate and tripalmitate. FFAs standards included arachidonic, cis-4,7,10,13,16,19-docosahexaenoic, elaidic, erucic, linoleic, linolenic, nervonic, oleic, palmitoleic, petroselinic, arachidic, behenic, decanoic, dodecanoic, hexanoic, lignoceric, myristic, octanoic, palmitic and stearic acid.

HPLC-ELSD chromatographic system comprised of Agilent 1260 Infinity module equipped with an autosampler (G132913 – 1260 ALS), evaporative light scattering detector (ELSD, G4218A), Quaternary Pump (G1311C) and thermostat (G1330B). Triglycerides were separated on Phenomenex Kinetex 250x4.6 mm 5 μ , following a method adapted from AOCS Official Method, Ce 5b-89 (AOCS, 2017). FFAs were separated on a Phenomenex Luna 3 μ 150x2.0 mm analytical column, following the method from Phenomenex Luna Applications: ID 1258. The signal was acquired and processed with ChemStation software. Temperature for both columns was 25°C. ELSD detector conditions were 40°C and gain of 8. Scientific grade nitrogen gas was used as a nebulizing gas. Resolution of FFAs was executed within 30 minutes under gradient conditions with a flow rate of 0.3 mL/min. TGDs resolution was achieved within 25 minutes at isocratic condition with a flow rate of 1.0 mL/min. The composition of the solvents and running program for the HPLC-ELSD method are summarized in Table 4-3 (FFAs) and Table 4-4 (TGDs). The volume of injection for standards and samples was 10 μ L. The identification of each compound was performed by comparing the retention time with the peak of corresponding standard.

Table 4-3 Solvent Program Gradient for Free Fatty Acids (FFAs).

Time (min)	Mobile phases	
	A ^a (%)	B ^b (%)
0	20	80
15	20	80
20	0	100
31	20	80

40

20

80

^aWater, ^bAcetonitrile**Table 4-4** Isocratic Solvent Program for Triglycerides (TGDs).

Time (min)	Mobile phases	
	A ^a (%)	B ^b (%)
0	50	50
25	50	50

^aAcetonitrile, ^bIsopropanol

4.3 Results

4.3.1 Evaluation of the product and modes of possible action

Evaluation of the product at the supplier recommended dose of 15 ppm, revealed a peak reduction in TSS of 28% after 24 hours when operated as an inhibition chemical (Figure 4-1) corresponding to a TSS concentration of 375±46 mg/L versus 519±64 mg/L for the dosed and control tests respectively. In comparison, when used as a rehabilitation chemical, a peak reduction of 53% was observed compared to the control at the time point of 72 hours corresponding to TSS concentration of 185±2 mg/L versus 394±38 mg/L respectively. At and beyond 14 days, the inhibition, rehabilitation and control trials all reached a comparable levels, likely due to exhaustion of nutrients in the batch experiments. Such observations concur with previous trials of the same product that reported a peak of efficiency at 24 hours from addition, and where both control and treatment reached similar levels after 60 hours. The trials were focussed on inhibition and consisted of a standard blend of aerobic bacteria inoculum (Polyseed) grown in triptic soy broth (TBC) solution (Podella et al., 2009).

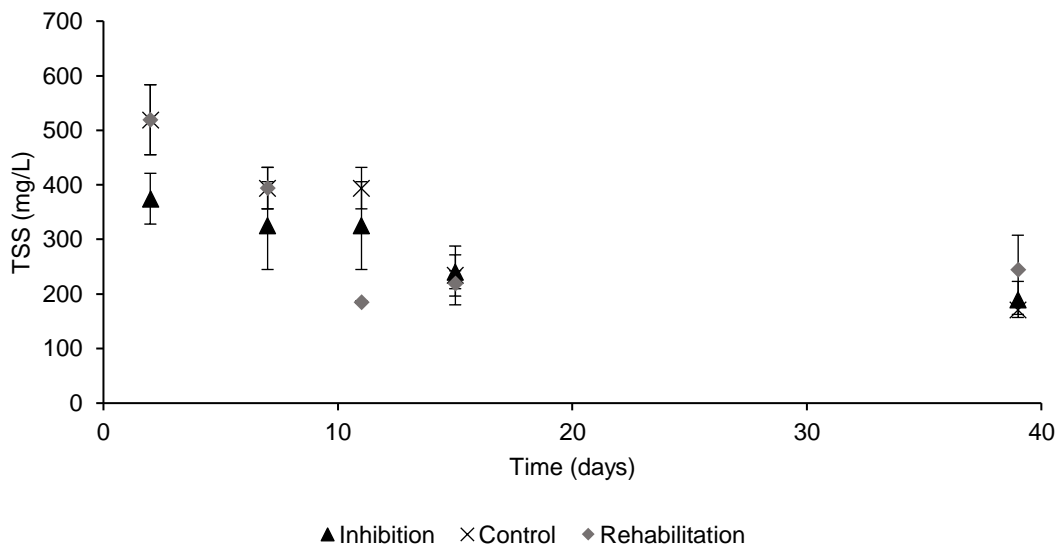


Figure 4-1 Comparison of the impact of dosing 15 ppm of the product on the total suspended solids concentration.

4.3.2 Uncoupler dose evaluation

Dose response trails based on a seven-day test period, revealed a reducing final mass with increasing dose in the case of inhibition (Figure 4-2). To illustrate, deposit mass decreased from 42 ± 6 mg to 32 ± 2 mg as the dose was increased from 3 ppm to 12 ppm corresponding to a TSS reduction of 420 ± 61 mg/L to 319 ± 22 mg/L respectively. Further increases in dose from 12 ppm to 20 ppm did not lead to an additional reduction in deposit mass which was 30 ± 4 mg at 20 ppm corresponding to 297 ± 37 mg/L TSS. In contrast, when used for rehabilitation, the impact was greater and was insensitive to dose (Figure 4-2). To illustrate, the addition of the uncoupler led to an on average 38% reduction in pre-formed deposit mass compared to the control (48 ± 8 mg), while masses of deposit degraded under 3 ppm and 20 ppm respectively were 24 ± 4 mg and 21 ± 9 mg, corresponding to TSS concentration of 243 ± 41 mg/L and 213 ± 86 mg/L (Figure 4-2). The reported TSS reductions observed in the current trials are consistent with findings from other applications, namely yield reduction when using activated sludge processes to treat wastewater. For instance, reductions of between 34-37% were reported using the same products as in the current study with doses of 20-120 ppm in an activated sludge plant (Shin et al., 2018). Whereas the

commercial supplier reported reductions of 25% at a dose of 3 ppm and 30% reduction at a dose of 15 ppm on different case study sites (Advanced BioCatalytics, 2023). Comparison to reported performance of chemical metabolic uncouplers for solids reduction in activated sludge indicate greater levels of reduction such as 38.6% (TCS), 39.3% (THPS) and 43.4% (DCP) (Li et al., 2016); 58.2% (pCP), 59.8% (oCP) and 80.8% (oNP) at 20 ppm dose (Fang et al, 2015). However, better performance has also been reported at very low doses such as 42.7% and 40% with 1 ppm addition of TCS and 2,6-dichlorophenol respectively.

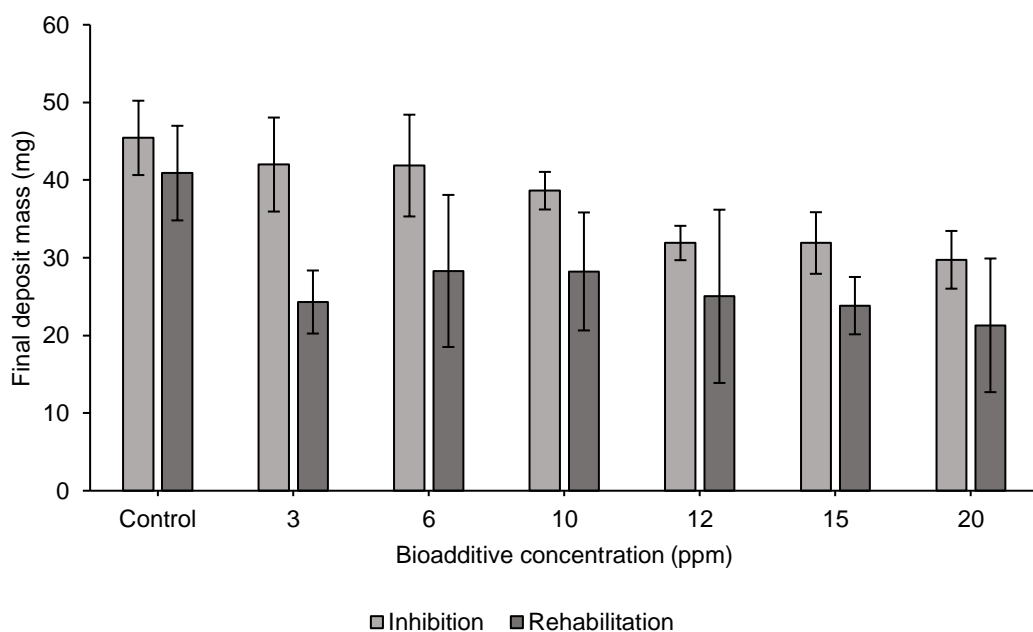


Figure 4-2 Final deposit mass on day 7 of uncoupler treatment at various concentration of the product.

The compositional analysis of the synthetic sewage solution before and after inhibition and rehabilitation trials showed no specific preferences towards any of the main organic fractions. Carbohydrates, lipids, and proteins in synthetic sewage were degraded simultaneously and no significant differences were noted between control and dosed samples regardless of product dose (Figure 4-3 and Figure 4-4). To illustrate, overall removal range between 88 ± 3 and $93\pm 5\%$ for FOG, 85 ± 1 and $87\pm 1\%$ for carbohydrate, 87 ± 3 and $90\pm 3\%$ for COD_f and 17 ± 12 and $54\pm 10\%$ for protein during the inhibition trials (Figure 4-3). The equivalent ranges during the rehabilitation trials were between $88\pm 1\%$ and 100% for FOG,

75±3% and 86±4% for carbohydrate, 85±3 and 92±1% for COD_f and 48±13 to 68±12% for protein (Figure 4-4). This is consistent with previous observation when using chemical uncouplers in that substrate removal efficiency was not significantly affected by the presence of uncouplers used in their study (TCP, Ye and Li, 2005; TCS, CDP, THPS, Li et al., 2016; TCP, Zeng et al., 2008, THPS, Guo et al., 2014, TCP, Strand et al., 1999; pNP, Low and Chase 1999; DNP, Mayhew and Stephenson, 1998 and TCS, Chen et al., 2002). To illustrate, COD removal rates in a continuous lab-scale activated sludge system treating synthetic wastewater were 88.5% in the control and 85.7% when dosed with 3 ppm of TCS (Ye and Li, 2005) The equivalent data in the current trial is an average COD_f removal of 85±6% in the dosed system compared to 87±3% for the control (Figure 4-3). The one exception to this was observed with regards to proteins where removal decreased at higher doses in the inhibition trials. To illustrate, protein removal in the control was 46±6% and this compared to average removals of 50±5% for doses between 3-10 ppm and 17±6% for doses between 12-20 ppm (Figure 4-3). Equivalent findings have been reported for chemical uncouplers dosed into activated plants where a reduction of up to 9% in the TN removal has been observed (Li et al., 2016; Ye and Li, 2005). Interestingly, the reduction in protein removal was only seen in the inhibition trials and was not replicated during the rehabilitation trials (Figure 4-4).

