CRANFIELD UNIVERSITY

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Biological FOG degradation: development of a standardised bioadditive protocol

School of Water, Energy and Environment
STREAM IDC

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This thesis is submitted in partial fulfilment of the requirements for the degree of EngD

ABSTRACT

Fat, oil and grease (FOG) rich effluents from commercial food service establishments (FSEs) have been identified as major contributors to sewer blockages. Under UK Legislation, all FSE kitchens should be fitted with an '*effective mean of grease removal*'. Currently, microbial bioadditives, one of the preferred FOG management options, do not have an industry-agreed testing methodology to prove their '*effectiveness*'. The primary goal of this research was to generate the scientific knowledge underpinning a testing protocol for FOG-degrading bioadditives.

Environmental conditions prevalent in FSE wastewater streams were identified in a characterisation of effluents from three catering outlets on Cranfield University campus and used as a basis for design of synthetic culture media for testing bacterial degradation.

To address interferences caused by high levels of surfactants in FSE wastewater, a novel FOG quantification method, based on the Gerber method used in the milk industry, was developed for the protocol. This method is efficient at recovering emulsified FOG, and also allows emulsified and free fractions to be quantified separately.

Microbial utilisation of different wastewater components was evaluated in batch degradation trials using a model bacterium, *Bacillus licheniformis* NCIMB 9375, and different synthetic wastewater compositions. The source of readily available carbon showed great influence on FOG-degradation response. FOG uptake was also influenced strongly by the carbon to nitrogen ratio in the media composition, with removal rate constants decreasing by over an order of magnitude from 0.0285 to 0.0026 h^{-1} when initial COD:N increased from 33:1 to 147:1.

A cost-benefit analysis of the currently available FOG management solutions suggest that bioadditives, as a standalone solution, are the best option in terms of total cost and space requirements, followed by bioadditives used with grease separators. Combining physical and biological treatments can enhance the

individual performance of both technologies and allow energy recovery from physically separated FOG.

Keywords: FSE, wastewater, Gerber, COD:N, bioaddition, bioremediation, *Bacillus licheniformis* NCIMB 9375

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ASM	Activated sludge models
BOD	Biological oxygen demand
BS	British Standard
CAPEX	Capital expenditure
CDW	Cell dry weight
CMC	Critical micelle concentration
Co	Cobalt
COD	Chemical oxygen demand
DCC	Dublin County Council
DI	Distilled (water)
DO	Dissolved oxygen
FA	Fatty acid
FFA	Free fatty acid
FOG	Fat, oil and grease
FSE	Food Service Establishment
GI	Grease interceptor
GRU	Grease Removal Unit
HEM	Hexane Extractable Material
ICP-MS	Inductively coupled plasma mass spectrometry

K	Potassium
LCFA	Long Chain Fatty Acids
LLE	Liquid-Liquid Extraction
LOD	Limit of detection
MDL	Method detection limit
ME	Mixed effluent
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
Na	Sodium
NCIMB	National Collection of Industrial Food and Marine Bacteria
NS	Nominal Size
OD	Optical density
OM	Organic matter
P	Phosphorous
pNP	p-nitrophenol
PQL	Practical quantification limit
$_{s}$COD	Soluble chemical oxygen demand
SDS	Sodium dodecyl sulphate
SPE	Solid Phase Extraction
SPH	Soy protein hydrolysate
SS	Suspended solids

TBA	Tributyrin agar
$_{t}$COD	Total chemical oxygen demand
ThCOD	Theoretical chemical oxygen demand
TN	Total nitrogen
WW	Wastewater
Zn	Zinc

1 Introduction

1.1 FOG deposition in sewers

Sewer blockages, attributed to hardened fat, oil and grease (FOG) deposits or 'fatbergs' have received much public and media attention in recent years. It has been estimated that, of around 370,000 sewer blockages annually in the UK, 80% are caused by FOG deposit accumulation (Water UK, 2018). Resultant sewer overflows pose public health, and environmental hazards through exposure to pathogens and hazardous chemicals, together with disruption to local businesses and services in association with sewer clearance works, which cost Water Utilities (and their customers) over £100 million annually (Water UK, 2018). Until relatively recently it was believed that FOG deposits were simply caused by cooling and solidifying of saturated fats and oils. Subsequent research (He et al., 2013, 2011; Keener et al., 2008; Williams et al., 2012) has identified the mechanism as a saponification reaction between metal ions, dominantly calcium, and free fatty acids liberated from triglyceride molecules through thermal hydrolysis during cooking (Choe and Min, 2007), or by lipase-catalysed microbial degradation as illustrated in Figure 1-1.

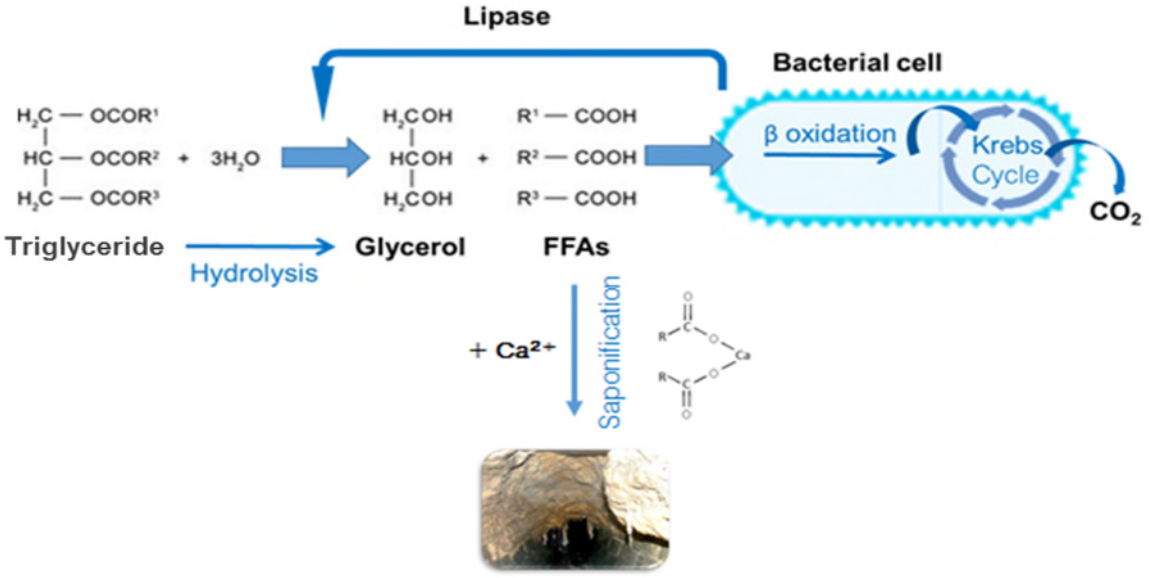

Figure 1-1 Pathways of microbial triglyceride degradation and FOG deposit formation

Although FOG entering the sewer system can originate from domestic, industrial or commercial premises, higher numbers of sewer blockages and flooding are usually recorded in urbanised areas with '*hot-spots*', generally near food service establishment (FSE) hubs (Keener et al., 2008). Accordingly, FOG management practices in commercial kitchens have become a focus for minimising sewer blockages, with FSEs encouraged by local authorities to separate FOG from wastewater at source, before entering the sewer system.

1.2 FOG management legislation

In the UK, FSE discharges fall under the Water Industry Act (1991); the Environmental Protection Act (1990); the Building Act (1984); and the Food Safety Act (1990). In particular, the Building Regulations 2010 (HM Government, 2010) section H1 2.21 states: "*Drainage serving kitchens in commercial hot food premises should be fitted with a grease separator complying with BS EN 1825-1:2004 and designed in accordance with BS EN 1825-2:2002 or other effective means of grease removal*." Since many small FSEs have neither the financial means nor physical space to install a suitable grease trap, the question arises as to whether FOG-degrading bioadditives can satisfy the requirements for '*other effective means of grease removal*' by providing a residual effect in sewers.

In 2008, as part of their FOG control programme, Dublin County Council (DCC) introduced a trade wastewater effluent licensing scheme whereby annual charges are set in response to FOG management practices within the establishment. The main requirements of which were the installation of an appropriately-sized, well-maintained grease trap, and discharge compliance of 100 mg/l. This was regulated under the discharge trade effluent, Section 16 of the Local Government (Water Pollution) Act 1977 (Wallace et al., 2017). Bacterial bioadditives were temporarily banned by DCC because microbial additions do not currently have a BS EN standard or an industry-agreed testing methodology, causing negative financial consequences for many bioadditives' producers. Products were subsequently permitted in conjunction with grease separators, but only after a DCC agreed field-demonstration of positive impact on FOG degradation over time (O'Dwyer, 2010).

Hence commercial suppliers of bioadditive products have a major and urgent financial interest in the independent development of a standardised test for the determination of their efficacy.

1.3 FOG management strategies in food service establishments

Current FOG management practices include: (1) kitchen management and staff training, (2) 'at source' treatments in the form of *physical separation* through grease interceptor/separator/trap devices or the use of *biological additions* (or bioadditives).

Fundamental to any FOG management strategy is staff training and employment of kitchen policies to minimise the amount of FOG and food waste introduced into the drainage system. Advice includes scraping and dry wiping of cooking utensils and service ware with kitchen towel prior to washing and using strainers in the sink to isolate food waste for disposal. In addition, there are also a range of technologies which can help remove FOG from the wastewater prior to discharge into the sewers based on physical or biological processes.

A typical grease interceptor/separator collects FOG by flotation following the principle of Stoke's law, whereby the difference in density between oil and water allows FOG droplets to rise to the surface. Grease separators vary in size according to their design working capacity based on wastewater flow rates. Smaller units, designed for low-flow rates, can be located under sinks or associated with individual kitchen appliances; whilst larger units can accommodate flows from the entire kitchen and may be located underground or outside. In both systems, FOG is retained inside the unit with the wastewater, and must be pumped out periodically to maintain its efficiency. Alternative to both systems, but still based on the physical principle, are grease removal units (GRU), where the oil fraction is actively removed and stored separately for recovery. While separator systems can be efficient in oil removal, they are typically only designed to remove oil droplets greater than 150 µm (API, 1990) and thus may fail to efficently retain dissolved and emulsified fats. Enhanced techniques, such as plate or tube settlers; air flotation; and chemical or electro-coagulation are available to achieve separation of smaller emulsified FOG droplets as summarised in Table 1-1. However high capital and operating costs typically limit employment of these advanced

technologies to large industrial operations and would not be viable for small to medium FSEs.

Biological additions, or bioadditives, comprise of consortia of microorganisms which produce lipolytic enzymes to hydrolyse and degrade FOG into benign end–products - carbon dioxide and water (Figure 1-1). In the sewage system, a substantial proportion of FOG is effectively degraded by indigeneous microorganisms playing a dominant role in sewers' biological processes (Brooksbank et al., 2007; El-Bestawy et al., 2005; El-Masry et al., 2004; Markossian et al., 2000; Tano-Debrah et al., 1999; Wakelin and Forster, 1997). As a result, there has been increasing interest in the addition of lipid-degrading microorganisms (also known as bioaugmentation) to naturally occuring microbial communities in sewers to enhance FOG removal (Brooksbank et al., 2007). The use of bioadditives to treat kitchen effluent has increased over the last decade, due to their relative ease of use and low cost compared to grease separators, and where spatial limitations preclude their installation. FOG degrading bioadditives are marketed as alternative solutions to grease traps or as enhancements to their efficacy.

Table 1-1 Physico-chemical treatment technologies for oily water treatment. Adapted from (Bande et al., 2008; Italtraco, n.d.)

Separation process	Minimum droplet size (µm)	Comments
Gravity separator - API	150	Economical & simple, but limited efficiency
Gravity separator – inclined/corrugated plates	45 – 60	Simple operation, but plates prone to clogging
Dissolved/dispersed air flotation	35 – 50	High power requirements. Chemical coagulants may be necessary.
Filtration	25 – 30	Power requirements. Chemical coagulants may be necessary. Backwash requires treatment.
Chemical coagulation	15 – 20	Costly chemical requirements and sludge disposal.
Membrane ultrafiltration	< 5	High power requirement. High costs of cleaning.
Electrocoagulation/ electroflotation	< 5	High efficiency/low cost. Electrodes require regular replacement.

FOGs discharged by FSEs are mainly derived from plants and animals, and consist of triglycerides composed of three fatty acid chains linked to a glycerol backbone (Figure 1-1). Hydrolysis is the first step in their biological degradation and is catalysed by enzymes called lipases, which liberate the fatty acids from the glycerol backbone allowing them to be uptaken by microbial cells for further degradation. The movement of fatty acids from the outside to the inside of the cell is regulated at gene level through the production of fatty acid transport proteins and fatty acid degradation proteins. The latter, fatty acyl coenzyme A (CoA) synthetases, converts free fatty acids into their acetyl CoA derivatives through β-oxidation, which can then be used in the tricarboxylic acid (TCA) cycle. The transport and the degradation proteins work together and are present at basal levels under nutrient-rich conditions. In the presence of high concentrations of long chain fatty acids (LCFAs), there is a specific induction of the fatty acid transport and degradation proteins which will increase their production (Black and DiRusso, 2003). The next step(s) of acetyl CoA degradation will vary depending on the oxygen availability. Under aerobic conditions, acetyl CoA can enter the Krebs cycle, whilst glycerol can be degraded *via* the glycolysis pathway. Whereas in typically anaerobic conditions acetyl CoA and glycerol are oxidised through fermentation (Hames and Hooper, 2005).

Most of the commercial supplements used in the UK are microorganisms selected for their efficacy in high and low oxygenic environments like sewers or separators. These products can contain either single microorganisms, or a mixture of microorganisms. Sporulating non-pathogenic *Bacillus* spp*.* are the most commonly used in FSEs products as they are usually safe foruse in kitchen environments and have a long shelf life without requiring refrigeration (Brooksbank et al., 2007) . Some bioadditives might contain enzymes, such as lipases, to improve triglycerides degradation and free fatty acids (FFA) concentration. The release of FFA will not only provide carbon and energy to the bioadded bacteria but also the non-lipase producing endogenous microorganisms, which will be able to access additional carbon sources.

The addition of oil-degrading bacteria has given contradicting results and in many cases has proved to be ineffective (Brooksbank et al. 2007; Mendoza-Espinosa & Stephenson 1996). Furthermore, bioaddition failure is often linked to the use of a single organism; scarce understanding of the environment; inadequate dispersion or access

to organic substrates; and other factors limiting biodegradation, such as inhibitors or toxic compounds (Watanabe et al., 2000). Whilst there is evidence that bioadditives can enhance FOG degradation, there is limited information on how this strategy is affected by the other wastewater components and the physico-chemical composition of the FOG content.

1.4 FOG bioaugmentation studies of commercial products

The majority of previous studies have adopted environmental parameters (pH, temperature, contact time, use of FOG as sole carbon source, optimum nutrient concentrations) to promote bacterial growth and/or enhance FOG removal, rather than reflecting real operational conditions. Many studies have investigated microbial lipolytic activity and/or FOG-degrading potential at lab scale (El-Masry et al., 2004; El-Bestawy et al., 2005; Tano-Debrah et al., 1999; Wakelin and Forster, 1997) but only a few have focussed on commercial bioadditives, with mixed results.

He et al. (2012) and Tang et al. (2012) reported enhancement in FOG removal at full scale for bioadded systems, whilst Brooksbank et al. (2007) found only one of 'several' commercial microbial supplements capable of significantly degrading fat and oil. Other studies found little difference in efficacy between bioadditives and native microbial populations (Loperena et al., 2006; Mendoza-Espinosa and Stephenson, 1996). Of two bacillus-containing commercial products tested by Tzirita and Quilty (2012), the efficacy of Product B was fully attributed to a fungal component, and Product A largely to a *Pseudomonas* species together with nutrient and surfactant additions. In all research reviewed significant FOG degradation was only achieved on timescales of hours, days or weeks whilst residence times in kitchen drains or grease separators might be limited to minutes or hours.

In four working GRUs, Carter (2005) observed reductions in retained grease between 2% and 81% in conjunction with bacterial additives. Poor performance (2%) was attributed to influent temperatures in excess of 40°C, and physical undersizing thereby highlighting the issue of achieving sufficient residence times in real-life environments.

According to Keener et al. (2008), sewer FOG deposits appear to accumulate between 50 - 200 m downstream of the point of discharge from an identified FOG contributor.

Phase 2 of the Orange County FOG Control study used cameras to monitor FOG deposit accumulation, over a six-month period after FSEs adopted bioadditive dosing. Although, in some cases, results indicated build-up was less than anticipated, simultaneous improvements in 'best management practices' by catering staff led the authors to conclude that improvements could not be attributed to the effect of bioadditives alone (Shaffer et al., 2006).

Moreover, in detergent-rich media, the chosen method of FOG quantification has great impact on the results' reliability. Extraction efficiency has often been ignored in these studies. For example, Brooksbank et al. (2007) determined, and accounted for, 18 – 50% apparent removal in sterile controls using solvent (dichloromethane) extraction, as a manifestation of under-recovery.

1.5 Factors influencing bioadditive performance

A considerable body of literature has arisen through a desire to optimise microbial lipase production for their use in a variety of industrial applications such as detergents and the food industry as reviewed in Jaeger and Reetz (1998) and Verma *et al.* (2012), with a more recent focus on industrial wastewater treatment to mitigate environmental pollution concerns. Only a fraction is reviewed here with a specific focus towards lipolytic activity in non-pathogenic *Bacillus spp.* owing to their suitability for use in food-processing environments (Brooksbank et al., 2007). Environmental conditions associated with a selection of prominent studies are summarised in Table 1-2.

A number of key factors can influence the ability of microorganisms to produce hydrolytic enzymes and degrade FOG. These include temperature; pH; contact time; the presence of co-substrates, providing alternative sources of carbon and energy; nutrients and the nature of FOG, with respect to type, concentration and particle size. The latter being strongly influenced by the presence of surfactants. However, previous degradation studies largely do not reflect the conditions which might be found in real FSE wastewater effluents. For example, trace elements are typically added to synthetic media at levels conducive to microbial growth and activity, and in fact have never been comprehensively characterised in FSE WW. The influence of co-

substrates on lipase activity has been investigated extensively, but rarely at concentrations typically found in kitchen wastewater.

Temperature and pH have received more attention, particularly with respect to identifying particular strains performing well at high pH and temperature in biological detergents. Experimental contact times tend to be set over a period of days or weeks, thus of little relevance to residence times actually available under real conditions. Finally, although field studies using real wastewater have more relevance, variability in WW composition complicates interpretation of the results.

1.5.1 Temperature and pH

Temperature and pH have strong influence on bacterial growth and activity. Mesophilic bacteria, which include most *Bacillus* species, tend to favour temperature ranges from 20 to 45ºC for growth and lipase production (Gupta et al., 2004). Exceptions include *B. licheniformis* MTCC-10498 producing optimum lipase activity at 55ºC (Sharma et al., 2012); and *Bacillus thermoleovorans* IHI-91 with an optimum growth temperature of 65ºC (Markossian et al., 2000). However, optimum lipase activity, for this bacterium was observed at 60ºC owing to more rapid thermal inactivation at the higher temperature (Becker et al., 1997). In contrast *Pseudomonas sp*. strain D2D3 favoured the lower mesophilic range with a FOG removal efficiency optimum at 30ºC (Shon et al., 2002); and a mixed culture featured in Tano-Debrah et al. (1999) producing best results between 20 and 25°C.

Although bacteria tend to favour neutral pH values (Gupta et al., 2004), lipase applications in the detergent industry has driven research to identify alkophilic strains. For example *Burkholderia cepacia* showed lipase activity over a pH range 7 – 12 (Rathi et al., 2001) and a thermophilic *Bacillus sp.* retained 74% activity up to pH 9 (Handelsman and Shoham, 1994). Thermophilic *B. coagulans* BTS-3 produced highest amount of lipase at pH 8.5, with the enzyme remaining stable over pH range of 8.0–10.5 (Kumar et al., 2005). Finally, Tano-Debrah et al. (1999) developed an inoculum producing the highest FOG removal at pH 9.5 which, they suggested, was due to enhanced emulsification in alkaline conditions.

Table 1-2 Selected FOG biodegradation studies using commercial products

Publication	Inoculum	Media	FOG type	Time	FOG degradation (%)	Calculated FOG degradation rate (mg/l/h)
Tzirita & Quilty (2012)	Commercial products based on a mix of *Bacillus spp*., *Pseudomonas spp*. and fungus.	Minimal, and enriched synthetic media (0.2 g/l yeast extract, 0.1g/l glucose)	Butter (7.5 g/l) or extra virgin olive oil (8 g/l).	7 – 14 days	Depending on the product and the FOG source, from 28% to 97%.	12.5 – 43.3
Loperena et al. (2006)	Commercial product comprising of *Bacillus spp*. and unidentified bacteria (Sybron Bi-Chem 1003FG); and activated sludge as native inoculum	Mineral medium	Butter oil (200-1000 mg/l) as sole carbon source.	80 hours	Commercial product: 82 and 95% Native inoculum: 78 and 95% for native inoculum.	Commercial product: 2 - 11.3 Native inoculum: 4 – 9.4 .
Tano-Debrah et al. (1999)	Mixed culture of 15 unidentified bacterial isolates from different WW samples (JAT inoculum)	Basal medium supplemented with glucose + various nitrogen sources	100 g/l mixture of lard, olive, salad, sesame, corn, peanut, grapeseed	14 days	11 – 86.5% depending on media supplement	32 – 257
Brooksbank et al. (2007)	Commercial product (F69 Organica) comprising of *Bacillus spp.*	Mineral medium – standard: 25 mgl glucose, 100 mg/l yeast extract	10 ml/l lard, rapeseed, sunflower or soya oil	21 or 28 days	37 - 62%	7.3 – 9.2 µl/l/h (approx. 6.7 – 8.5 mg/l/h)

1.5.2 FOG type and concentration

Different lipases often show specificity for different types of triglycerides or fatty acids. This specificity can be based on: (1) the position of the fatty acid (FA) on the triglyceride molecule (regiospecificity); (2) the FA chain length – true lipases hydrolyse long-chain fatty acids greater than C10, but can also show esterase activity towards short-chain FAs such as tributyrin (C4); and (3) the degree of saturation (the number of double bonds in the FA). Numerous studies have investigated the impact of FOG type as an inducer of lipase production (El-Shafei and Rezkallah, 1997; Lima et al., 2003; Sharma et al., 2012; Shon et al., 2002). Bora and Bora (2012) studied a lipase from a thermophilic *Bacillus* species and determined its specificity towards oil comprising of unsaturated fatty acids. This is in agreement with studies on FOG deposit composition which have been shown to contain a high proportion of saturated fats (He et al., 2011; Keener et al., 2008) residual from microbial degradation (Brooksbank et al., 2007; Williams et al., 2012). In contrast Shon et al. (2002) demonstrated equivalent high-removal efficiency for olive oil (unsaturated) and animal fat (saturated) by *Pseudomonas sp*. strain D2D3.

1.5.3 Surfactants

Surfactants (contraction for surface active agents) are amphiphilic compounds with two opposing portions, a hydrophobic tail and a hydrophilic head which extends outwards from the oil droplet. Surfactants act on oil droplets in two ways. As *emulsifying agents*, by reducing interfacial tension and facilitating the breakdown of bulk oil into smaller droplets; and as *stabilisers*, whereby the charged outer shells of the micelles hinder droplet aggregation and coalescence, thus maintaining small droplets in suspension.

Many of the microorganisms which are able to successfully utilise oil as substrate, also have the ability to produce biosurfactants, to enhance bioavailability of oily materials. These include rhamnolipids from *Pseudomonas aeruginosa*, surfactin from *Bacillus subtilis* and *B. pumilus,* and lichenysin from *B. licheniformis* (Mulligan, 2005). For example Zhang et al. (2009) demonstrated an increase in removal of frying oil from 10% to 93% when rhamnolipids were added.

The use of detergent is fundamental to cleaning procedures in FSEs: and undoubtedly impacts on grease separator efficiency according to Stokes' Law (Equation 1-1), wherein oil droplet rise rate (v) is proportional to particle diameter (d):

$$v = \frac{\left(\rho_p - \rho_f\right) \cdot g \cdot d^2}{18\mu}$$ **Equation 1-1**

Whilst surfactants hinder grease trap operation by generating and maintaining small droplet sizes (Chan 2010; Ducoste et al. 2008), the resultant increase in surface area/volume ratio enhances bioavailability to microbial FOG degradation (Zhang et al., 2009). To the authors' best knowledge, detergent/surfactant concentrations have never been quantified in FSE WW.

1.5.4 Impact of wastewater composition: co-substrates and nutrients

Literature pertaining the characterisation of FSE wastewater (WW) is sparse (Tarek N Aziz, 2010). The majority of previous studies report only on BOD, COD, FOG, SS and pH, with only He et al. (2012) including protein and carbohydrate. Yet, the presence of carbohydrates (Eltaweel et al., 2005; Takaç and Marul, 2008; Tano-Debrah et al., 1999) and protein (Ertuğrul et al., 2007) has been shown to either enhance or suppress lipolytic activity. Lipolytic activity was enhanced in the presence of up to1% peptone (Tano-Debrah et al., 1999); 0.5% yeast extract (Dharmsthiti and Kuhasuntisuk, 1998); and 1% glucose (Rathi et al., 2001), but decreased again at higher concentrations. It appears there may be a critical level of readily biodegradable carbon, and, in the case of protein also as a source of nitrogen, allowing bacteria to attain high population densities. Once the alternative carbon substrates are depleted, bacteria switch to utilisation of FOG.

1.5.4.1 Biodegradability

The principle of biodegradation is that organic matter (OM) acts as an electron donor, such that electrons are partitioned between a terminal electron acceptor (oxygen in the case of aerobic respiration) in order to generate energy for biomass synthesis, and the remainder incorporated into new biomass (Grady et al., 2011). Thus, all substrate carbon and electrons end up in new cells (anabolism/synthesis) or released as CO_2 (catabolism/respiration). Accordingly, OM degradation in wastewater treatment is

traditionally monitored as removal of biological oxygen demand (BOD) or, more usually, chemical oxygen demand (COD), as a measure of the amount of oxygen required in the oxidative degradation process. Both measures however have limitations. BOD assesses the amount of oxygen removed by a microbial population under standard conditions over a defined time period, typically at 20°C, over five days denoted as BOD_5. As such BOD values may not capture slowly hydrolysable OM such as FOG. COD tests have the advantage of yielding results within a few hours, but use strong chemicals to achieve oxidation of both organic and inorganic material, thus tending to overestimate biodegradable material. In reflection, the ratio of COD:BOD is typically used to evaluate biodegradability with values less than 2.5 considered suitable for biological treatment (Henze et al., 2001). As both COD and BOD measure oxygen demand, values reflect not only carbon concentrations but also the oxidation state of the material in question. For example, highly reduced compounds such as long chain fatty acid lipids, which are predominantly found in kitchen WW FOG, require more additional oxygen than partially oxidised compounds such as carbohydrates (Grady et al., 2011). Consequently, WW with high FOG concentration will contain more electrons and energy potential and thus a relatively higher COD than those with the same concentration of carbohydrates.

1.5.4.2 Influence of C:N:P ratio

The generalised formula for bacterial biomass of $C_5H_7O_2N$ indicates that bacteria take up carbon and nitrogen in a ratio of 5:1 in order to produce more cells. Under aerobic conditions roughly similar amount of carbon must be metabolised to generate the energy for assimilation such that a COD/BOD:N ratio of 20:1 is considered optimum for efficient microbial growth and biological treatment (Grady et al., 2011). Wastewater with lower ratios has an excess of nitrogen over that required for biomass synthesis, thus residual nitrogen will be left in solution and higher ratios may lead to incomplete carbon degradation. However, microorganisms also require energy for non-growth associated processes such as osmoregulation, cell maintenance and protein synthesis. Moreover microorganisms have demonstrated the ability to utilise more carbon than required through a variety of ‘wasteful’ processes including overflow metabolism whereby intermediate metabolites are excreted into the medium; shifts to less-efficient metabolic pathways, and futile energy-spilling reactions (Dauner and

Sauer, 2001). Bacteria can also respond to unbalanced nutrient availability by altering their internal composition (Egli and Quayle, 1986). Although bacteria vary considerably in cell size and elemental cellular composition according to microbial strain, Fagerbakke, Heldal and Norland (1996) confirmed a mean molar C:N ratio of 5:1 from a range of aquatic and cultured bacteria. While Vrede et al. (2002) linked cellular composition to substrate availability reporting that C-limited bacterioplankton had lower C:N wt/wt ratios (3.8 ± 0.1) than exponentially-growing (5.2 ± 0.2) or N-limited cells (7.5 ± 1.2). In both studies, a 4-fold reduction in CDW was observed between exponential and stationary phase cells.

Phosphorous (P) is also a crucial major nutrient with a typically cited optimum BOD:N:P of 100:5:1 for aerobic microbial wastewater treatment (Grady et al., 2011). However experimental design and literature reporting is inconsistent, with the vast majority of studies using COD and BOD interchangeably despite the fact that COD exceeds BOD by factors between 2.5 – 3.5 in high strength municipal wastewater (Henze et al., 2008). For example, COD:BOD ratios in FSE wastewater of 2.9 – 3.1 were reported in Wang et al. (2005); and 3.6 in Chung & Young (2013) yielding an apparently N-limited nutrient balance of BOD:N:P 100:1.25:3.2. Shon et al. (2002) observed a mean COD:BOD ratio of 1.75 yielding nutrient deficient BOD:N:P of 100:1.75:0.54. Nevertheless, FOG removal efficiency of 20% was observed within 24 hours, increasing to around 90% by 140 hours with the addition of *Pseudomonas* sp. strain D2D3 thereby supporting the premise that apparent nutrient deficiency can be accommodated given sufficient contact time.

1.5.5 Contact time

The efficiency of microbial FOG degradation is strongly influenced by the amount of time microorganisms remain in contact with the FOG in the wastewater. In grease traps contact times are on a scale of minutes to hours depending on size and flow rates. Aziz et al. (2012) estimated average residence times between 2.5 – 50 hours for a selection of oversized grease interceptors. Contact times for inline applications, will be significantly shorter necessitating a rapid microbial response for effective FOG removal. Maximum bacterial lipase production has been recorded during late log phase (Rathi et al., 2001); and stationary growth phase (Castro-Ochoa et al. 2005;

Sooch & Kauldhar 2013), suggesting there will be a delay in the initiation of FOG removal of some hours. However, the main activity for bioadditives is reputed to occur through development of biofilms on drain pipe surfaces extending into the sewer system. Domestic kitchen drain pipes had populations of 9.9 to 11.4 $\log_{10}$ cells/g capable of maintaining steady state population levels for over 160 days (McBain et al., 2003). Studies on microbial degradation in sewers have shown that substrate utilisation in biofilms on sediment surfaces (Chen et al., 2003) and pipe walls (Özer and Kasirga, 1995) is significantly higher than that associated with suspended populations. Potential contact time could be increased if FOG also adheres to tank or pipe walls in association with biofilms. Hence, we might expect a considerable, if not dominant, contribution to FOG degradation from the activity of biofilm communities in FSE wastewater systems.

1.6 Aim and Objectives

The research hypothesis tested by this thesis was that biological additions have different interactions with FOG when other carbon sources (carbohydrates and proteins) are available in discharged kitchen wastewater, hence the *in-vitro* assessment of their performance will need to include several conditions.

The aim of this work was to understand the biological processes associated with FOG degradation and removal from FSE wastewater streams. This understanding was then used to develop a standardised protocol for the determination of efficacy of FOG-degrading bioadditives.

This was achieved through the following objectives:

1. A comprehensive characterisation of wastewater from food service establishments to inform upon design of a realistic synthetic culture medium, and appropriate environmental conditions for experimental design.
2. The development of a novel method for quantifying FOG in emulsified systems.
3. The understanding of the action of microbial additives on organic components and kinetics of degradation using both synthetic and real wastewater.

4. The development of a standard protocol for testing the efficacy and kinetics of FOG degradation through the addition of microbial supplements to FSE wastewater.

5. The development of a practical guide for dosing regime tailored to the users' wastewater composition and sewers conditions.

6. The outline of a FOG management practice guide.

1.7 Thesis Structure

The steps involved in the delivery of this work are reported in the Thesis visualisation diagram (Figure 1-2). This thesis is divided into chapters formatted as papers for publication. An outline of the objective(s) addressed by each paper is shown below (Table 1-3)

Table 1-3 Objectives and Chapters

Chapter	Objectives addressed	Title/paper
1		Introduction
2	2/4	Determination of fat, oil and grease in food service establishment wastewater using a modification of the Gerber Method
3	1/4	A characterisation of Food Service Establishment wastewater
4	3/4/6	The impact of wastewater composition on microbial degradation of fat, oil and grease
5	4	Development of a protocol for testing FOG-degrading bioadditives for treating commercial kitchen wastewater
6	6	Considerations for selection of the most appropriate FOG management solution
7	5/6	Practical guide to effective biodosing
8		Conclusions and future work

All chapters were written by Caroline Gurd and edited by Dr Raffaella Villa and Prof Bruce Jefferson. All experimental work was undertaken at Cranfield University by

Caroline Gurd, with the exception of work carried out by MSc students supervised by Caroline Gurd in Chapter 2 (Carlos De Castro Rodriguez), and in Chapter 5 (Charlotte Christophe and Tololupe Elemo). ICP trace metals analysis in Chapter 3 by Richard Andrews, Cranfield University; and FOG droplet particle sizing in Chapter 3 by Martin Fairley, Cranfield University.

Chapter 1 is a general introduction and provides a brief summary of literature relevant to biological treatment of FOG in kitchen wastewaters.

Chapter 2 details the development and validation of a novel FOG quantification method specifically designed to overcome the interfering influence of surfactants which emulsify FOG in kitchen wastewater, and forms the basis of a paper published in the Water and Environment Journal (Gurd et al., 2018).

Chapter 3 reports on a comprehensive characterisation of effluents from three food service establishments on Cranfield University campus. This work has been submitted as a paper to the Journal of Environmental Management entitled "Characterisation of food service establishment wastewater and its implication for treatment".

Chapter 4 explores how varying concentrations of carbohydrates and protein impact on microbial FOG degradation when presented to bacterial populations as co-substrates. This chapter, together with data from Chapter 5, forms the basis of a paper submitted to Bioresource Technology entitled "Improving bioremediation of fat, oil and grease (FOG)"

Chapter 5 focusses on synthesising the findings from the previous chapters to develop a realistic test protocol for evaluating the performance of FOG-degrading bioadditives.

Chapter 6 proposes considerations for selection of the most appropriate FOG management solution in FSEs.

Chapter 7 discusses the implications of the work to FOG management and provides some practical guidance on biodosing in FSE drains and separators.

Chapter 8 presents overall conclusions and identifies further work that needs to be undertaken.

