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Research article

The role of lipids in activated sludge floc formation

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Abstract: Activated sludge is widely used to treat municipal and industrial wastewater globally and the formation of activated sludge flocculates (flocs) underpins the ability to separate sludge from treated water. Despite the importance of activated sludge flocs to human civilization there have been precious few attempts to rationally design fit for purpose flocs using a bottom-up approach based on a solid scientific foundation. Recently we have been developing experimental models for activated sludge floc formation based on the colonization and consumption of particulate organic matter (chitin and cellulose). In this study we lay the foundation for investigation of activated sludge floc formation based on biofilm formation around spheres of the lipid glycerol trioleate (GT) that form spontaneously when GT is introduced into activated sludge incubations. Sludge biomass was observed to associate tightly with the lipid spheres. An increase in extracellular lipase activity was associated with a decrease in size of the colonized lipid spheres over a 25 day incubation. Bacterial community composition shifted from predominantly Betaproteobacteria to Alphaproteobacteria in GT treated sludge. Four activated sludge bacteria were isolated from lipid spheres and two of them were shown to produce AHL like quorum sensing signal activity, suggesting quorum sensing may play a role in lipid spheres colonization and biodegradation in activated sludge. The development of this experimental model of activated sludge floc formation lays the foundation for rational production of flocs for wastewater treatment using lipids as floc nuclei and further development of the flocculate life-cycle concept.

Keywords: activated sludge floc; bacteria; lipid; glycerol trioleate; quorum sensing; biofilms

1. Introduction

The activated sludge process for wastewater treatment is a biotechnology fundamental to human health and represents a model high population density ecosystem for studying microbial interactions [1]. Activated sludge is primarily composed of microorganisms in aggregates or flocculates (flocs), enabling low-tech separation of biomass from the bulk aqueous phase by settling under gravity. Flocs

are composed of a dense microbial consortium in a matrix of extracellular polymeric substances [2] described by some as suspended biofilms or biofilms without surface association. It can be argued that human civilization is dependent on bacterial flocculate formation given the central role of this phenomenon in industrial and municipal wastewater treatment globally.

Despite the importance of activated sludge floc formation and function there remain significant knowledge gaps relating to how flocs form, function and change over time. Firstly, it is unclear how floc development is initiated. Secondly, the relative contributions of cell replication and cell recruitment to floc growth is not well characterised. Thirdly, there is no mechanistic microbiological description of the ultimate fate of a floc. Put another way, the "lifecycle" of activated sludge flocs is poorly described, possibly because heterogeneity between flocs is rife. A promising approach to addressing these knowledge gaps is to circumvent the challenge of heterogeneity through development and interrogation of experimental models of floc formation.

Previous investigations raised the question of whether activated sludge flocs are in fact surface associated biofilms anchored to particulate organic matter [3]. Activated sludge is a well-known source of extracellular cellulase, chitinase and lipase enzymes, suggesting that particulate organic matter (cellulose, chitin and lipids) represent major substrate resources for activated sludge bacteria [4]. Cellulose is the most abundant biopolymer on Earth and the principal component of toilet paper [5]. Chitin is the second most abundant biopolymer on Earth and addition of chitin or its deacylated derivative chitosan is common practice in wastewater treatment plants to improve sludge settling properties [6]. Municipal wastewater contains lipids at concentrations between 40 and 100 μg/L representing over 30% of the chemical oxygen demand [7,8]. Oleic acid is the most common fatty acid found in olive and sunflower oils commonly used in domestic settings [9].

In this study we developed an experimental model of activated sludge floc formation based on glycerol trioleate (GT) as a substrate. GT was added to activated sludge and observed to form small lipid spheres with which sludge biomass was tightly associated. Extracellular lipase activity was upregulated and a shift from Betaproteobacteria to Alphaproteobacteria was observed. Four bacteria associated with the GT spheres were isolated and identified and two of the isolates were shown to produce acylated homoserine lactones (AHLs). Foundation of this experimental model enables future research into lipid based floc formation and ultimately the rational construction of flocculates of desired function.