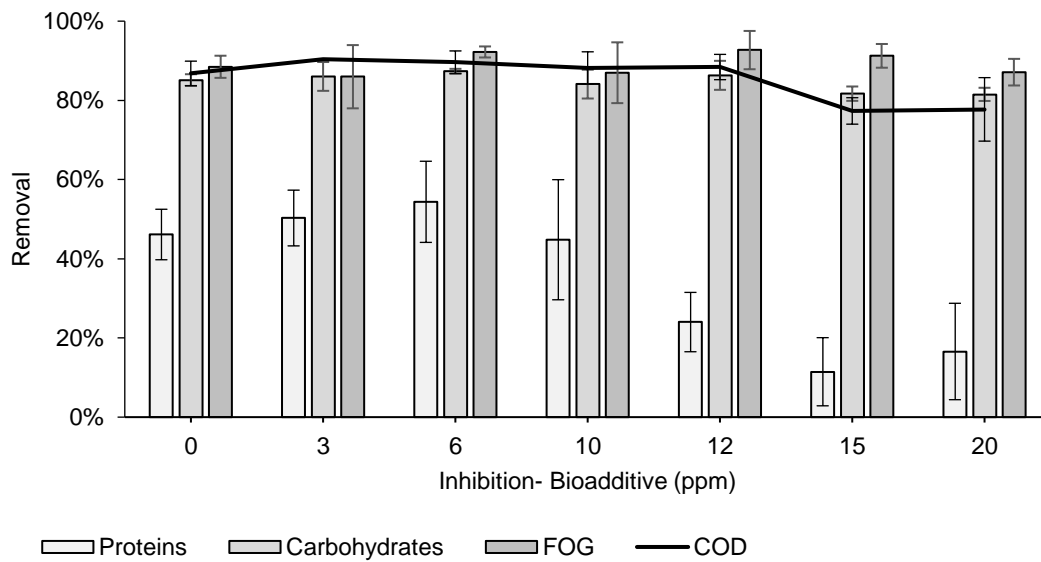


Figure 4-3 Uncoupler inhibition trial. Removal of organic compounds in treated oil-rich synthetic sewage.

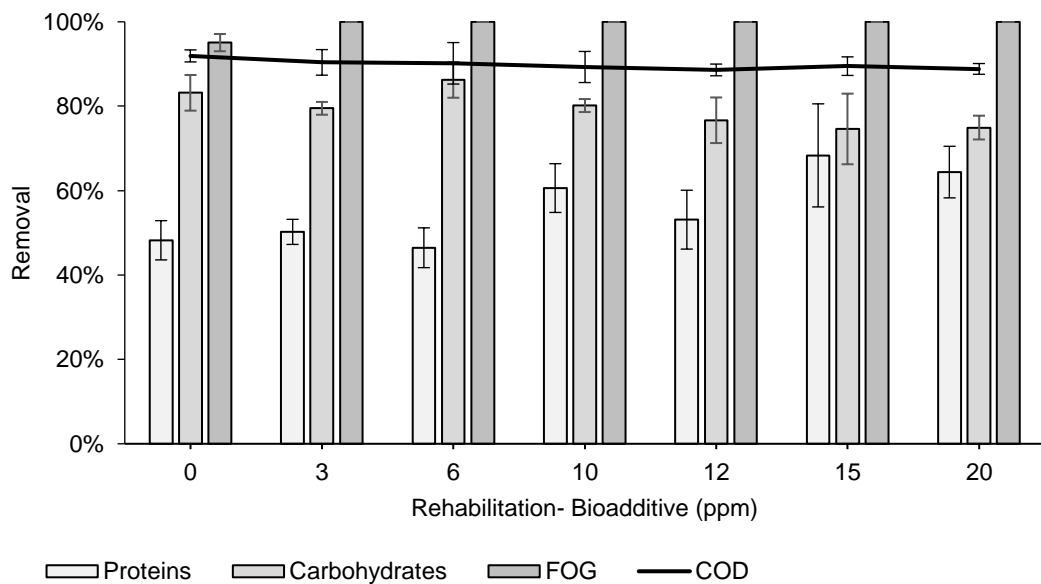


Figure 4-4 Uncoupler rehabilitation trial. Removal of organic compounds in treated oil-rich synthetic sewage with pre-deposited FOG.

One of the concerns related to bio-additives is that resultant increase in lipase generates high concentrations of FFAs and VFAs that simply move the FOG problems further downstream (Brooksbank et al., 2007). In the current case of uncoupler addition, no significant change in either FFAs and VFAs was observed

compared to the control and remained relatively low throughout for all doses trialled. To illustrate, during the inhibition trials, the uncoupler dosed samples contained 53 ± 23 mg/L FFAs and 18 ± 5 mg/L VFAs compared to 61 ± 44 mg/L FFAs and 16 ± 8 mg/L VFAs found in the control samples (Figure 4-5). In comparison, for the rehabilitation trials, no FFAs were measured in the dosed samples compared to 22 ± 5 mg/L in the control (Figure 4-6). In the case of VFAs, the dosed samples averaged 10 ± 3 mg/L compared to 7 ± 1 mg/L in the control.

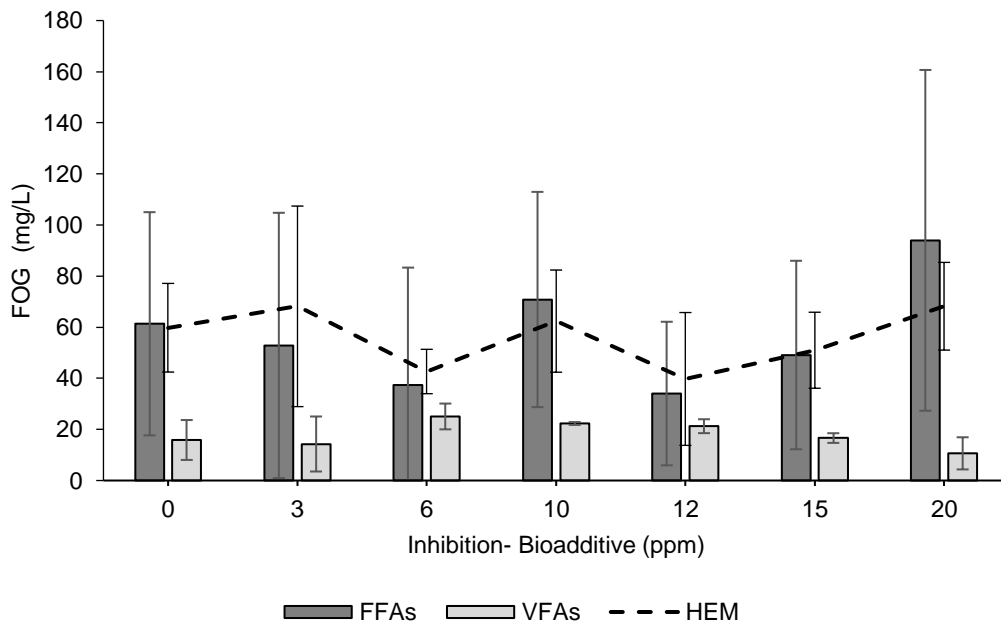


Figure 4-5 Uncoupler inhibition trial. FOG, FFAs and VFAs concentrations in treated oil-rich synthetic sewage. FOG as HEM material, FFAs and VFAs- HPLC analysis.

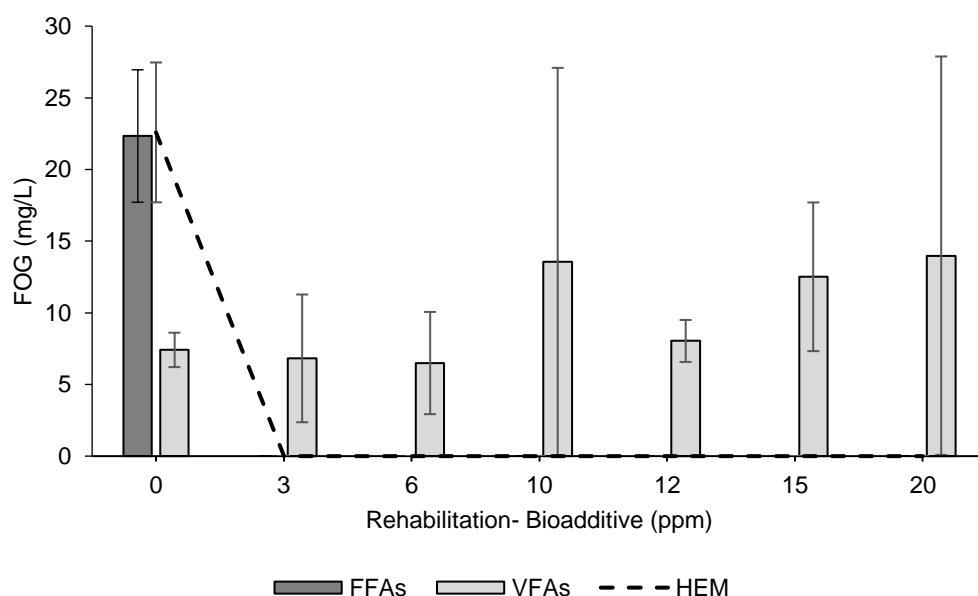


Figure 4-6 Uncoupler rehabilitation trial. FOG, FFAs and VFAs concentrations in treated oil-rich synthetic sewage with pre-deposited FOG. FOG as HEM material, FFAs and VFAs- HPLC analysis.

Ion chromatography analysis of liquid samples including chloride, nitrite, nitrate, bromide, lithium, sodium, ammonium, potassium, magnesium, calcium, and sulphate measurements showed no difference regarding ion concentration between control, inhibition, and rehabilitation trials, except for phosphate. Interestingly, the addition of uncoupler in inhibition trials appeared to enhance phosphate removal achieving nearly 100% compared to a $43 \pm 10\%$ reduction in the control samples. In addition, the dosed samples showed a slight increase of pH to 8.2 ± 0.1 compared to the control at 7.3 ± 0.3 . However, a similar trend was not observed for the rehabilitation trials where dosed samples appeared no different from the control samples, $40 \pm 5\%$ and $51 \pm 8\%$ respectively, nor a change in pH which remained similar to the control at 7.2 ± 0.2 . During the inhibition and rehabilitation trials an initial decrease and then increase of C/N ratio was observed when the uncoupler dose exceeded 10 ppm. The effect was more profound during the inhibition trials. To illustrate, the average C/N ratio increased from 2.9 ± 0.5 (3-10 ppm) to 4.7 ± 0.3 (12-20 ppm) compared to the control value of 3.3 ± 0.6 , while during the rehabilitation trials, the C/N ratio increased from 2.8 ± 0.2 (3-10 ppm) to 3.8 ± 0.6 , while the control C/N ratio was 2.8 ± 0.3 .

Table 4-5 Liquid samples analysis. Inhibition trials.

Bioadditive	C/N	COD_f	HEM	FFA	VFA	Calcium	Phosphate
ppm		mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0, start	6.2	1058 (87)	521 (106)	0.2	0	53 (1)	10
0, 7 days	3.3	138 (27)	60 (17)	61 (44)	16 (8)	37 (1)	6 (1)
3, 7 days	3	103 (9)	68 (39)	53 (52)	14 (11)	34 (5)	Not detected
6, 7 days	3.4	113 (38)	43 (9)	37 (46)	25 (5)	36 (1)	Not detected
10, 7 days	2.9	122 (43)	62 (20)	71 (42)	22(1)	36 (1)	Not detected
12, 7 days	4.5	128 (370)	40 (26)	34 (28)	21 (3)	36 (2)	Not detected
15, 7 days	5.0	258 (32)	51 (15)	49 (37)	17 (2)	41 (3)	Not detected
20, 7 days	4.4	229 (70)	68 (17)	94 (67)	11 (6)	34 (1)	Not detected

All experiments were performed in triplicate, and the data presented in Table 4-5 are three sample average, standard deviation is given in brackets.

Table 4-6 Liquid samples analysis. Rehabilitation trials.