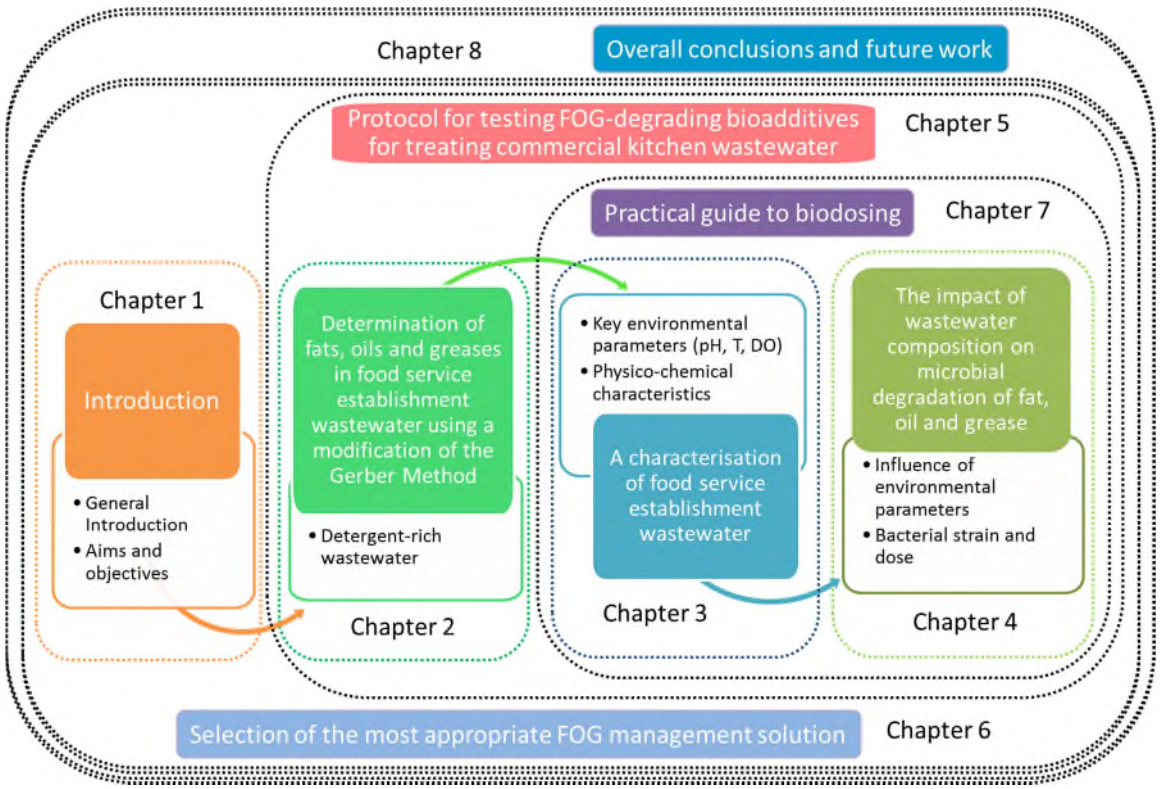

Figure 1-2 Visualisation of the Thesis structure

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2 Determination of fats, oils and greases in food service establishment wastewater using a modification of the Gerber Method

Gurd, C., Jefferson, B., Villa, R. and De Castro Rodriguez, C.

This chapter presents development of a novel method for quantifying FOG in wastewater containing high proportions of emulsified FOG. A brief overview of the method precedes the main text which forms the basis of a paper published in the Water and Environment Journal (Gurd et al., 2018).

2.1 Preface: Method overview

Accurate measurement of FOG in kitchen wastewater has been recognised as particularly challenging (Wang and Ducoste, 2012). Surfactants, such as protein and detergents, adsorb to form a protective layer around emulsified oil droplets thereby preventing solvent extraction leading to under-recovery of FOG (Aziz, 2010; Barton, 2012). Alternatively adsorbed protein may be co-extracted with FOG leading to over-recovery (Wang and Ducoste, 2012). The modified Gerber method was developed during this research to eliminate surfactant interference.

The new method is based on a Dairy Industry standard for measuring fat in milk (BSI ISO 2446:, 2008), The traditional method relies on bespoke butyrometers wherein milk is mixed with reagents which digest the proteinaceous milk fat globule membrane releasing emulsified fat. Fat content, as weight %, is measured from the height of a column of extracted oil on a graduated scale. However, even the more precise butyrometers designed for analysis of skimmed milk, can only measure in increments of 0.01% equivalent to 100 mg/l. Clearly this would be of little use for accurately measuring bulk wastewater samples. Accordingly, FOG is precipitated, isolated and condensed from larger sample volumes. Gerber butyrometer scales are calibrated to measure milk fat % based on a defined test portion (the exact volume of which varies internationally from 10.75 – 11 ml) and a milk fat specific gravity of 0.9 at the measuring temperature

of 65°C. For our purposes, the key attribute is that the scale is calibrated such that a reading of 1% fat is derived from an internal volume of 125 µl. Thus, the most accurate (0 - 1% scale) instrument, measures to 0.625 µl at a half-division reading.

In the modified method, illustrated in **Error! Reference source not found.**, sodium caseinate is added to wastewater and casein adsorbs to emulsified FOG droplets essentially forming synthetic milk fat globules. Subsequent reduction in pH causes the casein-FOG micelle to precipitate from the aqueous phase following the process of cheese curd production. The extracted solid is isolated by centrifugation, introduced into a Gerber butyrometer and processed to release the FOG which is measured on the graduated scale. FOG volume is converted to mass concentration (in mg/l) according to Equation 2-1, given that a 1% butyrometer reading is equivalent to 125 µl, and assuming a mean specific gravity of 0.89 g/ml over a range of edible oils (Esteban et al., 2012; H. Noureddini et al., 1992).

$$FOG = 1000 * \frac{butyrometer\ reading * 125 * 0.89}{mass\ bulk\ aqueous\ sample}$$ **Equation 2-1**

As this method is suitable for extracting emulsified FOG only, a separate step was incorporated into the overall method to allow quantification of any free FOG observed floating on the surface of the wastewater. This involves placing a pre-cut disc of oil-adsorbent hydrophobic material onto the liquid surface, at a temperature of 50ºC which allows solid animal fats to melt. The adsorbed FOG is eluted with hexane which is then evaporated leaving a FOG residue.

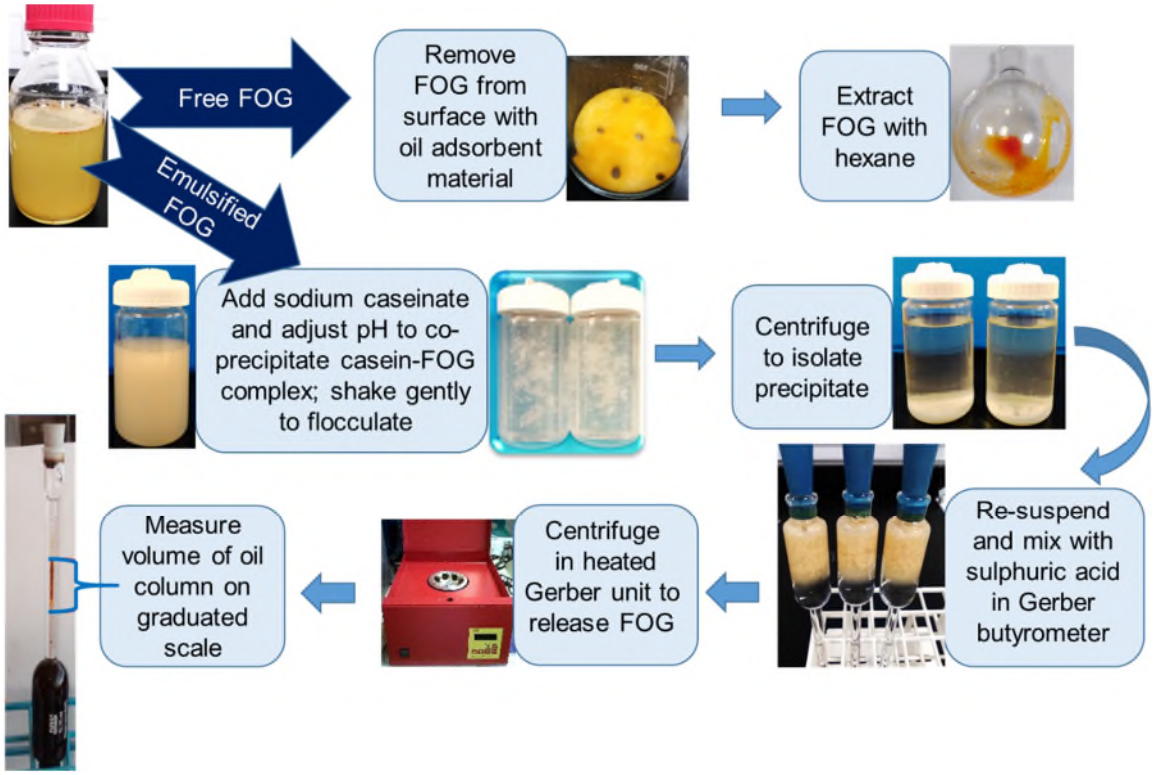

Figure 2-1 Illustration of key steps in modified Gerber quantification method for free and emulsified FOG

Abstract

Discharges from food service establishments (FSEs) are a major source of fat, oil and grease (FOG) which cause blockages in sewer networks. Previous research has identified that current methods are unsuitable for quantifying FOG in FSE wastewater owing to interference from surfactants in detergents, and protein from food residuals which emulsify FOG. A novel quantification method, based on the dairy industry Gerber method, has been developed which negates the impact of surfactants. Moreover, the method allows free and emulsified oil to be quantified separately providing greater insight into FOG management strategies. Trials in synthetic and real FSE wastewaters indicates the novel method is more reliable than standard liquid-liquid and solid phase extraction in FOG-rich systems.

2.2 Introduction

The term FOG (fat, oil and grease) encompasses a number of different materials (liquid and solid) and describes a heterogeneous group of chemicals including tri-, di- and mono-glycerides, sterols, non-volatile hydrocarbons, waxes and other complex lipids which exist in a combination of free and emulsified forms. Suspended and emulsified FOGs (FSE wastewater) are discharged into sewer systems from both domestic and commercial premises and are attributed to cause a significant proportion of the deposits (solidified and saponified) forming sewer blockages (Williams et al, 2012). Discharges from food service establishments are known to represent a major source of FOG deposits in sewers and hence are a focus for managing discharges. Current management practices of FSE wastewater include treatment in the form of biological additives to process the FOG material into benign end products; or passive grease separators to collect it. Current challenges associated with the cost and complexity of suspended and emulsified FOG measurement mean that surrogates are often preferred when selecting, designing and confirming performance. For instance, passive gravity separators commonly use light diesel oil as a surrogate in

validation tests although the specific gravity of the oil is significantly lower than typical FOG, raising questions over its applicability (Barton, 2012).

FOGs are insoluble in water but soluble in solvents (e.g. chlorinated fluorocarbons and alcohols) such that current measurement methods incorporate a solvent extraction step (BS EN 1825-1:2004; US EPA/R-98-002). In the UK, the standard method involves a liquid–liquid extraction (LLE) with 1.1.2-trichloro-1.2.2-trifluoroethane ($C_2Cl_3F_3$) which, due to environmental concerns, has been replaced with more appropriate options such as carbon tetrachloride (CCl_4) or n-hexane (C_6H_{14}). As each solvent potentially extracts different materials in addition to FOG, it is common practice to refer to the solvent used when expressing a concentration, for example, hexane extractable material (HEM). Quantification of the FOG content in FSE wastewater is normally based on gravimetric measurement of residuals post solvent evaporation, with reported alternatives utilising infrared spectroscopy or gas chromatography.

Previous investigations concerning current FOG levels and their treatment have indicated that predicted oil removal does not match measured oil removal (Lopez-Vazquez and Fall, 2004; Ducoste et al, 2008). Investigation into FOG recovery efficiency has demonstrated measurement interference when protein and/or detergent surfactant are present (Table 2-1). The surfactant molecules form micelles around the FOG droplets inhibiting hexane solvation; or proteins can be carried into the solvent increasing the total mass transferred. Furthermore, emulsion separation can be inhibited through foam formation during the agitation phase which may be ameliorated through centrifugation (Barton, 2012; Wang and Ducoste, 2012). Previous reports have suggested that SPE is more effective than LLE in the presence of surfactants (Lau and Strenstrom, 1997). This method involves use of a hydrophobic matrix that retains all non-aqueous components as the sample passes through the material. The retained oils are back eluted with an appropriate solvent (i.e. n-hexane) and the post-evaporated residual weighed (Wells et al, 1995). A benefit of such approaches is the ability to pre-concentrate enabling more accurate measurement of low concentrations of FOG. However, more recent studies have demonstrated that recovery remains low with SPE in

emulsified systems (Barton, 2012). For instance, the current research has concluded that surfactant concentrations of sodium dodecyl sulphate (SDS) beyond 40 mg/l inhibit recovery, with recovery levels as low as 20% once the SDS concentration exceeds 400 mg/l. Accordingly, solvent-based FOG measurements can both under and over-estimate the true content thereby influencing the understanding of true loads and the efficacy of the different treatment options. To illustrate, an over estimation of feed coupled to an underestimation of the effluent could considerably over estimate the removal efficiency of a system.

Table 2-1 Reported FOG recovery with different methods and interfering substances

Method	HEM recovery (%)	Interfering component	Reference
EPA 1664A (LLE-Hexane)	44-58	Protein	Wang and Ducoste, 2013
Modified LLE	111-117	Protein	Wang and Ducoste, 2013
Modified LLE method	47-63	Surfactants	Barton, 2012
SPE	91-92	none	Barton, 2012
SPE	30-50	Surfactants	Barton, 2012
SPE	63-78	Protein	Barton, 2012
Modified Gerber	92-104	Protein and surfactants	Davies et al, 2011

An alternative proposition is the Gerber method, which is an established method in the dairy industry for determination of the fat contents of raw and processed milks, and is used worldwide for applications such as payment testing and process standardisation (Kleyn et al, 2001). The fat within milk exists as a

stabilised emulsion due to protective casein protein coatings around the fat globules. Consequently, the sample is digested in sulphuric acid to break down the proteins releasing the fat, and isoamyl alcohol is added to facilitate phase separation. The contents are mixed in a specially designed butyrometer, and centrifuged to isolate the fat into the tube of the butyrometer where the percentage fat content is read from the graduated scale at a defined reading temperature of 65°C (BS ISO 2446:2008). The scale is based on a specific gravity of butterfat of 0.9 at the measuring temperature, and a predefined volume of milk (10.75 - 11 ml). Establishment of the consistency of the method through an international collaborative study concluded that the relative standard deviation of the measurement was 1.8% for low fat solutions (1 - 2% fat) and 0.6% for high fat (2 - 6% fat) solution (Kleyn et al, 2001).

The Gerber method represents a simple and inexpensive approach for determining fat contents, and as such has seen its use beyond just milk: with examples including cheese and meat products (de Langen, 1963); macadamia nuts (Rosenthal et al., 1985a) and avocado (Rosenthal et al., 1985b). A correction factor must be applied due to the difference in the specific gravity of the type of fat being measured. For instance, in the case of meat, a correction factor of 0.935 was applied based on the ratio of specific gravity of lard to butterfat (de Langen, 1963). Reported recoveries exceeded 99% at fat contents above 3% such that the author concluded that the Gerber method provides a more rapid and reliable measurement than traditional solvent extraction. Similarly, positive comparison between Gerber and solvent extraction has been reported in the case of avocados with a regression coefficient for the linear trend of $r = 0.928$ (Rosenthal et al., 1985b). Utilisation of the Gerber method for these systems relies on dissolution of the fat containing material and as such modification to the procedure has been required in terms of the temperature and the agitation time prior to measurement. For instance, in the case of the meat, the sample went through four cycles of agitation followed by resting in the water bath (de Langen, 1963).

The approach has also been adopted for fat measurement in wastewater from small dairy-based FSEs (Davies et al, 2011) requiring a modification where the fat was precipitated, isolated and condensed from larger samples to enable accurate determination. The approach was to lower the system pH to the isoelectric point (iep) of casein (pH 4.6). Casein-stabilised milk-fat globules precipitated and aggregated, and the isolated precipitate was processed through the standard Gerber method. The current paper extends such work to all FSE wastewater types by further modifications enabling the Gerber method to be applied to non-dairy based systems. In such cases, casein is added to the wastewater to promote co-precipitation of the emulsified material.

2.3 Materials and Methods

All reagents used were obtained from Fisher Scientific, UK, unless stated otherwise. Tests were performed on both synthetic and real FSE wastewaters. Emulsions were produced by emulsifying rapeseed oil (Tesco, UK) at different concentrations between 10 and 750 mg/l in distilled water to a total volume of 200 ml combined with analytical grade sodium dodecyl sulphate (SDS) in a 250 ml glass bottle. The impact of other potential matrix interferences was assessed by using a synthetic FSE WW containing soy protein acid hydrolysate (Amisoy, 400 mg/l), glucose (1200 mg/l), cornflour (Tesco, UK, 250 mg/l), sodium dodecyl sulphate (30 mg/l), Triton X-100 (14 mg/l), calcium chloride dihydrate (100 mg/l), potassium chloride (70 mg/l), magnesium sulphate heptahydrate (40 mg/l), ammonium chloride (10 mg/l), iron chloride (0.27 mg/l), zinc sulphate heptahydrate (0.16 mg/l), copper sulphate (0.07 mg/l), manganese sulphate monohydrate (0.045 mg/l), cobalt nitrate hexahydrate (0.002 mg/l) and ammonium heptamolybdate tetrahydrate (0.001 mg/l). Surfactants were measured with cell test kits (Spectroquant, Merck Millipore) quantified with a NOVA 60A Spectroquant photometer.

The other reagents used were sodium caseinate, Gerber sulphuric acid (density at 20°C is 1.816 $\pm$ 0.004 g/ml), isoamyl alcohol (density ranging between 0.808-0.818 g/ml), hydrochloric acid, n-hexane (HPLC grade), and 1% sodium

hydroxide. The density of rapeseed oil was determined by hydrometry per BS EN ISO 3675:1998 to be 0.889 ± 0.003 g/ml at 60°C. Real FSE wastewater was collected from local establishments on the campus of Cranfield University from a potwash sink and a commercial dishwasher.

2.3.1 Free oil determination

FOG in environmental samples often exists as free FOG floating on the sample surface or adhered to the sample container, together with an emulsified phase generated either mechanically, or chemically 'bound' to surfactant (detergent, protein and possibly carbohydrate). Whereas standard extraction techniques fail to recover emulsified FOG, the modified Gerber method can only extract emulsified FOG as it relies on co-precipitation with the interfacial casein layer which encapsulates the fat globules. Free floating oil is merely left on the sample surface. Consequently, free FOG measurement was performed separately to the emulsified phase.

Samples were heated to 50°C to ensure any animal fat was melted and surface oil was removed by adsorption onto discs cut from a hydrophobic meltblown polypropylene material (Serpro Ltd, UK) placed on the sample surface. After free oil removal, the aqueous phase was decanted, and weighed, for processing by the Gerber method. 40 ml of hexane was added to the original sampling container followed by gentle agitation to solvate FOG from both the disc and vessel surface. Following removal of the disc and phase separation, 20 ml hexane was extracted by pipette, evaporated and the residue weighed.

The oil adsorbent material is a cheap commercial product designed to remove hydrocarbon spills from water surfaces (Serpro Ltd 2009). As such, its suitability for removing extracting FOG from wastewater for quantification was unknown. Key considerations tested were:

- Suitability for allowing hexane extraction of adsorbed FOG
- Ability to adsorb vegetable oil and animal fats
- Impact of surfactants

Preliminary experiments revealed that a fine residue of small particles/fibres was observed to carry over from the disc material thereby contributing to the mass of HEM. Discs were preconditioned by soaking and flushing with hexane to minimise interference and determine a value for a method blank. Ten samples of disc material went through the procedure to ascertain a method blank reading, and the mean measured extracted material subtracted from subsequent sample measurements.

To determine the time required to elute adsorbed FOG from the discs, known masses of oil (~400 mg) were pipetted into 200 ml DI water in 250 ml glass bottles. Following introduction of hexane, discs were allowed to soak for varying lengths of time between 5 and 30 minutes.

To determine if the adsorbent material adsorbs animal fat as well as oil, known masses of rapeseed oil and lard were introduced into 200 ml DI water in glass bottles. Discs were applied to the surface, and samples held at 50°C for 30 minutes.

Potential interference from adsorbed surfactant was determined from triplicate blanks at SDS surfactant concentrations between 0 and 500 mg/l.

2.3.2 Modified Gerber method for emulsified FOG

Approximately 200 ml samples of synthetic emulsions or real FSE wastewater were weighed into transparent polycarbonate centrifuge bottles (250 ml Nalgene, Fisher UK). The FOG concentration step of emulsified phase extraction involves mixing 1 mL of 10% sodium caseinate solution into emulsified samples and reducing the pH through addition of hydrochloric acid dropwise until precipitated particle aggregation is visually confirmed, based on a 30 second mixing period. Visual observations determined that precipitation (an increase in turbidity) occurs over a broad range of pH around the iep. A narrower band, closer to the iep, lies within this range, in which precipitated particles rapidly aggregate and flocculate. Floc growth is facilitated by gentle mixing to keep sediments in suspension and encourage sweep flocculation - too high shear results in floc break-up leaving

small particles in suspension. Samples were placed horizontally on a rotary shaker operated at 90 - 95 rpm for 15 minutes or until the aqueous phase was clear. Then samples were centrifuged at 2000 g for 10 minutes (Sorvall Legend RT+, DJB labs, Newport Pagnell UK) which was repeated if small particles remined visually present in the supernatant. The supernatant was discarded, and 1% sodium hydroxide added to the precipitate and residual fluid to raise the pH to 7 to partially re-dissolve the casein generating a slurry for easy transfer. Three drops were sufficient in the synthetic medium, in association with prior pH adjustment to ~ 4. More was required at lower flocculation pH.

The method then follows the traditional Gerber method (BS ISO 2446:2008) with some modifications. The slurry was layered onto 10 ml of sulphuric acid in a 0 - 1% butyrometer (Funke Gerber, VWR, UK), followed by a few ml of DI water that were used to rinse down the walls of the centrifuge bottle. 1 ml of isoamyl alcohol was added and the total volume topped up with distilled water such that the liquid surface lies half-way up the butyrometer neck to facilitate setting the oil column on the graduated scale. An acid resistant bung was inserted and the butyrometer shaken vigorously for 90 seconds. The butyrometer was centrifuged in a heated Gerber unit (Funke Gerber Nova Safety, VWR, UK) for 10 minutes followed by tempering in a water bath at 65°C for 3 -10 minutes. The height of the oil column was measured by manipulating the bung to set the column base on a full-scale graduation. The cycle was repeated, and readings compared: if the second reading exceeded the first by more than 0.05, or if air bubbles or particulate material remained in the oil column, a third cycle was employed, and the final reading used for calculation of FOG.

An average specific gravity of 0.89 g/ml was used for all samples. The density of the rapeseed vegetable oil used for method development was measured with a hydrometer following the BS method (BS EN ISO 3675:1998) and was equal to 0.889 ± 0.003 g/ml at 60°C. Although the Gerber milk fat method specifies a reading temperature of 65°C, the value applied was the specific gravity at 60°C, given that the butyrometers inevitably cool slightly during reading (Day and

Grimes, 1918). The same value was used for real wastewater samples based on the data reported in Esteban et al. (2012) and Noureddini et al. (1992) who measured densities of various edible oils at different temperatures. The mean specific gravity of 12 oils tested at 60°C was 0.8909 ± 0.0045 g/ml. This value also agrees with the 0.9 g/ml reported by DEFRA (2002) for waste oil from oil/water separators (EWC Code 13 05 06).

The potential for surfactant interference was assessed on both free and emulsified FOG recovery by varying SDS concentrations between 0 and 500 mg/l. Recovery and detection limits were assessed by varying the initial oil concentration of the synthetic solution with rapeseed oil up to 735 mg/l.

2.3.3 Detection limits

Definitions of the detection and quantification limits were expressed as in the USEPA guidelines (40 CFR appendix B part 136). The limit of detection (LOD or method detection limit MDL) is defined as the minimum concentration that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. This was determined by seven low-concentration replicates for emulsified oil; and statistical distribution of ten method blank measurements for free oil according to Equation 2-2.

$$LOD = 3.3\ x\ S_{blank} \qquad \textbf{Equation 2-2}$$

Where S_{blank} is the standard deviation of the blank measurements

The limit of quantification LOQ (or practical quantification limit, PQL) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the test conditions, and was ascertained through analysis of seven replicates of low-oil concentration stock solution

2.3.4 Comparison to SPE and liquid-liquid methods

Samples of both synthetic and real FSE wastewater were co-analysed by LLE and SPE to compare with established methods based on US EPA 1664. In the

case of LLE, samples were adjusted to below pH 2 and transferred to a 1 l glass separating funnel. Three 10 ml portions of n-hexane were used to wash the bottle and combined in the separating funnel with a further 20 ml of n-hexane. The funnel was manually shaken for two minutes and then left to stand for thirty minutes. The aqueous portion was drained, and the solvent phase filtered through 10 g of anhydrous sodium sulphate into a preweighed 250 ml round bottom flask. The aqueous phase went through two more solvent extractions before the combined solvent system was dried in a rotary evaporator (Hiedolph Laborota 4000). The flasks were further dried overnight at 45 °C, cooled in a desiccator and weighed.

In the case of SPE, samples (adjusted to pH < 2) were passed through a solid phase extraction disc (Empore Oil and Grease, Sigma Aldrich, UK) in a vacuum filtration system. The discs had been rinsed with hexane, pre-conditioned with 10 ml methanol and rinsed with 30 ml DI water. The sample bottles were then rinsed progressively twice with 10 ml n-hexane which was passed through the disc to elute retained FOG. A final 10 ml hexane was used to rinse the filtration glassware and disc. The combined hexane extract was passed through sodium sulphate into a pre-weighed 100 ml round bottom flask and then measured as per the liquid-liquid method. In some samples of real WW, with high loads of suspended solids, a glass wool prefilter was employed to prevent clogging of the extraction disc. In addition, greater volumes of hexane were required to dissolve solidified FOG.

2.4 Results and Discussion

2.4.1 Free oil extraction method optimisation

Determination of the most appropriate approach to using the adsorbent material for free oil recovery was ascertained in relation to disc preparation, mixing intensity, contact time and the impact of surfactants. ‘No oil’ blank experiments initially revealed background measurements of fine particulate material up to 21 mg-HEM/disc. Pre-conditioning, by a five-minute soak in hexane and vacuum drying, reduced background HEM to 14.3 ± 1.3 mg/disc (n=6) which was further

reduced to 7.7 ± 2.5 mg/disc and finally 3.8 ± 2.1 mg/disc (n=10) by reduced shaking and additional rinsing respectively. Clearly variations in disc treatment create considerable variability in HEM measurements, which is exacerbated at low concentrations. Consequently, consistency in disc treatment, method blank determination and blank subtraction is recommended in calculating observed concentrations of free oil.

Validation against known masses of approximately 450 mg free oil in DI water revealed no significant difference in recoveries (100 ± 3%) for disc elution times between 5 - 30 minutes. The impact of surfactants on free oil recovery was elucidated by conducting a series of trials at different SDS concentrations between 0 and 500 mg/l, indicating a maximum additional mass of 2-3 mg HEM per per disc when the surfactant concentration reaches 500 mg/l. At, and below, 200 mg/l SDS no statistically significant difference was observed compared to the surfactant-free samples. Trials of free FOG recovery revealed recovery rates of 101 ± 1.5% for animal fat and 97 ± 6.4% for oil (Figure 2-2

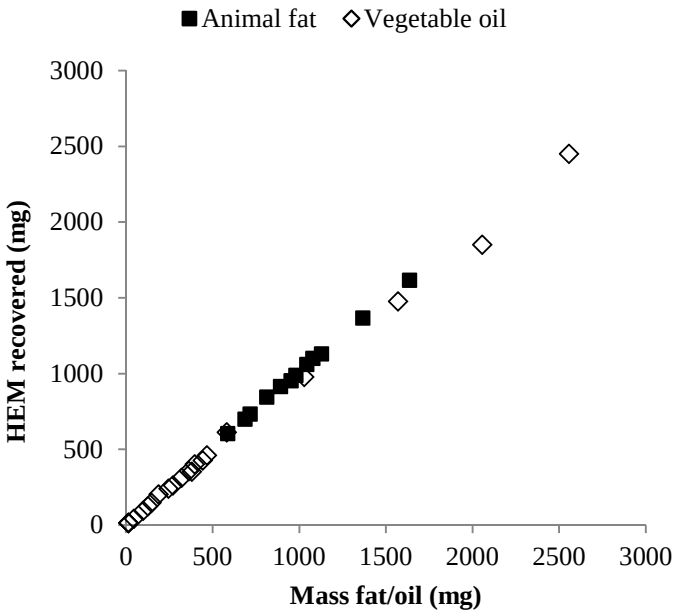

Figure 2-2). The maximum added animal fat level was 1637 mg/200 ml sample, equating to a concentration in excess of 8000 mg/l indicating that the approach is suitable for FSE wastewater samples. Indeed, it is posited that the use of a separate free oil measurement provides much greater insight into the load,

treatment and impact of FOG in FSE wastewater and downstream into the sewer than using traditional measurements which do not differentiate between the two phases.

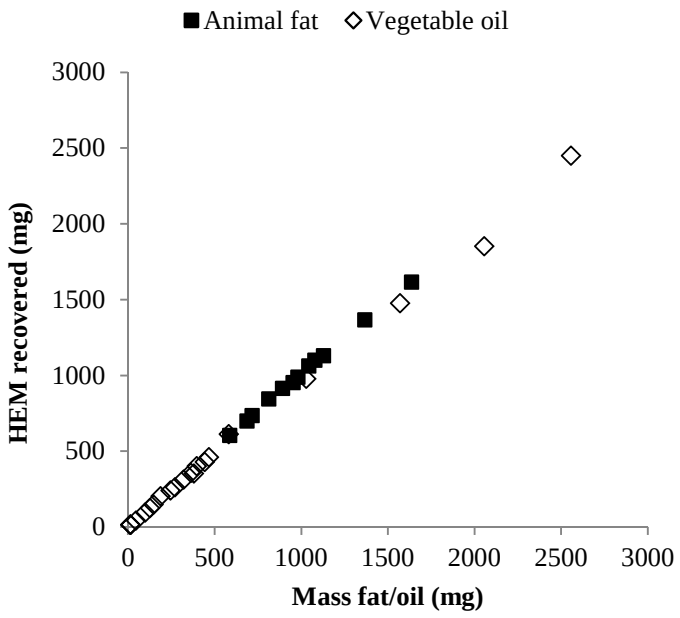

Figure 2-2 Free FOG recovery for animal fat ■ (n=12) and vegetable oil ◊ (n=29) from synthetic kitchen wastewater

2.4.2 Emulsified oil extraction

In the absence of surfactant, aggregation of the oil-caseinate mixture initiated at or close to the isoelectric point of casein, pH 4.6 (Figure 2-3). Addition of SDS surfactant reduced the iep of the mixture such that, at a SDS concentration of 100 mg/l, the required pH for visual aggregation varied from 2 to 3.8. At higher SDS concentrations of 200 and 500 mg/l the highest pH that visual aggregation occurred was between 1 and 2 (Figure 2-3). Typical surfactant concentrations in real FSE wastewaters vary widely; for instance, levels in the FSE samples used in the current study were 217 ± 119 mg/l from sinks and 26 ± 9 mg/l from dishwasher effluents (n=16). The levels are below the critical micelle

concentration (CMC) of SDS in both the current synthetic experiments and the real FSE wastewaters as the CMC of SDS varies between 7 and 9 mM as a function of pH (Rahman and Brown, 1983). Consequently, the observed changes in the pH required to induce precipitation reflect surfactant-casein interactions and direct inhibition through competition between casein and the surfactant for the surface of the oil (Demetriades and McClements, 2000).

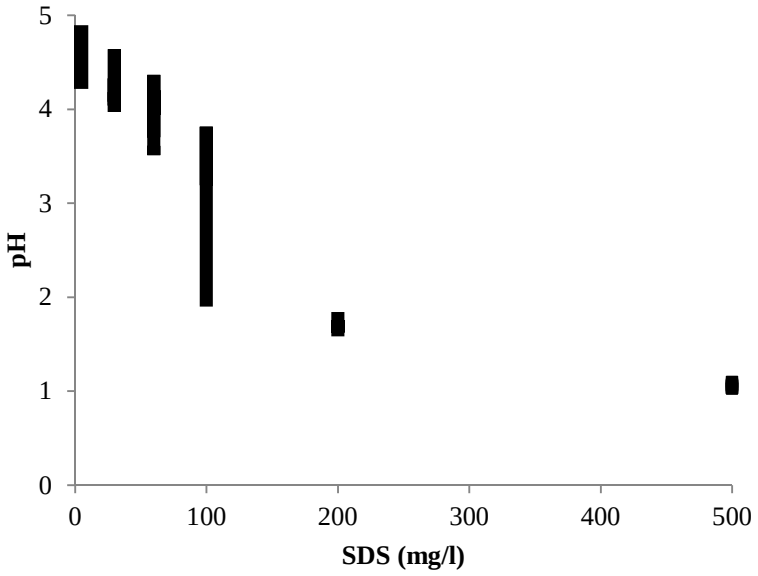

Figure 2-3 Impact of different sodium dodecyl sulphate (SDS) concentrations on the required pH range (indicated by bars) to induce aggregation of a synthetic oil-caseinate emulsion. Each bar comprises of data from between 20 and 50 measurements.

Aggregation in real FSE WW samples followed a similar correlation, for example dishwasher samples contained anionic surfactant concentrations of 2 ± 1 mg/l and consistently aggregated over a pH range between 4 and 4.4. Sink samples had higher, and more variable levels: aggregation was achieved between pH 3.3 and 3.8 for samples up to 183 mg/l, but adjustment to 1.7 was required for a sample measuring 278 ± 18 mg/l. The pH change is required for the oil replacement in the surfactant-casein interaction. The amount of hydrochloric acid required to precipitate and aggregate the oil-casein particles varies in all samples

and is visually confirmed once flocculation starts, based on a 30 second mixing period.

Emulsified oil recovery was tested over a range of initial oil concentrations from 10 – 735 mg/l Figure 2-4(A)). Recovery of 95 ± 4% was observed for concentrations exceeding 70 mg/l, decreasing at lower concentrations as a reflection of an offset around the minimum detectable concentration illustrated by a negative intercept of -10.9 mg/l (Figure 2-4(B)). Additional measurements from seven replicates of diluted stock emulsion revealed a limit of detection of 20 mg/l at a mean recovery of 27 ± 11%. The associated limit of quantification was determined to be 60 mg/l based on acceptable accuracy and precision of recovery at 80 ± 4% (n=7). However, the use of the precipitation stage enables a threefold pre-concentration (three bulk sample precipitates combined in one butyrometer) without a loss of accuracy such that the working LOQ can be reduced to 20 mg/l. This compares to LOD and LOQ for LLE of 1.4 and 5 mg/l based on surfactant-free trials of 1l samples (US EPA/R-98-002, 1999) with an acceptable recovery range of 78 - 117%. However, previous trials have demonstrated the reduced recovery of LLE in the presence of surfactant - detergent and protein - (Barton, 2012; Wang and Ducoste, 2012) suggesting that a balance between LOQ and recovery is required.