Bioadditive	C/N	COD_f	HEM	FFA	VFA	Calcium	Phosphate
ppm		mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0, start	6.2	1058 (87)	521 (106)	0.2	0	53 (1)	10
0, 7 days	2.8	154 (34)	62 (10)	61 (35)	7 (1)	33 (2)	6 (1)
0, 14 days	2.1	85 (9)	23 (5)	Not detected	7 (1)	36 (5)	9 (1)
3, 14 days	2.7	107 (37)	Not detected	Not detected	7 (5)	36 (3)	8 (2)
6, 14 days	2.7	102 (45)	Not detected	Not detected	7 (4)	35 (5)	7 (2)
10, 14 days	3.1	115 (25)	Not detected	Not detected	14 (14)	32 (2)	8 (0)
12, 14 days	3.2	121 (18)	Not detected	Not detected	8 (2)	35 (4)	8 (2)
15, 14 days	4.5	107 (21)	Not detected	Not detected	13 (5)	32 (1)	8 (1)
20, 14 days	3.6	116 (12)	Not detected	Not detected	14 (14)	33 (3)	7 (1)

All experiments were performed in triplicate, and the data presented in Table 4-6 are three sample average, standard deviation is given in brackets.

4.3.3 Physicochemical changes of FOG deposits induced by uncoupler during inhibition and rehabilitation trials. Mass balances.

Biological treatment of sewer FOG is often challenged by a lack of consensus regarding the efficacy of commercially available products. Moreover, there is lack of knowledge available regarding physicochemical changes caused to the nature of FOG deposits by these products. To resolve this larger scale trials were conducted to enable more complete quantification.

4.3.3.1 Liquid samples analysis

The analysis of liquid samples showed positive impacts of dosing the uncoupler solution on COD_c and TSS content of treated samples compared to the control (Table 4-7). To illustrate, at doses of 3, 12 and 20 ppm the COD_c at the end of the inhibition trials were 383±51 mg/L, 366±24 mg/L and 115±19 mg/L, respectively, compared to 660±153 mg/L in the control. Similarly, TSS concentrations were 108±28 mg/L, 121±13 mg/L and 110±20 mg/L for the 3 ppm, 12 ppm and 20 ppm doses, compared to the control value of 205±43 mg/L (Table 4-7). The data revealed a maximum reduction in COD_c and TSS of 76±6% and 46±4% relative to control. A similar response to bioadditive dose was observed during the rehabilitation trials. The COD_c values measured at the end of the experiment were 65±59 mg/L, 136±70 mg/L and 54±18 mg/L for doses of 3 ppm, 12 ppm and 20 ppm respectively, compared to 661±58 mg/L for the control. The equivalent TSS data were 45±20 mg/L, 61±26 mg/L, 76±12 mg/L for doses of 3 ppm, 12 ppm and 20 ppm, respectively, compared to 156±34 mg/L for the control. The data revealed a maximum reduction in COD_c and TSS of 92±4% and 72±10% relative to control.

4.3.3.2 Solid samples analysis

To evaluate the efficiency of the uncoupler solution addition to inhibit and rehabilitate FOG deposition, the mass of formed deposits under different modes of action was compared to a control. During the inhibition trial, FOG deposit mass formations of 0.9±0.1 g, 1.0±0.1 g and 0.9±0.1 g were observed for doses of 3, 12 and 20 ppm and compared to the control where 1.6±0.1 g of deposit was formed (Table 4-7). In comparison, dosing during the rehabilitation trials resulted in masses of 0.4±0.2 g, 0.5±0.2 g and 0.6±0.1 g for doses of 3 ppm, 12 ppm and 20 ppm, compared to 1.3±0.3 g for the control. In line with earlier observations, the efficacy of the product appeared to be insensitive to dose with effective impacts observed for even the lowest dose of 3 ppm. For instance, mass deposit reduction compared to the control at a dose of 3 ppm was 44% for inhibition and 71% for rehabilitation. In both cases these were the maximum result observed.

Table 4-7 Scale-up inhibition and rehabilitation trial. Liquid samples analysis.

Bioadditive	INHIBITION			REHABILITATION		
	COD _c	TSS	Deposit mass	COD _c	TSS	Deposit mass
	mg/L	mg/L	g	mg/L	mg/L	g
Control	660	205	1.6	661	156	1.3
	(153)	(43)	(0.1)	(58)	(34)	(0.3)
3 ppm	383	108	0.9	65	45	0.4
	(51)	(28)	(0.1)	(59)	(20)	(0.2)
12 ppm	366	121	1.0	136	61	0.5
	(24)	(12)	(0.1)	(70)	(26)	(0.2)
20 ppm	155	110	0.9	54	76	0.6
	(19)	(20)	(0.1)	(18)	(12)	(0.1)

All experiments were performed in triplicate, and the data presented in Table 4-7 are three sample average, standard deviation is given in brackets.

4.3.3.3 Changes in FOG deposit composition

Analysis of the inhibition trials showed that the amount of lipid degraded was similar regardless the dose, yielding removals of $94\pm 4\%$, $93\pm 4\%$ and $94\pm 2\%$ for 3 ppm, 12 ppm and 20 ppm, compared to control value of $85\pm 4\%$ (Figure 4-7). Similar results were observed with regards to carbohydrates, but enhanced removal of proteins was observed for dosed samples compared to the control. To illustrate, protein removal was $49\pm 28\%$, $46\pm 8\%$ and $51\pm 8\%$ for doses of 3 ppm, 12 ppm and 20 ppm respectively compared to $20\pm 4\%$ for the control sample. In comparison, during the rehabilitation trials, enhanced removal compared to the control was observed for lipid and protein but to a much lesser extent (Figure 4-8). To illustrate, lipid degradation levels of $83\pm 6\%$, $69\pm 11\%$ and $79\pm 4\%$ were observed for doses of 3 ppm, 12 ppm and 20 ppm, respectively, compared to $30\pm 21\%$ in the control. In the case of protein, removals of $32\pm 9\%$, $15\pm 4\%$ and $26\pm 11\%$ were observed for doses of 3 ppm, 12 ppm and 20 ppm respectively,

compared to the control at $13\pm 8\%$. This confirms the ability to utilise low doses which was also reported by Fan et al. (2014) where an increase in dose from 0.12 to 120 ppm resulted in only a 5.6% increase in FOG removal.

The oil-rich synthetic wastewater at the start of the experiments comprised of 4.38 g of lipid, 1.23 g of protein and 1.04 g of carbohydrate.

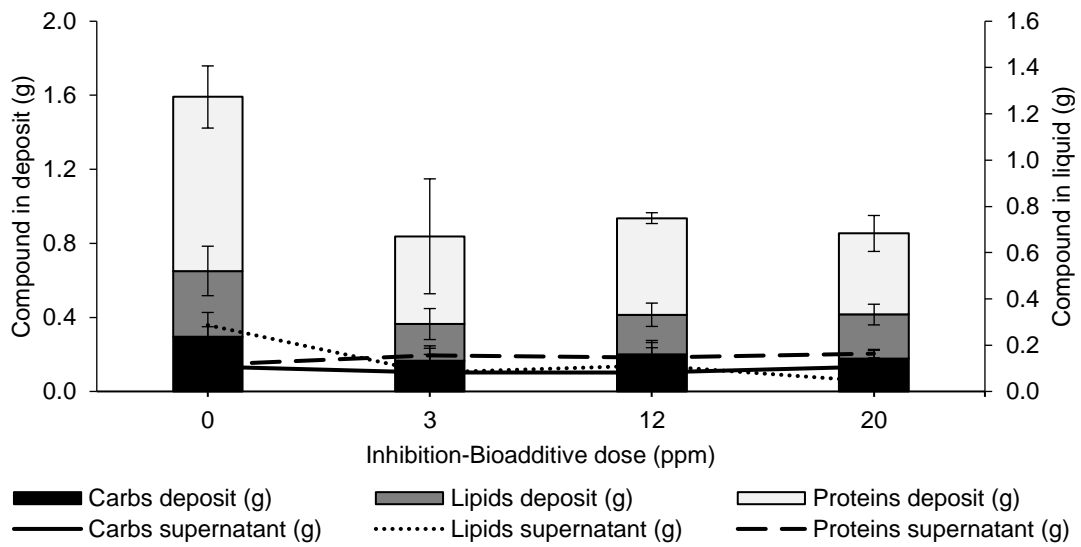


Figure 4-7 Inhibition trials. Changes in deposit composition and liquid phase compounds degradation due to action of the uncoupler.

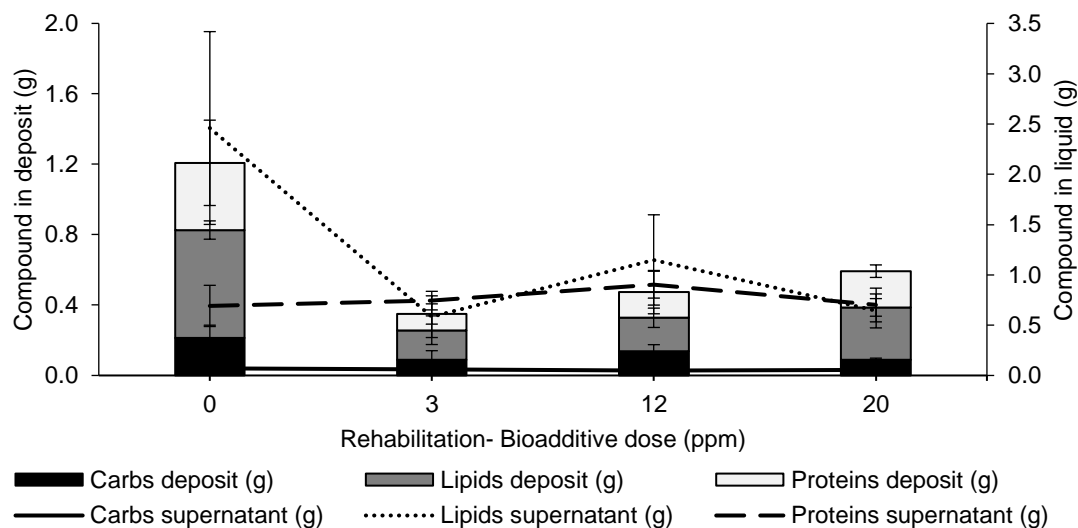


Figure 4-8 Rehabilitation trials. Changes in deposit composition and liquid phase compounds degradation due to action of the uncoupler.

4.3.3.4 Changes in Free Fatty Acids composition

No FFAs or VFAs were detected in the liquid samples upon completion of the inhibition and rehabilitation trials. The analysis of lipid extracts from the solid samples revealed that the fatty acids present were predominately linoleic acid (C18:2) with a contribution from oleic acid (C18:1). These are the main FFA present in the source oil used in the trials (rapeseed oil) which comprises 56.6-64.9% oleic acid, 17.1-20.9% linoleic acid and 4.2-5.0% palmitic acid. No difference was observed between the control and the dosed samples in terms of removal of either linoleic acid or oleic acid which were removed by 97% and 99.5% respectively. The difference in deposit mass then impacted the concentration of FFAs in the deposit. To illustrate, the incorporation of linoleic acid into the deposit resulted in lipid fractions of 117 ± 42 mg linoleic/g lipid, 131 ± 47 mg linoleic/g lipid and 115 ± 22 mg linoleic/g lipid for doses of 3, 12 and 20 ppm respectively compared to the control at 72 ± 24 mg linoleic/g lipid. Equivalent data for oleic acid was 65 ± 11 mg oleic/g lipid, 64 ± 27 mg oleic/g lipid and 68 ± 29 mg oleic/g lipid for the dosed samples compared to 46 ± 8 mg oleic/g lipid in the control. Corresponding data for the rehabilitation trials was 7 ± 2 mg linoleic/g lipid, 7 ± 3 mg linoleic/g lipid and 12 ± 3 mg linoleic/g lipid in the dosed samples compared to 4 ± 2 mg linoleic/g lipid in the control. In terms of oleic acid,

bigger differences were observed at 17 ± 5 mg oleic/g lipid, 21 ± 8 mg oleic/g lipid and 41 ± 6 mg oleic/g lipid for the dosed samples and 10 ± 2 mg oleic/ g lipid in the control.

4.3.4 Rehabilitation of synthetic and real FOG samples

Comparison of deposits as a function of age revealed that fresher deposits were more amenable to treatment. To demonstrate, deposits aged 7 and 14 days showed the highest deposit mass loss of $37\pm 2\%$ and $28\pm 4\%$ respectively compared to reductions of $17\pm 0\%$ for 28-day aged deposit and $10\pm 3\%$ for 90-day aged deposit. Overall, there was a decrease in deposit mass loss as a function of age with the 180-day aged and the real sample showing deposit losses of $5\pm 0\%$ and $2\pm 0\%$ respectively (Table 4-8). For synthetic deposits, the mass loss was predominately associated with changes in lipids and proteins which decreased by $46\pm 3\%$, $30\pm 10\%$, $33\pm 15\%$, $28\pm 10\%$ and $15\pm 8\%$ for protein and $55\pm 12\%$, $73\pm 12\%$, $38\pm 22\%$, $56\pm 21\%$ and $40\pm 11\%$ for lipids for deposit ages of 7, 14, 28, 90 and 180 days. In contrast, the real deposit, which was sampled from a sewer, was almost entirely made of lipids which decreased by $7\pm 4\%$. The different compositional make-up of the deposit impacted the associated C:N ratio which varied between 6:1 for the fresh deposit up to 12:1 for the 180-day aged synthetic deposit and 289:1 for the real deposit. Importantly, deposits with similar C/N ratios and similar compositions showed very similar deposit mass losses (e.g., day 14 and 28). Previous research with bioaugmentation products supports this observation where higher C:N ratios are less effective (Gurd et al, 2020). Further, sufficient protein availability has been reported to be beneficial for FOG removal (Kyong et al., 2002; Tano-Debrah et al., 1999). Moreover, the observed degradation response to aged synthetic and real FOG samples is consistent with previous lipolytic activity studies in multi-substrate media (Castro-Ochoa et al., 2005; El-Shafei & Rezkallah, 1997; Sharma et al., 2014) where authors observed bacterial preference for co-substrates utilisation to support growth followed by a switch to FOG utilisation during stationary phase growth. Similarly, Gurd et al. (2020) observed that the initial carbohydrate concentration had a strong influence on FOG degradation during stationary phase grow, concluding that this

compound will have an impact on the outcomes of FOG bioremediation. This is particularly important concerning the recent discovery of the role carbohydrates in FOG deposits formation (Jawiarczyk et al., 2023, Chapter 2). In summary, carbohydrates and organic nitrogen are known to impact the ability of microorganisms to degrade complex organics, such as FOG.

Table 4-8 Composition of treated deposits, initial and after treatment.

		Lipids	Carbs	Protein	C/N ratio	Deposit mass
		%DS	%DS	%DS	of deposit	reduction (%)
7	Fresh	21 (3)	19 (1)	58 (2)	6:1	
	Control	14 (6)	25 (1)	41 (6)	9:1	34 (7)
	Bioadded	14 (1)	25 (1)	47 (4)	7:1	37 (2)
14	Fresh	51 (14)	18 (0)	29 (12)	14:1	
	Control	25 (9)	18 (0)	25 (12)	18:1	22 (3)
	Bioadded	18 (3)	18 (0)	25 (9)	16:1	28 (4)
28	Fresh	43 (4)	29 (1)	25 (2)	16:1	
	Control	42 (11)	30 (0)	18 (2)	22:1	18 (1)
	Bioadded	31 (10)	30 (0)	20 (5)	21:1	17 (0)
90	Fresh	18 (4)	52 (0)	27 (4)	11:1	
	Control	15 (3)	57 (1)	21 (4)	14:1	9 (4)
	Bioadded	8 (3)	57 (1)	20 (3)	14:1	10 (3)
180	Fresh	1 (1)	78 (0)	21 (1)	10:1	
	Control	0 (0)	77 (1)	20 (1)	12:1	6 (0)
	Bioadded	1 (0)	77 (1)	20 (2)	12:1	5 (1)
Real	Fresh	90 (0)	7 (0)	1 (0)	289:1	
	Control	90 (3)	5 (0)	2 (0)	243:1	5 (3)
	Bioadded	88 (6)	5 (0)	1%	229:1	2 (0)

4.4 Discussion

The results of the current research have demonstrated the effectiveness of dosing a mixture of heat-shock yeast proteins and surfactants for FOG management. The solution works through the mechanism of energy uncoupling resulting in a reduction of TSS which was observed to be effective when used for either inhibition of deposit formation or rehabilitation of pre-formed deposit. The

surfactant components enhance the process by limiting aggregation or encouraging disaggregation and thereby maximising the contact area for degradation (Campo et al., 2007). Inclusion of surfactants has been previously reported to improve the effectiveness of lipase (Sultana et al., 2022) with one study showing a four times improvement compared to control without surfactant (Goldfeld *et al.*, 2015). Further, the results presented here support the view that FOG deposition is not driven by saponification as previously suggested but is instead initiated by starch-FFA complexation followed by accumulation of other components onto the formed precipitates (Jawiarczyk et al., 2023; Chapter 2). Recent work has shown the need for both FFAs and TSS to enable FOG deposit formation as no blockages were observed in an artificial sewer in the absence of TSS (Otsuka et al., 2020). Accordingly, the addition of uncoupler agents can enhance the activity of the native biological population, enabling degradation of available material with significantly reduced solids production and thereby inhibit deposition processes.

The effectiveness of the product was observed to be dose insensitive with the best performance observed at the lowest dose of 3 ppm (Figure 4-7 and Figure 4-8). This is consistent with previous studies, where large increases in dose have been shown to generate little or no increase in FOG removal (Fan, 2014; Sultana et al., 2022). This reflects that the uncoupler is not driving a specific chemical reaction but is rather altering the metabolic pathways of the native microbial community. This is supported by the fact that similar TSS reductions are reported for use of the same product applied to treatment of wastewater using the activated sludge process (Shin et al., 2018). This extends to chemical uncouplers where similar levels of TSS reduction of 30 – 50 % have been reported when used with the activated sludge process (Yang et al., 2006; Fan, 2014; Li et al., 2016). The current work extends this application to degradation of FOG.

In addition, the work has shown the importance of nutrients in enhancing the degradation processes yet the concentration of these and other constituents are often not reported during investigations into the efficacy of bio-additives for FOG removal (He et al., 2012). The carbon to nitrogen ratio is of particular importance

(expressed as either COD:N or C:N), with higher values known to reduce FOG degradation rates in dispersed systems (Gurd et al., 2020). Where nutritional conditions are in balance (e.g. COD:N of 20; Grady et al., 2011), catabolism and anabolism are tightly coupled and the majority of energy generated is utilised for microbial growth (Gurd et al., 2020). This is because just enough energy is provided from carbon degradation to allow all available nitrogen to be co-synthesised into new biomass. The other factor that impacts FOG removal is its competition with other available substrates of use by native microbial communities, such that FOG removal is hindered by the presence of more easily metabolised carbohydrates and proteins (Gurd, et al., 2020). Both aspects were observed in the current study where low levels of deposit reduction were observed as either carbohydrate removal increased (as seen with the increasing age of the synthetic deposit) or very high C:N ratios within the deposit (as with the real sample). Overall, this emphasises the importance of tracking all components in the system, especially when conducting laboratory trials to ensure that results can be translated to the field trials which often does not occur.

In addition to the mass reduction, visible changes to deposits during the synthetic sewage trials were observed when the uncoupler was dosed. Specifically, formed deposits were less adhesive to the walls and produced smaller aggregates compared to those observed for the control. The implication of this is that changes in the compositional make-up of the deposit altered the adhesion properties of the deposit, which will translate in practice to making the deposit easier to remove from the sewer walls, thus reducing the time and hence cost associated to manual cleaning.

4.5 Conclusions

The results have proven the potential for dosing a product based on a mixture of heat-shock yeast proteins and surfactants for the application of FOG management in sewers. The product was shown to be effective at low doses for both deposit formation inhibition and rehabilitation of preformed deposit. The product operated as an energy uncoupler enabling the native wastewater bacteria to degrade the available substrate while minimising biomass production.

The ability to degrade FOG was shown to be influenced by the other constituents within the system such that deposit degradation was reduced when the C:N ratio was high or residual carbohydrates were present. Importantly, this emphasises the need to measure carbohydrates, proteins and nutrients when investigating the potential for biological degradation of FOG.

The results also demonstrated that the efficacy of the product was insensitive to the dose applied, congruent with its mechanism of action. Consequently, the findings raise the possibility of employing yeast proteins extracts with surfactants to achieve minimalization of FOG deposit formation in sewers, as well as providing a promising means of deposit reduction through the addition of a low dose of the product. Additionally, the application of the product was observed to change the characteristics of the FOG deposit, such that it should be easier to remove, which translates to reduced time requirements when cleaning sewers.

4.6 Acknowledgement

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5 Chapter 5 Economic assessment of dosing an energy uncoupler with surfactants for Fats, Oils, and Grease (FOG) management in sewers.

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Abstract

Effective management of Fat, Oil, and Grease (FOG) deposits in sewers presents a significant challenge for the water industry in the UK and worldwide due to its potential to impact customer satisfaction, business operation and regulatory repercussions. Despite the efforts, no uniform approach has been established so far, and existing measures remain insufficient.

This paper conducts an economic evaluation of a heat-shock protein uncoupler combined with surfactants within the context of maintaining sewer systems against FOG accumulation. Initial findings indicate that dosing of the uncoupler to sewers is economically viable providing the addition of the uncoupler 1) decreases the frequency of both planned and unplanned cleaning procedures by slowing down FOG buildup in the sewers compared to no dosing, and therefore leading to the prospect of implementing more effective maintenance schedules, 2) facilitates the cleaning process by modifying the adhesion properties of deposited FOG, such that its removal becomes simplified.

Furthermore, the added value becomes apparent in the reduction of blockages and the mitigation of associated external and internal flooding events. By doing so, the dosing of the uncoupler holds a potential to help in circumventing penalties linked to ODIs imposed by the UK's water sector regulator, Ofwat. Moreover, the performance of assets can be enhanced, leading to a prolonged operational lifespan.

Keywords: FOG management, sewer blockages, sewer maintenance, economic evaluation, biodosing, uncoupler

5.1 Introduction

Municipal sanitary sewer networks are a significant, and indispensable component of the public infrastructure. Comprising of pipes, conduits, pumping stations, and force mains, the sewer network functions to gather and convey wastewater from residential, industrial, and commercial origins to treatment

facilities (Arbour, 1995; Guo et al., 2013). The public tends to overlook the intricacies of a properly functioning wastewater collection system, often taking it for granted, and inadvertently misusing it by improperly disposing inappropriate wastes down the drains and toilets such as fats, oils and greases (FOG) as well as waste chemicals and food stuffs (Williams et al., 2012). Underperformance of sanitary sewer systems is frequently attributed to the accumulation of FOG deposits, underlining the need to optimize the operation and maintenance of these systems (Wallace et al., 2017). Data from around the globe suggests FOG as the cause of pipe blockages to different degrees, ranging from between 21% in Australia (Marlow et al, 2011; Sultana et al, 2022); 40-50% in the USA (Keener et al, 2008); 55-60% in the UK (Scottish Water, 2012; Williams et al, 2012); 60% in Hong Kong (Chan et al, 2010) to a high of 70% in Malaysia (Husain et al, 2014).