Analysis for the free oil measurements indicated accuracy and precision of 97 ± 6% and estimated LOD of 36 mg/l from statistical analyses of method blanks, although this will vary according to disc preparation. As recovery remained within a range of 80 – 107% down to 36 mg/l the LOQ can be assumed equivalent to LOD.

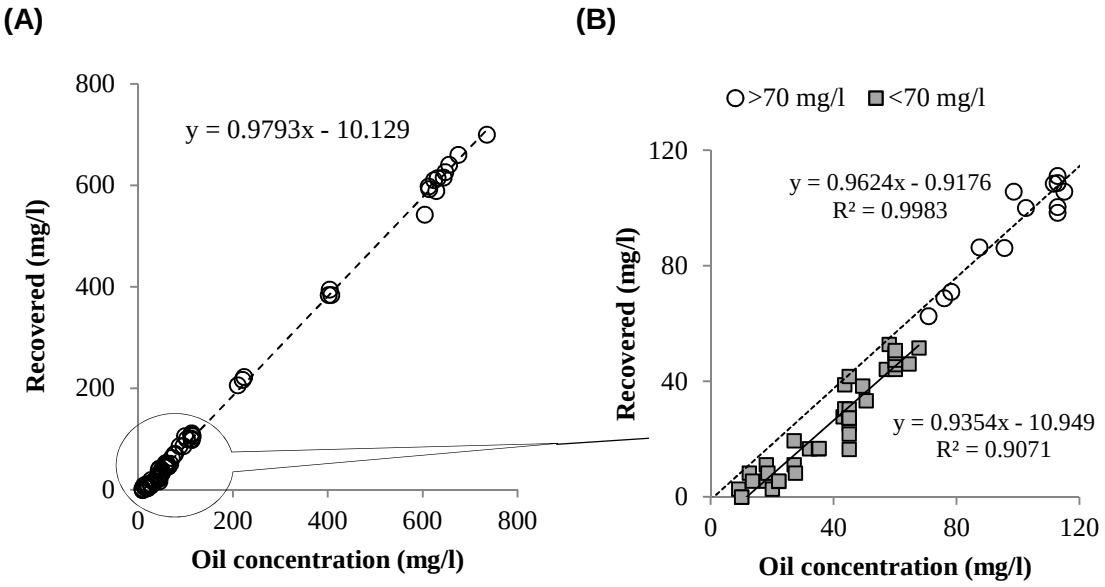

Figure 2-4 Emulsified FOG recovery as a function of FOG concentration in synthetic kitchen wastewater over a range of initial oil concentrations from 10 – 735 mg/l (n = 71) (A). (B) magnifies the lower range in (A) to highlight recovery reduction below initial concentrations of 70 mg/l (grey boxes).

2.4.3 Dual method validation

The use of the Gerber method for emulsified oil, coupled to a separate measurement of free oil through pre-adsorption, enabled total FOG recovery levels of 101 ± 2 % across all surfactant concentrations tested (0- 500 mg-SDS/l) at a total initial oil concentration of approximately 2000 mg/l (Figure 2-5(A)) and 100 ± 7% at 500 mg/l (Figure 2-5(B)). The trial in Figure 2-5(A) demonstrated that the majority of the oil was recovered as free oil from samples generated by manual agitation (shaken for two minutes) with a maximum emulsified fraction of only 16% observed. To ensure a greater fraction of persistent emulsified oil, a subsequent trial was conducted where samples were prepared by stirring vigorously for 60 minutes on a magnetic plate with a lower oil concentration of 500 mg/l (Figure 2-5 (B)). In addition, pH was adjusted to < 2 to reflect typical environmental preservation procedures for sampling. Much greater levels of emulsified oil were observed in the mechanically emulsified samples, which

equated to between 52% and 93% of the recovered oil when the surfactant concentration was 200 mg/l or less. Overall recovery was less consistent but remained between 96 ± 4% to 107 ± 5% at surfactant concentrations ≤ 200 mg/l and 88 ± 4% at 500 mg/l (Figure 2-5(B)). Such recovery levels are consistent with trials on a dairy based FSE using the Gerber method alone which reported recovery levels of 92 - 104% (Davies et al, 2011). Comparison to trials on low fat milk (1 - 2%) reveals a relative standard deviation across lab trials of 1.8% (Kleyn et al, 2001) suggesting that modification of the method to enable use across FSE has not adversely impacted on the recovery appreciably. Comparison to previous trials in wastewater reveals far better recovery than with LLE or SPE in the presence of surfactants where recovery levels were 47 – 63% and 30 - 50% respectively (Barton, 2012).

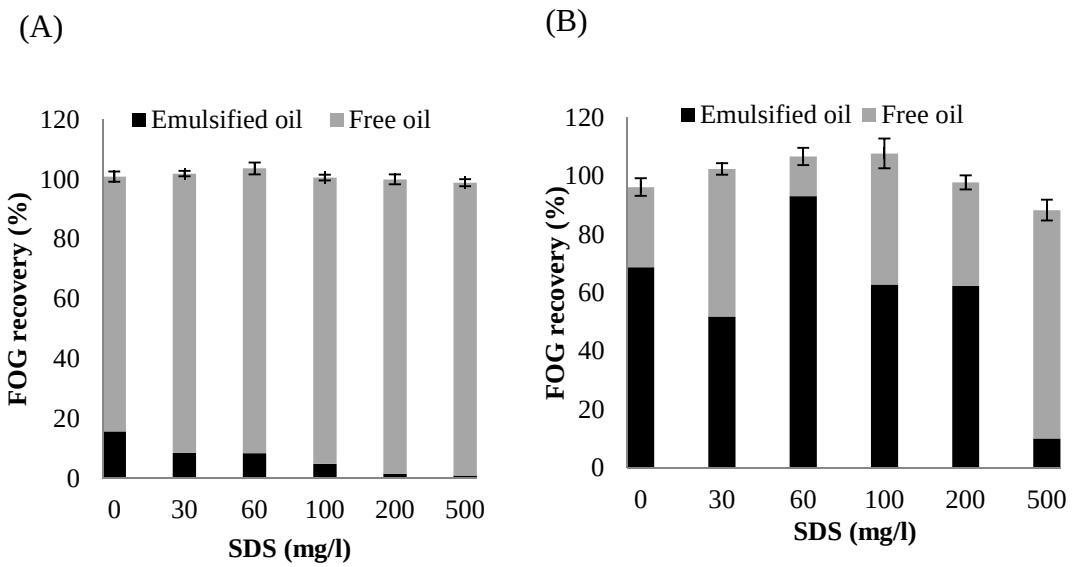

Figure 2-5 FOG recovery (free and emulsified) as a function of sodium dodecyl sulphate (SDS) surfactant concentration in (A) manually mixed (oil concentration 2000 mg/l) and (B) mechanically mixed systems (oil concentration 500 mg/l). Bars represent means measurements from triplicate samples, error bars indicate standard deviation of total recovery.

2.4.4 Comparison with standard methods

Comparison of the Gerber method and LLE on a series of synthetic FSE samples with varying initial oil concentration revealed significantly poorer recovery in the case of LLE (Figure 2-6). For instance, at initial oil concentrations of approximately 10 and 30 mg/l recovery levels were 279% and 477% for the LLE and 51% and 59% for the Gerber method. In contrast to the considerable over-estimation at low oil concentrations, the LLE method tended to under estimate oil levels for samples at initial oil concentrations of between 50 and 400 mg/l. In these cases, the LLE recovery varied between 49 and 74% whereas the recovery level for the Gerber method was 85% at 50 mg/L and then between 96-99% thereafter.

At low oil concentration of 30 mg/l and 10 mg/l, the SPE extraction showed higher recovery levels than the Gerber method: 99% vs 49% and 146% vs 51% respectively. This suggests that the LOQs for the two methods are around 50 mg/l for the Gerber and 30 mg/l for SPE. Overall, at concentrations at, or higher, than 100 mg/l Gerber extraction was more accurate and precise than SPE, with recoveries of 96 – 99% and 66 – 91% respectively. Overall SPE proved more reliable than LLE at all oil concentrations.

Comparison to real FSE wastewater reveals less difference between the Gerber and LLE methods, although overall the LLE still underestimated the values compared to the Gerber method, and showed greater variation within the triplicate measures (Figure 2-7). To illustrate, the fourth sample of the FSE wastewater generated the greatest difference with the Gerber method recording a FOG level of 141 $\pm$ 11 mg/l compared to 68 $\pm$ 29 mg/L with the LLE.

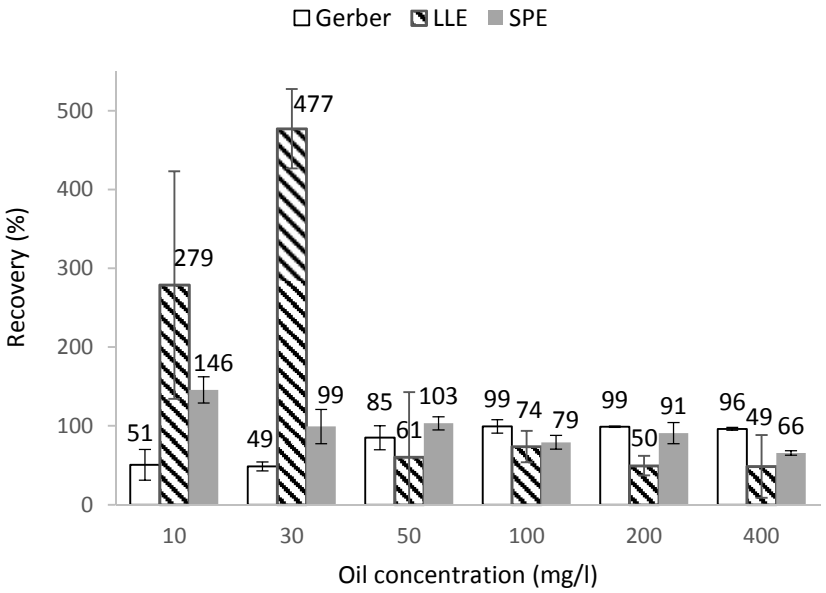

Figure 2-6 Comparison of oil recovery for the modified Gerber method, liquid-liquid extraction (LLE) and solid phase extraction (SPE) for synthetic FSE samples (n=3).

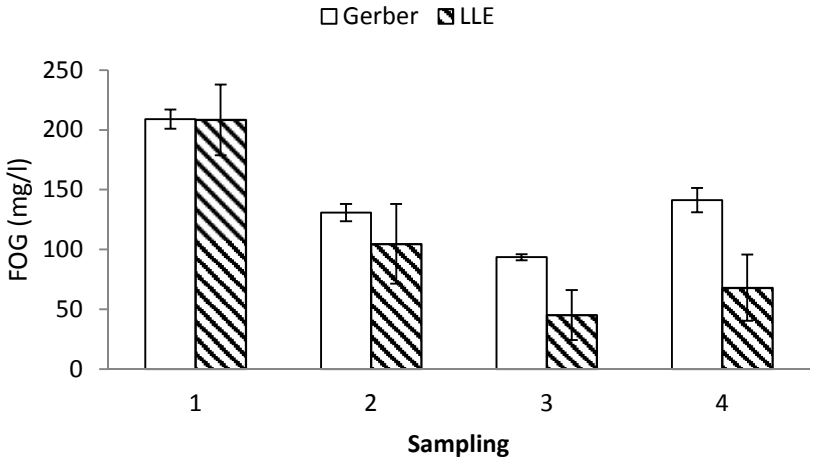

Figure 2-7 Comparison of the Gerber method and LLE for real FSE wastewater. Bars represent means of triplicate measurements with error bars as standard deviation.

Analysis of the combined Gerber and free-oil method in comparison to SPE for real FSE sink and dishwasher effluents revealed generally lower mean levels when using SPE compared to the combined method (Figure 2-8). In the case of the sink the combined method indicated that the FOG levels varied between 237 ± 18 mg/l to 2023 ± 441 mg/l compared to 268 ± 57 and 1640 ± 1242 mg/l for the SPE method. Although the combined method detected more mean FOG in four of the six samples, variability between triplicates was high, with most of the standard deviation associated with the free oil fraction which varied between 83 ± 23 mg/l (sample A) and 1569 ± 462 mg/l (sample D). A marked difference in performance was observed in samples from the dishwasher, from which the combined method extracted significantly more FOG than SPE (between 50 and 300%) from all eight samples, with recorded values ranging from 38 ± 4 to 477 ± 64 mg/l; and 24 ± 4 to 304 ± 44 mg/l respectively. Sample C revealed higher levels than SPE at 38 ± 4 and 24 ± 4 mg/l respectively despite being below the emulsified method LOQ of 60 mg/l. Enhanced performance in the dishwasher samples is likely associated with the higher degree of emulsification: free oil was only detected in two of the eight samples at 21 and 9% of total.

Comparison between the two real wastewater sources illustrated in Figure 2-8 revealed that the major difference in the FOG levels were due to differences in the free oil fraction with emulsified levels remaining similar. To illustrate, the emulsified oil concentration in the sink varied between 124 ± 39 mg/l and 454 ± 79 mg/l compared to between 38 ± 4 mg/l and 432 ± 36 mg/l for the dishwasher. The current data is consistent with previously reported ranges of between 256 and 1485 mg/l measured across four FSEs using SPE (Barton, 2012), 15 - 256 mg/l for a range of FSEs (Converse et al, 1984), and 730 - 1310 mg/l for four different FSE cuisines (Stoll and Gupta, 1997). However, the current data reveals the level of variation that can occur from sample to sample and due to differences in quantification method. Perhaps more importantly the dual method enables easy separation of free and emulsified oil levels in samples, which enables better understanding in relation to treatment in passive gravity separators or with biological additions that can convert the FOG to benign end products.

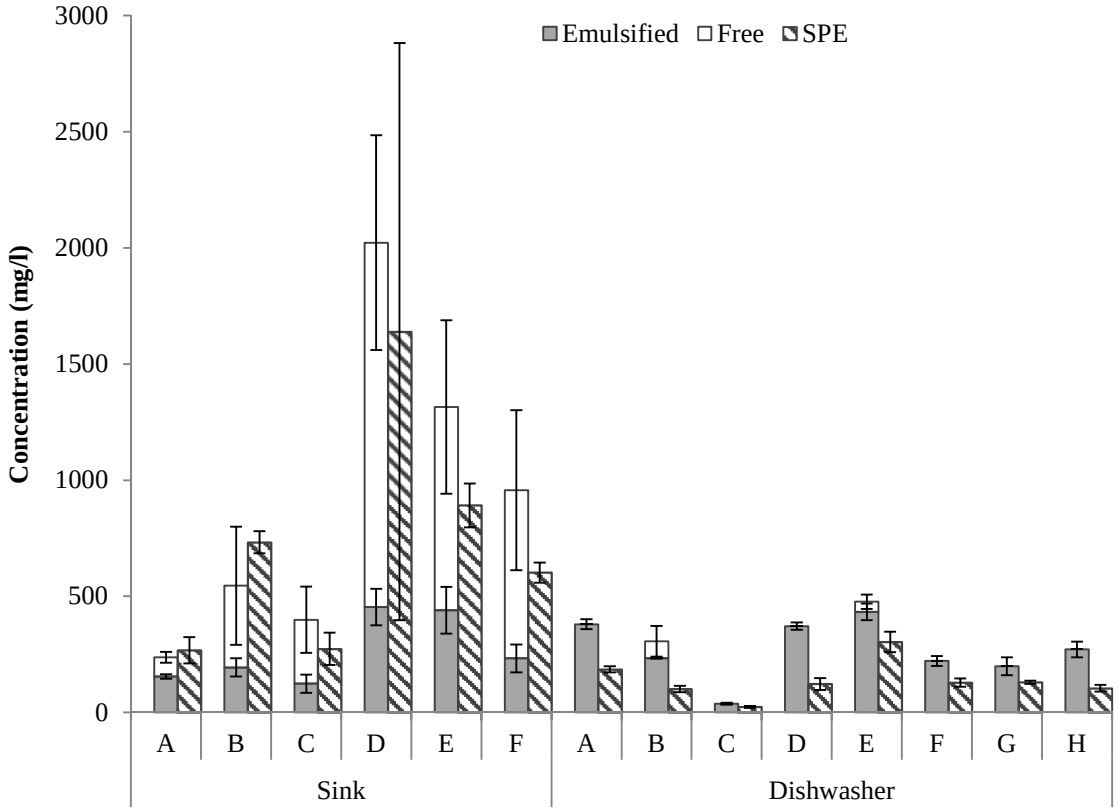

Figure 2-8 Comparison of the dual Gerber extraction method of free and emulsified FOG; and solid phase extraction (SPE) for 6 real sink, and 8 dishwasher FSE wastewater samples collected over 1 month. Data are presented as means of triplicate grab samples with error bars indicating standard deviation.

2.5 Conclusion

The development of a modified Gerber method for FOG measurements in FSE wastewater, coupled with a free oil pre-measurement, has been demonstrated to enable more consistent FOG recovery levels than typically observed in the current standard methods. Importantly, the addition of a casein precipitation stage has enabled application to non-dairy systems and negated the impact of surfactant on the reliability of FOG measurement in FSE wastewaters experienced in the other methods. Whilst the LOQ of the new method is higher

than standard liquid-liquid extraction techniques it has both excellent recovery and precision (95 ± 4% for emulsified oil and 97 ± 6% for free oil) down to below the 100 mg/l FOG level making it suitable for discharge monitoring. The technique is simple, inexpensive and rapid in comparison to standard methods enabling more consistent sampling to be undertaken. Furthermore, the simple separation of free and emulsified oil contents proposed in this method provides an opportunity to greatly enhance insights into management options and support innovation in the sector.

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3 A characterisation of food service establishment wastewater

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Abstract

Fat, oil and grease (FOG) contained in food service establishments' wastewater have been identified as major contributors to sewer blockages. FOG management in commercial kitchens relies on physical and/or biological treatments to remove FOG from wastewater streams. Optimisation for both kind of processes is influenced by wastewater (WW) composition and environmental conditions including temperature, pH, and oil droplets characteristics. A comprehensive characterisation of wastewaters from three food service establishments was undertaken to generate data to evaluate appropriate FOG management strategies. Two novel analytical methods were used to quantify the proportion of emulsified FOG and associated droplet size. On average 67% of FOG was emulsified with droplet sizes less than 100 µm, well below the removal capabilities of conventional grease interceptors but easily removed using biological means. From the WW composition results, a formula for predictive modelling was derived to represent average organic matter composition for kitchen wastewater as $C_{20}H_{38}O_{10}N$. Additional detailed chemical data were assembled for supporting future research in biodegradation experiments to reflect more realistic conditions than those used in previous studies.

3.1 Introduction

Effluent discharges from commercial food service establishments have been identified as major contributors to sewer blockages. Fat, oil and grease (FOG) undergoes chemical reactions in the sewer network leading to the build-up of hardened, saponified deposits. It has been estimated that FOG deposits contribute to around 80% of 370,000 sewer blockages annually in the UK, with sewer clearance works costing Water Utilities (and their customers) around £100 million (Water UK, 2018). Current FOG management practices include 'at source' treatment in the form of physical separation through grease interceptor (GI) or separator devices and/or the use of biological additives to degrade FOG into benign end-products. Previous studies

on food service establishment (FSE) wastewater composition have been driven by an interest in either physical or biological remediation. In both processes, particle size plays a pivotal role in their remediation potential. However, this parameter has hardly ever been considered in previous works. A detailed classification of oil-water emulsions present in oily wastewater has been provided by the American Petroleum Institute (1990). This classification, as reported in **Error! Reference source not found.**, defines particle size, emulsion stability and separation time under gravity for four main categories of particle ranging between 1 and 150 µm. According to this classification conventional gravity separators will only effectively remove oil droplets greater than 150 µm (American Petroleum Institute, 1990). For example, kitchen clean-up practices often include a dishwasher or an automatic self-washing appliance. The WW discharged from these appliances has been identified as particularly challenging for remediation processes based on physical separation as surfactants, high temperature/pH, and mechanical agitation will yield emulsions with mean droplet sizes less than 20 µm, classified as chemically stable oil-water emulsions (Chan 2010; Chung & Young 2013). Moreover, it is also likely that peak discharges of hot dishwasher wastewater in separators will entrain previously separated FOG into GI effluent. On the other hand, emulsified lipids are much more bioavailable to microorganisms. Therefore, a combination of the two processes could potentially enhance the overall FOG removal by exploiting the different remediation mechanisms. The different droplet size groups found in FSE WW and their potential for remediation are summarised in **Error! Reference source not found.**.

Comprehensive data on FSE WW characteristics is sparse. Kommalapati (2001) reported this lack of information 15 years ago, at the time, he identified the need for data from real high strength wastewater for the design of novel treatment systems. The majority of previous studies report only on BOD, COD, FOG, suspended solids and pH. A selection of data from the most comprehensive studies are given alongside results for this study in Table 3-4. Nutrient concentrations have rarely been investigated despite their crucial importance in organic matter biodegradation. Nitrogen (N) was reported in Wang et al. (2005), and N and phosphorous (P) in Nisola et al. (2009), although neither paper state the sampling point and therefore whether the effluent was mixed with sewage. Chung & Young (2013) recorded significantly

lower N concentrations in dishwasher effluent (3.8 ± 1.6 mg/l), compared to 30 – 100 mg/l in domestic wastewater (Henze et al., 2008). No comprehensive data has been found on trace elements. Protein and carbohydrate concentrations are reported in only one study (He et al., 2012) despite their potential impact on FOG degradation by providing a preferable substrate source (Eltaweel et al. 2005; Ertuğrul et al. 2007; Takaç & Marul 2008).

Table 3-1 Classification of oil in water emulsions according to their droplets size for removal or remediation. ***Modified from Bande et al.(2008), American Petroleum Institute (1990) and Brakstad et al.(2015)***

Droplets Size (µm)	Emulsion type	Emulsion Stability	Separation Time	Bio-degradation Rate
≥150	Free oil - Low miscibility with water, rapidly rises to the surfaces	Macro emulsion Weak	≤10 minutes	√
20-150	Dispersed or mechanically emulsified oil – Produced by the water flow and stabilised by electrical charges	Micro-emulsion Moderate	Hours	√√
1-20	Chemically emulsified oil - Miscible with water, stabilized by surfactants.	Micro-emulsion Strong	Days	√√√
≤1 (colloidal)	Dissolved oil - water is translucent and transparent. Removal by filtration, gravity settling is impossible.	Very Strong	Weeks	

Finally, no data on detergent surfactant concentrations was found in the literature reviewed, despite their potential influence on FOG emulsification and droplet size (Chan, 2010; Chung and Young, 2013). This study aims to address this gap by presenting unique insight into the degree of FOG emulsification generated by different washing-up techniques using two novel analytical methods. The modified Gerber FOG quantification method described in Chapter 2 distinguishes between free and emulsified FOG; and provides insight into the relative proportions of easily-separable FOG, which is amenable to removal in GIs, and emulsified FOG that may require alternative techniques such as bioremediation. A novel optical-based method developed at Cranfield University (Fairley, 2017) was employed in tandem to measure

FOG droplet size. The results presented in this work will provide a greater understanding of key environmental influences on FOG management strategies with respect to both physical and biological remediation.

3.2 Materials and methods

All reagents used were purchased from Fisher Scientific UK unless otherwise stated.

3.2.1 Sampling procedure

Wastewater grab samples were collected from 3 FSEs on Cranfield University Campus, UK serving traditional cafeteria-style food. In each location, washing-up procedures included a potwash sink for manual cleaning of cooking equipment; and a commercial dishwasher for food service items which require thermal sanitisation. The dishwashers investigated typically operated on a two-minute wash cycle, during which detergent and water at approximately 60ºC is pumped upwards through nozzles to blast food debris from items loaded on to racks. At the end of each cycle 3 litres of clean water, at greater than 82ºC, are sprayed from above to rinse and achieve heat sterilisation, and approximately 3 litres of the wash water are discharged. Wash water is recirculated continuously until the entire wash tank (30 – 40 L) is discharged at the shift end. Ware-washing procedures for the two dishwashers investigated differed. In one establishment items were loaded on to racks and rinsed with a flexible hose situated over a sink to remove bulk debris prior to loading into the dishwasher. WW samples were taken from an access point in a pipe downstream of the pre-rinse sink and dishwasher, and the timing manipulated in order to capture both end of dishwasher cycle discharge and pre-rinse water from the sink. Henceforth these samples are identified as ‘mixed effluent’ (ME). The dishwasher in the second FSE was simply loaded with dirty items without prior cleaning: grab samples were taken directly from the wash tank. Similarly grab samples were taken from the potwash sink in the third FSE.

Initially 8 sets of samples were taken from each of the 3 FSE sampling points – potwash sink; dishwasher tank; and mixed effluent from dishwasher and pre-rinse sink, over a two month period (summarised in Table 3-2) and analysed for a

comprehensive range of characteristics summarised in Table 3-3. Seven additional samples were tested for BOD_5 and COD to estimate biodegradability potential. Temperature was measured at source by a commercial digital temperature probe. In total six grab samples of approximately 400 ml were taken for each sampling event in glass bottles with PTFE-lined lids, 3 for FOG quantification and 3 for the other characteristics. Samples were transported to the lab for preparation within ten minutes and preserved according to relevant Best Practice (BSI Standards, 2012).

A second sampling campaign focussed on characterising FOG with respect to quantifying the fractions of free and emulsified FOG using the novel modified Gerber method, together with emulsified FOG particle size distribution, pH and surfactants. Eight sample sets (each comprising of 7 separate grab samples 3 for FOG; 3 for FOG droplet size; and 1 for pH and surfactants) were taken from the same potwash sink, and the same dishwasher (minus the pre-rinse water) as the mixed effluent described previously. FOG and particle size analysis was initiated within 20 minutes of sampling. To avoid confusion, the second datasets are referred to as sink 2, and DW2 (Table 3-2).

Table 3-2 Summary of locations, effluent source and characteristics analysed for two FSE wastewater sampling campaigns undertaken on Cranfield University Campus

Location	Effluent source	Effluent notation	Characteristics analysed
Sampling campaign 1			
FSE 1	Dishwasher and pre-rinse mixed effluent	ME	See Table 3-3
FSE 2	Dishwasher tank	DW1	See Table 3-3
FSE 3	Potwash sink	Sink 1	See Table 3-3
Sampling campaign 2			
FSE 1	Dishwasher tank	DW2	FOG by modified Gerber method, droplet size, pH and surfactants
FSE 3	Potwash sink	Sink 2	FOG by modified Gerber method, droplet size, pH and surfactants

3.2.2 FSE WW analysis

All of the first sample sets were analysed for the following: chemical oxygen demand (COD), suspended solids (SS), dissolved oxygen (DO), temperature, pH, FOG content, free fatty acids, ammonia, total nitrogen, total phosphorous and phosphate, surfactants, carbohydrates, micronutrients and heavy metals. A summary of the methodologies used, and their references are reported in Table 3-3.

3.2.3 FOG quantification by the modified Gerber method

FSE WW contains high concentrations of detergents. Traditional solvent-based extractions for FOG will produce high errors (as reported in Chapter 2). A modification of the Gerber method, employed for fat quantification in the dairy industry, was used for FOG quantification (Gurd et al., 2018). In addition to total FOG quantification, this method allows free FOG floating on the surface, and emulsified FOG to be measured separately. In brief, a disc of oil-adsorbent material was floated on the WW surface and sample bottles placed in a hot box at 50°C for 30 minutes to keep solid fats melted. Adsorbed FOG was eluted from the discs with hexane and determined by gravimetric analysis. Remaining emulsified FOG, and that bound to suspended solids, was extracted by the addition of sodium caseinate which adsorbs to the emulsified droplets and induces coagulation-flocculation. FOG was isolated by pH induced co-precipitation and centrifugation, and re-extracted from the solid sediment by a modification to the Gerber milk fat determination method (BSI ISO 2446:2008).

3.2.4 FOG droplet size

Particle size distributions were determined by optical-based sizing conducted on a Flowcam VSI – Portable, with external syringe pump (Meritics, Dunstable,UK). FOG droplets were distinguished from other particles of food debris through an image processing filter based on defined criteria for circle fit, transparency and edge gradient (Fairley, 2017).

Table 3-3 Characteristics and analytical methods for FSE wastewater samples from a potwash sink, dishwasher, and mixed effluent from a dishwasher and pre-rinse sink. All measurements were performed in triplicate

Characteristic	Method	Reference
FOG as hexane extractable material (HEM)	Solid phase extraction (SPE) with Empore Oil and Grease discs and n-hexane extraction gravimetric analysis.	EPA 1664B, 1999
Free fatty acids	Resolvation of HEM residue in propanol for microtitration with potassium hydroxide to phenolphthalein end point	Rockwood et al. (1947)
Suspended solids (SS)	Vacuum filtration through a 0.45µm Whatman filter paper, and gravimetric analysis	(APHA 1989)
Chemical oxygen demand (COD) total; and soluble from SS filtrate ammonia (NH_4) total nitrogen (TN) phosphate (PO_4) total phosphorous (TP) surfactants – anionic, nonionic, cationic	Spectroquant cell test kits measured with a NOVA 60 A Spectroquant photometer	Spectroquant® NOVA 60
Carbohydrate	DuBois phenol-sulphuric acid method (glucose as standard)	Dubois et al. (1956)
Micronutrients: Na, K, Ca, Mg, Mn, Zn, Mo, Co, Cu	Inductively coupled plasma mass spectrometry (ICP-MS) on a dual channel Perkin Elmer NexION 350D ICP-MS following microwave digestion (MarsXpress system)	Eduok et al. (2017)
BOD_5	According to BS EN 1899-1:1998.	British Standards Institute (1998)

3.3 Results and discussion

Results are in broad agreement with data from previous studies where available, as presented in Table 3-4. Suspended solids, were generally higher than literature values but this may reflect sampling directly at source whereas some sedimentation may have occurred in the pipework in previous studies. FFA content - mean 1.38 wt% as oleic acid, was similar to the 1 – 2% in waste cooking oil (Montefrio et al., 2010), indicating that cooking had effected a degree of lipid thermal hydrolysis.

A notable difference in pH was observed between wastewater from the potwash sink (6.8 – 7.3) and the two dishwasher sources (10.5 – 12.0) due to the high concentrations of sodium hydroxide in dishwasher detergent. Previous studies found typically neutral pHs, apart from values up to 10.4 and 11.7 in Ducoste et al. (2008) and He et al. (2012) respectively, and 10.6 - 11.7 specifically from dishwasher effluent in Chung & Young (2013). Anionic surfactants were extremely high in the potwash sink (120 – 820 mg/l) compared to dishwasher effluents (12 – 80 mg/l) as a reflection of controlled automatic dishwasher detergent dosing, versus manual application in the sink. Both ranges are considerably higher than in domestic discharges: 59 ± 41 mg/l and 11.1 mg/l for a kitchen sink and dishwasher respectively (Friedler, 2004).

Domestic sewage is typically rich in ammonia (NH_4) with concentrations of 20 – 75 mg/l contributing to a range of 30 – 100 mg/l for total nitrogen (Henze et al., 2008). Whilst total nitrogen (TN) in the FSE effluents was similar (24 – 79 mg/l), NH_4 concentrations were considerably lower: ranging from 0.7 – 7.4 mg-N/l suggesting that the majority of nitrogen is derived from organic material. Total COD tends to considerably higher concentrations (3188 ± 1192 mg/l) than ranges in municipal sewage of 500 – 1200 mg/l (Henze et al., 2008), and at the upper end of ranges, or exceeding those previously determined in FSE WW. Protein concentrations were estimated from the difference between TN and NH_4 multiplied by a standard nitrogen conversion factor of 6.25. Results reported in Figure 3-1 demonstrate that concentrations of FOG (439 ± 272 mg/l); carbohydrate (840 ± 780 mg/l); and protein (266 ± 84 mg/l) were highly variable both between and within the different effluent datasets. In general carbohydrates constituted the largest organic fraction in almost all samples apart from the potwash sink where FOG dominated.

Trace element concentrations, presented in Table 3-5, indicate considerable variability in sodium (Na) and potassium (K) as a reflection of sample source. Elevated Na in both dishwasher sources (572 ± 388 mg/l) compared to the sink (75 ± 30 mg/l) reflected sodium hydroxide in both dishwasher detergents. Similarly, higher K in the mixed effluent (219 ± 335 mg/l), relative to 19.4 ± 8.4 mg/l and 17.2 ± 7.5 mg/l in the sink and dishwasher respectively, was due to potassium hydroxide in that particular detergent. Relatively lower calcium concentrations in the dishwasher samples (13.6 ± 12.2 mg/l), reflected employment of a water softener system which may also have contributed to high Na. Based on theoretical microbial nutrient requirements suggested by Burgess et al. (1999), all samples were severely deficient in Cobalt (Co) and Molybdenum (Mo); and some samples deficient in manganese (Mn) and zinc (Zn). Phosphorous (P) is also an essential macronutrient and is typically required for biomass synthesis, together with nitrogen, in a N:P ratio of 5:1. A mean ratio of 4 :1 for the FSE WW suggests that most samples might also be deficient in phosphorus.

Table 3-4 Wastewater characteristics from three FSEs (n = 8) on Cranfield University campus (this study) in comparison to previous work. Concentrations in mg/l.

Source	Sample point	Temp (°C)	DO	pH	SS	FOG	FFAs (wt%)	BOD	sCOD	tCOD	NH_4-N	TN	PO_4-P	TP	Surfactants (mg/l)			Protein	Carbo-hydrate	Reference
															Anionic (MBAS)	Non-ionic (Triton X-100)	Cat-ionic			
University campus restaurant	Potwash sink	31 - 46	7.4 - 8.7	6.8 - 7.3	217 - 1791	253 - 1067	0.65 – 4.62	856	530 - 2685	1482 - 5363	0.8 - 7.4	26 - 79	3.8 - 12.5	4.4 - 17.1	121 - 817	6.2 - 39.8	0.09 - 0.56	158 - 477	202 - 961	This study
University campus café	Dishwasher	47 - 57	2.0 - 6.1	11.3 - 11.8	261 - 1094	89 – 850	0.84 – 1.27	1193 - 2247	717 - 3647	2305 - 4952	1.2 - 5.0	32 - 63	4.6 - 15.3	8.8 - 17.5	29 - 80	12.0 - 26.0	0.10 - 0.51	193 - 363	699 - 3997	
University campus cafe	Mixed effluent - pre-rinse & dishwasher	37 - 55	5.6 - 7.0	10.5 - 12.0	272 - 912	112 - 494	0.83 – 2.70	598 - 1399	309 - 1790	1250 - 3847	0.7 - 3.4	24 - 65	4.7 - 10.7	8.0 - 28.9	12.0 - 16.0	9.1 - 12.3		146 - 401	108 - 735	
Chinese restaurant	Drain entrance			6.6 - 8.0	13.2 - 246	120 - 172		58 - 1430		292 - 3390										(Chen et al., 2000)
Western restaurant	Drain entrance			6.9 - 9.5	152 - 545	52.6 - 2100		489 - 1410		912 - 3500										
American fast-food	Drain entrance			6.3 - 7.2	68 – 345	158 - 799		405 - 2240		980 - 4240										
Student canteen	Drain entrance			6.8 – 8.8	124 - 1320	415 - 1970		545 - 1630		900 - 3250										
UC bistro	Drain entrance			6.0 - 8.2	359 - 567	140 - 410		451 - 704		1500-1760										
Restaurant	Kitchen sink							536		936	4.6			15.6						(Surendran & Wheatley, 1998)
Tubitak restaurant	Not stated			6.8 - 8.3	140 - 2060	102 - 2650				305 - 3325										(Gunes, 2007)

O Barbecue restaurant	Not stated	15.6 ± 0.3		6.9 ± 0.5	1260 ± 61	894 ± 37			4280 ± 50.7		121.± 3.1	16.2 ± 0.4					(Nisola et al., 2009)
Restaurant	Sewer			6.0 - 6.2	270 - 450	250 - 520			2700 - 3000		68 - 98 (TKN)						(Wang et al., 2005)
Restaurant	Dishwasher			10.6 - 11.7	43 - 506	173 - 650			456 - 1588	0.16 - 0.76	1.63 - 5.74	7.8 - 10.8					(Chung and Young, 2013)
Various restaurants	Grease traps			6.6	1350	921			1400	7.56	14	4.33					(Shon et al., 2002))
Retirement home	GI influent	20 - 60	1.3 - 7.1	5.2 - 10.5		6 - 13620		1000 - 2000	3000 - 4000						231 ± 653	608 ± 827	(He et al., 2012)
Restaurant	GI influent	20 - 52	3.0 – 8.2	4.9 – 11.7											255 ± 641	470 ± 888	
Thai and Japanese restaurant	Not stated			6.7	1380	825	7400	21300									(Stoll and Gupta, 1997)
Western restaurant	Not stated			7	1100	1310	9200	20900									
Thai and western	Not stated			7.3	2160	730	6000	20900									
Asian Cafateria	Not stated			7.1	2400	1110	3330	8100									
Chinese restaurant	Drain entrance			6.1-8.0	250 - 650	500 - 4700	600 - 2500	750 - 5800									(Kang et al., 2011)
Restaurant/ hotel	Drain entrance			5 - 8.1	2 - 2800	0 - 3800	3 - 1600	4 - 410									(Nakajima et al., 1999)
Restaurant	Drain entrance			5.6 - 6.4	146 - 268	66 - 132	39 2-705	870 - 1445									(Converse et al., 1997)
Restaurant	Drain entrance			6.6 - 7.0	41 - 92	34 - 49	109 - 316	559 - 723									
Restaurant	Drain entrance			5.8 - 6.3	248 - 582	89 - 256	670 - 1058	963 - 2414									

Restaurant	Drain entrance		5.7 - 6.8	115 - 439	71 - 140	155 - 532	444 - 1070						
Bar/Grill	Drain entrance		6.0 - 7.0	42 - 132	2.9 - 96	104 - 218	424 - 495						
Restaurant	Sink	27		720		1460		6	74	31	74		(Siegrist *et al.* 1976)
Restaurant	Dish-washer	38		440		1040		4.5	40	32	68		

DO – dissolved oxygen; SS – suspended solids; FFAs – free fatty acids; BOD_5 – 5-day biological oxygen demand; $_sCOD$ – soluble chemical oxygen demand; $_tCOD$ – total chemical oxygen demand; NH_4-N – ammoniacal nitrogen; TN – total nitrogen; PO_4-P – phosphate (as phosphorous); TP – total phosphorous

Table 3-5 Trace elements in wastewater from three FSEs on Cranfield University Campus (n= 8) compared to literature data for domestic kitchen wastewater

Source	Na	K	Ca	Mg	Pb	Cr	Fe	Ni	Mn	Zn	Cu	Co	Mo
	mg/L				µg/l								
Sink	75.0 ± 29.7	19.4 ± 8.4	43.7 ± 2.4	5.97 ± 0.57					13.3 ± 12.4	91.1 ± 62.1	27.0 ± 6.9	0.488 ± 0.073	0.614 ± 0.465
Mixed effluent	430 ± 293	219 ± 335	45.2 ± 6.7	5.58 ± 0.57					15.1 ± 5.1	60.3 ± 20.3	36.4 ± 5.6	0.480 ± 0.065	0.719 ± 0.372
Dishwasher	714 ± 436	17.2 ± 7.5	13.6 ± 12.2	2.57 ± 1.43					20.5 ± 18.9	94.0 ± 61.9	24.7 ± 11.7	0.455 ± 0.114	0.869 ± 0.509
Domestic kitchen WW (Eriksson et al., 2002)	29 -180	19 - 59	13 - 30	3.3 - 7.3	62 - 140	72 - 130	600 - 1200	<25	31 - 75	120 - 1800	68 - 260	<0.12	

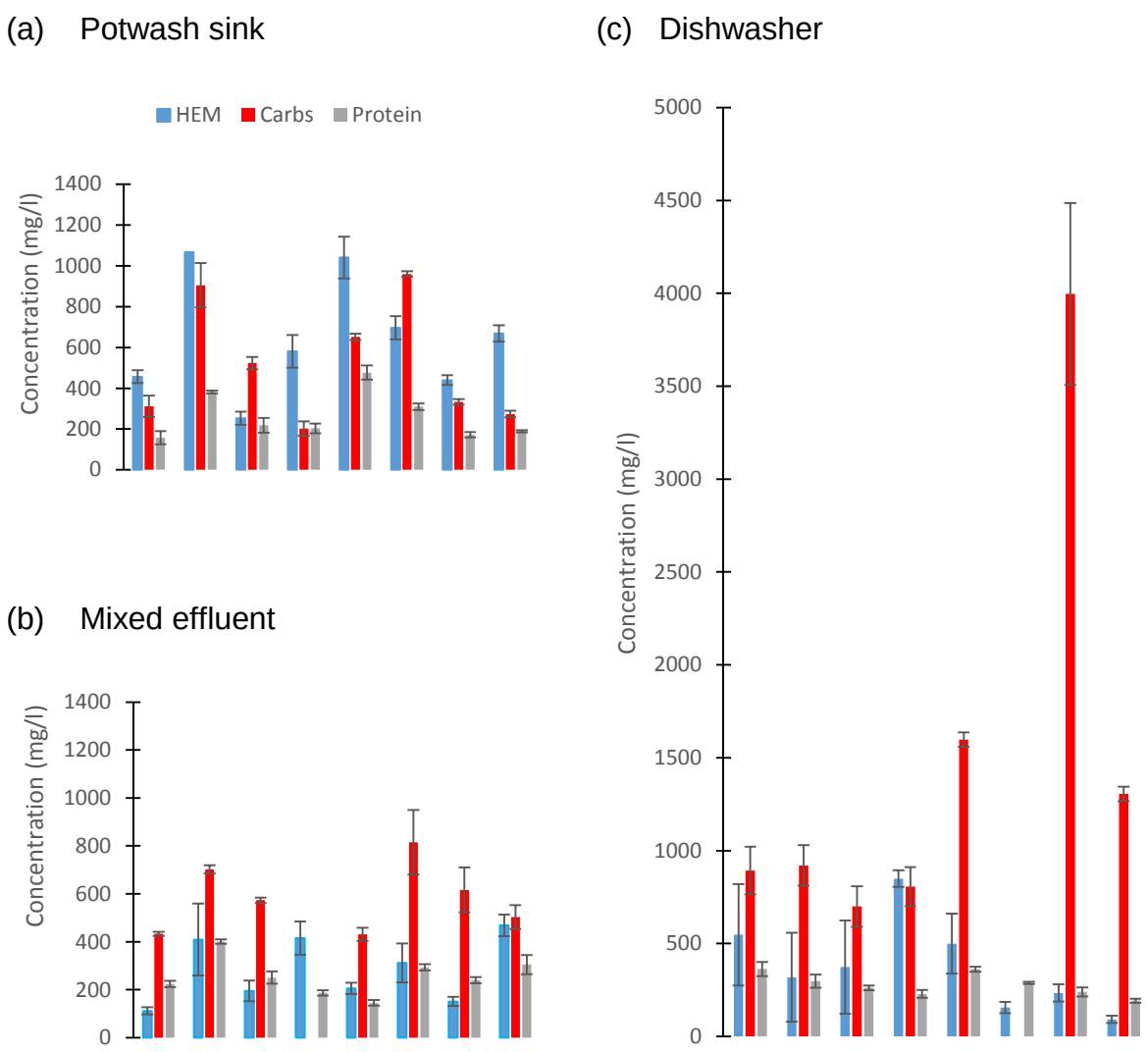

Figure 3-1 Fractions of FOG (as HEM), carbohydrate and protein (estimated from TN) in wastewater from a potwash sink (a), mixed effluent (b) and a dishwasher (c) from three FSEs on Cranfield University Campus. Carbohydrate measurements for samples 4 and 6 in (b) and (c) respectively were not obtained. Data bars represent means of triplicate measurements with error bars as standard deviation.

3.3.1 Biodegradability potential

Values of Biological Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD) and the measured wastewater composition were used to predict the water biodegradability of the FSE WW samples in different ways. Firstly, through the traditional method of the BOD/COD ratio which compares the amount of organic material which can be oxidised by biological processes, typically over 5 days – BOD_5, with the total amount

of material (organic and inorganic) which can be chemically oxidised, such that high ratios reflect easily biodegradable compositions. Secondly, through the COD:N ratio which estimates the relative proportions of carbon and nitrogen available for assimilation into new biomass. Low ratios tend to leave excess nitrogen in the wastewater once all the carbon has been removed, while high ratios will likely result in poor carbon degradation. Thirdly, a molecular formula for wastewater organic material was estimated from individual measurements of FOG, protein and carbohydrate which allowed an assessment of the stochiometric oxidation reactions involved with biological degradation.

3.3.1.1 BOD_5/COD ratio

WW biodegradability is often estimated by the ratio of these two values as BOD/COD. WW with a BOD/COD ratio >0.5 is considered to be easily treatable by biological processes, whereas values <0.3 suggest the presence either of organic matter that is hard to biodegrade, or of toxic substances inhibiting the microbial activities (Tchobanoglous *et al.*, 1991). A mean of 0.42 ± 0.09, comparable to those reported in other studies (Table 3-4), suggests that the samples lay within the moderately biodegradable range. However, limitations of this method are that some organics, such as FOG, may not be biologically degraded under the conditions of the BOD_5 test (5 days at 20ºC). Thus, the ratio may not reflect the potential for FOG removal.

3.3.1.2 COD:N ratio

The use of COD value to represent the bioavailability of mixed organic matter (OM) is preferred over the C content, so as to reflect variations in oxidation state of different substrates and thus the number of electrons available for donation. For example carbohydrate, protein and FOG have COD equivalences of 1.07, 1.5, and 2.88 mgCOD/mg-substrate respectively (Grady et al., 2011), yet contain on average 44, 53 and 77% carbon (Burnham, 2010) yielding COD:C ratios of 2.4, 2.8 and 3.7 respectively in accordance with degree of reduction. A benchmark COD:N ratio of 20:1 is generally considered optimum for biological treatment of domestic WW. The ratio of COD:N for the FSE WW samples was 63 ± 13 for the sink and mixed effluents, and higher at 85 ± 18 for dishwasher discharges, suggesting a significant N deficiency in all samples. However, studies have shown that when substrate carbon is in excess of

growth requirements (high COD:N ratios), microbial metabolic processes shifts towards surplus energy production, thus cell yields are reduced and higher COD:N ratios can be accommodated (Dauner et al., 2001; Egli and Quayle, 1986).

3.3.1.3 Stoichiometry and bioenergetics of microbial metabolism

Knowledge of the organic matter (OM) composition with respect to the major organic fractions, allowed a more detailed evaluation of the FSE WW biodegradability. In our samples, on average carbohydrates constituted the largest organic fraction (54%), followed by FOG (29%), whilst proteinaceous material contributed to only 17% of OM. Molar fractions of C, H, O and N were estimated for each sample using standard molecular formulae for protein ($C_{16}H_{24}O_5N_4$), carbohydrate (CH_2O), and FOG ($C_8H_{16}O$) (Grady et al., 2011) to derive a stoichiometric formula of $C_{100}H_{190\pm3}O_{50\pm15}N_{4.9\pm1.4}$ which simplifies to $C_{20}H_{38}O_{10}N$. indicating a relatively high carbon to nitrogen (C:N) ratio of 20 as compared to commonly used domestic WW compositions. One such formula, $C_{10}H_{19}O_3N$, reflects a protein-rich composition (Gray, 2004), whilst the composition used in activated sludge models (ASM), $C_{18}H_{19}O_9N$, derives from roughly equal proportions of protein, carbohydrate and fat (Henze et al., 2001).

The principle of biodegradation is that organic matter acts as an electron donor such that electrons are partitioned between a terminal electron acceptor (oxygen in the case of aerobic respiration) to generate energy for biomass synthesis, and the remainder incorporated into the biomass. Thus, reduced substrates contain more electrons and can generate more energy. As all substrate carbon and electrons end up in new cells (anabolism/synthesis) or released as CO_2 (catabolism/respiration), the relationship between energy production and cell yield (Y) can be expressed by combining redox half-reaction equations. Following the approach developed by Rittmann and McCarty (2001) for wastewater treatment modelling, substrate utilisation for both energy and growth can be expressed by combining half-reaction equations for substrate electron donors (R_d) and acceptors - oxygen (R_a) and new cell material (R_c) according to Equation 3-1.

$R = f_e^* R_a + f_s^* R_c - R_d$ **Equation 3-1**

Where f_e and f_s are the fraction of substrate electron equivalents partitioned into energy and synthesis respectively such that f_s represents Y on an electron equivalent basis.

In treatment terms, the maximum rate of oxidation occurs during maximum biological growth. Under balanced nutritional conditions catabolism and anabolism are tightly coupled such that cell growth yield is near to the maximum theoretical value (Y_{max}). However, in practice, observed yields (Y_{obs}) are usually lower than Y_{max} as carbon energy is also required for non-growth associated processes. Standard half-reaction equations for the FSE WW and associated literature data are presented in Table 3-6, together with the calculations derived therefrom in this study.

A half-reaction for OM in FSE wastewater was derived from the generic formula provided in Araujo Granda et al., (2016) (Equation 3-2), and is expressed in **Equation 3-3**.

$$\frac{(n-c)}{d}\ CO_2 + \frac{c}{d}\ NH_4^+ + \frac{c}{d}\ HCO_3^- + H^+ + e^- = \frac{1}{d} C_n H_a O_b N_c + \frac{(2n-b+c)}{d}\ H_2O$$ **Equation 3-2**

Where $C_nH_aO_bN_c$ represents organic substrate and d = (4n + a – 2b - 3c)

$$\frac{19}{95}\ CO_2 + \frac{1}{95}\ NH_4^+ + \frac{1}{95}\ HCO_3^- + H^+ + e^- = \frac{1}{95} C_{20} H_{38} O_{10} N + \frac{31}{95}\ H_2O$$ **Equation 3-3**

To investigate the potential impact of the high COD:N ratio on substrate uptake and growth, a maximum growth yield value of 0.67 for f_s (Gray, 2004), and the half reactions for oxygen and cell mass from Table 3-6 were substituted into Equation 3-1. The full reaction equation for growth in Equation 3-4, estimates that the maximum cell yield of 3.18 mmoles-cells/mmole-substrate would require an additional 2.18 mmoles

(30.5 mg/l) of nitrogen. Following the same calculation, the C_{10} and C_{18} domestic compositions would only require an additional 9.5 mg-N/l and 18.8 mg-N/l respectively, which is typically easily provided by available NH_4-N. In contrast the FSE WW only provided 2.7 ± 1.6 mgNH_4-N/l, thus maximum cell yield and carbon removal efficiency could not be achieved in wastewater with this composition without addition of nitrogen.

$$C_{20}H_{38}O_{10}N + 7.84O_2 + 2.18NH_4^+ + 2.18HCO_3^- => 3.18C_5H_7O_2N + 6.27CO_2 + 13.32H_2O$$ **Equation 3-4**

Nevertheless, microbial populations have the ability to adjust their metabolic processes in response to nutrient deficiency/carbon excess, by using more carbon to generate energy and less carbon, and thus less nitrogen, for biomass synthesis as cell yields decrease relative to carbon uptake (Dauner et al., 2001). The metabolic balance between growth and respiration at the point where nitrogen deficiency can theoretically be accommodated by available NH_4-N - 0.18 mmoles (2.52 mg-N/l) was calculated for when f_s = 0.25 (**Equation 3-5**), which represents a cell yield of 0.3 mgcells/mgsubstrate (Equation 3-6), and by mass balance, corresponds to a COD:N uptake ratio of 46:1.

$$C_{20}H_{38}O_{10}N + 17.81O_2 + 0.18NH_4^+ + 0.18HCO_3^- => 1.18C_5H_7O_2N + 14.25CO_2 + 15.31H_2O$$ **Equation 3-5**

$$\text{Yield} = \frac{\text{moles cells} * \text{MW}}{\text{moles substrate} * \text{MW}} = \frac{1.18 * 113}{1 * 452} = 0.30\ \text{g/g}$$ **Equation 3-6**

Table 3-6 Half reaction oxidation equations and associated data

	Molecular formula	Half-reaction	Theoretical oxygen demand (ThOD) (mg/mg substrate)	$f_{s\ max}$	Gibbs free energy ΔG half-reaction (kJ/e^-eq)
FSE WW	$C_{20}H_{38}O_{10}N$	$^{19}/_{95}\ CO_2 + {}^{1}/_{95}\ HCO_3^- + {}^{1}/_{95}\ NH_4^+ + H^+ + e^- \Rightarrow {}^{1}/_{95}\ C_{20}H_{38}O_{10}N + {}^{31}/_{95}\ H_2O$	1.68	0.67	36.08
Domestic WW	$C_{10}H_{19}O_3N$	$^{9}/_{50}\ CO_2 + {}^{1}/_{50}\ HCO_3^- + {}^{1}/_{50}\ NH_4^+ + H^+ + e^- \Rightarrow {}^{1}/_{50}\ C_{10}H_{19}O_3N + {}^{9}/_{25}\ H_2O$	1.99	0.67	31.80
Domestic WW	$C_{18}H_{19}O_9N$	$^{17}/_{70}\ CO_2 + {}^{1}/_{70}\ HCO_3^- + {}^{1}/_{70}\ NH_4^+ + H^+ + e^- \Rightarrow {}^{1}/_{70}\ C_{18}H_{19}O_9N + {}^{28}/_{70}\ H_2O$	1.42		32.0
Protein	$C_{16}H_{24}O_5N_4$	$^{8}/_{33}\ CO_2 + {}^{1}/_{50}\ HCO_3^- + {}^{2}/_{33}\ NH_4^+ + H^+ + e^- \Rightarrow {}^{1}/_{50}\ C_{16}H_{24}O_5N_4 + {}^{9}/_{25}\ H_2O$	1.5	0.64	32.22
Carbohydrate	CH_2O	$^{1}/_{4}\ CO_2 + H^+ + e^- \Rightarrow {}^{1}/_{4}\ CH_2O + {}^{1}/_{4}\ H_2O$	1.07	0.72	41.84
Fats and oils	$C_8H_{16}O$	$^{4}/_{23}\ CO_2 + H^+ + e^- \Rightarrow {}^{1}/_{46}\ C_8H_{16}O + {}^{15}/_{46}\ H_2O$	2.88	0.59	27.61
Oxygen (R_a)	O_2	$¼\ O_2 + H^+ + e^- \Rightarrow ½\ H_2O$			-78.14
Bacterial cells (R_c)	$C_5H_7O_2N$	$^{1}/_{5}\ CO_2 + {}^{1}/_{20}\ HCO_3^- + {}^{1}/_{20}\ NH_4^+ + H^+ + e^- \Rightarrow {}^{1}/_{20}\ C_5H_7O_2N + {}^{9}/_{20}\ H_2O$	1.42		

Data adapted from (Grady et al., 2011; Gray, 2004; Henze et al., 2008, 2001)

3.3.2 Physical properties of FOG

FOG concentration data from the second characterisation campaign are summarised in Table 3-7 indicating a clear difference in FOG fractionation between free and emulsified phases for the different effluents – sink 2 and DW2. Although emulsified FOG concentrations were similar (291 ± 90 and 309 ± 155 mg/l in the dishwasher and sink respectively), significantly more free FOG was observed in the sink samples (570 ± 481 mg/l), such that the emulsified fraction constituted 42 ± 16% of total compared to 94 ± 9% in DW2. FOG droplets were smaller in dishwasher samples (median diameter 18 ± 1 µm, mean max 67 µm) compared to the sink (median 27 ± 5 µm, mean max 98 µm) (

Figure 3-2). FOG becomes emulsified through the action of surfactants and mechanical agitation during washing-up – however total detergent surfactant concentrations were, as in the previous characterisation, considerably lower in DW2: 26 ± 8 mg/l compared to 217 ± 112 mg/l in sink 2 (Table 3-7), and thus could not account for the higher degree of emulsification in the former. It is most likely that a combination of high temperature and pH (11.58 ± 0.19), and constant mechanical agitation as wash water is recycled within the dishwasher over a period of hours, generated more stable emulsions associated with smaller FOG droplet sizes.

Table 3-7 Wastewater characteristics from a commercial dishwasher and potwash sink sourced from two FSEs on Cranfield University Campus. Data are means of 8 samples ± standard deviation

Characteristic		DW2	Sink 2
FOG (mg/l)	Free	22 ± 32	570 ± 481
	Emulsified	291 ± 90	309 ± 155
	Total	313 ± 92	879 ± 583
Surfactants (mg/l)	Anionic	2.1 ± 0.7	180 ± 98
	Non-ionic	24 ± 8	37 ± 19
pH		11.58 ± 0.19	6.76 ± 0.20
Median droplet size (µm)		18 ± 1	27 ± 5

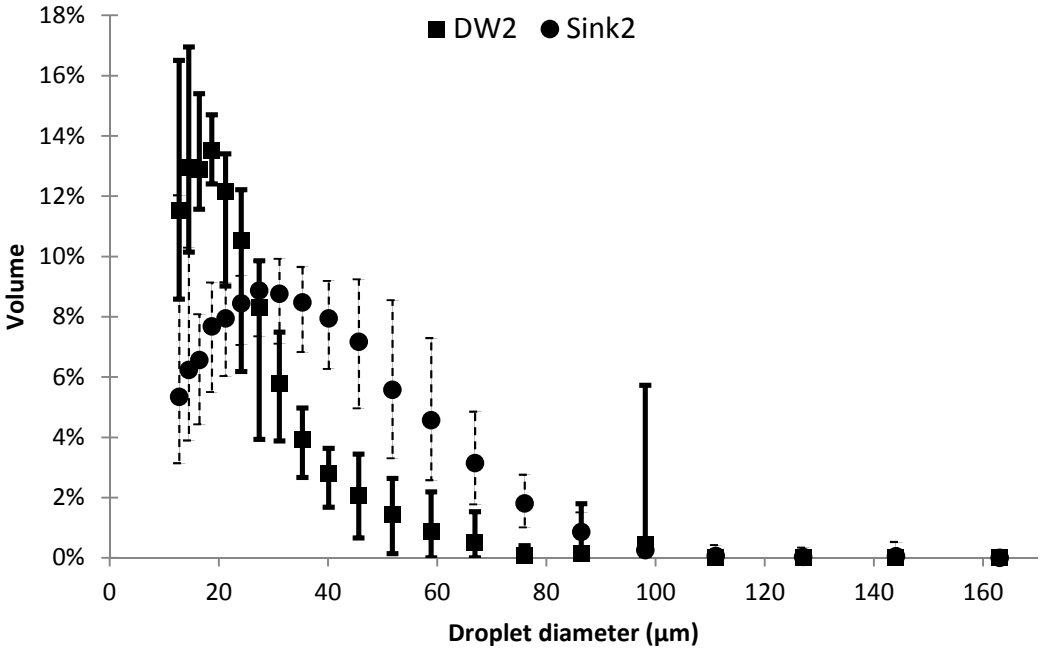

Figure 3-2 FOG droplet size distribution in sink and dishwasher effluents FOG droplet diameter, range 11.2 – 163 µm. Error bars represent ranges from triplicate measurements of 8 samples.

3.3.3 Implications for FOG management

Grease interceptors rely on the principle that FOG droplets coalesce and rise, allowing FOG to be skimmed from the surface. According to Stokes' Law, smaller particles have lower rise rates and remain in the aqueous phase for longer time periods. In this study, FOG quantification and droplet size determination were co-ordinated such that analyses were performed simultaneously for each sample. Thus, FOG droplet size (Figure 3-2) and the relative proportions of the free and emulsified fractions (Table 3-7) appear to be directly linked, representing a temporal 'snapshot' in a dynamic system. For example, smaller FOG droplets in dishwasher effluents reflect a more stable emulsion and mostly remain in suspension for, at least, the duration of the free oil extraction step of the Gerber analysis resulting in high emulsified fractions. These data reflect a sampling-to-measurement time of approximately 45 minutes for both procedures, and is thus comparable to recommended minimum contact times of 30 minutes for passive grease separator design (Tchobanoglous et al., 1991). Only 7 ± 10% of total FOG resided on the surface in the dishwasher samples. Of the residual emulsified fraction more than 99% of droplets were smaller than 67 µm in diameter suggesting that minimal FOG is likely to be removed from dishwasher effluent by physical methods. Moreover, 99.7% of emulsified FOG droplets in all the sink samples were ≤ 98.1 µm: well below grease interceptor design capability of 150 µm (American Petroleum Institute, 1990), suggesting that around half of total sink FOG might also not be amenable to gravity separation. Based on mean emulsified FOG fractions of 291 mg/l and 309 mg/l for DW2 and sink 2 respectively, reliance on conventional grease separator devices may allow approximately 300 mg/l FOG to be discharged to the sewer network. In contrast biological FOG degradation is facilitated by emulsification which increases the available surface to volume ratio for microbial/enzyme attachment. However their ability to degrade FOG efficiently could be restricted by limitation in certain trace elements (Burgess et al., 1999), and macronutrients as suggested by high COD:N:P ratios of 100:1.9(±0.6):0.5(±0.2) demonstrating potential nitrogen and phosphorous limitation compared to the industry accepted optimum of 100:5:1

(Grady et al., 2011). The data suggests that grease separators are an ineffective means of managing FOG in dishwasher effluents but could easily remove nearly 60% (570 mg/l) of total FOG from sink wastewater. Thus, combining physical and biological treatments would not only allow significant amounts of FOG to be collected for energy recovery, but would reduce the carbon load for biological degradation. Theoretical oxygen demand (ThOD) was estimated for emulsified sink fractions, according to Equation 3-7, assuming mean values of 309 mg/l for FOG; protein (263 mg/l) and carbohydrate (520 mg/l) from the first characterisation together with associated theoretical OD conversion factors from Table 3-6. This allowed for an adjusted ThOD:N:P ratio of 100:3.4:0.7 to be estimated using mean TN (45mg/l) and TP (9.4 mg/l) from the first dataset, which suggests an increase in biodegradability potential in free FOG-separated sink wastewater.

$$ThOD = (FOG \times 2.88) + (protein \times 1.5) + (carbs \times 1.07) \quad \textbf{Equation 3-7}$$

Theoretically, total carbon removal is possible at high COD:N ratios in association with low microbial yields, a crucial determining factor will be whether microbial communities show a particular organic substrate preference, such that FOG removal might be inhibited by the presence of more easily-metabolised carbohydrates and protein. An additional challenge to microbial treatment is the highly alkaline conditions in excess of pH 11 effected by dishwasher detergents. Alternatively, more neutral formulations might be used.

3.4 Conclusions

FOG-rich wastewater from commercial food service establishments is a major contributor to sewer blockages, promoting ‘at source’ FOG management strategies. Wastewater samples were collected from three commercial food service establishments to reflect different washing-up strategies and analysed for a comprehensive range of characteristics which might influence both physical and biological treatments. In addition, the use of two novel analytical methods

were employed which allowed discrimination between emulsified and free surface FOG; and between emulsified FOG droplets and particles of food debris. FOG droplet size exerts a strong influence on removal strategies and is thus a pivotal factor for identifying the best removal technology. Smaller emulsified droplets are harder to remove by physical separation yet enhance microbial degradation due to their increased surface area. On average 94% of FOG in dishwasher effluents, and 42% in a potwash sink comprised of emulsified droplets with diameters below 100 µm, equating to a mean concentration of 300 mg/l and, therefore, is most unlikely to be removed by conventional grease separators devices. In addition, carbon/nutrient balances suggest that biological treatment efficiency might be limited by nitrogen, phosphorous and trace element deficiency. As such, combining physical and biological treatments in wastewater with high proportions of free FOG would allow easily-separable FOG to be physically removed, thereby reducing carbon loading and associated nutrient limitation on biological treatment.

The findings in this study provide useful information for evaluating the best FOG management strategies for FSE wastewater by demonstrating the difference in FOG loading and fractionation generated by different washing-up procedures. The proposed organic matter composition formula $C_{20}H_{38}O_{10}N$ can be used for predictive modelling of biological treatment processes, and the detailed chemical data for designing parameters for biodegradation experiments to reflect more realistic synthetic wastewater compositions than have been used previously.

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4 The impact of wastewater composition on microbial degradation of fat, oil and grease

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Abstract

In addition to high concentrations of fat, oil and grease (FOG), wastewater from food service establishments contains high quantities of carbohydrates and proteins, which could also contribute towards blockages in sewer networks. Carbohydrates and organic nitrogen are also known to impact on the ability of microorganisms to degrade complex organics, such as FOG. This can have obvious implications for their biological remediation of kitchen wastewater and hence FOG deposition in the sewage system. Microbial utilisation of varying concentrations of oil, carbohydrates and protein in synthetic kitchen wastewater was evaluated in batch degradation trials using a model bacterium, *Bacillus licheniformis* NCIMB 9375. Concentrations of the individual organic fractions were varied over ranges comparable to real commercial kitchen effluents. In all tests, lipase production and FOG removal were always initiated during stationary phase. This suggests the need for a pre-growth stage of the bioadditions before dosing or further time for the development of a biofilm on the drains/pipes surface. The source of readily available carbon (e.g. carbohydrates or sugars) showed great influence on FOG-degradation response. For example, lipid removal was reduced in the presence of glucose compared to equivalent concentrations of particulate starch. FOG uptake was also influenced strongly by the carbon to nitrogen ratio in the media composition, with removal rate constant decreasing by over an order of magnitude when initial COD:N increased from 33:1 to 147:1.

4.1 Introduction

Alongside physical processes (i.e. grease separators) for managing emissions of fat, oil and grease (FOG) from food service establishment wastewater (FSE WW), bioadditive products, consisting of different bacterial strains with an affinity for lipid degradation, can be added to the drains to enhance natural biodegradation. Over the years, the addition of oil-degraders to FOG-rich wastewater has produced variable results , in many cases, has proved to be ineffective (Mendoza-Espinosa and Stephenson, 1996; Brooksbank et al., 1997, Wakelin and Forster, 1997). Bioremediation failure is often linked to the use of a single organism, scarce microbial adaptation to the environment, inadequate dispersion or access to easily degradable organic substrates and other factors limiting FOG biodegradation, such as inhibitors or toxic compounds (Watanabe et al., 2000). For example, FSE WW comprises largely of food residues, thus providing microbial populations with alternative energy and carbon sources, such as carbohydrates and protein, which can be more easily metabolised by the cell than FOG. In addition, triglycerides, the dominant FOG components in kitchen WW, have a very low aqueous solubility compared to other carbon sources and can be more difficult to access by the microbial cells. Triglycerides must first be broken down into their constituent long chain fatty acids (LCFA) and glycerol by hydrolytic lipase enzymes before they can be transported through cell walls. The break-down and the movement of triglycerides from the outside to the inside of the cell is regulated at gene level through the production of fatty acid transport proteins and fatty acid degradation proteins. These two groups of protein work together and are present at basal levels under nutrient-rich conditions. In the presence of high concentrations of LCFAs, their production can be increased, and thus triglycerides digestion (Black and DiRusso, 2003).

The effect of organic matter composition in lipid degradation, has been investigated through two fields of research: optimisation of industrial lipase production, and removal of FOG from wastewater. Lipase production, can be enhanced (Hasan et al., 2006), reduced or completely inhibited by particular carbohydrates and organic nitrogen sources and concentration (Eltaweel et al.

2005; Ertuğrul et al. 2007; Takaç & Marul 2008). Rathi et al. (2001) found optimum lipolytic activity from *Burkholderia cepacia* at 1% glucose over a test range of 0.25 – 2.5% (2.5 – 25 g/l) in the presence of 1.5% oil. El-Shafei and Rezkallah (1997) demonstrated a dramatic impact on lipase activity from *Bacillus cereus* and *Bacillus coagulans* when fodder yeast medium was supplemented with a glucose range of 0.15 – 1.2%. They found that lipase production was completely inhibited at the low and high concentrations with an optimum at 0.6%. These studies suggest that a critical minimum amount of glucose might be required for bacterial growth, whereas too much negated the need to utilise oil as a substrate. However, such studies typically used considerably higher substrate concentrations, oil and carbohydrates, than in most real WW. Moreover, lipase production does not necessarily lead to microbial removal of hydrolysed free fatty acids. In a mixed bacterial culture, Tzirita and Quilty (2012) determined that *Bacillus* species were responsible for lipase production, but only non-lipolytic *Pseudomonas putida* was capable of assimilating the free fatty acids. Previous lab-based FOG degradation studies, have similar limitations in translating the results to real FSE wastewater compositions by either using FOG as the sole carbon source (Becker et al. 1999; Loperena et al. 2006); or arbitrary concentrations of other substrates - either higher (Tano-Debrah et al., 1999), or in atypical proportions (Brooksbank et al., 2007; Tzirita and Quilty, 2012).

The ratio of carbon to nitrogen has a strong influence on microbial growth (Grady et al., 2011), thus the afore-mentioned experiments showing differences in lipase response (inhibition or enhancement) to varying concentrations of organic carbon and nitrogen may, in fact, reflect their relative ratios in the media. Microorganisms utilise carbon substrates through oxidation as a source of energy (catabolism) or, together with nitrogen, for growth and product formation (anabolism). Under balanced nutritional conditions catabolism and anabolism are tightly coupled such that the amount of energy generated by carbon oxidation is dominantly used to fuel growth, leading to cell growth yields near maximum theoretical values. In wastewater treatment, nutrient balances are typically assessed based on COD concentrations rather than carbon alone, so as to reflect variations in oxidation

state of different substrates, and thus the number of electrons available for donation in oxidation reactions. For example, one mole of generic carbohydrate, $(CH_2O)n$, n=1, contains 4 available electrons resulting in a COD:C ratio of 2.67 according to Equation 4-1, whilst one mole of palmitic acid, $C_8H_{16}O$, can donate 46 electrons giving a COD:C ratio of 3.83. Thus, more reduced lipids contain more electrons and can provide more energy on a carbon for carbon basis than carbohydrates, or indeed proteins.

$$\frac{no.of\ electrons\ x\ 8\ gCOD}{moles\ C\ x\ molecular\ weight\ C} \qquad \textbf{Equation 4-1}$$

Typically a COD:N ratio of 20:1 is recommended for efficient treatment of WW of domestic composition (Grady et al., 2011). This ratio provides enough carbon to allow all available nitrogen to be co-synthesised into new biomass together with just the right amount of carbon energy to drive the process. Nevertheless, microbial populations have the ability to adjust their metabolic processes in response to nutrient deficiency/carbon excess, whereby the balance between synthesis and energy shifts towards surplus energy production under carbon excess conditions (Dauner et al., 2001). Thus, organic matter in WW with COD:N greater than 20:1, such as that discharged by FSEs, can potentially be fully degraded without additional nitrogen but at reduced cellular yields.