FOG inputs into sewers come a range of sources including food service establishments (FSEs), such as food manufacturers, restaurants, grocery stores, bakeries, hospitals, hotels, mall food courts, as well as residential establishments such as apartment complexes and high-density housing, and single houses. FOG deposition diminishes the capacity of wastewater collection systems as well as causing septic tanks to clog, properties to flood, and untreated sewage to enter local waterways and ecosystems (Wallace et al., 2017; Gross et al., 2017; Iasmin et al., 2014; Williams et al., 2012).

The financial implications of FOG-related issues are substantial and include: 1) costs associated with cleaning, repairing, and replacing sewer lines, 2) impact on maintenance and equipment at sewer pumping stations, 3) effects on equipment and maintenance at wastewater treatment plants, 4) generation of odours and contribution to the formation of hydrogen sulphide, and 5) financial penalties for internal and external sewer overflows as well as sanitary sewer overflows (SSOs) (Ducoste et al., 2008; He et al., 2011; NEIWPCC, 2003; Williams et al., 2012). The financial penalties (or rewards) are delivered from the regulator for England and Wales, OFWAT, via the outcome delivery incentives (ODIs) introduced during the regulatory price review process PR14. These are based on designated performance commitment levels which if surpassed can lead to financial rewards

in the form of "outperformance payments" or conversely, "underperformance payments" as a penalty if performance falls below the expected level. In AMP7, the management of FOG-related ODIs holds significant importance (Ofwat, 2023) with a focus on internal sewer floodings, pollution incidents, and customer satisfaction. To illustrate for the year 2020/2021, water companies in England and Wales lost £150 million in revenue for not meeting performance targets with respect ODIs that FOG influences (Figure 5-1) (Anglian Water, 2022; Dwr Cymru Welsh Water, 2022; Northumbrian Water, 2022; Severn Trent, 2022; South West Water, 2022; Southern Water, 2022; Thames Water, 2022; Wessex Water, 2022; Yorkshire Water, 2022). Among the sector's 16 companies, 11 faced net penalties because of underperforming according to the ODI framework. Consequently, these companies were required to make reductions to customer bills for the year 2023/24. Some of the most significant penalties within these ODIs were incurred for not meeting targets related to pollution, internal sewer flooding, and compliance with water treatment works (GWI, 2022). Sewer flooding is split into internal which is defined as sewage backup and overflow within a building or property, causing wastewater to enter indoor spaces and external which relates to flooding onto streets, sidewalk, or open areas (Scottish Water, 2022).

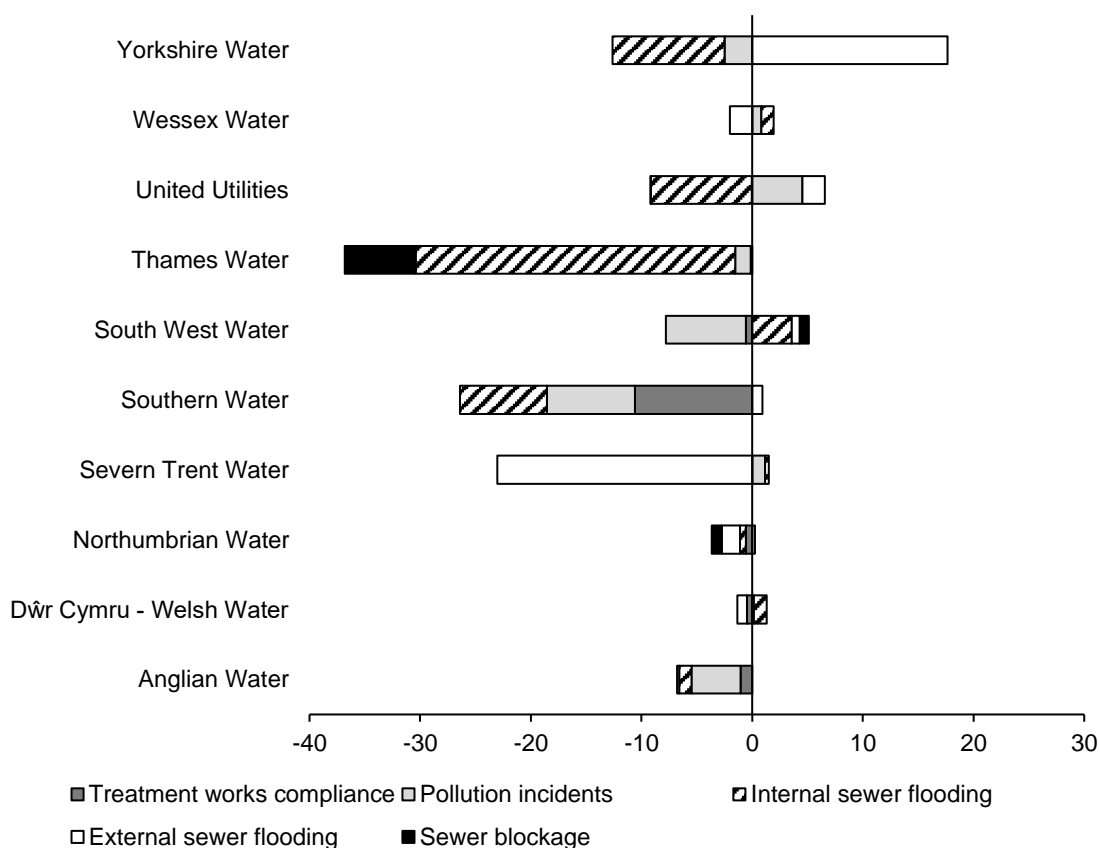


Figure 5-1 FOG related ODIs incurred by Water and Sewerage Companies (WaSC) in England and Wales during 2021-2022.

5.2 Mitigation and management practices

The most effective approach to addressing FOG issues continues to be source control, achieved through a combination of retention of used cooking oils, FOG physical separation from discharged wastewater, i.e., grease traps and interceptors, along with regulatory and educational initiatives (Wallace et al., 2017). However, uptake of good practice is unlikely to be universal and a certain amount of FOG will always inevitably find its way into the sewer system (Gurd et al., 2019). In addition, FOG input from domestic households is normally not managed and has recently been estimated to constitute an average input into the sewer of 2.3 kgFOG/household/year (Collin et al., 2021). However, it is where high concentrations of FOG enter the system in close proximity such as streets with high densities of FSEs, that are the main concern as the input levels of FOG

far exceed the natural degradation capacity of the sewer and represent the highest risk locations for blockages.

Maintenance involves combinations of physical inspection and manual cleaning. Physical inspections encompass visual inspections (including lamping), as well as closed-circuit television (CCTV) assessments. Visual testing offers a cost-effective means of assessing blockages, pipe dimensions, materials, and overall conditions. Through CCTV surveys, it becomes possible to comprehensively evaluate the pipes' integrity and state, identifying issues such as breaks and leaks. Furthermore, these surveys offer valuable insights into blockages, pinpointing their precise locations and enabling the collection and archival of a visual history of the sewer system for future reference. The main purposes physical inspection delivers are: 1) detect blockages that may lead to backups and overflows, 2) identify recurring problem zones, aiding in planned maintenance scheduling, 3) establish a baseline for future comparisons to track build-up and degradation rates, and 4) support legal actions against persistent FOG polluters.

The primary goal of the cleaning process is to eliminate all materials that obstruct the interior surface of the pipe, thereby restoring its capacity and is predominately achieved through either mechanical or hydraulic cleaning (Wallace et al., 2017; Williams et al., 2012). Hydraulic cleaning methods, like jetting, employ the force and velocity of water to cleanse the sewer pipe's invert and walls. On the other hand, mechanical approaches involve the use of equipment such as rodders, bucket and cable machines, scrubbers, and pigging devices equipped with a range of attachments. These tools manually break down FOG buildup, and extract materials from the pipes (NEIWPC, 2003).

Sewer cleaning can be either proactive or reactive. Scheduled proactive cleaning, often supported by CCTV inspections, aims to eliminate FOG before blockages arise. This approach can be further enhanced by on-demand cleaning, especially in areas susceptible to frequent blockages, often referred to as FOG 'hot spots'. Unscheduled cleaning involves reacting to reported blockages with the aim of restoring the pipe's capacity and alleviating sewage backup into properties and/or

overflows (Table 5-1). While mechanical sewer maintenance is commonly employed, it is not without its limitations. For instance, equipment utilizing cutters and brushes designed to come into contact with the pipe wall, proves to be incompatible with plastic pipes (NEIWPC, 2003). These tools have the potential to generate scratches or friction on the inner surface of the pipe, elevating its roughness, therefore reducing its flow characteristics. While power rodders might be considered for cleaning plastic pipes with careful usage, there always remains the inherent risk of the cutter deflecting from the FOG blockages, thereby damaging the pipe.

Table 5-1 Manual FOG cleaning methods (EPA, 1999; NEWPCC, 2003).

Cleaning method	Description	Advantages	Disadvantages
Jetting	The most widely employed manual sewer maintenance method. It relies on the power and speed of water to cleanse the inside of sewer pipes.	<ul style="list-style-type: none"> • Highest flexibility among manual cleaning methods with the minimal personnel requirements. • Eliminates the necessity for manually removing extracted FOG from the manhole by utilizing vacuuming. • Allows adjustable water stream angles for enhanced cleaning efficiency. • Self-contained with all necessary tools on the truck or trailer. 	<ul style="list-style-type: none"> • Demands operators with high skill levels and thorough training. • Mishandling can lead to significant harm to operators or sewer mains. • Needs different nozzles and hoses for effective use. • Costly
Rodders	Tools are specifically designed to cut or scrape materials from the walls of the pipe.	<ul style="list-style-type: none"> • Requires minimal equipment, making it a cost-effective choice in scenarios where advanced machinery might not be accessible. 	<ul style="list-style-type: none"> • The most labour-intensive mechanical cleaning method. • Limited in terms of efficiency and scalability for larger and more extensive sewer systems.
	Hand rodding	<ul style="list-style-type: none"> • Requires minimal equipment, making it a cost-effective choice in scenarios where advanced machinery might not be accessible. 	<ul style="list-style-type: none"> • Limited in terms of efficiency and scalability for larger and more extensive sewer systems.
	Small engine powered rodders	<ul style="list-style-type: none"> • Cost-effective option. • Effective for cleaning in smaller systems where larger equipment faces access limitations. 	<ul style="list-style-type: none"> • Limited in terms of efficiency and scalability for larger and more extensive sewer systems.

<p>Larger mechanical power rodders are equipped with a reel for carrying steel rods and an engine that generates the force needed to rotate, push, and pull these steel rods.</p>	<ul style="list-style-type: none"> • Capable of clearing various obstructions in sewer lines. • Efficient at removing grease and FOG blockages. • Costs less than hydraulic cleaning approaches. 	<ul style="list-style-type: none"> • Tools lack deposits-moving capability. • Best suited for solidified grease. • Operates on drivable surfaces. • Demands operators with high skill levels and thorough training. • Mishandling can lead to significant harm to operators or sewer mains. 	
<p>Bucket machine</p>	<p>System pulls a special bucket through the pipe to gather material, which is subsequently physically removed from the pipe.</p>	<ul style="list-style-type: none"> • Very powerful • Offers optimal cleaning with minimal chance of operator errors • Design offers through cleaning, leaving no debris behind. 	<ul style="list-style-type: none"> • Highly labour-intensive • Mainly suitable for targeted cleaning tasks, particularly for clearing significant blockages from larger sewer systems.
<p>Cable machine</p>	<p>A cable is pulled through the main sewer line between manholes, carrying a cutter, brush, or bucket cleaning the sewer as the cable is pulled back through.</p>	<ul style="list-style-type: none"> • Highly efficient for larger diameter sewers where other equipment may be less effective. 	<ul style="list-style-type: none"> • Cable drag operations are labour-intensive and require the longest setup time to complete the work.