Analysis of the current literature identified clear research gaps with respect to assessing the potential for biological FOG management specific to FSE wastewater. This study aims to close some of these gaps by delivering a more thorough evaluation of bacterial response to variations in organic substrates under more realistic conditions than previously used by monitoring:

- microbial FOG-removal over time in response to alternative organic substrates (carbohydrates and protein) in controlled synthetic media reflecting real concentrations in FSE wastewater, as identified in Chapter 3.
- co-monitoring uptake of carbohydrates and protein to gain a better understanding of overall microbial substrate utilisation.

- co-monitoring bacterial population growth to correlate substrate utilisation with growth stages.

Degradation experiments were conducted in batch reactors using synthetic culture media to simulate FSE WW as determined from a characterisation of FSE effluents reported in Chapter 3. All media compositions contained a basal mineral medium and surfactants, in addition to oil, carbohydrate and an organic nitrogen source. In each experiment, initial concentration of oil, carbohydrate or protein was varied over ranges identified in Chapter 3. In addition, the impact of carbohydrate type was investigated using either glucose as a readily-bioavailable carbon and energy source, or cornflour to represent starch in particulate form which, like FOG, requires enzymatic pre-hydrolysis to enable microbial uptake.

4.2 Materials and methods

Chemicals were obtained from Fisher Scientific, UK unless otherwise stated.

4.2.1 Synthetic wastewater preparation

Based on the FSE wastewater characterisation in Chapter 3, a basal medium was designed containing trace elements and surfactants (Table 4-1). 500 ml of culture media was made up in 1L Erlenmeyer flasks. Solutions of glucose, and/or cornflour (Tesco, UK) were added at varying concentrations as a source of carbohydrate, and soy protein acid hydrolysate (Amisoy) as both a nitrogen and carbon source. Flasks were autoclaved at 121ºC for 20 minutes. Media was buffered to pH 7 by addition of 30 ml/l of sterile 1M sodium phosphate buffer after autoclaving to prevent precipitation of calcium phosphate. 30 mg/l of the anionic surfactant sodium dodecyl sulphate was also added post-autoclave to prevent thermal degradation. Filter-sterilised rapeseed vegetable oil (Tesco, UK) was added dropwise to the flasks with a tared sterile transfer pipette. Concentrations of the organic components were varied to achieve the test parameters in Table 4-2.

Table 4-1 Base media composition containing trace elements and surfactants in mean concentrations identified in real FSE wastewater in Chapter 3

Component	Concentration (mg/l)
Calcium chloride dihydrate	100
Potassium chloride	70
Magnesium sulphate heptahydrate	40
Ammonium chloride	10
Iron chloride	0.27
Zinc sulphate heptahydrate	0.16
Copper sulphate	0.07
Manganese sulphate monohydrate	0.045
Cobalt nitrate hexahydrate	0.002
Ammonium heptamolybdate tetrahydrate	0.001
Triton X-100 (non-ionic surfactant)	14
Added post autoclave:	
Sodium dodecyl sulphate (anionic surfactant)	30
Sodium phosphate buffer	30 mM

Table 4-2 Test conditions for substrate, bacterial dose and incubation times compared to ranges in real FSE WW identified in Chapter 3

Variable parameter	Range in FSE WW (mg/l)	Concentration (mg/l)	Incubation time (h)	Constant parameters (mg/l)
Rapeseed oil	Total FOG: 200 – 2000 Emulsified FOG: 120 - 540	150, 300, 450, 600, 800	8, 16, 38, 62	Glucose 1200 Starch 220 SPH 400 (TN 52) Dose 5E5 cfu/ml
Glucose	Total carbohydrate: 100 - 4000	100, 500, 1000, 2000, 4000	8, 16, 38, 62	Oil 580 Starch 0 SPH 400 (TN 52) Dose 5E5 cfu/ml
Cornstarch	Total carbohydrate: 100 - 4000	100, 500, 1000, 2000, 4000	8, 16, 38, 62	Oil 580 Glucose 0 SPH 400 (TN 52) Dose 5E5 cfu/ml
Soy protein hydrolysate, SPH, (as total nitrogen)	TN: 24 – 80 Protein: 146 - 477	20, 40, 60, 80 124, 248, 373, 497	8, 16, 38, 62	Oil 580 Glucose 500 Starch 500 Dose 5E5 cfu/ml

4.2.2 Bacterial inoculum

Bacillus licheniformis NCIMB 9375 was purchased from the UK national culture collection (NCIMB) as previous studies have indicated that this strain is capable of metabolising a variety of organic carbon and nitrogen sources (Voigt et al., 2007, 2006). Stock cultures were maintained on nutrient agar (Oxoid CM0003) at 4°C and subcultured at least monthly.

4.2.3 Analytical methods

FOG quantification

Oil concentration was quantified using the modified Gerber method described in Gurd et al. (2018) (Chapter 2) with some modifications. Preliminary experiments showed that the free oil extraction step was not necessary since shaking the synthetic WW overnight generated a stable emulsion. However, after incubation of the bacterial culture upwards of 24 hours, there was a progressive build-up of indigestible material which hindered oil separation in the butyrometers by amalgamating with oil at the base of the scale. Thus, a portion of the FOG could not be extracted leading to false-positive results for FOG removal. To reduce interference, samples taken beyond the 16-hour time points were precentrifuged at 10000g for 20 minutes to remove bacterial biomass before the supernatant was decanted into a new centrifuge bottle for the precipitation/flocculation stage. Details of method optimisation are described in Chapter 5.

Total soluble nitrogen

Organic nitrogen is taken up by bacterial cells and incorporated into new biomass. Hence total nitrogen concentrations in a biological system may remain fairly constant. Consequently, given that soy protein hydrolysate (SPH) is initially soluble, nitrogen uptake was monitored as soluble total nitrogen (TN) in samples filtered through a 0.45 µm syringe filter. Samples were analysed with a Shimadzu TOC-V analyser, calibrated with TN standard solution. Equivalent soluble protein was calculated by multiplying by a conversion factor of 6.37 determined from the amino acid/TN ratio provided by the manufacturers (Sheffield Bioscience, 2014).

Carbohydrates

Glucose and starch concentrations were quantified spectrophotometrically by the Dubois method on a Jenway 6300 Visible spectrophotometer at 490 nm as described in Chapter 3.

Bacterial growth

Bacterial growth was monitored by spread plate technique on Oxoid nutrient agar (CM0003). Plates were incubated at 25ºC for 24 hours and those with between 30 and 300 colonies were considered statistically significant. Optical density (OD) was found to be an unreliable method for monitoring biomass owing to optical interference from the oil emulsion; nor was cell dry weight (CDW) dependable due to suspended solids in the medium, particularly starch. For example, emulsified oil removal, and hydrolysis of particulate starch into soluble components, would manifest as apparent biomass reduction through OD and CDW methods respectively. CDW was determined for the glucose-only dataseries according to standard methods.

Lipase activity

Lipase activity was measured spectrophotometrically on a Tecan infinite 200Pro microplate reader. A calibration curve was generated using p-nitrophenol (pNP) standards prepared in 0.2 M 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (adjusted to pH 7.5). 40 µl of each standard solution and 60 µl of MOPS was pipetted into 96-well microplates and incubated at 37°C in an incubator shaker for one hour at 200 rpm. 5 µl of 1M sodium carbonate was added to each of the microplate wells and left for 3 min to stop the reaction. The absorbance of the p-nitrophenolate ion was measured at a wavelength of 405 nm. 0.5 mM pNP substrates of butyrate and octanoate were prepared in MOPS. 40 µl of substrate, 20 µl of buffer and 40 µl of unknown sample were pipetted into microplate wells and treated as per the standards. Absorbance measurements were also taken for substrate and sample blanks. Enzyme activity, expressed as nmoles/ml*min (mU/ml), was calculated from Equation 4-2 using the slope of the standard curve, and absorbance adjusted for both substrate and sample blanks. All measurements were performed in triplicate.

$$Activity = \frac{Adjusted\ absorbance}{slope * time}$$ **Equation 4-2**

4.2.4 Degradation trials

Each experiment was performed in three independent runs, each comprising of batches of 16 – 20 flasks, allowing for triplicate analysis of four or five different substrate concentrations at four separate time points as defined in Table 4-2. Following media preparation, flasks were shaken overnight to emulsify the oil before dosing with 1 ml of bacterial inoculum to achieve an *in-situ* dose of 5 x 10^5 cfu/ml. *Bacillus* inocula were precultured in nutrient broth to an optical density (OD_{600nm}) of 0.43 equivalent to 2.5 x 10^8 cfu/ml. Doses were confirmed by spread plating. Culture were then incubated at 40ºC at a shake speed of 150 rpm in an incubator shaker (SciQuip Incu-Shake TL6-5R, SciQuip UK) for periods up to 62 hours. Samples were taken for analysis at 8, 16, 38, and 62 hours - whole flasks were taken and sampled immediately for cell counts, and 100 ml subsamples analysed in duplicate for FOG. Further subsamples were stored at -18ºC for carbohydrate, TN and lipase activity analysis. Sterile controls were treated in the same manner without inoculation.

4.2.5 Data analysis

All measurements were done in triplicate, unless explicitly reported. Apart from the glucose variation trial where one batch failed, data are presented as means of triplicate independent experiment with errors estimated as standard deviation. All numerical and statistical analyses were done in Microsoft Excel.

4.3 Results and discussion

4.3.1 Effect of varying initial FOG concentration

Minimal FOG removal was observed (above an extraction efficiency of 95 ± 4% achieved in sterile control flasks) during the log phase of bacterial growth (Figure 4-1(a)), with maximum FOG removal rates after 16 hours incubation. This growth-related response is consistent with previous lipase activity studies in multi-substrate media (Castro-Ochoa et al., 2005; El-Shafei and Rezkallah, 1997; Handelsman and Shoham, 1994; Sharma et al., 2014) suggesting a bacterial preference for co-substrates to support growth followed by a switch to FOG utilisation during stationary phase. Das et al., (2010) determined that *B. coagulans* RK-02 produced maximum lipase activity after exhaustion of glucose which had supported log phase growth. Similarly, in the present work, soluble carbohydrate concentrations decreased rapidly from 1200 mg/l during log phase reaching almost identical concentrations (50 ± 9 mg/l) after 16 hours across all data series (not shown). Bacterial populations fell by approximately half from 8 to 16 hours before recovering as a reflection of diauxic growth (Figure 4-1(a)).

(a) (b)

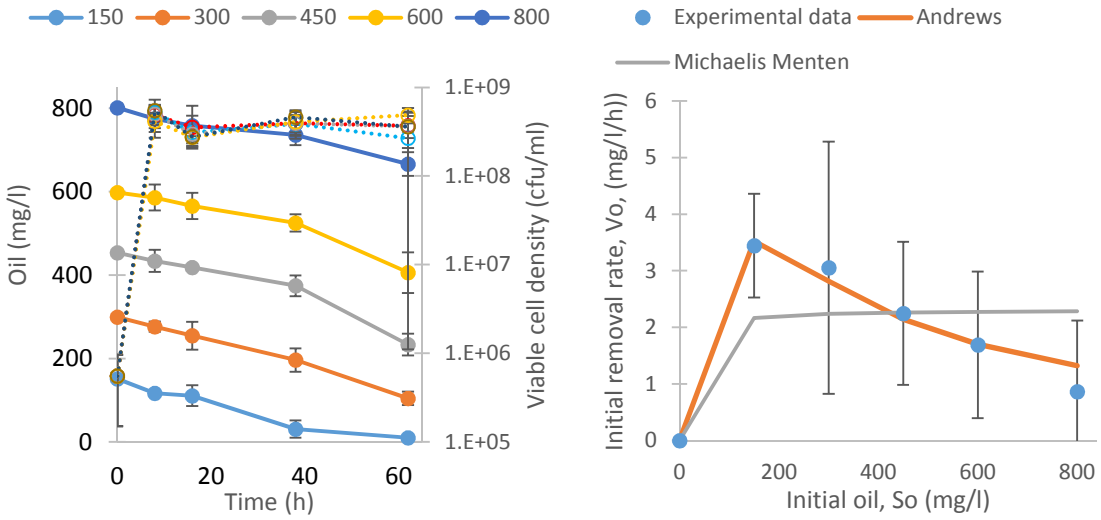

Figure 4-1 (a) Time course of rapeseed oil removal (solid lines) and bacterial growth (dashed lines) for *B. licheniformis* 9375 in synthetic wastewater with different initial oil concentrations from 150 to 800 mg/l. (b) FOG removal rate data fitted to the Michaelis Menten, and Andrew's substrate inhibition enzyme kinetic models calculated using 'Solver' in Microsoft Excel. Data are presented as means of triplicate independent experiments with error bars as standard deviation.

Figure 4-1(a) also suggests variable FOG removal rates between the data series. 'Initial' removal rates, v_o, were defined as removal rate between 16 – 38 hours, when carbohydrate was largely exhausted leaving FOG as the limiting carbon substrate. Removal rates were fitted to two common enzyme kinetic models: the Michaelis Menten model (Equation 4-3); and Andrews substrate inhibition model (Equation 4-4) which includes an additional function to take into account a negative inhibitory effect of high concentrations of substrate or reaction products.

$$v = \frac{V_{max}\, x\, S}{K_M + S} \qquad \textbf{Equation 4-3}$$

Where:
v is FOG removal reaction rate (mg/l*h)
V_{max} is the maximum possible removal rate under substrate saturation (mg/l*h)
S is substrate concentration (mg/l)
K_M is the Michaelis Menten constant

$$v = \frac{V_{max}\ x\ S}{K_s + S + \frac{S^2}{K_i}}$$ **Equation 4-4**

Where:
K_s is the half saturation constant (mg/l)
K_i is the inhibition constant (mg/l)

Despite considerable variability around mean values, the FOG removal data appears to be a better fit for the Andrews substrate inhibition model (Figure 4-1(b). This yields kinetic constants of V_{max} 29 mg/l*h; K_s 524 mg/l; K_i 39 mg/l and a critical fat concentration to inhibition (when v = V_{max}) of 143 mg/l according to Equation 4-5.

$$s = \sqrt{K_s\ K_i}$$ **Equation 4-5**

This degree of substrate inhibition is considerably higher than previously determined (Becker et al., 1999; Lima et al., 2003). Loperena et al. (2006) calculated a critical fat concentration of 360 mg/l for substrate inhibition to growth, over a range of initial fat concentrations from 200 – 1000 mg/l. Jung et al. (2002) reported a marked drop in COD removal from dairy wastewater by activated sludge from 86%, 75% to 0% with FOG supplementation of 400, 600 and 800 mg/l respectively.

4.3.2 Influence of co-substrates

4.3.2.1 Carbohydrates: glucose and starch

Culture media were made with a fixed oil concentration, and either glucose or corn starch as a source of carbohydrate. Initial concentrations of 100, 500, 1000, 2000, 4000 mg/l were used to reflect the range observed in real FSE WW.

Bacterial cell yield

To gain an estimate of cell yield, cell dry weights (CDW) were determined at the 8-hour sampling point, for the glucose media cultures, which was assumed to be

roughly equivalent to the end of log-phase growth. Mean CDWs were fairly consistent (0.20 – 0.24 g/l) in all series apart from at 100 mg/l which was only 0.09 g/l. However, cell numbers were lowest in the 1000 mg/l series – 2.5 x 10^8 cfu/ml compared to 3.6 x 10^8 cfu/ml at 100 mg/l and over 6 x 10^8 cfu/ml at the higher concentrations. Consequently, cell dry weight varied with changes in carbohydrates. The smallest cells weighed 247 fg/cell from cultures containing 100 mg/l of initial glucose; this value is consistent with growth of smaller cells under carbon limitation (Figure 4-2(a)). Similarly, cells grown in cultures containing 2000 and 4000 mg/l of glucose are consistent with cells grown under nitrogen limitation, 359, and 321 fg/cell respectively. An apparent optimum of 919 ± 137 fg/cell was achieved using 1000 mg/l of glucose. Similar results were reported by Vrede et al. (2002), who also found a mean 75% cell weight decrease in C-limited bacterioplankton, and a less dramatic decrease of 35% under N-limitation compared to exponentially growing cells.

Cell yields (mass of new cell biomass/mass substrate consumed) were calculated in terms of COD units for both biomass and removed substrate. COD equivalent conversion factors, from Table 3-6, of 2.88, 1.07, 1.5 and 1.42 were used for FOG, carbohydrate, protein and biomass respectively. The data in Figure 4-2(b) shows mean observed growth yield (Y_{obs}) under glucose limitation in the 100 mg/l data series as 0.76 mg-cellCOD/mg-substrateCOD – very similar to the maximum true yield for growth on carbohydrate, 0.71 mg-cellCOD/mg-substrateCOD as predicted by bioenergetic models used in wastewater treatment (Grady et al. 2011). These models also predict that maximum carbon use efficiency is achieved at a COD:N ratio of 20:1. In this work, the associated relative uptake of theoretical (calculated) COD:N is estimated at a somewhat higher value of 25 ± 3 (Figure 4-2(b)). When available glucose concentration was increased above 1000 mg/l, bacterial populations responded by increasing carbon catabolism over anabolism, reflected in COD:N uptake increasing up to 50:1, in association with cell yields dropping to 0.23 mg-cellCOD/mg-substrateCOD (Figure 4-2(b)).

A strong negative correlation (R = - 0.84) between Y_{obs} and COD:N uptake, illustrated in Figure 4-2(b), indicates that the parameter of COD:N uptake is a

useful tool for monitoring bacterial response to available substrate in the absence of direct biomass yield measurements.

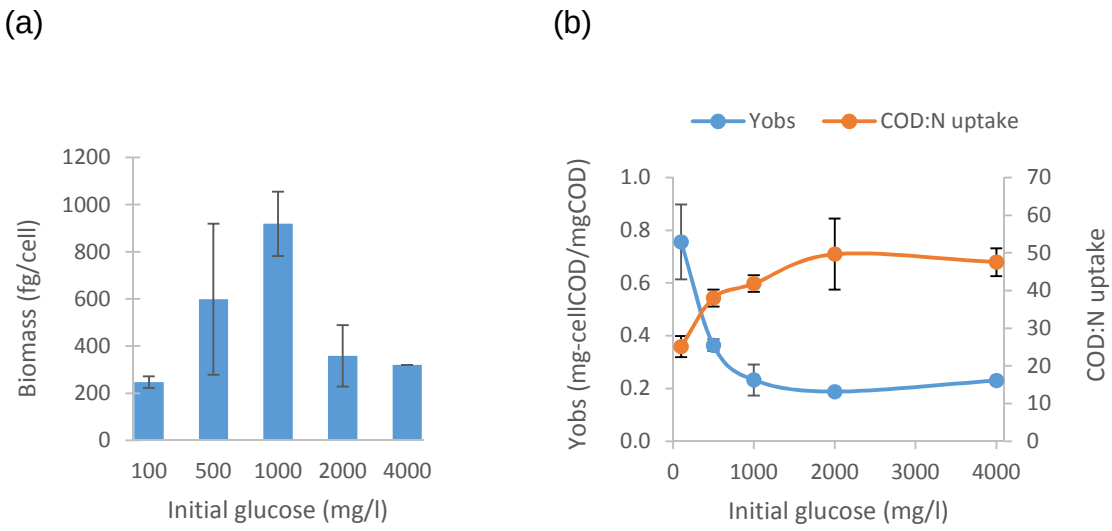

Figure 4-2 Bacterial growth at different concentrations of initial glucose: (a) cell dry weight, (b) cell yield and relative uptake of theoretical COD and N. Data represent means of duplicate independent experiments with error bars as standard deviation.

Removal rates for the different organic fractions, normalised to COD units, are presented in Figure 4-3. In the glucose media dataset (Figure 4-3(a)), growth was almost exclusively supported by glucose, with minimal contribution from FOG (0 – 7.7 mgCOD/l/h). In contrast, growth in the starch media is supported by a more even distribution of FOG, starch and protein (Figure 4-3(b)). Total log-phase substrate removal rates were considerably lower (18 – 58 mgCOD/l/h) than in the presence of readily-metabolisable glucose (21 – 204 mgCOD/l/h). Both datasets show positive correlation between increasing initial carbohydrate concentrations and both COD uptake rates and COD:N uptake. For example, up to 10-fold more glucose removal was achieved by diverting excess carbon into energy production: 16 mgCOD/l/h and 164 mgCOD/l/h for media with 100 and 2000 mg-glucose/l respectively (Figure 4-3(a)).

Carbohydrate uptake rates were considerably lower in starch compared to glucose media: 0.3 – 32 mgCOD/l/h and 16 – 164 mgCOD/l/h, respectively. Since a minimum lag of 4 hours was observed before particulate starch began to be hydrolysed into soluble fragments <45µm (not shown) it is likely that early growth was predominantly supported by readily available amino acids provided by the soy protein hydrolysate. In all conditions, log-phase growth was largely supported by readily available substrates, it is therefore important to evaluate the metabolism during stationary phase of growth to evaluate the real impact of carbohydrates on FOG degradation.

Stationary phase metabolism

Comparison of COD removal rates in Figure 4-3(c) and (d) shows that carbohydrate type had a strong influence on FOG degradation during stationary phase. FOG removal was limited in the glucose media (2 - 12 mgCOD/l/h), particularly in association with low bacterial populations in the 100 mg/l series, and by residual glucose availability at high initial COD:N in the 4000 mg/l series. Higher FOG uptake rates in the starch media (14 – 24 mgCOD/l/h) were consistent with the production of enzymes associated with the metabolism of less biodegradable carbon sources in response to readily-available carbon limitation (Egli, 1995). For example, Voigt et al. (2007) demonstrated up-regulation in a number of genes associated with metabolism of alternative carbon sources, including amino acids and lipids, when *B. licheniformis* 9375 cells were subjected to glucose starvation. Given that glucose had been completely exhausted after the first 8 hours in the 100, 500 and 1000 mg/l cultures we might have expected to see a similar response to carbon limitation ie. triggering of enzymes to degrade FOG. This was clearly not the case in our experiments. Thanh et al. (2010) demonstrated that *B. licheniformis* grown on 4.4 mM glucose (~800 mg/l) secreted around half this amount as acetate and other overflow metabolites into the medium, which were subsequently consumed during stationary phase. As COD:N uptake during log phase in this study was in excess of the 20:1 ratio for growth requirements we can assume a similar response was triggered in our

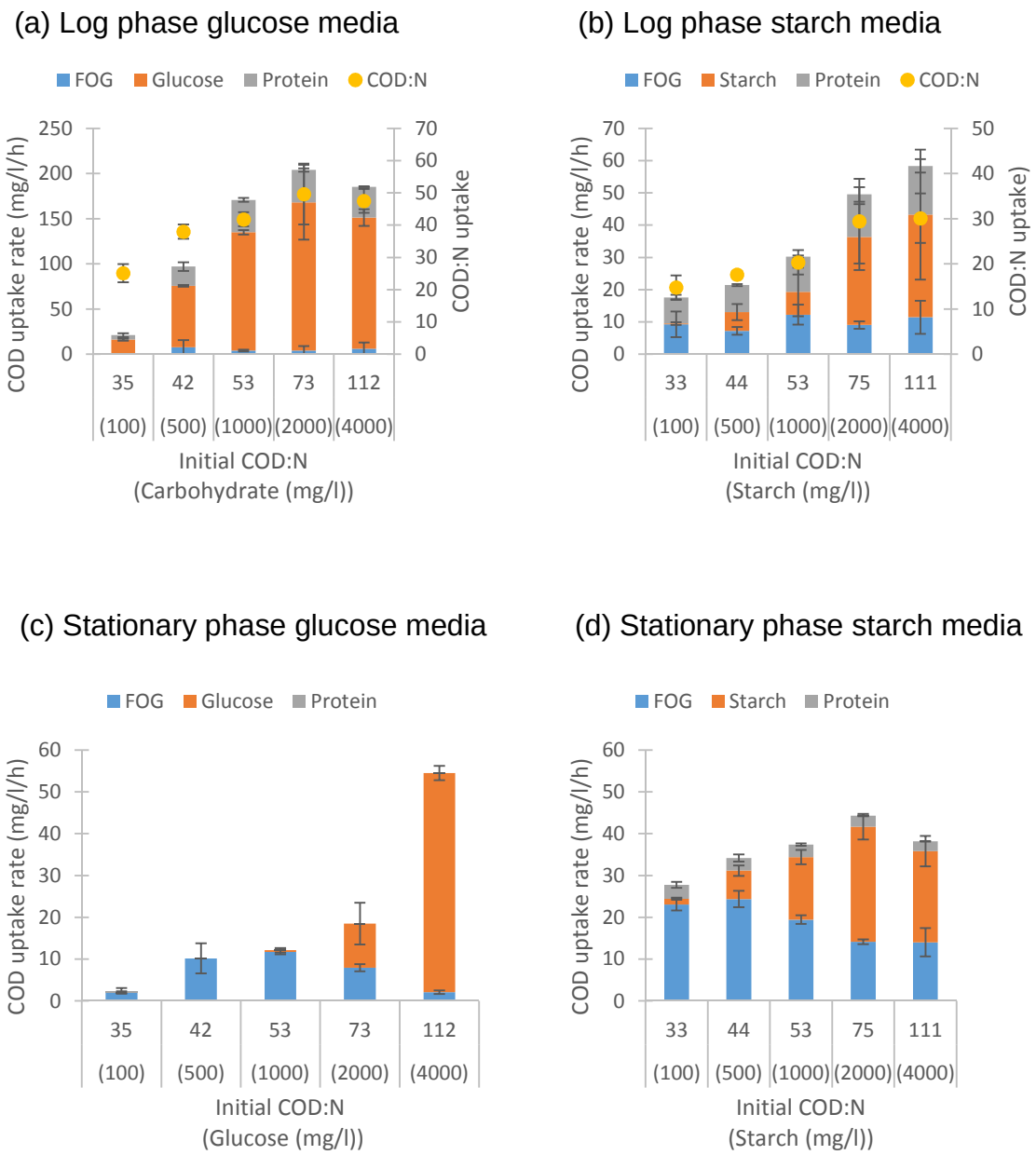

Figure 4-3 Theoretical COD uptake rates calculated for individual substrate fractions (bars), and COD:N uptake rates during growth phase (orange circles), for media with different initial concentrations of glucose (a) and (c); and starch (b) and (d). X-axes denote initial COD:N ratios, together with carbohydrate concentrations in parentheses. Log phase is defined as 0 – 8 hours; and stationary phase as 8 – 62 hours. Data represent means of duplicate independent experiments with error bars as standard deviation.

systems. Hence, the presence of more readily bioavailable overflow metabolites limited the need for FOG hydrolysis. In fact, removal time-course data (not shown) suggests that FOG uptake may have been delayed rather than repressed, in that uptake rates increased during the course of the experiment. For example, maximum removal rates of 8.7 ± 0.2 mg-FOG/l/h (24.9 ± 0.6 mgCOD/l/h) were observed from 38 - 62 hours in the 1000 mg-glucose/l series. Removal remained repressed at the highest concentration, reaching only 1.2 ± 0.4mg-FOG/l/h. In the glucose-limited 100 mg/l series, lower substrate consumption (estimated as 14 ± 4 fg-COD/cfu/h) was likely insufficient to generate enough maintenance energy to support stationary phase population levels, as suggested by their decline over the course of the experiment. Apart from the glucose-excess 4000 mg-glucose/l series, total COD uptake rates were consistently higher in the starch media (28 – 44 mgCOD/l/h) resulting in stable mean stationary phase populations. In fact, COD uptake increased at the three lowest starch concentrations from log to stationary phase, although growth profiles, and continued N uptake, suggest that there may have been some overlap, with log phase extending beyond the 8-hour sampling point. Yet, even when calculated between 16 and 62 hours, rates remained between 26 – 34 mg-COD/l/h (48 – 148% of log-phase values) and approximating to a cell uptake rate of 45 – 78 mg-COD/cfu/h.

It should be noted that starch was never completely exhausted even at the lowest initial concentration: residual concentrations varied from 17 – 69% suggesting that FOG became the preferred substrate once lipolytic enzymes were produced.

4.3.2.2 Protein variation

Batch experiments of FOG degradation with varying Amisoy soy protein hydrolysate (SPH) content, were conducted under the same environmental conditions as those reported in Section 4.3.2.1, but with media comprising of constant carbohydrate concentrations of 500 mg/l each of glucose and starch. SPH concentrations were varied (150, 300, 450, and 600 mg/l), to achieve initial TN concentrations of 20, 40, 60 and 80 mg/l, with associated amino acid

concentrations of 124, 248, 373 and 497 mg/l, calculated as 82.8 wt% from the manufacturers' information datasheet (Sheffield Bioscience, 2014).

Variations in SPH yielded considerably less variation in total initial COD than the previous experiments (3002 – 3535 mgCOD/l), thus facilitating distinction between the influence of carbonaceous substrate concentration and relative proportions of carbon and nitrogen. Lower mean COD uptake at COD:N 147 shown in Figure 4-4(a) is skewed by delayed carbohydrate uptake in association with slower bacterial growth in one of the triplicates, which may have reflected different conditions in that particular flask. In contrast to the positive correlation illustrated in Figure 4-3, total COD uptakes rates showed a moderate negative correlation (R = -0.67) with initial COD:N, presumably due to decreasing nitrogen availability. However, associated COD:N uptake increased from 31 to 50 in response to increasing carbon excess. In contrast to the 500 mg/l (COD:N 42) series in glucose media where glucose was exhausted in the first 8 hours, total mean carbohydrate concentrations only decreased to 638 ± 33 mg/l. Assuming that no starch was removed, this infers that 28% of glucose remained unconsumed. Accordingly, at a comparable COD:N ratio (45:1), total COD and COD:N uptake were slightly lower (88 mgCOD/l/h and 31:1) than in the equivalent glucose cultures - 97 mgCOD/l/h and 38:1.

Mean stationary phase FOG uptake rates were up to 21% higher than at comparable initial COD:N ratios in the starch media. For example, 26, 23 and 17 mg/l/h at COD:N 45, 56 and 80 compared to 24, 19 and 14 mg/l/h at COD:N 44, 53 and 75 in the starch media. Yet both data sets show a similar negative correlation between FOG uptake and initial COD:N. Given that carbohydrates were of a constant initial concentration, the results are indicative of COD:N influence. Apart from the most N-limited series (COD:N 147) N uptake continued beyond 8 hours. Whilst continued N uptake in the starch dataset was associated with an approximate doubling in cell numbers between 8 and 16 hours, cell growth in this experiment was not significant. Mean stationary phase populations were smaller with increasing COD:N, thus, although COD removal rates per cell were similar in COD:N 45, 56, 80 cultures (67 – 74 fgCOD/cell/h), increasingly

higher populations led to higher bulk removal (Figure 4-4(c)). The highest carbon-excess cultures at COD:N 147 appeared to also demonstrate a cellular response with lower total COD uptake of only 47 fgCOD/cell/h.

(a) Log phase (b) Stationary phase (c) Cellular uptake

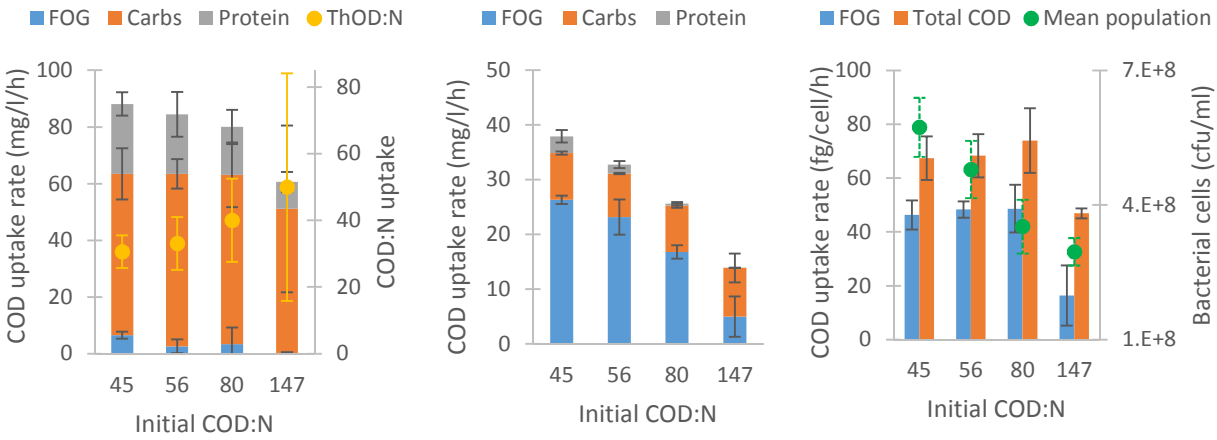

Figure 4-4 COD uptake rates calculated for individual substrate fractions (bars), together with total COD:N uptake rates (orange circles) under varying concentrations of organic nitrogen during (a) log phase (0 – 8h) and (b) stationary phase (8 – 62h); (c) COD uptake rates per cell in stationary phase, and mean stationary-phase bacterial population concentrations (green) at different initial COD:N ratios. Data represent means of triplicate independent experiments with error bars as standard deviation.

Enzymatic lipolytic activity

The enzymatic lipolytic activity was measured on octanoate and butyrate pNP substrate. Lipase production was barely detectable at 8 hours (Figure 4-5). Butyrate-specific activity increased during stationary phase and showed a strong negative correlation between initial COD:N and activity: R = - 0.87 and R = - 0.70 at 32 and 68 hours respectively, such that more enzyme was produced in N-rich media. Highest activity was clearly achieved at lower initial COD:N in the pNP octanoate assay whilst activity remained low for carbon-rich media.

(a) (b)

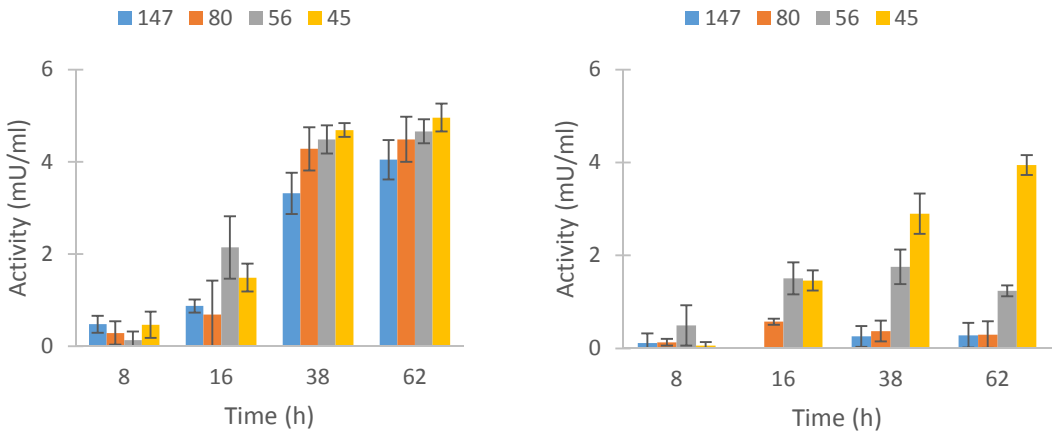

Figure 4-5 Lipase activity with time for (a) butyrate-specific enzyme and (b) octanoate-specific enzyme in synthetic wastewater at different initial COD:N. Data represent means of triplicate independent experiments with error bars as standard deviation.

4.3.3 Impact of co-substrates on FOG removal

Little or no FOG removal was observed during the exponential growth phase under any combination of organic substrates. Log-phase growth was largely supported by readily-biodegradable glucose where present, or by a combination of starch, protein and FOG in starch-only media. FOG removal rates generally increased during stationary phase but remained suppressed in glucose-rich media.

FSE WW is typically rich in organic matter and relatively low in nitrogen compared to mixed domestic effluents (Henze et al., 2008). The optimum nutrient balance for efficient WW treatment is generally assumed to occur at an initial COD:N ratio of 20:1. Lower ratios are said to be carbon-limiting leaving residual nitrogen in solution, whilst higher, nitrogen-limiting ratios can be accommodated through partitioning more carbon energy into non-growth associated processes (Dauner et al., 2001). These criteria were closely met at the lowest concentration in the

glucose media experiment, wherein near maximum growth yields were achieved in association with COD:N removal of 25 ± 3. With increasing concentration, more COD energy was dissipated resulting in lower yields and COD:N uptake up to a maximum of 50. The same response was observed in the other experiments with starch and mixed carbohydrate compositions.

First order FOG removal rate constants (k), were calculated for all substrate conditions for the 16 – 62 hour time period, and reported in Figure 4-6. All data sets showed similar negative correlation between FOG removal and initial COD:N such that FOG uptake was favoured by nitrogen-rich environments.

FOG removal was consistently lowest in the glucose media wherein it was likely that stationary-phase maintenance energy was supplied by overflow metabolites excreted during log phase as reported in Thanh et al. (2010), thus delaying the need for FOG hydrolysis. Despite an initial COD:N of 35 in the 100 mg-glucose/l cultures, bacterial growth was poor due to limitation of readily-available carbon, leading to low FOG k values (0.001 h^{-1}) during stationary phase.

FOG k was up to an order of magnitude higher when glucose was replaced by starch. Maximum starch removal occurred in early stationary phase followed by an apparent switch to FOG as the preferred substrate after 16 hours.

Although C:N should have little influence on COD uptake in stationary phase, given that nitrogen is not required for biomass assimilation, it is suggested that residual N availability may have an influence on enzyme production. TN was removed more slowly in the starch media during log phase (1.2 – 1.9 mg/l/h) than in glucose media (2.6 – 4.1 mg/l/h excluding the 100 mg/l series), leaving more nitrogen in solution. Nitrogen uptake continued throughout stationary phase whilst populations declined from a maximum at 16 hours, indicating that N was used for enzyme synthesis allowing continued hydrolysis of starch and FOG. Similarly, N uptake during early stationary phase in the protein variation media was not associated with significant bacterial growth but was associated with increasing enzyme activity.

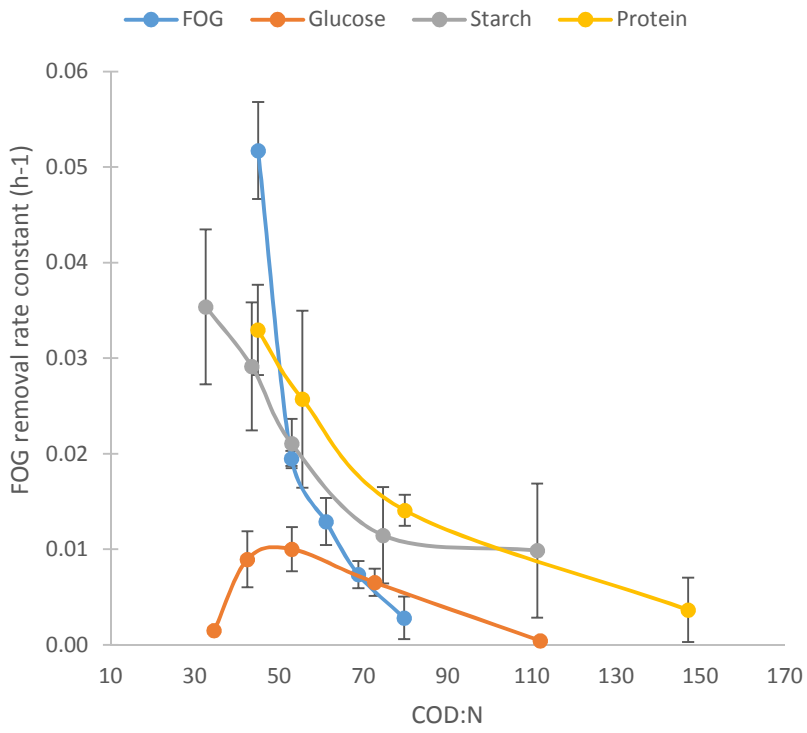

Figure 4-6 First order FOG degradation rate constants (16 – 62 hours) in synthetic media with varying concentrations of FOG (blue), glucose (red), starch (grey) and protein (orange). Data represent means of triplicate independent experiments with error bars as standard deviation (duplicates only for the glucose dataset).

Microbial response is governed by the ratio of readily-available carbon and nitrogen according to the model proposed by Schimel & Weintraub (2003) which was developed for microbial decomposition in soils, where microorganisms are commonly limited by readily-available carbon. Those authors propose that enzyme synthesis is prioritised, and biomass production will only ensue when the carbon and energy requirements for more enzyme synthesis and cellular maintenance are met. Given that FOG contains more electrons than starch as reflected in their relative COD factors (2.88 and 1.07 respectively), and thus delivers more carbon and energy it is suggested that bacteria in our systems, would favour the synthesis of lipase(s) rather than starch-degrading enzymes.

This would also explain the relatively higher rates of FOG uptake in stationary phase when both fractions remained.

A marked elevation in carbon uptake was observed in media with easily biodegradable carbohydrate (glucose) compared to more slowly-hydrolysable starch Figure 4-3(a) and (b). For example, total COD uptake of 171 ± 1 mg/l/h in glucose media compared to 30 ± 5 mg/l/h under the same initial conditions (1000 mg/l carbohydrate, COD:N 53). Nitrogen was not completely exhausted under any conditions, and thus would appear not to be a limiting factor. However, the data shows that only 56 ± 4% of initial N concentrations were removed, which is consistent with the proportion of free amino acids in the soybean protein hydrolysate used for the media (Sheffield Bioscience, 2014). This indicates that the remaining 40% of nitrogen, available in the form of peptides, was less accessible for microbial uptake and anabolism. Accordingly, biodegradability of both organic carbon and nitrogen should be considered when evaluating microbial response to COD:N

4.4 Conclusion

The influence of wastewater composition on degradation of oil was investigated in batch tests, using mixed substrate synthetic media and*Bacillus licheniformis* NCIMB 9375 as reference microorganism. Key findings were:

- In media where substrate components did not interfere with dry mass measurements, a strong negative correlation was identified between cell yields and COD:N uptake, which could be useful for monitoring bacterial biomass yields in absence of direct measurements.
- FOG removal rate constants correlated negatively with initial COD:N in all media compositions.
- FOG removal was minimal during log-phase bacterial growth in all WW compositions, with highest removal typically observed after 14 - 16 hours of incubation in association with increased lipase activity.

- FOG removal rates decreased with increasing COD:N in almost all variations of wastewater composition.
- In the absence of glucose, or relatively low concentrations in mixed carbohydrate media, higher FOG uptake in stationary phase was typically associated with higher population levels. Lowest populations levels were observed in media with high initial COD:N suggesting that available nitrogen was limiting to growth.
- Lowest FOG removal was observed in the presence of readily-available glucose, particularly in association with low bacterial populations at low glucose concentrations; and residual glucose at higher concentrations.
- FOG removal rates were higher when glucose was replaced by starch as carbohydrate source. Maximum starch removal rates were observed between 8 and 16 hours followed by an apparent switch to FOG uptake
- Residual nitrogen, surplus to growth requirements in low initial COD:N compositions, may have allowed for a greater amount of enzyme production, and thus FOG hydrolysis, during stationary phase.
- The influence of glucose repression, and the residual concentrations of nitrogen, suggest the importance of considering ratios of readily-available carbon to readily-available nitrogen rather than COD:N ratio.

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5 Development of a protocol for testing FOG-degrading bioadditives for treating commercial kitchen wastewater

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Abstract

The use of bioadditive products comprising of bacteria with an affinity for degrading Fat, oil and grease (FOG) is becoming a common management strategy for reducing the amount of FOG discharged in wastewater from food service establishments (FSEs), and resultant blockages in sewer networks. Yet, there is currently no standardised method to quantitatively assess their efficacy. The few assessment methods reported in literature are inconsistent in testing conditions used, and more so in their results. This paper reports the development of a simple laboratory-scale test protocol for microbial bioadditions using *Bacillus licheniformis* NCIMB 9375 as a model FOG-degrading bacterium. A synthetic FSE wastewater was designed to represent realistic characteristics of food service establishment wastewater in terms of organic composition, trace elements and surfactant concentrations. The synthetic culture medium had a high theoretical COD:N ratio of 62:1, typical of real FSE WW, thus presenting a relatively challenging composition compared to previous studies. FOG removal rates for the *Bacillus*, and a proprietary multi-strain bioadditive, were found to be comparable to their performance at similar COD:N ratios in real kitchen wastewater, thereby validating the suitability of the test as an evaluation tool.

5.1 Introduction

Effluent discharges from commercial food service establishments have been identified as major contributors to sewer blockages caused by saponified FOG deposits. It is estimated that, of around 370,000 sewer blockages annually in the UK, 80% are caused by FOG accumulation, with sewer clearance works costing Water Utilities around £100 million (Water UK, 2018). The UK Building Regulations 2010 state that every FSE should employ effective means of grease

removal (HM Government, 2010). In this context, FOG-degrading bioadditives are marketed as alternative solutions to grease traps, or as enhancements to their efficacy.

Previous studies on microbial lipolytic activity have investigated FOG-degrading potential at lab scale (El-Masry et al., 2004; El-Bestawy et al., 2005; Tano-Debrah et al., 1999; Wakelin and Forster, 1997). Some were conducted using FOG as a sole carbon source (Loperena et al., 2006; Mendoza-Espinosa and Stephenson, 1996; Wakelin and Forster, 1998) thereby ignoring the influence of alternative organic substrates. A few have focussed on commercial bioadditives with mixed results. Brooksbank et al. (2007) established that only one of 'several' commercial microbial supplements, was capable of significantly degrading fat and oil. Tzirita and Quilty (2012) reported that a commercial bioadditive product containing *Bacillus* species was unable to degrade butter within 13 days, yet supplementation with *Pseudomonas putida* yielded 97% degradation after 7 days, despite only the *Bacillus* species exhibiting lipolytic activity. Of two *Bacillus*-containing commercial products tested by Tzirita and Quilty (2012), the efficacy of one product was fully attributed to a fungal component, whereas the other largely to a *Pseudomonas* spp. together with nutrient and surfactant additions. However, in both studies, the absence of *Bacillus* FOG degradation was also associated with lack of bacterial growth, suggesting environmental conditions may have been unsuitable for these strains, rather than an inherent inability to degrade FOG. Moreover, the absence of both FOG degradation and bacterial growth observed by Tzirita and Quilty (2012), and poor performance in Brooksbank et al. (2007) may have reflected the low concentrations of glucose (100 mg/l) in their culture media, as discussed in chapter 4.

Other studies found little difference in efficacy between bioadditives and native microbial populations (Loperena et al., 2006; Mendoza-Espinosa and Stephenson, 1996). In working grease retention units (GRUs), Tang et al. (2012) determined enhanced FOG removal, whilst Carter (2005) observed reductions in retained grease between 2% and 81% in conjunction with bacterial additives. Poor performance was partially attributed to physical under-sizing thereby

highlighting the issue of achieving sufficient residence times for full degradation in real-life environments. Significant positive impact was also reported by He et al. (2012).

An effective means of FOG quantification is crucial to delivering reliable evaluation of FOG degradation (Brooksbank et al., 2007).These authors determined 18 – 50% under recovery in sterile controls using solvent (dichloromethane) extraction which if unaccounted for, would manifest as a false-positive for FOG removal. Measuring FOG by solvent extraction techniques is particularly challenging in kitchen wastewater due to interference from surfactants such as detergent (Barton, 2012); and proteins (Tarek N Aziz, 2010; Davis et al., 2011; Wang and Ducoste, 2012). The modified Gerber method described in Gurd et al. (2018) was developed to overcome this specific challenge.

This study aimed at developing a reliable method to test the performance of FOG-degrading bioadditives under realistic and reproducible environmental conditions. A number of factors were considered to have potential influence on bacterial activity and FOG-degrading ability, including culture medium, level of bacterial dose, contact time, pH and temperature. This paper explains how these variables were controlled within the bioadditive test protocol to reflect realistic characteristics of FSE wastewater streams. In addition, a FOG quantification method suitable for FSE wastewater, was optimised for the suggested testing conditions.

5.2 Material and methods

5.2.1 Bacterial inocula and reference strains

Reference bacteria were obtained from the UK National Collection of Industrial Food and Marine Bacteria (NCIMB): *Bacillus megaterium* NCIMB 8508; *B. licheniformis* NCIMB 9375; *B. subtilis* NCIMB 3610 and *B. pumilus* NCIMB 1522. Cultures were maintained on nutrient agar (Oxoid CM0003) at 4°C. A multi-strain commercial grease-degrading formulation was also used.

Lipolytic potential was screened by culturing the strains on tributyrin agar plates composed of nutrient agar with 1% v/v tributyrin (Kumar et al., 2012). Plates were inoculated with a streak of bacterial culture grown in tributyrin-supplemented nutrient broth, and incubated at 5, 20, 30 and 37 °C for eight days. Lipolytic activity was assessed by measuring the diameter of the zone of clearance (width of clear zone less width of bacterial growth zone).

5.2.1.1 Standardised *Bacillus* inoculum preparation

Correlation curves were constructed to provide the relation between OD_{600} and bacterial cell counts (cfu/ml) to ensure consistency of dose. Bacteria were preactivated by transferring a single colony into a 500 ml duran bottle containing 400 ml of sterile nutrient broth allowing a 3:1 liquid to headspace ratio, and incubated at 30°C without shaking (Hall et al., 2013). About two hours prior to the desired inoculation time, the culture was split between two sterile 500 ml conical flasks stoppered with foam bungs to promote aerobic conditions, and shaken at 37°C and 150 rpm in an incubator shaker (SciQuip Incushake). Periodic samples were taken for spectrophotometric OD measurements at 600 nm (Jenway 6800 UV/Vis). Cultures grown to an OD of 0.43 contained approximately 2.5 x 10^8 cfu/ml in late log-phase. Actual doses were confirmed by spread plate counts.

5.2.1.2 Dose level

To investigate if initial dose would influence substrate degradation, batch experiments were conducted using different doses of inocula to cover a range of values, 10^4 – 10^7 cfu/ml, identified through literature and bioadditives producers' recommendations. Doses of *B. licheniformis* NCIMB 9375 were varied by inoculating flasks with different amounts of the same inoculum calculated to achieve initial bacterial counts of , 10^4, 10^5, 10^6 and 10^7 cfu/ml. To compensate for residual nutrients in the nutrient broth culture medium, doses were made up to the same volume with residual filter-sterilised medium. As minimal FOG removal had been observed before 16 hours previously (Chapter 4), sampling times were changed to 14, 34, 42 and 62 hours. A commercial product was also

tested by dosing different volumes directly into the culture media without preculturing.

5.2.2 Synthetic wastewater design

An appropriate synthetic FSE WW was based on a characterisation of three sources of FSE WW detailed in Chapter 3, together with findings from Chapter 4.

Stock solutions were made up (Appendix A) and diluted to yield a mineral medium containing calcium chloride dihydrate (100 mg/l), potassium chloride (70 mg/l), magnesium sulphate heptahydrate (40 mg/l), ammonium chloride (10 mg/l), iron chloride (0.27 mg/l), zinc sulphate heptahydrate (0.16 mg/l), copper sulphate (0.07 mg/l), manganese sulphate monohydrate (0.045 mg/l), cobalt nitrate hexahydrate (0.002 mg/l) and ammonium heptamolybdate tetrahydrate (0.001 mg/l). 30 mg/l sodium dodecyl sulphate and 14 mg/l Triton X-100 were added as anionic and non-ionic surfactants respectively. Glucose and cornflour were used as a source of carbohydrates; soy protein acid hydrolysate (Amisoy) as a source of organic nitrogen and carbon; and vegetable oil (rapeseed, Tesco, UK) as a model FOG.

5.2.3 Degradation trials experimental procedure

5.2.3.1 Degradation in synthetic wastewater

Aliquots of filter-sterilised rapeseed vegetable oil (Tesco, UK) were weighed, via a tared transfer pipette, into shake flasks (1l), containing 500 ml of autoclaved (121ºC, 15 psi, 20 minutes), synthetic FSE WW. Exact FOG concentrations were determined with respect to media weight assuming 1kg equal to 1 litre. Oil was allowed to emulsify by shaking overnight in an incubator shaker at 150 rpm. Flasks were inoculated with bacterial cultures under aseptic conditions at the desired dose. Oxic conditions were promoted by stoppering flasks with a foam bung and continuous shaking. Flasks were incubated under controlled environmental conditions and removed for analysis at defined contact times. Sterile control flasks were treated in the same manner, without inoculation. Each experiment was conducted independently in triplicate.

5.2.3.2 Degradation in real wastewater

Bulk samples of wastewater were collected from two different food service establishments on Cranfield University campus. Sample containers were shaken vigorously to emulsify and homogenise the free FOG fraction. Subsamples of 500 or 250 mls were transferred into conical shake flasks and inoculated with either *Bacillus licheniformis* or the bioadditive product at a dose of 5E5 cfu/ml since bioadditive products are typically dosed at 10^4 – 10^5 cfu/ml in commercial applications. Flasks were cultured as in the synthetic WW trials. Degradation was monitored over a period of 160 hours. In one series of experiments wastewater samples were spiked with starch or ammonium chloride to alter the COD:N ratio.

5.2.4 Analytical techniques

Bacterial growth was monitored by spread plate colony counting. Carbohydrate content was determined by DuBois phenol-sulphuric acid method (glucose as standard). Total nitrogen on a Shimadzu TOC analyser. pH was measured with a Jenway pH meter. Lipase activity was measured spectrophotometrically as described in Chapter 4. FOG was determined using the modified Gerber method (Gurd et al., 2018) with some modifications as described below.

Oil in solid samples was measured by accelerate solvent extraction (ASE) on a Dionex ASE 200 extractor according to the 'fat in meat' method (Dionex, 2011), using hexane as solvent and extraction conditions set to static extraction time 2 minutes, flush 50% and nitrogen purge 100 seconds.

5.2.5 Data analysis

All testing and analyses were done in triplicate, and errors were estimated as sample standard deviation using Microsoft Excel.

5.3 Results and discussion

5.3.1 FOG quantification: method modifications

Oil concentration was quantified using the modified Gerber method described in (Gurd et al., 2018) with some further adjustments. These changes were

necessary to accommodate the potential diverse growth patterns and responses to the media of the microbial species to be tested by the proposed method.

5.3.1.1 Organic matter-oil mix: pre-centrifugation step

Preliminary experiments showed that the free oil extraction step was not necessary in the synthetic media since shaking the synthetic WW overnight generated a stable emulsion. However, after 24 hours incubation of some *Bacillus* cultures a progressive build-up of organic matter started to hinder oil separation in the butyrometers by combining with oil at the base of the scale as illustrated in Figure 5-1(a). This is likely to be the effect of bacterial attachment to emulsified FOG droplets.

To reduce interference, samples were pre-centrifuged to separate the bacterial biomass before the supernatant was decanted into a new centrifuge bottle for the precipitation/flocculation stage. A combination of centrifugation speeds and time were investigated to determine the optimum conditions for this new step. Results varied depending on bacterial population levels and culture age. However, the results of two experimental runs revealed that nearly three times more FOG was extracted after cultures were pre-centrifuged at 10000g for 20 minutes than at 5000g for 10 minutes (Figure 5-1(b and c)).

Potential FOG ‘losses’ from aqueous samples through pre-centrifugation were quantified in a separate experiment. Centrifuge sediments were recovered, dried and analysed for oil content by accelerated solvent extraction (ASE). Mean concentrations of oil partitioned into the sediments, calculated based on aqueous sample volume, were 36.2 ± 10.3 mg/l and 31.5 ± 12.5 mg/l for cultures of the commercial product and *Bacillus licheniformis* respectively. Although the data indicates that the precentrifugation stage will generate a degree of false-positive for microbial FOG removal from aqueous samples, there was no trend towards increased oil in sediment concentrations between sampling times of 14 – 62 hours, thus relative FOG removal can be assumed to be constant. Moreover recovery values were identical to those in undosed controls, 96 ± 3%, suggesting that any impact was within the error of method recovery limits.

Sample pre-centrifugation at 10000g for 20 minutes is therefore recommended for the test protocol.

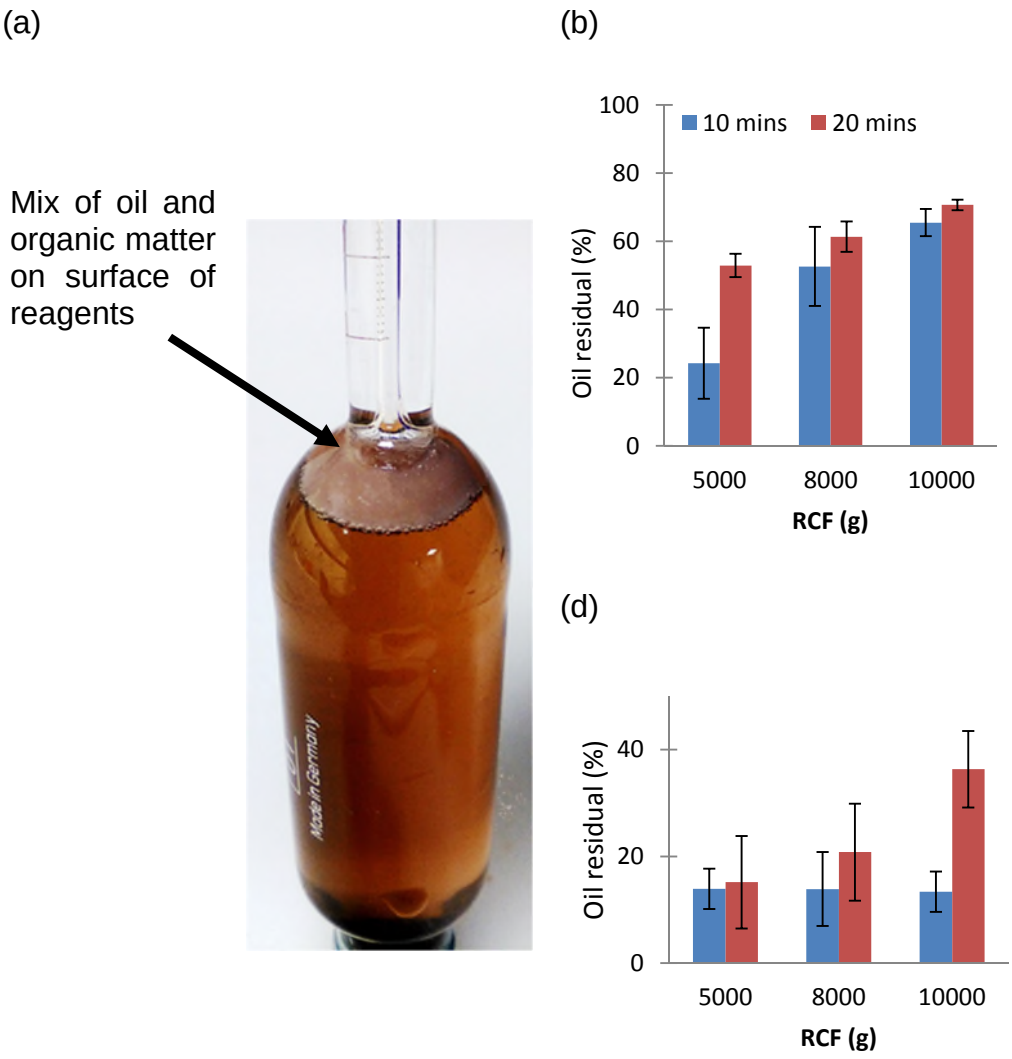


Figure 5-1 (a) Oil and organic matter mix hindering separation and extraction of FOG in a butyrometer. (b) and (c) FOG extracted under varying pre-centrifugation conditions (relative centrifugal force and time) in *Bacillus* cultures.

5.3.1.2 Casein-oil particles separation

Although emulsions shaken for 24 hours proved stable to coalescence during centrifugation, in some samples caseinate-stabilised oil particles creamed to the surface, some of which remained as a film of yellowish material adhered to the

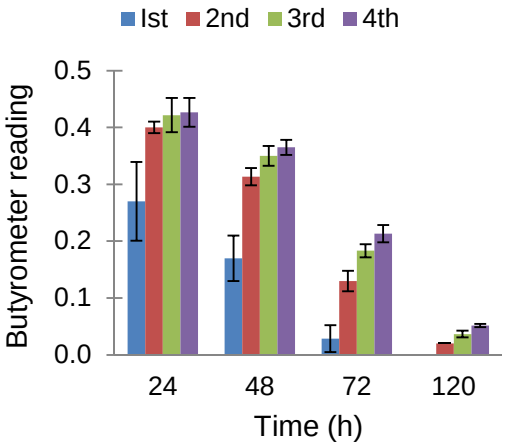
bottles following decantation. When analysed separately, this creamed material was estimated to comprise of up to 30% of recoverable oil and thus would result in significant under-recovery if ignored. This residual material was easily rinsed off into the second centrifuge bottle by means of squirting DI water from a wash bottle around the inner surface. It is likely that enhanced oil recovery at the higher centrifugation speeds and duration was linked to a greater degree of emulsified oil separated from the pellet. For example Chow and Ho (2002) determined that emulsified oil droplets stabilised by natural surfactants could be creamed from

5.3.1.3 Optimisation of number of centrifugation cycles

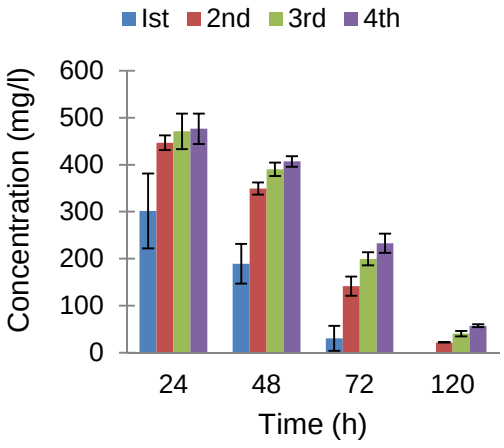
Despite pre-centrifugation, organic matter still caused interference in some samples after incubation for 48 hours. Triplicate 100 ml subsamples were taken from two series of flasks inoculated with the commercial product after 24, 48, 72 and 120 hours. Readings were taken for each replicate over four cycles of centrifugation and tempering (10 minutes centrifugation and a maximum of 10 minutes tempering in a water bath). Direct butyrometer readings are presented in Figure 5-2 (a) and (c) together with derived apparent concentrations in (b) and (d). A significantly lower concentration was derived from the first reading at each time point with a high degree of variability. Increased readings between 2nd and 3rd cycles resulted in increased mean concentrations of 19 and 48 mg/l at 24 and 48 hours, respectively, with readings, and concentrations, fairly stable between 3rd and 4th cycles (5 and 12 mg/l). By 72 hours apparent mean concentrations increased by 55 and 33 mg/l at the 3rd and 4th reading. The decreased impact at 120 hours is likely due to lower concentrations of residual oil and/or lower bacterial population levels. Although the data suggest that four reading cycles would be best in all case, the British Standard for the original Gerber method (BSI ISO 2446: 2008) allows for repeat readings up to a maximum processing time of 60 minutes for homogenised milk wherein the structure of the recombined milk fat globule membrane confers enhanced stability to emulsions. The casein-surfactant-FOG droplet complex generated during the extraction process is similar in structure to recombined milk fat globules. However the recommended time would be exceeded by increasing the maximum processing time from 3 to 4

cycles (60 – 80 minutes), thereby potentially allowing the production of other oil soluble compounds and artificially increasing the measurement (BSI ISO 2446:, 2008).

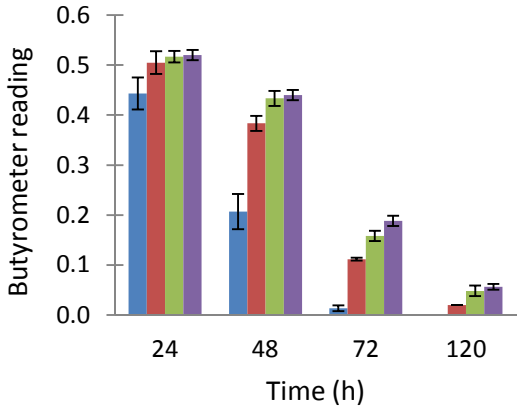
(a) Series 1 butyrometer reading



(b) Series 1 calculated concentration



(c) Series 2 butyrometer reading



(d) Series 2 calculated concentration

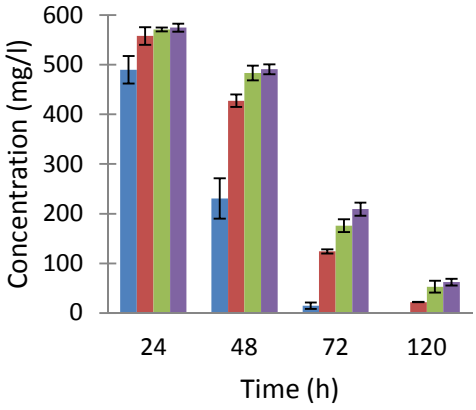


Figure 5-2 Impact of multiple cycles of centrifugation and incubation for two series of flasks inoculated with a commercial product, on oil measured on the butyrometer scale (a) and (c); and the subsequent effect of apparent concentration (b) and (d). Data are presented as means of technical triplicate measurements.

Accordingly, a maximum of three readings is recommended for the test protocol with the caveat that residual concentrations may be underestimated where

cultures are incubated for time periods above 48 hours. For example, 48-hour samples analysed for 3 cycles were 3 ± 2% lower than the 4th cycle reading, whilst the difference was 15 ± 1% and 22 ± 10% for incubation periods of 72 and 120 hours, respectively.

5.3.2 Protocol parameters

5.3.2.1 Temperature and pH

Temperature of FSE WW in Chapter 3 - mean of 54°C in dishwasher effluents, 37°C in the sink were consistent with reported ranges for grease interceptor influents (Aziz et al., 2012b; He et al., 2012). Moreover the authors determined that high temperatures tended to be maintained between influent (45°C) and effluent (42°C) (Aziz et al., 2012b). Accordingly, a controlled temperature of 40°C was set for the test protocol.

Although dishwasher effluents showed considerably higher pH (11.6 ± 0.2) compared to neutral values in a potwash sink (7.0 ± 0.3): a protocol value of 7 was set in order to facilitate comparison of a wide range of microbial supplements as most bacteria favour neutral pH. A rapid decline from pH 7 to around 5 when *Bacillus* strains were cultured in unbuffered media was mitigated by incorporation of 30 mM sodium phosphate buffer, allowing pH to be controlled at 7 ± 0.5.

5.3.3 Synthetic WW design

A synthetic culture medium was designed based on the data from the FSE WW characterisation in Chapter 3 and the evaluation experiments in Chapter 4. Design objectives were to reflect both a realistic composition, and to produce a simple and reproducible test which would yield measurable results within a reasonable time frame. Organic components compared to means measured in the real FSE WW are provided in **Table 5-1**. Previous experiments presented in Gurd et al. (2018) revealed that standard solvent-based FOG quantification methods, such as liquid-liquid and solid phase extraction, failed to extract emulsified FOG from kitchen wastewater. However, the new Gerber method was also subject to under-recovery at concentrations below 70 mg-FOG/l.

Consequently, FOG concentrations were chosen to reflect the higher mean value of 596 ± 498 mg-FOG/l as determined by the Gerber quantification method, so as to maintain concentrations within the most accurate recovery range over the course of the experiment. Total carbohydrate composition was set at 1000 mg/l comprising of equal concentrations of readily-available glucose and more slowly hydrolysable starch, together with 400 mg/l soy protein hydrolysate.

Table 5-1 Synthetic WW organic components compared to FSE WW

Ingredient (mg/l)		FOG	Carbohydrate	Total organic nitrogen	Formula	COD:N
FSE wastewater		440 ± 272	839 ± 780	45 ± 14	$C_{20}H_{38}O_{10}N$	58.3 ± 0.04
Synthetic wastewater		590	1000	52	$C_{22}H_{42}O_{11}N$[a]	62.5[a]
Vegetable oil (rapeseed) (Tesco)	590	590	-	-		
Soy protein hydrolysate (Amisoy)	400	-	-	52		
Cornflour – 88% starch (Tesco)	568	2.8[b]	500	< 1[b]		
Glucose	500	-	500	-		

[a] In addition 2.7 mg/l nitrogen from ammonium chloride is accounted for in the molecular formula and COD:N ratio. [b] Calculated from nutritional data

5.3.4 Bacterial inocula and reference strains

Four *Bacillus* strains, purchased from the UK national culture collection (NCIMB), were screened for lipolytic potential on tributyrin agar (TBA). *B. pumilus* NCIMB

1522 showed best overall enzyme activity producing clearance zones with mean diameters of 5, 9 and 11 mm after incubation for eight days at 20, 30 and 37ºC respectively, but performed poorly in subsequent degradation tests with rapeseed oil in aqueous media. *B. licheniformis* also showed good activity towards tributyrin at 37ºC (6 mm clearance) and was 4 times more efficient at degrading rapeseed oil, with mean degradation rates of 7.6 mg/l/h compared to 1.8 mg/l/h for *B. pumilus.* These results highlight limitations of using TBA for screening true lipase activity as it does not reflect the ability to hydrolyse long-chain fatty acids which dominate edible fats and oils and also form the major fatty acid components of FOG deposits in sewers (Williams et al., 2012). Nor does extracellular triglyceride hydrolysis guarantee uptake of FFAs into bacterial cells. Moreover *B. licheniformis* 9375 has been shown to possess a number of genes associated with metabolism of various carbohydrates, amino acids, lipids and proteins (Voigt et al., 2007), producing a variety of extracellular enzymes including proteases and peptidases and enzymes for carbohydrate degradation (Voigt et al., 2006).