The alternative is to use chemical or biological dosing to support or replace mechanical cleaning, or to avoid blockages forming in the first place. Typical chemicals examples include hydroxides, caustics, and neutralizers (NEIWPC, 2003) or biological solutions such as enzymes, bacteria, bio-additives (Gurd et al., 2020; Lauprasert et al., 2017). They are considered appealing due to their relatively lower costs compared to manual removal, ease of administration, resilience to environmental changes, and long-term safety for both humans and the environment (Brooksbank et al., 2007). The systems rely on continual dosing and the reported results to date indicate mixed performance such there is uncertainty over the performance of biological dosing (Gurd et al., 2020).

In the current research, an alternative dosing agent has been suggested in the form of a commercially available solution containing mixture of yeast protein extract (uncoupler) and surfactants (Chapter 4). FOG minimization through the dosing of the product has been shown to operate through enhanced removal of total suspended solids (TSS) and nutrients, without an increase in biomass generation (Jawiarczyk et al., 2023, Chapter 4). The product was shown to work as either an inhibition or rehabilitation solution with the latter including observed change in the deposit character such that it should reduce the requirements for manual cleaning and hence reduce the associated costs. To date there is a paucity of independent literature concerning the efficacy and dose requirements of the product with recent trials conducted by a UK water company and information available from the commercial supplier (Table 5-2). The water company trials of the product support the claims that the product will reduce cleaning requirements and will facilitate easier cleaning. Overall, doses of between 2-10 mg/L were used resulting in around a 50% reduction in maintenance requirements. In addition, dosing the product for sewer cleaning was also reported to limit FOG build up at sites previously at risk (Table 2). In the case of pumping stations, dosing of the product reduced the rate of deposit accumulation and the resultant frequency of site visits from weekly to monthly. Other non-FOG applications include benefits seen in activated sludge, anaerobic digestion and industrial wastewater treatment where commensurate gains are

report but using higher doses of up to 20 mg/L (Advanced BioCatalytics, 2023; Baldrige and Podella, 2005; Goldfeld et al., 2015).

Table 5-2 Beneficial examples of heat shock yeast proteins uncoupler dosing to wastewater.

Case Study	Application	Aim	Dose	Improvements	References
Sewers, FSE, UK	Sewers; dosing to manhole downstream of FSE	FOG accumulation control	3 mg/L	<ul style="list-style-type: none"> Limited FOG build up, 5-15% of pipe diameter No flooding events (6 months trial) No blockages reported (6 months trial) 	Internal report, 2018
Sewers, Retail Park and FSEs area	Sewers; dosing to sewer coming directly from the kitchen	FOG accumulation control. Reduction in external flooding events.	2-10 mg/L	<ul style="list-style-type: none"> Limited FOG build up, 5-15% of pipe diameter 50% reduction in maintenance requirements Enhanced, easier cleaning facilitated by softer nature of deposits No flooding events (6 months trial) 	Internal report, 2018
Sewers, Retail Park and FSEs area	Sewers; dosing to sewer coming directly from the kitchen	FOG accumulation control. Reduction in external flooding events.	2-10 mg/L	<ul style="list-style-type: none"> 65% reduction in maintenance requirements No reactive cleanses (6 months trial) No flooding events (6 months trial) 	Internal report, 2018

Case Study	Application	Aim	Dose	Improvements	References
Sewers, Busy car park	Sewers, shaft tank	FOG accumulation control. Prevention of sewage overflows into nearby watercourse during storm.	2-10 mg/L	<ul style="list-style-type: none"> • 50% reduction in maintenance requirements • Rehabilitation of pre-deposited material (1 month from the start of the trial) • No reactive cleanses (6 months trial) • No spilling events (6 months trial) 	Internal report, 2018
Pumping Station, UK	Sewer pumping station	FOG accumulation control.	2-10 mg/L	<ul style="list-style-type: none"> • Reduction in energy demand • 50% reduction in maintenance requirements. • Enhanced, easier cleaning facilitated by softer nature of deposits • No emergency calls for maintenance 	Internal report, 2018
Municipal WWTP, JAX Heights, Florida, US	Activated sludge	Sludge reduction.	3 mg/L	<ul style="list-style-type: none"> • Enhanced and consistent performance of bioadded train (control and bioadded train) • 45% air supply reduction • 25% sludge disposal costs reduction • Total savings/product cost 2:1 	Advanced BioCatalytics, 2023
Municipal WWTP, City of Arvin, US	Anaerobic Digestion	Sludge reduction	4 L/ day	<ul style="list-style-type: none"> • 25% sludge production reduction 	Advanced BioCatalytics, 2023

Case Study	Application	Aim	Dose	Improvements	References
Municipal WWTP, Clark County, Nevada Sanitation District, US	Wastewater Lagoons	Grease and scum elimination. Odour elimination	3 mg/L (no aeration)	<ul style="list-style-type: none"> No observable presence of grease or scum withing 4 of commencing the treatment No odour 	Advanced BioCatalytics, 2023
Industrial WWTP, Walworth, Wisconsin- Kikkoman Foods, Inc. Soya Sauce producer	Plant excided the permitted discharge limits	Treatment efficiency improvement,	18 mg/L	<ul style="list-style-type: none"> 60% BOD reduction 45% TSS reduction 65% surcharges reduction 	Advanced BioCatalytics, 2023
Industrial WWTP, Minneapolis, Minnessota- Rahr Malting Large producer of malt for the brewing industry	Activated sludge	Sludge reduction.	15 mg/L	<ul style="list-style-type: none"> 30% sludge production reduction 25-40% energy demand reduction Lower aeration demand; less air and shorter cycles BOD, TSS, ammonia, and phosphorus reduction 	Advanced BioCatalytics, 2023

Case Study	Application	Aim	Dose	Improvements	References
Industrial WWTP, Oxnard, California- Smuckers's Strawberry Processing Plant	Aerobic digester	Odour reduction.	10 mg/L	<ul style="list-style-type: none"> No odour <p>Year one:</p> <ul style="list-style-type: none"> 50% sludge production reduction 32% energy demand reduction <p>Year two:</p> <ul style="list-style-type: none"> 60% sludge production reduction 50% aeration electric expenses reduction 	Advanced BioCatalytics, 2023
Industrial WWTP, Meat Processing Plant	Activated sludge	Improvement in treatment of wastewater heavily laden with grease and blood proteins. Odour reduction.	20 mg/L	<ul style="list-style-type: none"> 50% BOD reduction 66% TSS reduction Significant odour reduction Improved wastewater clarity No sludge builds up in the surge tanks Maintenance costs reduction 	Advanced BioCatalytics, 2023

Case Study	Application	Aim	Dose	Improvements	References
Industrial WWTP, Fibreglass Manufacturing Plant	Clarifiers	Elimination of pervasive organic odours. Elimination of grease balls and scum.	Not provided	<ul style="list-style-type: none"> • Cleared clarifier water • Disappearance of grease balls 	Advanced BioCatalytics, 2023
Industrial WWTP, Fruit processing plant	Activated sludge	Sludge reduction. Aeration costs reduction.	Not provided	<ul style="list-style-type: none"> • 48.3 % reduction in aeration costs • 60% reduction of sludge disposal costs • 51.4% overall savings 	Advanced BioCatalytics, 2023

5.3 Economic evaluation

The benefits observed by dosing the product are a reduction in the rate of FOG deposition which reduces the frequency of manual cleaning, inspection, and electricity consumption of the pumps (inhibition) and/or reduces the difficulty of cleaning and hence reduces the cost associated with manual cleaning (rehabilitation). Additional benefit is associated to the reduction in blockage associated with dosing in terms of the levels of “underperformance payments” associated with FOG related ODIs such as the external sewer flooding.

The dosing of the product involves the inclusion of dosing equipment and the cost associated with the product itself. Typical dosing units include a kiosk that contains a chemical storage tank, typically 30-50 L, and a dosing valve which is manually adjusted to deliver the required dose. Reported dosing concentrations range between 2-10 mg/L for the recent FOG trails and up to 20 mg/L for the other applications (Table 5-2). The kiosk is assumed to be housed near the input to the sewer, typically near the local FSE.

An economic assessment of the cost and benefits of dosing the product has been undertaken for a generic example system comprising a pumping station, association NRV and a length of sewer. The system is associated with an area containing several FSEs and other buildings such that it receives a combined sewage with a high FOG content with a maximum flowrate of 12 L/s. Based on the finding of the current work, a dose of 3 ppm is used throughout as no additional benefit was observed when dosing higher levels (Chapter 4). The assumed benefits of dosing the product are a roughly halving of the frequency of most cleaning activities congruent with reported benefits (Table 5-2). The economic data used is adjusted from an internal water company report that conducted trials on the product (Table 5-3). The numbers have been adjusted to avoid commercial sensitivity and have inflated to the current date through a cumulative inflation factor of 1.2 (MacroTrends, 2023).

Table 5-3 List of assumptions used for the economic analysis (Internal report, 2018).

Parameter	Value (£)	Pre dosing frequency	Post dosing frequency
Site visit for quick visual inspection	100	weekly	monthly
CCTV scan	350	2 per sewer clean	2 per sewer clean
Sewer line cleanse	1,500	3 months	6 months
Cleanses of the pumping station wet wall	1,400	2 months	4 months
Cleanse of Non-Return Valve (NRV)	3,000	6 months	12 months
Product cost per litre (L) of solution	10-100		
Annual cost of the dosing unit including installation, depreciation, running costs and maintenance per site	650		

The total maintenance costs, total dosing costs, and annual savings were then calculated as follows, where n is annual frequency, an C is the cost per event:

$$\begin{aligned}
 \text{Total maintenance costs} = & n_{\text{site visits}} \times C_{\text{site visits}} + n_{\text{CCTV}} \times C_{\text{CCTV}} \\
 & + n_{\text{sewer cleanse}} \times C_{\text{sewer cleanse}} + n_{\text{wet well cleanse}} \times C_{\text{wet well cleanse}} \\
 & + n_{\text{NRV cleanse}} \times C_{\text{NRV cleans}}
 \end{aligned} \tag{5-1}$$

$$\text{Total dosing cost} = (\text{Cost}_{\text{per Litre}} \times \text{Annual volume of dose used}) + \text{Annual cost of dosing unit} \tag{5-2}$$

$$\begin{aligned}
 \text{Annual savings (£)} = & (\text{Total maintenance costs}_{\text{pre dosed}} - \text{Total maintenance costs}_{\text{post dosed}}) \\
 & - \text{Total dosing costs}
 \end{aligned} \tag{5-3}$$

$$\text{Annual savings (\%)} = \frac{\text{Annual savings (£)}}{\text{Total maintenance costs}_{\text{post dosed}} + \text{total dosing costs}} \tag{5-4}$$

5.4 Economic assessment

For the generic case highlighted above the total maintenance costs before dosing were £28,400 per year and this reduced to £12,800 per year due to the impact of dosing the product. Accordingly, the annual cost reduction in maintenance due to the dosing of the product was £15,600. The product unit cost was set as a variable to establish the breakeven price which was £13/L (Figure 5-2). The equivalent annual saving was 60%, 45%, 24% and 9% when the product unit cost was £5/L, £7/L, £10/L and £12/L respectively.

The impact of dosing still has significant uncertainty associated with it and longer-term trials are required to reduce this uncertainty and establish long term benefits. Analysis of the impact of changing frequency of cleaning due to the dosing revealed that the breakeven price changed to £19/L if the impact of dosing was to extend by 50% and £22/L if it extended the frequency to 200%. The expected cost of the product is between £15-20/L such that the economic assessment indicates that dosing the product will likely be economically viable based on changing the maintenance frequency.

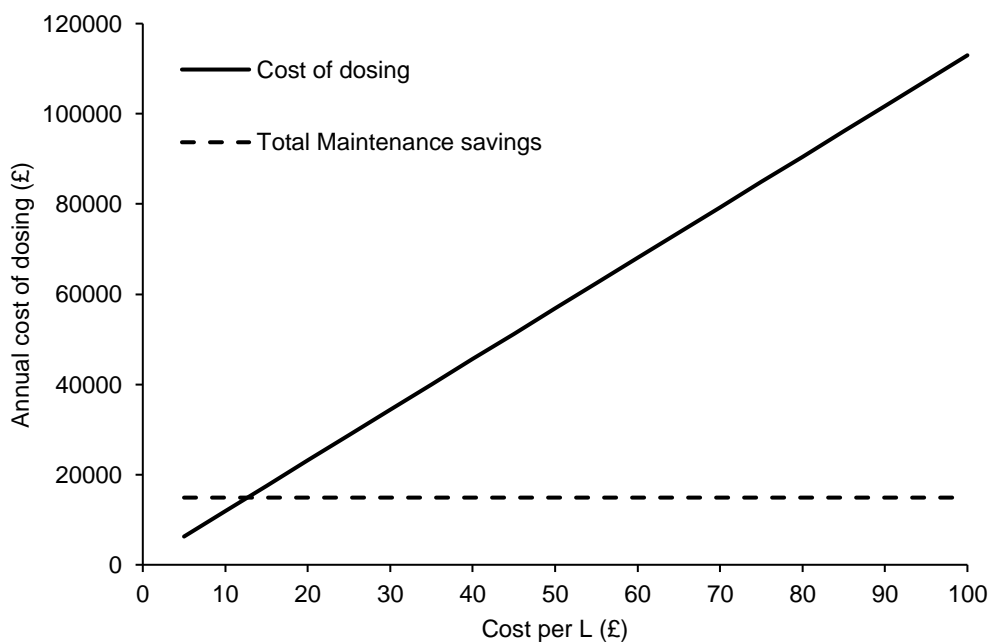


Figure 5-2 Cost assessment for product dosing.

5.5 Added value

The economic analysis excludes two other items highlighted to provide savings which can be viewed as added value. Firstly, dosing the product has been reported to loosen the deposit and prevent it from hardening (Table 5-2). This reduces the effort needed to clean the wet well or sewer and should reduce the total maintenance cost even further. To illustrate, the cleaning of the wet well, sewer and NRV represent 78% of the total cost based on the frequency assumed post dosing (Table 5-3). Accordingly, a reduction in the cleaning cost due to the activity being easier has potential to generate an increase in the breakeven cost. To illustrate, if the cleaning components reduce in cost by 25% (from £10,200 to 7,650) the breakeven price would increase to £16/L. In addition, it has been reported that the electrical demand for pumping has been reduced by 20 kWh/month, equating to annual energy saving of 240 kWh which equates to a saving of £52/year per pump based on the current industrial sector electricity cost of £0.21/kWh (Department for Energy & Net Zero, UK, 2023).

The final area for financial benefit is related to the ODI and the potential reduction in the costs associated with “underperformance payments”. Assuming that continual dosing of the product reduces the total number of blockage and flooding events then substantial saving could be achieved that outweigh those generated by reduction in the maintenance. The specific value of the ODIs are company specific and so generic analysis is not possible. Illustration of the potential is seen by comparing four different companies value for the ODIs associated with external and internal sewer flooding, blockages and pollution incidents (Table 5-4).

Table 5-4 FOG related Outcome delivery incentive rates for incidents surpassing the companies' AMP targets.

Water Company	External sewer flooding (£)	Blockages (£)	Internal sewer flooding (£)	Cat 3 Pollutions (£)	References
Severn Trent	19,400	2,079	42,820	53,900	Ofwat, Severn Trent (2018)
Thames Water	26,165		90,000	130,000	Ofwat, Thames Water (2015, 2018)
Southern Water			75,260	20,571	Ofwat, Southern Water (2018)
South West Water	1,000- 4,000		89,000	10,300– 19,300	Ofwat, South West Water (2018)

The available data and set ODIs vary significantly across the WaSC companies in the UK, but all show that each occurrence imposes a considerable financial burden on the companies involved. In the majority of cases this value is likely to exceed the estimated costs of biosoding. Furthermore, the cost of underperformance across an entire AMP 5-year period associated with FOG-related incidents such as internal sewer flooding and pollution incidents, holds the potential to become significant. Conversely, prevention of such incidents through enhanced maintenance and fewer blockage occurrences offers considerable financial incentives. Moreover, it's important to recognize that the impact of ODIs extends beyond immediate financial implications through its influence on the future price review (Ofwat, 2023).

5.6 Conclusions

An effectively managed wastewater collection system relies on a well- organized and structured approach to its operation, maintenance, and rehabilitation. Central to this approach are inspections and cleaning, which play a pivotal role in upholding the efficient functionality of the sewer network, minimizing disruptions, and extending its overall lifespan. The often cause of suboptimal performance and deterioration of sanitary sewer system is the buildup of FOG deposits. In this

context, considering biological sewer FOG maintenance as an alternative or complement to mechanical cleaning methods holds the potential to simplify sewer maintenance efforts and lower the associated costs.

At the suggested dosage of 3ppm, utilizing the product at its current price remains economically viable when factoring in a 50% reduction in maintenance frequency and a 25% decrease in cleaning time requirements compared to standard practices. Furthermore, the product generates financial advantages through decreased electricity consumption, resulting in a reduction of 20 kWh per pump per month in usage demands. Based on cost comparison, the benefit of dosing compared to business as usual, has the potential to reduce the annual cost of sewage cleanse by 60%, 45%, 24% and 9% when the assumed product unit cost is £5/L, £7/L, £10/L and £12/L respectively.

The application of the uncoupler for dosing holds the potential to alleviate financial strains linked to FOG deposition in sewers particularly pertinent in scenarios involving: 1) increased operational expenses due to frequent cleaning interventions in regions where source control poses a challenge such as high density FSEs area or domestic FOG, 2) reactive maintenance in response to blockages and pollution incidents, 3) increased capital expenditures for asset replacement due to pipe bursting and pumps clogging 4) penalties and regulatory fines due to incidents of internal and external flooding, along with pollution events.

Furthermore, the consistent application of uncouplers within sewers and pumping stations could extend its positive impact downstream. This encompasses potential enhancements in effluent quality, improvements in biological processes at wastewater treatment plants, and a potential decrease in overall WWTP maintenance costs, as indicated by commercial trials at WWTPs. However, these assertions would require validation through comprehensive research and economic evaluation.

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6 Chapter 6 Implications of the work – Practical guide to effective sewer Fats, Oils, and Grease (FOG) management with biologically based energy uncoupler solutions.

The research presented in the thesis led to a number of key observations related to the mechanisms of FOG deposition and the potential efficacy of dosing a mixture of heat-shock yeast proteins (energy uncoupler) and surfactants to reduce or eliminate FOG problems in sewers:

- (1) The predominant mechanism of initial deposit formation is the complexation of complex carbohydrates such as starch with FFA acids.
- (2) Deposit growth is the result of the interactions between proteins, carbohydrates and FFAs.
- (3) Accumulation of oil onto deposits is driven post formation and is not part of the deposition process.
- (4) Large excesses of calcium that are commonly observed in FOG deposits reflect calcium bridging with starch or FFAs, such that calcium competes with FFAs for amylose binding sites rather than saponification.
- (5) Addition of a mixture of heat-shock yeast proteins (energy uncoupler) and surfactants demonstrated a clear and observable reduction in the rate of deposit formation ($45\pm 3\%$) and reduction in pre deposited mass ($61\pm 10\%$) compared to control.
- (6) The impact of the uncoupler product was insensitive to dose such that the maximum benefit was observed with lowest dose of 3 mg/L.

Consideration of these observations with the reference to current understanding associated with FOG deposition and its control led to two key implications:

- (A) Modification to the existing model of FOG deposition

The current model of FOG deposition requires amendment to better reflect the processes that are critical in deposit formation. The new proposed model of deposition consists of a 2-stage process: (1) initial precipitation that is driven by complexation of wastewater compounds such as starch and FFAs and (2) subsequent growth caused by fats, lipids, protein, and calcium aggregation on the preformed core (Figure 6-1).

The current model highlights shortcomings in existing FOG mitigation methods, which primarily concentrate on eliminating fats, while overlooking the crucial impact of other significant wastewater components such as starch or the breakdown of fats into FFA acids. Further, the new model indicates that the treatment could be aligned with the stage of deposit formation, potentially leading to improved efficacy of FOG treatment options. To illustrate, the management strategy could be focused on addressing specific compounds that contribute to initial precipitation (1st stage) and/or subsequent growth (2nd stage). This approach might involve the introduction of specialized enzymes (lipase, carbohydrase and/or protease), bioadditives or bioaugmentation products. This intervention would aim to either decrease the initial concentration of compounds contributing to initial precipitation, such as starch and FFAs in oil-rich wastewater, i.e., inhibition, or to facilitate the breakdown of unreacted lipids, proteins and saponified fats to prevent their aggregation on preformed core, and to enhance the degradation of pre-deposited FOG, i.e., rehabilitation.

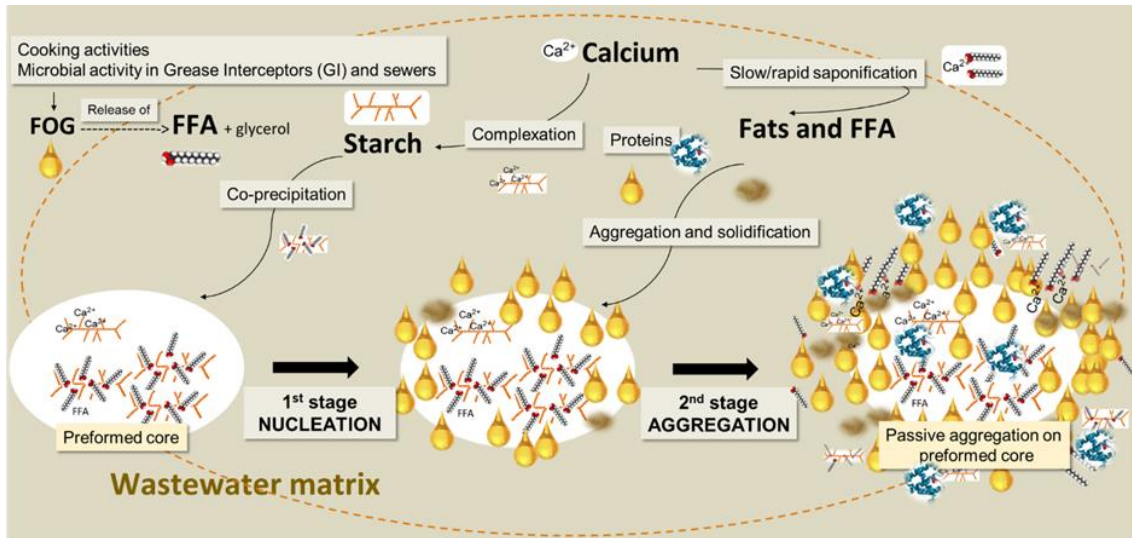


Figure 6-1 Novel model of FOG deposits formation in sewers (Chapter 2).

Presently, the industry lacks a standardized and universally accepted testing methodology for evaluating biological approaches to manage FOG in sewer systems. A general lack of knowledge and consensus exists regarding the assessment and comparison of existing products. This is mainly due to the diverse operating principles of various biological products, such as enzymes, bioaugmentation, or biostimulation. While there has been advancement in research devising testing protocols for kitchen wastewater that consider its unique composition, a comparable procedure for treating oil-rich wastewater and/or FOG deposits in sewers has yet to be established. Whereas the successful degradation of FOG-rich wastewater has been achievable under laboratory conditions, the applicability of these results to a real sewer environment remains elusive. Unsuccessful implementation is often driven by lack of comprehension regarding the composition of treated wastewater.