As such *B. licheniformis* 9375 was capable of metabolising a variety of organic carbon and nitrogen sources, enabling assessment of their impact as alternatives to FOG, and was consequently selected as the reference strain for protocol development.

5.3.4.1 Dose level for the testing protocol

Bioadditive products are typically dosed at 10^4 – 10^5 cfu/ml in commercial applications (Osprey Biotechnics, n.d.; Zebec Biologicals, n.d.), while academic studies have employed higher starting doses, 10^5 – 10^7 cfu/ml (Brooksbank et al., 2007; Tano-Debrah et al., 1999; Tzirita and Quilty, 2012). Accordingly, dosing levels of 10^4 – 10^7 cfu/ml were tested using *B. licheniformis* 9375 and a commercial bioadditive product. Variable doses were achieved by inoculating different volumes (0.02 – 20 ml per flask) from the same bacterial culture grown in nutrient broth. In order to eliminate any influence of nutrient carry over from the pre-culture, all doses were made up to 20 ml with filter sterilised culture. Residual

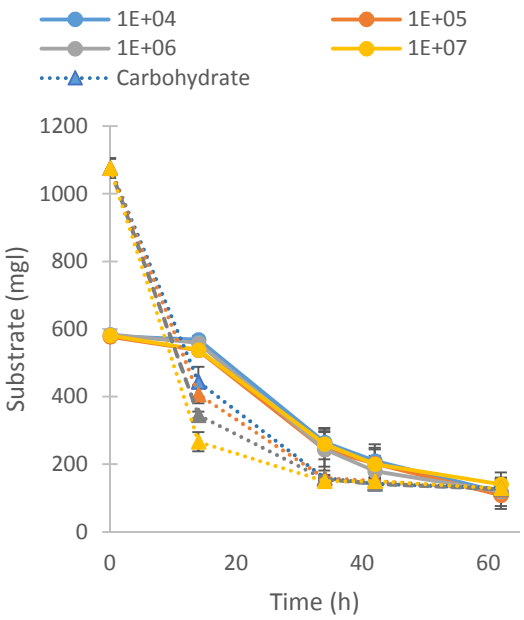
soluble nitrogen was higher than anticipated resulting in a theoretical COD:N ratio of 42 whereas the synthetic wastewater composition ratio was designed to be 62.

Addition of monoculture of reference strain

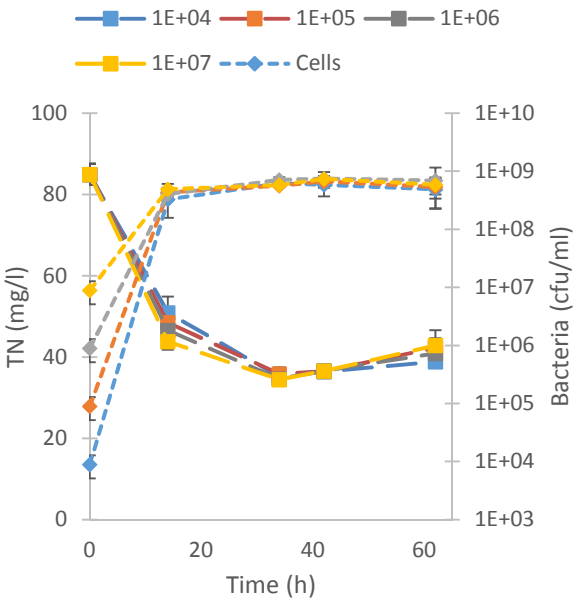
At a fixed medium concentration, varying the dosing level of *B. licheniformis* between 10^4, 10^5, 10^6 and 10^7 cfu/ml had no significant impact on maximum population density ($6.4 \times 10^8 \pm 2.4 \times 10^8$ cfu/ml) or FOG removal (**Figure 5-3**(a) and (b)). Maximum FOG removal rates of 14.9 ± 0.8 mg-FOG/l/h were observed between 14 and 34 hours, followed by deceleration up to 62 hours such that overall stationary phase removal was 9.5 ± 0.4 mg-FOG/l/h). In both cases the lower end of the range was associated with the highest dose. These rates compare favourably with those for the wastewater with the most similar composition and COD:N ratio (45) in Section 4.3.2.2 in Chapter 4. Using a dose of 5×10^5 cfu/ml, maximum removal rates of 14.0 ± 0.9 mg-FOG/l/h were observed between 16 and 38 hours; and mean stationary phase rates of 9.3 ± 0.3 mg-FOG/l/h from 16 – 62 hours. This suggests that the different type of proteins in the nutrient broth dose, compared to the soy hydrolysate, did not alter the bacterial response to initial COD:N.

By 14 hours, residual carbohydrate concentrations had decreased from 1000 mg/l to 443, 407, 346, 267 mg/l with increasing dose. Assuming that the readily metabolisable fraction, 500 mg/l glucose, was removed first, the results indicate that starch metabolisation was initiated earlier at higher doses. Lipase enzyme activity shows a similar trend whereby, at 14 hours, butyrate-specific enzyme activity was approximately 3, 4 and 5-fold higher; and octanoate-specific enzyme 5, 6 and 7 times higher with increasing dose **Figure 5-3**(c) and (d). For all doses, enzyme activities reached similar levels by 34 hours (4.8 ± 0.9 and 3.2 ± 0.2 mU/ml for butyrate and octanoate respectively) which persisted for up to 62 hours. Similarities in the time course, and magnitude of lipase production is consistent with FOG removal. In combination, the data suggest that production of enzymes for both starch and FOG hydrolysis were triggered earlier in response to more rapid glucose exhaustion for higher inoculum doses.

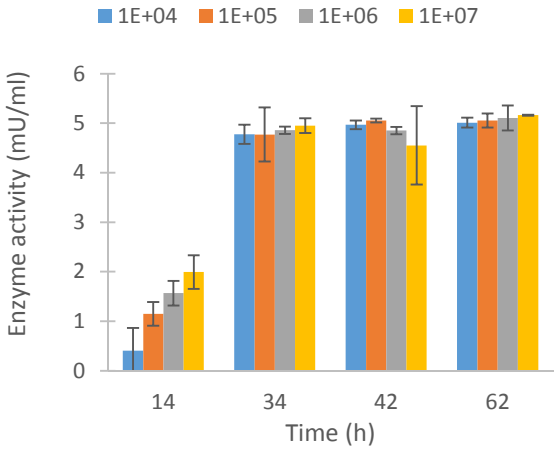
(a) FOG and carbohydrate



(b) TN and growth



(c) Butyrate-specific lipase activity



(d) Octanoate-specific lipase activity

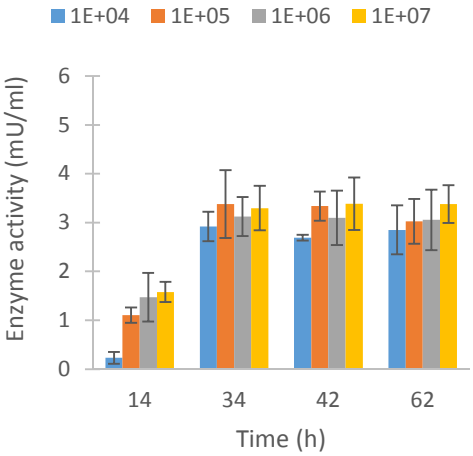


Figure 5-3 Substrate removal profiles and lipase activity for *B. licheniformis* in synthetic wastewater at doses of 10^4 (blue); 10^5 (red); 10^6 (grey); and 10^7 (orange) cfu/ml: (a) FOG (circles, solid lines); carbohydrate (triangles, dashed lines); (b) bacterial growth (diamonds, dotted lines) and total nitrogen (squares, long-dashed lines); (c) and (d) butyrate-specific and octanoate-specific lipase activity, respectively. Data represent means of triplicate independent experiments with error bars as standard deviation.

Addition of a multi-strain commercial product

A second experiment was done using a multi-strain commercial bioadditive product which was dosed directly into the wastewater without pre-culturing in nutrient broth. Consequently, the synthetic WW composition remained unaltered with a COD:N ratio of 61.2 ± 0.2. Figure 5-4(b) shows that growth rates were slower for the commercial product cultures: reaching population densities of up to an order of magnitude lower than *B. licheniformis* at 14 hours. Growth continued beyond 14 hours, with populations roughly doubling by 34 hours.However, mean cell numbers for the commercial products, during stationary phase (defined as 14 – 62 hours), were only $2.7 \times 10^8 \pm 6.3 \times 10^7$ cfu/ml - approximately half those in the *Bacillus* cultures ($6.2 \times 10^8 \pm 1.4 \times 10^8$ cfu/ml). These results are consistent with the trend towards decreasing populations under more N-limiting conditions observed in Chapter 4. Similarly to observations for single strain addition, maximum FOG removal rates were observed between 14 and 34 hours (Figure 5-4(a)), with an apparent correlation with increasing dose. However, there was no significant decrease in residual FOG concentration between the 34 and 42 hour sampling points, in fact mean concentrations were 38 mg/l higher in the 10^7 cfu/ml series, which suggests that variability in bacterial population composition between different flasks may have led to some difference in FOG removal response. When averaged over the whole stationary phase period of 14 – 62 hours removal rates were not significantly different for the different dosing levels, but were approximately half those in the *Bacillus* cultures: 4.7 ±1.1, 5.5 ± 0.7, 5.1 ± 1.7, 4.1 ± 0.2 mg-FOG/l/h for doses of 10^4, 10^5, 10^6 and 10^7 cfu/ml respectively. This response is consistent with smaller populations having lower maintenance energy requirements and thus removing less carbon. Population levels were, on average only 56% of those in *Bacillus* cultures in the most comparable medium composition in Chapter 4 at COD:N 56 (4.8E+08 ± 6.4E+07 cfu/ml), in association with mean FOG removal rates 58% of 8.5 ± 1.5 mg-FOG/l/h.

The lack of a dose related response is in contrast to several previous studies where maximum lipase activity was observed within a much smaller range of

doses (Baharum et al., 2003; Hasan et al., 2006; Salihu et al., 2011; Sharma et al., 2012; Thakur et al., 2014; Veerapagu et al., 2013). These authors proposed, that lower doses led to lower populations, whereas cultures with higher doses were subject to nutrient and dissolved oxygen limitation. For example, Masomian et al. (2010) compared a range of inoculum sizes from 1 – 9% (v/v) of *Aneurinibacillus thermoaerophilus*: lipase activity and population levels in 48 hour cultures increased as doses increased from 1 – 7%, with both parameters decreasing in a 9% inoculum.

Overall, the data supports a relationship between population size and FOG uptake. Higher uptake rates were observed in the *Bacillus* cultures relative the commercial product which was most likely due to a lower COD:N ratio in the former, 42:1 as opposed to 62:1. However, there is no evidence to suggest that inoculating with different size doses impacted significantly on population levels, FOG removal or lipase production.

Variations in dosing level had little effect on FOG removal. Thus, dosing according to manufacturers' instructions is recommended for the test protocol, as results for different products dosed at different levels should be comparable.

(a) Commercial product FOG and carbs

(b) Commercial product TN and growth

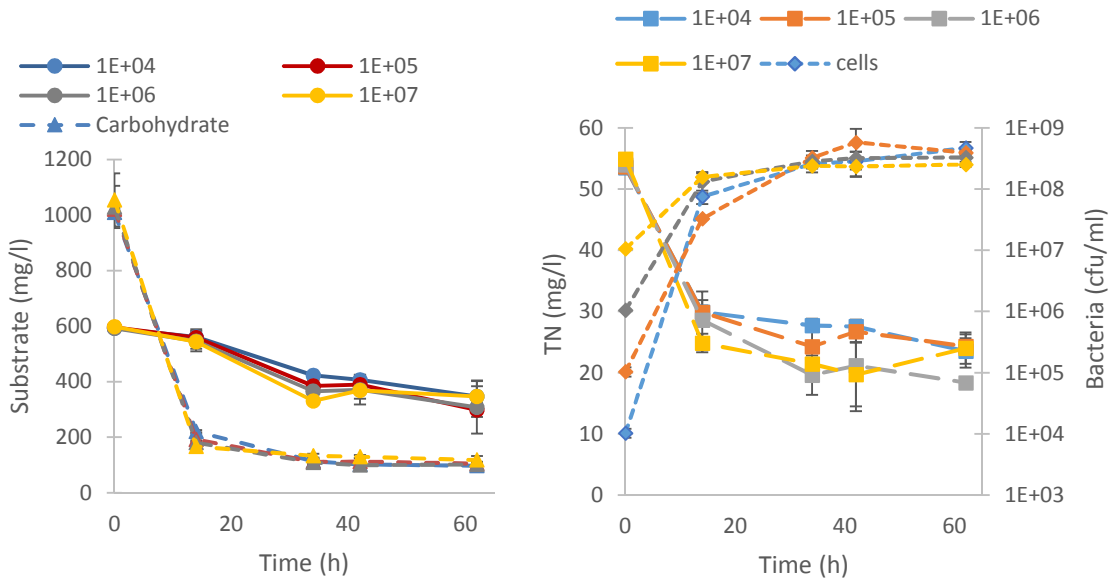


Figure 5-4 Substrate removal profiles for a commercial bioadditive product in synthetic wastewater at doses of 10^4 (blue); 10^5 (red); 10^6 (grey); and 10^7 (orange) cfu/ml: (a) FOG (circles, solid lines); carbohydrate (triangles, dashed lines); (b) bacterial growth (diamonds, dotted lines) and total nitrogen (squares, long-dashed lines). Data represent means of triplicate independent experiments with error bars as standard deviation.

5.3.5 Contact time for the test protocol

Comparable FOG removal rates were determined from extended experiments up to 120 hours dosed at 10^6 cfu/ml, in *Bacillus licheniformis* cultures in a different synthetic media composition with an initial COD:N of 47. Maximum rates of 13.5 ± 0.2 mg/l/h were observed between 24 and 48 hours. Rates decelerated thereafter with a mean rate of 9.3 ± 1.8 mg/l/h over 24 – 72 hours, and an average rate of 5.7 ± 0.0 mg/l/h up to 120 hours. FOG removal rates were more variable in cultures of the commercial product (initial COD:N 53) and showed less marked deceleration over the course of the experiment. Removal rates over a 24 – 72 hour period were 5.9 ± 2 mg/l/h, again comparable with the mean 14 – 62 hour rate of 4.9 ± 0.6 mg/l/h in the shorter trials. In both experiments, almost complete

removal was achieved by 120 hours: 90% for the commercial product and to undetectable levels for the *Bacillus,* yet an indication of FOG-degrading efficacy could be established during early/mid stationary phase. Therefore, sampling times of 16, 40 and 64 hours are suggested to combine technically feasibility with capturing maximum rates of FOG removal

5.3.6 Bioadditive performance in real FSE wastewater

Degradation experiments were done using *B. licheniformis* and the commercial bioadditive in real FSE wastewaters from two different food service establishments. In one trial the wastewater was amended by adding starch or ammonium chloride to vary the COD:N (2). Variability was observed in the FOG removal profiles which was likely due to difficulties in achieving an initial homogenous distribution of FOG between sub samples taken from the bulk sample. For example, Figure 5-5 indicates little change in total FOG concentration over the first 40 hours. At 16 and 40 hours, observed emulsified FOG decreased from an initial concentration of 708 mg/l to 608 and 609 mg/l, yet observed free FOG concentrations apparently increased from 433, 504 and 516 mg/l. It is likely that there was more free FOG initially in the individual flasks measured at 16 and 40 hours, which masked total FOG removal. No free FOG was detected at 160 hours although initially free FOG had been observed visually in all flasks, suggesting that it had become emulsified due to the production of microbial biosurfactants which increase bioavailability of FOG. In other trials variability resulted in an initial dip in mean FOG at 16 hours followed by an increase up to the 64-hour sampling time. These technical issues highlight the benefits of using a synthetic wastewater with known FOG concentrations in each flask to facilitate the evaluation of FOG removal.

To smooth out variability, FOG removal rate constants (k) were calculated for the whole experimental period rather than just for stationary phase. Initial theoretical COD:N ratios were estimated using COD conversion factors of 2.88, 1.07 and 1.5 for FOG, carbohydrate and protein respectively (Grady et al., 2011), where protein was estimated from TN using the standard conversion factor of 6.25.

Data were plotted in Figure 5-6 together with FOG k for *B. licheniformis* and the bioadditive product in synthetic wastewater compositions determined in Chapter 5. Despite very different initial compositions, summarised in 2, FOG removal response for the bioadditive in real WW showed a clear negative relationship with initial COD:N, following a parallel trend line to experiments with *Bacillus* in synthetic WW. FOG k was lowest at highest COD:N: 0.0053 h^{-1} at COD:N 138 and 150 for *Bacillus* cultures, and was not significantly different from results in synthetic media at COD:N 147. Moreover, the results were very similar to the commercial product in real WW (0.0049 and 0.0055 h^{-1} at 138 and 150 respectively). Similarly FOG k for commercial product cultures in the test protocol synthetic composition lay on the real WW trend line at COD:N 61, and the value of 0.0222 h^{-1} at COD:N 47 was also not significantly different from real WW - 0.0207 h^{-1} at COD:N 39.

Overall the data suggest that FOG removal in synthetic media is representative of that achieved in real FSE wastewaters of varying compositions: the dominant influence being the initial ratio of COD:N.

Table 5-2 Initial concentrations of FOG, carbohydrate and nitrogen in real wastewater used in the degradation trials

FOG (mg/l)	Carbohydrate (mg/l)	TN (mg/l)	COD: N
698 ± 61	222 ± 11	16 ± 1	150
1141 ± 77	883 ± 16	33 ± 1	138
356 ± 78	3028 ± 236	56 ± 3	86
356 ± 78	3028 ± 236	*122 ± 6	39
356 ± 78	#4654 ± 186	56 ± 3	117

*Amended with additional ammonium chloride

#Amended with additional starch

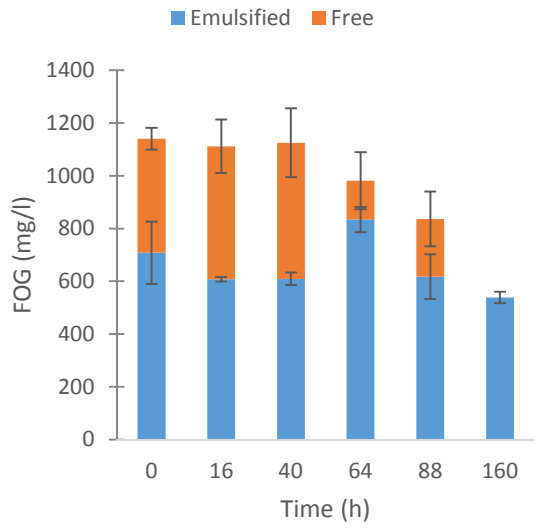


Figure 5-5 Relative proportions of free and emulsified FOG in cultures of a commercial bioadditive product in FSE wastewater.

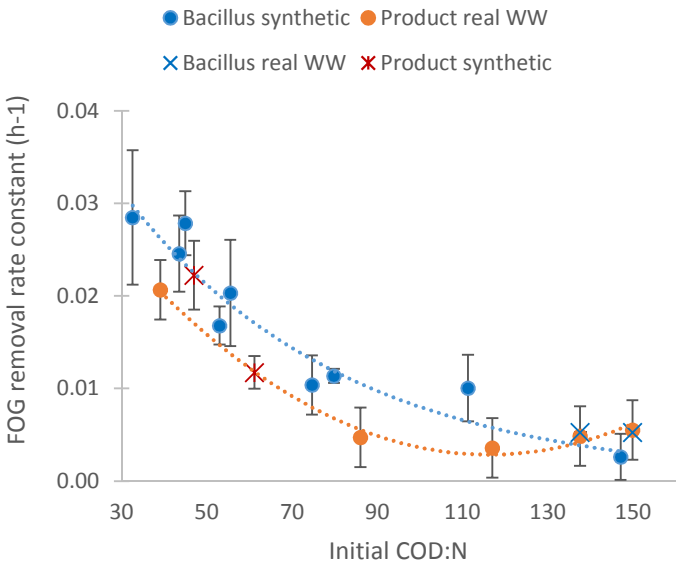


Figure 5-6 FOG removal rate constant for *B. licheniformis* and a commercial bioadditive product cultured in real and synthetic wastewaters of varying initial composition detailed in 2.

5.4 Proposed Protocol methodology

Leading on from the results presented in Section 5.3 we propose a Protocol methodology for testing the efficacy of FOG-degrading bioadditive products designed for commercial use in FSE environments.

5.4.1 Protocol Test media

In order to test the versatility of bioadditives in response to wastewater with varying composition, it is proposed that products be tested across a range of COD:N ratios from 20:1 to 140:1. Keeping the organic matter composition constant, COD:N is varied by amendment with ammonium nitrate according to Table 5-3. FOG removal will be reported as first order removal rate constant in h^{-1} (mean and standard deviation of triplicates) as a graph in the form of Figure 5-7. The testing should be run in parallel with *B. licheniformis* NCIMB 9375, used as a reference bacterium to validate the testing conditions, for which first order removal rate constants should be between 0.004 and 0.04 for COD:N ratio of 140:1 and 20:1, respectively.

Table 5-3 Proposed media composition for the Bioadditive Test Protocol

(a)

Organic component (mg/l)	
Vegetable oil (rapeseed) (Tesco)	590
Soy protein hydrolysate (Amisoy)	140
Cornflour – 88% starch (Tesco)	568
Glucose	500
Total theoretical COD (mg/l)	2943

(b)

COD:N	**NH_4NO_3 (mg/l)**	**Total nitrogen**
20	360	147
60	80	49
100	24	29
140	0	21

5.4.2 Proposed Protocol procedure

1) Prepare synthetic FSE WW medium according to Appendices A and B and transfer 500 ml into 51 x 1l pre-weighed shake flasks:
    - 3 for each time point and each COD:N ratio for the bioadditive to test (36)
    - 3 for each time point at a chosen COD:N ratio with *B. licheniformis* NCIMB 9375) (12)
    - 3 blank controls, to be extracted at the end of the trial to verify the extraction yields of the Gerber method (3)

For ease of space management, we recommend three separate runs with one flask for each condition (COD:N). This would result in 5 samples to be analysed per each time point.

2) Autoclave before adding buffer (pH7) and sodium dodecyl sulphate surfactant.
3) Transfer weighed masses of filter-sterilised vegetable oil dropwise into each flask using a tared sterile transfer pipette. Record mass.
4) Place flasks in an incubator shaker set at 40ºC and 150 rpm for a few hours to emulsify the oil.
5) Remove and inoculate with bioadditive according to manufacturers' instructions under aseptic conditions and replace in incubator. Three flasks are not inoculated as blank controls.
6) At each time sampling point (16, 40 and 64 hours) remove four flasks (one of each COD:N ratio) plus one for *Bacillus* control.
7) Weigh each flask to determine the total volume of medium (assuming 1 kg = 1l).

8) Weigh triplicate subsamples of approximately 100 ml into 250 ml polycarbonate centrifuge bottles, and centrifuge at 10000 g for 20 minutes.

9) Decant aqueous phase into separate centrifuge bottles, ensuring that any residual creamed material from around the medium surface level is rinsed into the new bottle by means of a DI water wash bottle.

10) Add 1 ml of 10% sodium caseinate solution and mix rapidly with an inert stirrer. Reduce pH to around 4.2 by adding 1M HCl and stir gently until the formation of large aggregates is observed. The optimum flocculation pH may vary – pH can be fine-tuned with 0.1M HCl and 1% NaOH. Rinse any adhered material from the pH probe and stirrer back into the bottle.

11) Place bottles horizontally on a platform shaker and gently shake (80 - 90rpm) to keep sediments in suspension for 15 mins, or until the aqueous phase is clarified. Higher shaking speeds cause floc breakage.

12) Centrifuge at 2000g for 10 mins to isolate the precipitate

13) Decant supernatant from the bottle leaving as little liquid as possible (a few mls only).

14) Raise pH to ~ 7 with 1% NaOH (depends on flocculation pH: 3-4 drops is sufficient for flocculation at pH ~ 4). Sediment removal from bottle surface is facilitated by laying the bottle horizontally to enable liquid contact with the precipitate.

15) Use a 5/10 ml pipette with a wide tip to recover the precipitate and pipette slurry gently in a layer onto sulphuric acid in a 0 – 1% Gerber butyrometer. Rinse any residual material bottle with a few mls DI water and add to the butyrometer. Add 1ml isoamyl alcohol followed by DI water, if required, to bring the level up to ⅓ – ½ way up the neck. Insert basal bung and shake vigorously for 90 secs – the butyrometer will become very hot.

16) Centrifuge butyrometers in a heated Gerber unit for 10 mins and temper in a water bath held at 65°C for 3 – 10 mins before reading the volume of

extracted oil from the graduated scale. There should be no air bubbles or undigested material in the oil column. Repeat centrifugation, tempering and reading. If the difference between 1st and 2nd readings exceeds 0.05%, or if air bubbles are present in the oil column, repeat again. The final reading is recorded for FOG quantification.

17) FOG concentration (mg/l) is quantified according to Equation 5-1.

$$FOG = 1000 * \frac{butyrometer\ reading * 125 * 0.89}{mass\ bulk\ aqueous\ sample}$$ **Equation 5-1**

Equation

18) Report data as first order removal rate constant in h-1 (mean and standard deviation of triplicates) as a graph in the form of Figure 5-67.

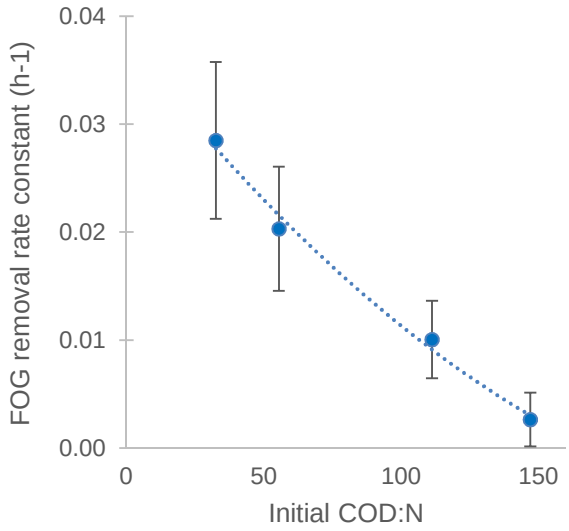


Figure 5-7 Example of results from testing protocol

5.5 Conclusion

A protocol to quantify the FOG-degrading ability of bioadditives used in FSE wastewater treatment was developed and validated. A synthetic culture medium was designed to represent realistic characteristics of food service establishment wastewater in terms of organic composition, trace elements and surfactant concentrations. The benefits of using a synthetic medium with known concentrations of oil were highlighted by experiments in real FSE wastewaters, where FOG removal response was less well defined, due to technical difficulties in achieving a homogeneous distribution of free oil amongst the reaction flasks.

Using synthetic and real wastewaters of varying compositions, a clear negative relationship was determined between FOG removal and initial COD:N over a range of 33:1 to 150:1. FOG removal rate constants were comparable in synthetic and real WW with similar COD:N for cultures of *Bacillus licheniformis* NCIMB 9375, and a commercial bioadditive product. A protocol culture medium is proposed to test the efficacy of bioadditive products over a range of COD:N from traditionally accepted optimum conditions (20:1), up to 140:1 to present more challenging conditions compared to previous studies.

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6 Considerations for selection of the most appropriate FOG management solution

Poor FOG management in commercial kitchens leading to sewer blockages can incur environmental and financial costs both to the individual business, and the wider locality, and FSEs are obliged by law to minimise FOG discharges. Preventing FOG disposal down the drains is undoubtably the best solution: practices such as dry wiping of plates and utensils prior to washing up should be encouraged by raising staff awareness. However, inevitably some FOG will be discharged in wash water. Physical or biological technologies provide a secondary barrier by removing FOG from wastewater before it enters the sewer network. Selection of the best technological strategy is primarily dependent on financial resources; space availability; FOG concentration and degree of emulsification; and the volume of wastewater produced.

6.1 FOG management technologies

6.1.1 Passive grease separators

The British Standard (BS EN 1825 2002) describes how to select an appropriately sized grease separator to achieve sufficient residence time to allow for passive gravity separation as the less dense FOG floats to the surface over time. As such, maximum wastewater flow rate, such as when a full sink is discharged, is a crucial determining factor. Likely flow rates can be estimated by the size, type and number of appliances connected to the separator. Separators are deemed to work efficiently until the working volume is reduced by the accumulation of solid sediments at the bottom of the tank and/or the floating FOG layer reaches 25%, at which point they will require pumping out by a licensed contractor (Wallace et al., 2017). The amount of FOG/solids loading in the wastewater can be estimated from the meals served per day. Based on the design criterion, BS EN 1825 compliant separators are often too large to be retrofitted within a commercial kitchen and must usually be situated externally or underground. Under BS EN 1825, calculations based on a number of criteria yield different categories of nominal size (NS) for grease separator selection. For example an NS1 device, tested at a 1 l/s flow rate, has a design volume of 380 L, although smaller nominal sizes may still comply with the testing requirements at lower flow rates (Fairley, 2018).

Smaller separators may or may not comply with BS EN 1825, but may satisfy the Building Regulations 2010 criterion for "*effective means of grease removal*". These separators are typically employed where space requirements prevent installation of a larger model, or where capital investment (CAPEX) is prohibitive. Being smaller they allow shorter retention times, separate FOG less efficiently, and need to be emptied more frequently, typically every 2 – 4 weeks, than larger capacity BS EN 1825 separators which are typically emptied 3-4 times a year.

6.1.2 Grease removal units (GRUs)

Grease removal units are typically smaller than BS EN 1825 gravity separators. The major difference, is that when FOG floats to the surface it is continually removed into a separate container. Units can be passive, where floating FOG flows from the tank into an external collection vessel, or automated whereby a mechanical skimmer facilitates FOG removal (Wallace et al., 2017). Thus, working volume is primarily only decreased by sediment accumulation, reducing the emptying frequency compared to similarly-sized passive separators. GRUs need to be maintained by the kitchen staff on a daily and weekly basis, to include emptying the FOG collection container, emptying the solids strainer basket and cleaning the skimmer blades and outlet valve.

6.1.3 Biological dosing systems

Bioadditive products comprise of non-hazardous bacterial populations selected for their ability to degrade FOG as well as other organic components present in wastewater. FOG-degrading bacteria produce extracellular enzymes that catalyse the breakdown of triglyceride molecules into glycerol and free fatty acids, which are then taken up by the cells and used for growth or oxidised to generate energy, carbon dioxide and water. The most common biological dosing systems typically comprise of wall-mounted units containing packs of bioadditive fluid which is automatically dispensed either by drip feeding, or by a timer set to dispense a daily dose at a time of low- or no-wastewater flow, usually overnight. Some systems feature an alarm to indicate that the fluid pack needs changing, which might be monthly based on a 5 L sized pack. Products are usually dosed directly into the drainage pipe work downstream of a FOG effluent source and upstream of a grease separator. Care should be taken not to site systems too close to appliances that discharge very hot

effluents as excessive heat could kill the bioadditives (British Water, 2018). As with the performance of grease separators, the efficacy of biological treatment is influenced by contact time, and hence by the wastewater flow rate.

Although biological systems can be used as a standalone inline system, they are frequently used in conjunction with passive gravity separators. Combining the two technologies enhances the efficacy of both systems. Free FOG is easily removed by physical separation while biological treatment favours emulsified FOG. Grease separator tanks can reduce flow rates and thus allow additional contact time for microbial activity. In return, the microbial action of FOG/organic degradation can slow down the accumulation of FOG and solids and, as a result, reduce maintenance requirements and frequency of pumping (British Water, 2018).

6.2 Selection of site-specific FOG management solutions

Selection of the best FOG management strategy should be based on the most optimal solution under the specific operating conditions of a particular food service establishment. Each technological solution has various associated strengths and weaknesses, as summarised in the SWOT analysis in Table 6-1, and the Optioneering considerations in Table 6-2, which should be considered when addressing the site-specific needs of any catering establishment. The ultimate aim is to minimise FOG discharges to prevent *drain blockages*, which would directly affect the FSE operations, and *sewer blockages*, with a more widespread impact on neighbouring premises and which might result in costly prosecutions by local authorities.

Specifically, the UK Building Regulations 2010 prescribe that FSEs be fitted with a BS EN 1825 compliant grease separator “or other effective means of grease removal”, whilst BS EN 1825 defines grease separator size on a number of criteria, but mainly wastewater flow rates and FOG-loading. As these factors are rarely known for a catering establishment, two common methods for separator size selection, which consider wastewater flow and FOG-loading are based on either (1) number and type of appliance; or (2) meals served per day, operating hours and kitchen type (eg. restaurant, take-away, hotel etc.). However, the two calculation methods can produce significantly different results. For example, a small restaurant with a dishwasher and a sink, serving 100 meals per day, might require a separator with a working volume of

0.75 m^3 or 1.5 m^3 according to the two above mentioned methods (Fairley, 2017). Thus, even following the Standard calculations might lead to selection of an inappropriate separator in practical terms. Problems following separators under-sizing include insufficient contact time for FOG to separate, allowing a proportion to be discharged, but also increase frequency of emptying and cleaning, producing additional operating costs.

Although a suitably-sized device according to the Standard would appear to be the best option, a major limitation faced by many establishments is the space required for its installation and access for maintenance, which may simply not be available in certain premises. In these cases, an alternative option is for an under-sized separator, for meeting the "other effective means of grease removal" requirements stated in the plumbing regulations. Flows can be split between multiple separator devices fitted to different appliances. An important issue with smaller separators is hot discharges from dishwashers. Particularly, when a full dishwasher wash tank is emptied for cleaning, large volumes of hot water can be discharged rapidly into the separator and wash out previously retained FOG. This is a greater problem for small separators, as devices with larger working volumes will allow for thermal balancing within the tank.

GRUs are available in a range of sizes and are designed to be easier to maintain, with removable FOG collection vessels and solids filter baskets. Cleaning should be managed frequently, often daily to maintain system efficiency and minimise odour generation. Further consideration should be given to facilities for hygienic storage of the collected FOG to prevent attracting pests, and to the costs associated with disposal via a licensed waste oil carrier.

However, even the most appropriately sized grease separators will struggle to remove emulsified FOG. Performance testing for BS EN 1825 separators is based on a model wastewater comprising of water and light diesel oil with a degree of mechanical agitation producing an unstable emulsion, and are thus designed to remove oil droplets of diameter greater than 150 µm (American Petroleum Institute, 1990). The current work has identified that the majority of oil droplets in FSE effluents from sinks and dishwashers are considerably smaller than the API test limitation of 150 µm. For example, wastewater from a commercial dishwasher investigated in Chapter 3 was

dominated by emulsified FOG (94 ± 9% of total) with 99% of droplets smaller than 67 µm. Samples from a potwash sink showed higher proportions of free oil floating on the surface (59 ± 16%) which should be easily removed by gravity separation, with a maximum droplet size in the emulsified fraction of 98 µm. On average 68% of total FOG from both sources was emulsified, with a mean concentration of 300 mg/l. Reliance on conventional BS EN 1825 separator devices might allow all the emulsified FOG to be discharged to the sewer network. In contrast, emulsified FOG is more amenable to microbial degradation – smaller droplets have a larger surface area to volume ratio, allowing for the attachment of bacteria and enzymes. Consequently, regardless of size, there is always a case for biological dosing to enhance FOG removal efficiency within passive grease separators and GRUs.