The newly introduced FOG deposit model highlights the numerous potential binding pathways between wastewater components that contribute to deposit formation. This underscores the significance of comprehensively tracking all components within the system, particularly during laboratory trials, to enable effective translation of results to real-world scenarios. This research provides the evidence that recreating FOG deposition in controlled laboratory condition, closely mimicking the real deposits, is achievable. As a result, this establishes

evidence that testing biological products can effectively occur by utilizing oil-rich synthetic sewage. By performing mass balances within this context, the ability to replicate variations in treated wastewater and deposits becomes more accurate, enabling a consistent and precise assessment of both current and future biological products. Hence, this approach could serve as a refinement to currently underdeveloped laboratory-based testing procedures.

(A) The potential of dosing uncouplers for management of FOG deposition in sewers.

The concept of inducing metabolic path shift of indigenous wastewater microorganisms leading to decrease in TSS and improved nutrient uptake (in accordance with findings from Chapter 1), without associated biomass generation increase provides a promising means of deposits inhibition and rehabilitation. Beneficial impacts are achievable with addition of a series of low concentration doses of the uncoupler product, without significant system modifications and at a comparatively low cost. The presented work represent the first systematic trial of the product for FOG management applications.

The reduction in lipid, protein and carbohydrate content within the FOG deposits, particularly prominent in the context of proteins (inhibition) and lipids and proteins (rehabilitation) attributable to uncoupler's impact have provided support to the view that FOG deposition does not primarily stem from saponification. Consequently, it becomes essential to redirect management practices to consider all wastewater components such as proteins, carbohydrates, and lipids- to effectively manage FOG deposit formation in sewers. Moreover, it has been observed that the ability of microorganisms to degrade FOG is intertwined with the presence of other nutrients in the system that are frequently omitted or overlooked during bioremediation practices. For instance, deposit degradation was hindered by high C:N ratio and high compositional homogeneity of the deposits. Overall, the use of the uncoupler product can provide a viable substitute or complementary measure for conventional sewer cleaning methods. The reduction in deposit formation provides compelling evidence that maintenance practices could be less frequent, leading to decreased inspection expenses (due

to reduced frequency) and a diminished need for cleaning activities (slower built up). Furthermore, the modification in the deposit's composition led to a decrease in its adhesion properties. This alteration can potentially simplify the process of removing the deposit, resulting in a reduction in the time and costs associated with manual cleaning when required.

7 Chapter 7 Conclusions and Future Work

7.1 Conclusions

The overall findings of this research propose a new model of FOG deposition consisting of two stages: (1) initial precipitation driven by complexation of wastewater compounds such as starch and FFAs and (2) subsequent growth caused by fats, lipids, protein, and calcium aggregation on the preformed core and is accompanied by compositional changes as the deposits age. The process of FOG deposition can be inhibited and/or rehabilitated through the dosing of a uncoupler product that contains a mixture of heat-shock yeast proteins (energy uncoupler) and surfactants.

The specific conclusions in relation to the original objectives are as follows:

Objective 1: To understand how FOG related deposits form in sewer networks

In contrast to the generally held view, the results of this research query the validity of the existing notion that saponification is the leading attributer in FOG deposit formation. Instead, the current work suggests an alternative novel model that follows a two-step process: 1) initial precipitation driven by complexation of wastewater compounds such as starch, FFAs and (2) subsequent growth caused by fats, protein, and calcium aggregation. Importantly, the rate of deposition is controlled by the initial starch concentration followed by factors drawing excess calcium and protein towards the deposit core.

FOG formation can be successfully replicated under controlled laboratory conditions with synthetic formula providing a robust matrix for currently undeveloped laboratory-based testing procedures and development of novel solutions.

Objective 2: To produce a state of the art critical review to investigate the mechanism of chemical uncoupling and its application in reducing biomass yield in biologically driven processes, with a potential focus on bioremediation of FOG deposits in sewers.

The use of uncouplers reduces the generation of biomass associated with substrate utilisation. Translating the current understanding of uncoupling to the case of FOG deposition and rehabilitation suggests the predominate mechanism is likely to be true metabolic uncoupling. This mechanism intensifies nutrients and TSS removal from wastewater, by increasing the percentage of active bacteria over the total microbial population. Further, uncoupler dosing should be effective for both inhibition and rehabilitation of existing deposits.

Objective 3: Establish the potential to manage FOG deposits through the dosing of an uncoupler.

Dosing of the uncoupler product is capable of both inhibiting and rehabilitating FOG deposits. Trials with an uncoupler based on heat treated yeast protein extract with added surfactants was able to function effectively as an inhibitor of deposit formation ($45\pm 3\%$) or rehabilitation ($61\pm 10\%$) of preformed deposit. The action of the uncoupler was seen in terms of mass reduction as well as changes in the compositional make-up of the deposit with a specific shift towards a significant reduction in proteins (inhibition) and proteins and lipids (rehabilitation). A critical factor in the effectiveness of the uncoupler has been shown between the initial C:N ratio, composition of the deposit and its degradability. This indicate that the effectiveness of dosing uncouplers will have some site specific features that require testing to determine the required dose and potential impact.

The uncoupler was effective at low doses of 3 ppm, beyond which minimal additional benefit was observed. The dosing of the uncoupler can be conducted

in a financially beneficial manner where the costs of dosing are covered by the reduction in maintenance costs that are achieved through a reduction in frequency of cleaning required.

7.2 Future Work

In the course of this project further areas for research have been identified:

The experimental work primarily involved synthetic sewage and artificial FOG deposits to ensure replicability and comparability of the outcomes. Whilst artificial FOG deposits do not fully represent the varied composition of wastewater and FOG deposits across the sewer system, they represent a viable means for studying deposit formation and synthetic FOG that closely mimics real deposits. This enables controlled and reproducible approaches to be used for optimizing bio-additive dosing and understanding its efficacy. This crucial step is pivotal in progressing the current approach into a standardized testing methodology. The procedure should encompass a thorough quantification and characterization of the lipid, protein, and carbohydrate fractions present within both the wastewater and FOG deposits. It is essential to identify representative sites within the sewer system, ensuring they encompass a diverse range of conditions and FOG concentrations. To further investigate the evolving nature of the deposits over time and to track changes in deposits makeup due to biodosing, it is imperative to consistently gather samples from the same location at predefined intervals. To ensure the uniformity of solid samples, those should be subjected to pre-treatment before analysis, while the adherence to the standardized sampling and data interpretation will allow to maintain accuracy throughout the investigation.

Dynamic experimentation should be explored to simulate real-time deposit formation under varying flow conditions and composition of wastewater, enabling a more comprehensive understanding of accumulation mechanisms. Fourier Transform Infrared Spectroscopy (FTIR) offers a powerful tool for analysing the chemical composition of FOG deposits, with the ability to differentiate between carbohydrates, proteins, and fats. Within the fat fraction, FTIR can further distinguish free fatty acids (FFAs), saponified fats, and unsaponified triglycerides based on their unique spectral fingerprints. By integrating FTIR analysis, the percentage contribution of these components to deposit formation over time could be quantified, providing insights into the evolution of deposit layers under different conditions. This approach, employing both synthetic sewage and real

wastewater, would enhance the understanding of deposit dynamics and inform strategies for optimised bio-additive dosing and deposit management in sewer systems.

There is undoubtful correlation between indigenous wastewater bacteria and FOG deposit formation. Deposition and accumulation of FOG is likely facilitated by the deposition, growth, and metabolism of the bacteria within biofilms attached to sewer walls and suspended flocs. Understanding the relationship between indigenous wastewater bacteria and FOG is crucial for further developments in effective mitigation measures. To gain a comprehensive understanding of this relationship it is imperative to integrate microbiological data with physicochemical parameters and operational conditions. Expanding the knowledge requires a focus on analysing samples collected from diverse points within sewer system, encompassing locations with varying degrees of FOG buildup. The analysis of samples should focus on parameters such as microbial community composition, nutrients content and FOG concentration. To gain valuable insights into the identification and quantification of bacterial species residing within samples extracted from both FOG-enriched and FOG-deficient environments, along with a deeper understanding of their actual gene expression patterns, techniques such as DNA sequencing and metagenomics should be employed.

Furthermore, lab-controlled experiments offer another possible avenue for exploration. The controlled bioreactors comprising synthetic sewage as media and with various nutrients ratios and FOG concentrations could be inoculated with (1) indigenous wastewater bacteria, (2) isolated and enriched microbiota consortia from FOG-rich environment, and (3) FOG bioaugmentation consortia. Monitoring the resulting changes in microbial communities, nutrient removal, and FOG formation over time could culminate in development of predictive models forecasting FOG formation based on microbial composition and activity incorporating factors like nutrients availability, FOG concentration and hydraulics.

While rehabilitation has emerged as an appealing approach for FOG management, potentially surpassing the effectiveness of the inhibition, our trials with aged synthetic and real FOG deposits highlighted the intricate correlation

between initial C:N ratio, composition of the deposit and its degradability. To gain deeper insights, further work should conduct additional tests encompassing diverse samples from different points within the sewer network. This expanded investigation will shed light on how deposit composition impacts its degradability and will consequently offer more comprehensive guidance to water companies. Specifically, trials should be conducted from samples with different initial C:N ratios, age and compositional makeup i.e., lipids, proteins and carbohydrates, and be collected from various locations on sewer network such as drains, sewers, pumping stations, shaft tanks and WWTP. This approach will ensure the incorporation of the full spectrum of FOG deposit variability, which is inherently linked to its specific location within the sewer infrastructure.

The results indicate a promising avenue for adapting FOG management approaches by aligning them with the distinct phases of deposit formation. It is acknowledged that the examined uncoupler is not substrate specific, i.e., lipids, proteins, or carbohydrates, but instead causes metabolic shift across the wastewater microorganisms, reflected in changed deposit make-up, with the most profound effect on protein and lipid degradation. Further investigation is recommended to explore the potential benefits of targeting specific compounds that play a pivotal role in the initial precipitation and subsequent growth. This should involve introducing specific enzymes, bioadditives, or bioaugmentation products to reduce the initial concentration of targeted compounds like starch and FFA in oil-rich wastewater (inhibition), or promoting the breakdown of unreacted lipids, proteins and saponified fats both in fresh and aged deposits (rehabilitation).

Furthermore, the key insights have emerged from Chapter 2, shedding light on the multifaceted mechanisms through which uncouplers can operate, inducing alterations that lead to reduced biomass production. To further advance uncoupler research for FOG mitigation, it is worth considering the exploration of intermittent dosing of uncouplers to explore the potential of the diverse mechanisms of action that can occur. Additionally, the investigation of synergies between different uncouplers could prove valuable. For instance, an innovative approach could involve employing two distinct uncouplers to achieve inhibition.

The first uncoupler could induce true metabolic uncoupling, to efficiently remove compounds from wastewater. Simultaneously, the second uncoupler could disrupt the attachment of biofilms to the pipe wall, thereby impeding the formation of FOG deposits. In the context of rehabilitation, one of dosed uncouplers could target metabolic maintenance, leading to changes in the composition of EPS that are responsible for binding substrate to deposits, whereas the second uncoupler could trigger biofilm fragmentation and detachment from the wall.

Finally, in the light of the inconsistent outcomes deriving from field trials involving bioadditives, it is recommended to address the current limited understanding of their functioning within the sewer environment. This limitation is primarily attributed to the execution of the field trials within operational sewer systems, where there is no benchmark control. To overcome this, it is advised to undertake a pilot-scale sewer loop study, utilizing authentic oil-rich wastewater, and incorporating both biodosing and control lines. This approach would enable testing of the product's performance across diverse seasons and wastewater temperatures, timeframes, and varying levels of FOG, carbohydrates, and proteins. This comprehensive assessment would bridge the gap between laboratory findings and full-scale application, ultimately providing a more informed basis for conducting an accurate economic evaluation.