When using biological treatment as a standalone inline system, consideration should be taken as to identify the optimum dosing and location for the addition to achieve maximum efficiency. For example, dosing points should be sufficiently downstream of appliances discharging hot water to prevent thermal shock, which may inactivate or kill bacterial populations. Moreover, the use of sanitising chemicals may need to be revised, for example applying concentrated bleach directly down a sink would effectively destroy bioadditive populations.

To summarise, the dominant considerations involved in FOG management technology Optioneering are cost, space requirements, wastewater flow rates and the volume of FOG produced. In addition, Table 6-2 provides an assessment of the nature of the wastewater in terms of temperature, solids and FOG emulsification. Small droplets of emulsified FOG cannot be removed efficiently by physical means but enhance the action of biological products. However, as this study has shown, the presence of other forms of more easily-biodegradable organic matter can influence microbial FOG removal. The ratio of COD:N was found to have a significant influence, with FOG removal rates decreasing with increasing ratios. Many bioadditive products also contain nutrients, including nitrogen which reduces the COD:N ratio to enhance FOG uptake.

Table 6-1 SWOT (strengths, weaknesses, opportunities and threats) analysis for FOG management technologies

	Strengths	Weaknesses	Opportunities	Threats
Large grease separators (BS EN 1825 compliant)	- Long contact times for physical separation - Proven removal efficiency - Well established technology worldwide - BS/EN standard	- Large footprint - High CAPEX and OPEX - Maintenance/cleaning requirement (licensed contractor) - Potential odour formation	- Separation of solids - Use of material(s) for energy recovery - Reduction in general COD due to contact time by natural microbial activity	- Emulsified oil not removed by the system
Small grease separators (non BS EN 1825 compliant)	- Small footprint - Lower CAPEX than large separators	- Short contact times for physical separation - Potential odour formation - Maintenance/cleaning requirement (licensed contractor)	- Separation of solids - Use of material(s) for energy recovery	- Emulsified oil not removed - Lack of standardisation (BS std) - Potential growth of pathogens in kitchen environment - FOG washout/remobilisation with high temperature inflow
Grease removal units (GRU)	- Small footprint - Low OPEX	- High CAPEX - Short contact times for physical separation - Potential odour formation - Maintenance/cleaning requirement (contractor and in-house) - Electrical and mechanical requirements	- Separation of solids - Use of material(s) for energy recovery	- No current design / performance standards recognised in the UK - Emulsified oil not removed - Potential for break-down of moving mechanism - Potential growth of pathogens in kitchen environment - Potential for pest infestation
Inline biodosing	- Minimal maintenance requirements - Simple technology - Low OPEX+CAPEX costs - Minimal space required	- Lack of design parameter - Unknown contact time - Action impacted by wastewater quality/kitchen cuisine	- Reduction in general COD - Emulsified oil removed by the system	- Lack of standardisation (BS std) - Temperature and pH variability - Chemicals - Maintaining biomass - Wastewater composition - Free oil removal unknown
Biodosing in a grease separator	- OPEX+CAPEX costs (depending on separator size) - Some space required - Longer retention times than inline dosing	- Potential high CAPEX and OPEX if applied to BS1825 size separators - Space requirements	- Reduction in general COD - Reduced cost of maintenance and frequency of cleaning - Reduced odour formation - Emulsified and free oil removed	- Lack of standardisation (BS std) - Temperature and pH variability - Chemicals - Maintaining biomass - Wastewater composition

Table 6-2 Optioneering considerations for FOG management strategy selection

Option	Physical Requirements	Wastewater requirement	Pros/cons
Do Nothing	None		- Low cost in the short term - Likely that more failures will occur in the future leading blockages/reactive remediation/fines
Physical separation: Grease separators (outdoor/BS EN 1825)	- Suitable for large establishments - Suitable for high WW flow rates - Large footprint required - Access required for pumping/cleaning - Not suitable for kitchens with a high number of automatic washing equipment (containing mainly emulsified oil)	- Not suitable for effluents with highly emulsified oil - Suitable for high BOD/COD/FOG/solids	- Expensive to install and maintain - Regular cleaning is required for good removal efficiency - FOG recovery for energy
Physical separation: Small grease separators (indoor/under sink) & GRUs	- Small space required - Not suitable for kitchens with a high number of automatic washing equipment (high temperature effecting separation)	- Not suitable for effluents with high FOG loading or emulsified oil - Not suitable for high solids loading - Not suitable for WW at high temperature	- Easier to install and maintain - Relatively low-cost technology - FOG recovery for energy
Biological: inline dosing	- Minimal space required - Dosing point should be well downstream from any source of heat or u-bends - Not suitable for high flow-rate (low contact time)	- Suitable for effluents with highly emulsified oil - Biological processes can be slow inline - Products available to work at high pH or temperature - COD:N ratio should be balanced to optimise removal	- Pre-growth of bioadditions might be required - Easy to install and maintain - Relatively low-cost technology
Biological: biodosing in a grease separator	- Small space required - Suitable for medium flow-rate (adequate contact time) - Contact time available for activation of non-live products	- Suitable for water with highly emulsified oil - Products available to work at high pH or temperature - Physical separation of FOG in the systems will improve the COD:N ratio	- Easy to install and maintain - Relatively low-cost technology - FOG recovery for energy

6.2.1 Cost benefit analysis

Table 6-3 provides a range of likely costs associated with different FOG management technologies, including capital expenditure (CAPEX), annual operating expenditure (OPEX) and an estimate for total expenditure averaged over five years (TOTEX). Estimates for installation of the various technologies was unavailable, although we can assume that under-sink small separators and biodosing systems will incur significantly lower costs than installation of a large outdoor or underground unit.

Firstly, the option not to implement any strategy would result in frequent drain blockages, for example one pub reported having to jet clean their drains every 4-5 months prior to adopting a biodosing system (Mechline, 2018). However, premises which fail to manage FOG discharges might not only block their own drains, potentially causing costly flooding incidents, but also impact the local sewer network. In two recent cases successful prosecutions were brought against catering establishments by Severn Trent Water under Section 111 of the Water Industry Act. Despite numerous inspections, and repeated requests to address the issue the companies refused to take action, and were ultimately ordered to pay fines, including costs, of £5,495 and £9,266 (BBC, 2018; Seymour, 2016).

Passive grease separators have long been the traditional choice for many FSEs. A internet review of costs and services from various UK suppliers revealed prices varied depending on size and construction materials: different grades of stainless steel of HDPE. Small separators ranged from £ 203 for a 20 litre model with a design flow rate of 0.45 l/s suitable for an establishment serving 10 – 15 meals per day (UK Grease Traps Direct, 2017), to £972 for a 100 litre device, 2.75 l/s flow rate which could serve 2 large sinks and a dishwasher (Aqua Cure Ltd, 2016a). Clearly, the smaller model would be ideal for a very small establishment, such as a small B&B, whilst the larger model might be suitable for a small café or restaurant. Larger separators, more suitable for outdoor installation, ranged from £827 for a 482 litre model capable of accommodating 8 large sinks and the waste

from 640 meals per day (UK Grease Traps Direct, 2017), to £4200 for an 11,000 litre unit (Clearflow Ltd, 2018). Within these ranges, there is flexibility for combining multiple smaller units to substitute for a larger model. However, the major costs associated with grease separators are for pumping out the retained FOG and solids on a regular basis, which should be carried out by a licenced waste carrier. Typically, smaller units require emptying monthly, whilst larger units might last for 3 months provided they are not over-loaded. At typical costs of £150 - £200 per pump out for small units, rising to £600+ for the largest separators, annual operating costs are well in excess of the original capital expenditure. Moreover, the increased frequency of pumping for smaller units results in similar OPEX costs to larger models. Consequently, for medium to large premises where CAPEX might be reduced by substituting two or more smaller separators for a larger unit, increased OPEX will likely negate any initial savings. For example, 5-year TOTEX for units larger than 100 L capacity were estimated from £7,900 to £16,200, compared to £9,200 - £13,000 for each sub-100 L unit (Table 6-3). Clearly, unless space availability is prohibitive, a single large separator could be more cost-effective than multiple smaller models.

In contrast, GRUs are more expensive to purchase (£2160 - £5800) but are associated with lower OPEX as the frequency for pump outs is decreased by continuous removal of FOG. However, savings in contractor pumping costs is balanced to an extent by costs of staff maintenance, which is estimated at 10 minutes per day at the National Minimum Wage totalling around £500 per year, which, arguably, would not be considered an additional cost to a business owner. On a specific like-for-like basis, comparison of a small separator and a GRU is summarised in Table 6-4 using data provided by Aqua Cure Ltd (2016a), who estimated pump out intervals of three weeks and three months for a similarly-sized passive separator and a GRU respectively. This comparison reveals a clearer contrast, with a GRU offering potential savings of nearly £2700 when total operating costs are averaged over three years, although additional costs associated with disposal of grease recovered form GRUs during daily maintenance were not accounted for in this calculation. Similarly, Table 6-4

indicates that biodosing into a small separator can achieve savings of over £4800 over three years by increasing the pump out frequency from three weeks to two months (Aqua Cure Ltd, 2016a). Table 6-3 suggests that inline biodosing alone is potentially the cheapest option of all the technologies compared to small separators or GRUs with a 5-year TOTEX range of £2900 - £4400 for a single system. However, as with substituting multiple small separators for one large unit, an FSE might need to dose at more than one location to achieve optimum FOG management.

Table 6-3 Ranges of capital and operating costs for FOG management technologies

	CAPEX[1] (£)	**OPEX (one year) (£)**	**TOTEX (5 years) (£)**
Do nothing	-	5495 – 9266[2]	Multiple fines/ business closure
Small grease separators (≤ 100 l)	175-972	1800-2400 (assuming monthly pumping frequency)	9,200-13,000
Large grease separators (outdoor/BS1825)	827-4200+	1400-2400 (assuming quarterly pumping frequency)	7,900-16,200
Grease removal units (GRU passive or mechanical)	2160-5800	960-1160[3] (assuming quarterly pumping frequency for small units, and biannual for large units + daily in-house labour costs)	8,000-10,800
Inline biodosing	222-560	480-780	2900-4400
Biodosing in a small grease separator	222-560 (dosing system) + 175-972 (trap)	250-780 (dosing) 450-600 (assuming a three times-a-year pumping frequency)	3900-8500

[1] Excludes installation cost which can be significant for large separators. [2] Examples of fines resulting from prosecution (BBC, 2018; Seymour, 2016). [3] Excludes costs associated with waste FOG collection

Table 6-4 Comparison between similar sized GRU and passive separator with and without biodosing

		CAPEX (£)	OPEX (one year) (£)			TOTEX (3 years) (£)
			In-house maintenance & power	Pump out costs	Bioadditive	
Small GRU		2784	667	700		**6967**
Small passive separator		550	-	2975		**9655**
Small passive separator with biodosing	Separator	550	-	1050	-	**4800**
	Dosing	222	20		246	

Data adapted from Aqua Cure Ltd (2016a, 2016b)

6.2.2 Sustainability

An additional consideration unaccounted for in the cost-benefit analysis is that of sustainability. Recovery of FOG in separators offers the opportunity for energy recovery by incineration, anaerobic digestion and biodiesel production. De Castro Rodriguez (2016) estimated that annual FOG recovery could potentially power 66,000 or 236,000 homes through incineration and anaerobic digestion; or provide biodiesel for 188,000 cars. FOG recovered daily from GRUs is of higher purity than 'brown grease' from grease separators and could potentially be considered as 'yellow grease' from waste cooking oil. Yellow grease has a lower FFA and water content than brown grease and is less contaminated with food waste, and thus is more cost-effective for biodiesel production (Wallace et al., 2017). A recent UK Government report by the Department for Transport (2018)

reported that used cooking oil and brown grease accounted for 40% and 1.8% of 1,306 million litres of biofuel produced between April 2017- April 2018.

6.3 Conclusion

On balance, biodosing systems as a standalone solution appears to be the best option in terms of total cost and space requirements, followed by biodosing into a separator. In terms of efficacy, grease separators offer best performance on the free oil fraction whilst emulsification enhances bioavailability for microbial populations. Thus, combining the two treatments will have a synergistic effect on total FOG removal. When considering environmental impact, biodosing into a separator or GRU offers the additional benefit of yielding energy recovery from physically separated free oil. For larger establishments, with available space, a single large separator or GRU might be cheaper than multiple smaller units, and operating costs could be potentially reduced further with biodosing.

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7 Implications of the work – Practical guide to effective biodosing

Consideration of the above conclusions allows us to summarise our findings and produce some practical guidance on effectively dosing bioadditives to kitchen drains or separators.

Current dosing practices are based on ‘number of meals served per day’ or ‘number and type of appliances discharging into the drains’ and do not take into account parameters such as the wastewater composition or microbial environmental working requirements by which bioadditions work.

A more appropriate dosing guide should take into consideration the following:

7.1 Activity of the bioadditive

The FOG-degrading ability of bioadditives should be known in order to guide effective biodosing. Although widely used to manage FOG emissions, microbial additions do not currently have a BS EN standard or an industry-agreed testing methodology. A proposed testing protocol is summarised in Chapter 5. In contrast to previous published works, the proposed protocol is set-up to account for (1) the wastewater composition to treat and (2) the recovery yields of the FOG quantification method (reported in Chapter 2).

Activity should be reported as first order removal rate constant in h^{-1}, calculated as the mean and standard variation of the triplicate measurements. The testing should be run in parallel with *B. licheniformis* NCIMB 9375, used as reference bacteria and first order removal rate constants should be between 0.004 and 0.04 h^{-1} for COD:N ratio of 140:1 and 20:1 respectively.

7.2 Kitchen set-up

A pre-dosing kitchen survey is required for planning the dosing exercise. Many kitchen appliances are sources of FOG including combi ovens and steamers as well as sinks and dishwashers (Cermakova et al., 2018). Their location and

discharges within the kitchen drainage system can greatly influence bioadditives' action. Furthermore, a number of appliances do not discharge directly in the drains, but will generate high volumes of FOG-rich discharges on cleaning eg. rotisserie ovens, deep fat fryers, kettles, bratt pans, wok stations. The use of disinfectant/sanitising chemicals, which will kill or inhibit microorganism should also be considered. In addition, drainage system layout and sharp bends could produce bottlenecks - points of high FOG concentration, which will need to be addressed separately.

Wastewater discharges, flow rate and volumes, will affect contact time and the ability of microorganisms to degrade and/or to attach and create biofilms. Dosing should be done in periods of low- or no-flow (e.g. overnight) to allow microbial populations to grow and establish. Consideration should also be given to combining biological dosing with physical grease separation, since combining the two methods can enhance the individual performance of both. Physical separation will remove easily-separable free FOG, thereby reducing the organic loading on the bioadditives: leaving them to focus on the emulsified fraction.

7.3 Composition of the wastewater

Temperature and pH have important influences on microbial activity. Most bacteria favour mesophilic temperature (25 - 45ºC) and neutral pH. Dosing points should be sufficiently far downstream of appliances that discharge hot water. Effluents with high pH, such as those from dishwashers, could be accommodated by selection of bioadditives with high pH tolerance, or by substituting neutral pH detergents for typically caustic compositions.

The results of this work has shown that COD:N ratios in FSE WW can vary from 30:1 to 150:1 and that these values are considerably higher than the reported optimum ratio of 20:1 for wastewater degradation. The current work has also demonstrated the importance of the COD:N on FOG removal. Higher FOG removal rates were observed in media with lower COD:N. For example, uptake rates per cell were similar over a COD:N range 45 – 80 but population levels decreased which translated into lower overall FOG uptake. At highest tested ratio

(147) there was a 3-fold decrease in per cell uptake, together with smaller populations overall FOG uptake 5 times lower than at COD:N 45.

Phosphorous levels were also less than the optimum of COD:P 100:1. Mean ratio in this work was 100:0.52±0.24, which is comparable with previous studies (Chung and Young, 2013; Krishnan et al., 2008; Shon et al., 2002). The potential influence was not tested in this work as it was always in excess on account of the phosphate buffer in the test media.

Although WW composition is highly variable day to day, water composition (COD:N:P) and FOG concentrations should be tested before starting to use bioadditives. This will help to identify:

- Kitchen practices to minimise FOG loading such as training of kitchen staff in 'best practice' is recommended. For instance, mean concentrations of around 300 mg/l of FOG were determined in WW samples from a dishwasher. An anomalously low value of 38 mg/l was observed when a different staff member was on duty who was diligent about dry wiping of plates, resulting in almost a 10 times reduction.
- Emissions from specific premises. For example, a bakery, likely to have high concentrations of carbohydrates, and potentially sugars, which might inhibit FOG removal.
- Bioadditive products could contain additional nutrients to reduce COD:N, or offer an optional nutrient supplementing product.

7.4 Quantity of bioadditive to be used and contact time

Our work showed that there was no additional benefit derived from varying doses between 10^4 and 10^7 cfu/ml in batch culture. All populations reached similar levels in a given wastewater composition within 8 hours. Thus, no further recommendations can be given other than to follow guidance on dosing levels provided by the manufacturers.

A common concern amongst bioadditive sceptics is that there will be insufficient contact time for bioadditive populations to act on the FOG. Moreover, the present work identified that FOG removal does not begin until stationary phase, with a lag time of at least 14 - 16 hours. This suggests that there may be a requirement for a pre-culturing phase prior to dosing. Indeed, some bioadditive systems are designed to administer the product following pre-culturing in a separate tank. However, the ability of bacteria to form biofilms on waste pipe walls also allows them to exist as 'pre-activated' populations in a variety of physiological states including stationary-phase state (Rani et al., 2007). Biofilms initially form because many bacteria have hydrophobic cell surfaces and thus they are attracted to surfaces by electrostatic interactions (Arnaouteli et al., 2016). FOGs are also hydrophobic and tend to adhere to the pipe walls. This brings FOG in direct contact with the biofilm layer thereby increasing contact time indefinitely provided they can be degraded at less than, or the same rate, as deposition.

Moreover, cell hydrophobicity allows the bacteria to rapidly adhere to oil droplets – a characteristic measured by the bacterial adhesion to hydrocarbon (BATH) test (Rosenberg, 1984). Once adhered, bacterial degradation can continue even after emulsified oil in the wastewater has flowed through the drainage system into the sewer network.

In this work, highest FOG removal rates for *B. licheniformis* of 8 – 9 mg/l/h were observed in media with different compositions mixed at COD:N ratios of 56:1 and 45:1 respectively, and at 9.5 ± 0.4 mg/l/h at COD:N 42:1. FOG degradation rates needs to be at least equal to adherence rates on a biofilm surface to prevent net accumulation of deposit. Although this was beyond the scope of this work it is assumed that these conditions will be met during periods of inactivity in the kitchen, such as overnight.

Investigations into microbial removal of organic matter in sewer environments has found biofilms show a high degree of metabolic activity, with cell counts in the biofilm layer higher than in those in activated sludge wastewater treatment plants (Chen et al., 2003; Lemmer et al., 1994). Moreover, (Chen and Leung, 2000)

determined that oxygen uptake rates and ATP contents were twice as higher in sewer sediments than in the sewage phase indicating that biofilm populations levels and microbial activity were double those in the wastewater phase.

By inference, FOG removal rates in drain pipes might be three times higher than those observed in the aqueous phase in this work. Consequently, it is advisable to locate a dosing system as far up the kitchen drain system as possible in order to maximise the extent of biofilm coverage and allow more time for planktonic bacteria to adhere to emulsified droplets.

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8 Conclusions and Future Work

FOG management in commercial food service establishments is pivotal to minimising sewer blockages. This research aimed to provide insight into how wastewater composition affects selection of the most appropriate solution for reducing FOG emissions. A novel method was developed and validated for quantifying emulsified and free FOG separately. This method revealed a significant proportion of emulsified FOG in FSE wastewater, which may not be removed by conventional grease separators.

On the other hand, biological treatment is challenged by potential nutrient deficiency and high ratios of carbon to nitrogen. Evaluation of FOG degradation in synthetic wastewater of varying composition confirmed the hypothesis that biological addition has different interactions with FOG when other carbon sources (carbohydrates and proteins) are available in discharged kitchen wastewater. It also confirmed that an *in-vitro* assessment of their performance will need to include several testing conditions, specifically different COD:N ratio and carbon sources. The specific conclusions in relation to the original objectives that lead to this overall conclusion are:

Objective 1: to generate a comprehensive characterisation of wastewater from food service establishments to inform upon design of a realistic synthetic culture medium, and appropriate environmental conditions for experimental design (Chapter 3).

- FSE wastewater composition was highly variable in terms of concentrations of FOG (100 – 2000 mg/l); carbohydrate (100 – 4000 mg/l); and total nitrogen (16 – 79 mg/l) which was mainly derived from protein.
- COD:N ratios varied over a range of 30:1 to 150:1, suggesting that organic matter removal by biological treatment might be limited by nitrogen deficiency.
- Most samples were also deficient in phosphorous and some trace elements.

- FOG was more emulsified in wastewater from a dishwasher (94% of total FOG) than in a potwash sink (42%).
- On average, emulsified FOG concentration was 300 mg/l (68 ± 30% of total) and was associated with droplet sizes less than 100 µm, meaning that a significant amount might not be removed by physical separation.

Objective 2: to develop of a novel method for quantifying FOG in emulsified systems (Chapter 2).

- The novel method facilitates a more accurate assessment of FOG concentrations in FSE wastewater than standard solvent-based methods which fail to extract emulsified FOG.
- The method also allows for free oil which floats on the surface, and emulsified FOG to be quantified separately thereby providing unique insight into FOG management strategies.

Objective 3: To understand the action of microbial additives on organic components and kinetics of degradation using both synthetic and real wastewater (Chapter 4)

- FOG removal by *Bacillus licheniformis* NCIMB 9375 was inhibited in synthetic media with readily-biodegradable glucose as a carbohydrate source as opposed to hydrolysable starch. Rates converted to COD equivalents varied from 2 – 12 mg-COD/l/h in glucose media, and 14 – 24 mg-COD/l/h in starch media at equivalent concentrations.
- The dominant influence on FOG removal in synthetic and real wastewater was the initial ratio of COD:N. Removal rate constants decreased by an order of magnitude as COD:N increased over a test range of 33:1 to 150:1 in synthetic and real wastewaters of varying compositions.
- FOG removal was minimal during log phase bacterial growth with the main period of removal after 16 hours of incubation.

- Lower COD:N ratios were generally associated with lower stationary phase population levels and thus lower maintenance energy requirements leading to lower FOG removal.

Objective 4: To develop a standard protocol for testing the efficacy and kinetics of FOG degradation through the addition of microbial supplements to FSE wastewater (Chapter 5).

- A realistic synthetic wastewater needs to be used to address daily variations of COD:N ratio (Chapter 3 and Chapter 4).
- Results from the synthetic wastewater were validated using real wastewater (Chapter 5).
- At the tested conditions, varying the dosing level of the reference *Bacillus* strain, and a commercial bioadditive product, between 10^4 and 10^7 cfu/ml had minimal impact on FOG removal.
- First order FOG removal rate constants were found to be a better metric than removal rates, for comparing FOG removal response in media with different compositions.

Objective 5: To propose a practical guide for dosing regime tailored to the users' wastewater composition and sewers conditions (Chapter 7)

- Kitchen set-up, wastewater composition and contact time are key considerations when planning a dosing regime.
- Future research needs to be undertaken to further understand how bioadditives interact with FOG in drainage systems.

Objective 6: To outline a FOG management practice guide (Chapter 6)

- Kitchen set-up, space requirements, capital and operating expenditure and wastewater characteristics were identified as key considerations when selecting a FOG management strategy.

- Biodosing was considered to be the cheapest option (CAPEX and OPEX), requiring the least space for installation, and with minimal maintenance requirements.
- Biodosing into a grease separator or GRU has a synergistic effect on the efficacy of both technologies and reduces OPEX for physical treatment.

8.1 Future work

This research focussed on the *in-vitro* removal of FOG by suspended bacteria in the aqueous phase of synthetic FSE kitchen wastewater to establish a testing protocol. The protocol was designed using a culture collection bacterium (*Bacillus licheniformis* NCIMB 9375) with known lipolytic activity, as a standard reference. The work included a trial with a commercial bioadditive with synthetic and real FSE wastewater to validate the protocol conditions. The work did not cover the activity of the bioadditives in sewers nor their interaction with the native sewers' microbial communities.

Several areas for further work have therefore been identified:

8.1.1 FOG behaviour in sewers

FOG is present in wastewater in different forms: suspension, emulsion or in aggregation with other components. Further work should cover the behaviour of FOG in mixed effluents (e.g. sewage) with particular regard to how much of the FOG material adheres to the pipe walls/biofilm from the wastewater stream. The different forms of FOG material could have a very distinctive behaviour once in the sewers and hence have very different biodegradability potential. In addition, how stable is this deposit?

8.1.2 Bioadditive biofilms in sewers

The main activity of bioadditives is assumed to occur in a biofilm layer on the pipe surfaces enabling direct contact with adhered FOG and long contact times.

Further work is required to understand the mechanism of biofilm formation of the microbial bioadditions, and their interaction with the inherent biofilm. The work should also address the level of dosing required to establish such a biofilm and the time frame for its formation. Finally, a long-term experiment should aim to assess the resilience of these biofilms over time (e.g. how long do we have to dose for? And how strong is the biofilm?).

8.1.3 Action of bioadditives in sewers

Microbial action in FOG-laden sewers can be defined as bioremediation (e.g. removal of existing blockages) or maintenance (e.g. keeping the clean pipes flowing). The two type of actions are very different and will entail separate strands of future research work.

Wastewater flows have an impact on the contact time between FOG and microorganisms, both in suspension and in biofilms. Additional research should aim to identify the activity of bioadditives in flowing conditions and at different dissolved oxygen levels. Can bioadditives degrade the FOG at a faster rate than it is deposited?

Biofilm action can occur on the flowing wastewater or at the deposited FOG level. If so, what is the rate of FOG removal per unit area of pipe biofilm? Moreover, when does the removal occur? Only in periods of no-flow?

8.1.4 Interactions between bioadditive bacteria and native wastewater populations

This work only briefly covered the interaction between bioadded and native microorganisms. Future work should also take into account not only the lipolytic degradative action of the bioadditions, but also the interaction between the different microbial groups. As reported in chapter 4, the lipase-producing ability of a bioadded *Bacillus* sp might help the degradative action of other species otherwise unable to hydrolyse lipids. In addition, very little is known about the level of addition required to achieve a positive interaction between the added

bacteria and the existing biofilm population, or the level required to maintain viable populations in this ‘new’ environment.

APPENDICES

Appendix A Preparation of synthetic FSE wastewater

A.1 Basal mineral medium

Make up the following stock solutions (A-C) and store at 4°C

A.1.1 (A) Macronutrients stock solution: dilute the following in 500ml DI water

Compound	mg
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	5120
Potassium chloride (KCl)	3510
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	2100
Ammonium chloride (NH_4Cl)	510

A.1.2 (B) Trace elements stock solution:

- First make a 100 ml solution of:

Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$) – 68 mg

Cobalt nitrate hexahydrate ($Co(NO_3)_2.6H_2O$) – 156 mg

- Add 1 ml of the above solution in with the following and make up to 1 litre

Compound	mg
Iron chloride (FeCl3)	266
Zinc sulphate heptahydrate ($ZnSO_4$.7H2O)	164
Copper sulphate ($CuSO_4$)	68
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	46

A.1.3 (C) Surfactant:

Triton X-100 (specific gravity 1.065) - a 1:15 dilution

A.1.4 Phosphate buffer stock solution (1 M):

Dibasic sodium phosphate (Na_2HPO_4) – 71 g in 500ml DI to include 38ml 5M HCl to adjust to pH ~6.6 => 30mM buffer in synthetic

Autoclave and store at room temperature.

A.2 Combine with organic components

A.2.1 Concentrated stock solution

Make up a 10 x concentrated stock solution in 1 l DI water according to Table A-1.

Table A-1 Synthetic wastewater stock solution used in the experiments reported in Chapter 5.

Ingredient	Mass (mg)	Volume (ml)
Soy protein acid hydrolysate (Amisoy)	4000	
Cornflour (Tesco: % w/w Carbs 88, Protein 0.4, Fat 0.5)	5682 (yields 500 mg/l starch)	
Glucose	5000	
Macroelements stock solution (A)		100
Trace element stock solution (B)		10
Triton X-100 dilution (C)		2

A.3 Prepare media in flasks

1. Dilute 50 ml aliquots into 500 ml DI water and transfer in to 1l shake flasks
2. Insert foam bung, cover with aluminium foil and autoclave flasks at 121°C for 20 minutes.
3. Under aseptic conditions add 0.15 ml 10% SDS solution to each flask – yields 30 mg/l (laboratory protocols advise adding after autoclaving to prevent thermal degradation).
4. Under aseptic conditions add 15 ml 1M sodium phosphate buffer to each flask (yields 30mM, pH7). Added after autoclaving to prevent precipitation.
5. Add weighed aliquots of filter-sterilised vegetable oil with a sterile plastic transfer pipette (~300 mg/flask).
6. Place in incubator shaker for at least 4 hours to emulsify.

Appendix B Preparation of Bioadditive Protocol test media

Follow instructions for preparing media in Appendix A, substituting Table B-1 (below) for Table A-1. Add aliquots of 1% ammonium nitrate (NH_4NO_3) solution to each 500 ml media as prescribed in Table B-2.

Table B-1 Synthetic wastewater stock solution for the Bioadditive Test Protocol (made up to 1L)

Ingredient	Mass (mg)	Volume (ml)
Soy protein acid hydrolysate (amisoy)	1400	
Cornflour (Tesco: Carbs 88%)	5682 (yields 500 mg/l starch)	
Glucose	5000	
Macro-elements stock solution (A)		100
Trace element stock solution (B)		10
Triton X-100 dilution (C)		2

Table B-2 Ammonium nitrate additions to media in Table B-1 to achieve different COD:N ratios

COD:N	NH_4NO_3 (mg/l)	Volume of NH_4NO_3 (1% soln.) per 500 ml flask (ml)
20	360	18.0
60	80	4.0
100	24	1.2
140	0	0

Summary of Thesis corrections – Caroline Gurd, s181225

Chapter	Section	Corrective action
	Abstract	• Clarification, punctuation and grammar • Emphasised benefits of combined treatments • Re-trimmed to 300 words
1. Introduction		• Clarification, punctuation and grammar throughout
	1.2 FOG management legislation	• Previously section 1.5
	1.3 FOG management strategies in food service establishments	• Last paragraph of Section 6.1 moved here. • Further clarification • Staff training emphasised
	1.5.3 Surfactants	• Figure (1.2) removed
	1.7 Thesis Structure	• Paper submissions added • Fig 1.3 text amended 'General Introduction' replaces 'Literature Review'
	References	• Corrections and formatting
2. Determination of fats, oils and greases in food service establishment wastewater using a modification of the Gerber Method		• Clarification, punctuation and grammar throughout
	2.1 Preface: Method overview	• New section 2.1 (Method overview) added to provide brief overview of modified Gerber FOG quantification method supplementary to published paper, including visual representations: Figures 2.1 and 2.2
	2.2 Introduction	• Chunk of text moved
	2.3 Materials and methods	• Additional text to explain procedures better
	2.4 Results	• Free oil method moved up front (2.4.1) for continuity • Figure captions elaborated to provide more detail and tick marks added to y-axes • Fig 2.4 A & B swapped over. Data ranges in B altered to correlate with the text. Caption text altered for clarification. • Fig 2.8 'gap' between sample sets on x-axis removed
	3. Conclusion	• Recovery efficiency quantified
	References	• Corrections and formatting
3. A characterisation of food service establishment wastewater		• Clarification, punctuation and grammar throughout
	3.2.1 Sampling procedure	• Figure 3.1 removed – gave false impression that mixed effluent was combination of potwash sink and dishwasher, when actually was from a separate pre-rinse sink. • Text and Table 3.2 added to provide clarification of sampling locations and procedure

	3.3 Results	• Figure captions elaborated to provide more detail and tick marks added to y-axes • Concentration ranges amended to include variability
	3.3.1 Biodegradability potential	• Text added to introduce following parameters for assessment
	3.3.1.3 Stoichiometry	• Moles changed to mmoles to match mg/l concentrations
	3.3.3 Implications for FOG management	• Text reworked to better explain nutrient deficiency, and COD:N:P ratios. • Discussion changed to focus on adjustment of COD:N:P ratio for sink samples only (rather than sink and DW combined) as the carbon load would only be significantly affected by free FOG separation in sink effluents • Equation 3.7 added to provide calculation of theoretical oxygen demand
	3.4 Conclusions	• Modified with respect to changes in Section 3.3.3
	References	• Reformatted and corrected
4. The impact of wastewater composition on microbial degradation of fat, oil and grease		• Clarification, punctuation and grammar throughout
	Abstract	• Clarification of different WW composition/components
	4.1 Introduction	• Reformatted – subsections removed; text amended to develop 'funnel' format towards study objectives
	4.2.1 Synthetic wastewater preparation	• Base media composition reformatted into Table 4.1 • Protein concentrations added to Table 4.2
	4.3 Results	• Figure captions elaborated to provide more detail and tick marks added to y-axes
	4.3.2.1 Carbohydrates: glucose and starch	• Subtitle ' Bacterial growth' changed to 'Bacterial cell yields' to emphasise focus of text • Figure 4.3. X-axis amended to express carbohydrate concentrations as well as COD:N
	4.4 Conclusions	• Restructured to provide more clarity
	References	• Reformatted and corrected
5. Development of a protocol for testing FOG-degrading bioadditives for treating commercial kitchen wastewater		• Clarification, punctuation and grammar throughout
	5.3 Results	• Figure captions elaborated to provide more detail and tick marks added to y-axes
	5.3.1.3	• Heading changed
	5.3.6 Bioadditive performance in real FSE wastewater	• Fig. 5.6 – data in (b) replaced with separate Table 5.2 + standard deviations added
	5.4 Proposed Protocol methodology	• New section replaces subsections 5.3.7 and 5.3.8. This presents our proposals for a Standard Test Protocol for use in the

		bioadditive product industry (hence future tense in text). • Fig 5.8 deleted as is now presented as Fig 2.1 in Chapter 2
	5.5 Conclusions	• Mistake corrected
	References	• Reformatted and corrected
6. Considerations for selection of the most appropriate FOG management solution		• Clarification, punctuation and grammar throughout
	6.1 Introduction	• Deleted and replaced by brief summary • Last paragraph moved to Introduction 1.3
	6.2.1	• Title changed to 'Passive grease separators' • Merged with Section 6.2.2
	6.4 Conclusions	• Elaborated
	References	• Reformatted and corrected
7. Implications of the work – Practical guide to effective biodosing		• Clarification, punctuation and grammar throughout
8. Conclusions and Future Work		• Clarification, punctuation and grammar throughout • Last 2 bullets from Objective 2 moved to Objective 1 • Additional bullet point added to Objective 6 • Additional text to highlight novelty of work