2. Materials and Method

2.1. Sampling and laboratory incubations

Activated sludge samples were acquired from St. Mary's municipal wastewater treatment plant for establishment of cultures in the laboratory. The treatment plant is located in western Sydney serving a population of app. 160000 in a catchment area of 84 km^2 and discharges into the Hawkesbury-Nepean River. On a daily basis the plant processes 35 million litres of wastewater. The biological process is conducted in three stages (Anaerobic, Anoxic and Aerobic) and the sludge samples collected for this study were taken from the Aerobic stage.

Enrichment cultures consisted of 50 mL activated sludge in 250 mL erlenmeyer flasks incubated at 37 °C and 140 rpm. Quadruplicate cultures were treated with 1% glyceryl trioleate (Sigma-Aldrich T7140-10G). Samples were taken over a 25 day period, consisting of 0.5 mL for

DNA extraction and 0.5 mL for lipase assays. Sterile filtered (0.22 μm Millipore) activated sludge supernatant was added back to incubations after sampling to maintain original volumes.

2.2. Microscopy

Lipid spheres were removed with tweezers and washed in phosphate buffered saline (PBS) before transferring to a glass slide. Spheres were stained with 5 μL SybrGreen and imaged with an Olympus Fluorescence Microscope.

2.3. Molecular microbial community analysis

DNA was extracted using a xanthogenate-SDS based method [10]. Sludge samples were centrifuged at 16,100 rcf for 5 min in a 1.5 mL eppendorf tube before removing supernatant and pellet storage at − 20 ˚C. For DNA extraction from GT spheres, semi-solid lipid droplets were removed with tweezers and washed in PBS, transferred to a 1.5 mL eppendorf tube and stored at − 20 ˚C. Thawed pellets were re-suspended in 0.2 mL PBS for extraction. Extracted DNA was resuspended in molecular biology grade water. DNA was quantified via NanoDrop (NanoDrop ND-1000 Spectrophotometer) as well as Qubit (Invitrogen, Qubit 2.0 Flourometer), as per standard protocol for broad range DNA detection.

The PCR for denaturing gradient gel electrophoresis (DGGE) was set up with the primers 357FGC and 907R (10μM) [11] for 40 μL as follows: 20 μL EconoTaq Master mix (Lucigen), 12.48 μL molecular water, 2 mM of each primer, 7.7mM bovine serum albumin and 5–50 ng template. The PCR protocol was the following: 2 min at 95 °C then 30 cycles of denaturing phase: 30 sec at 94 °C, annealing phase: 30 sec at 54 °C, extension phase: 1 min 30 sec at 72 °C, followed by a final extension phase: 10 min at 72 °C and storage at 4 °C.

The DGGE was conducted using the BioRad DCode system and protocol for 50% to 70% denaturing gradient on a 6.7% arcylamide gel. Electrophoresis was conducted at 75 V and 60 °C for 16.5 h in 1× TAE. The gel was stained with SybrGold for 20 min and imaged on a BioRad Gel Doc XR Imaging system and BioRad Image Lab software. Bands of interest were selected from the DGGE and excised and re-run on DGGE PCR to test their integrity before sequencing.

The sequencing PCR was set up for the 357F primer at 20 μL as follows: 1 μL BigDye terminator V3.1, 20–50 ng PCR product, 0.32 μL primer, 3.5 μL 5× sequencing buffer and made up to 20 μL with molecular water. The PCR protocol was the following: 26 cycles of denaturing phase: 10 sec at 96 °C, annealing phase: at 5 sec 50 °C, extension phase: 4 min at 60 °C, followed by and storage at 4 °C. The samples were cleaned up by addition of 5 μL of 125 mM EDTA and 60 μL 100% (v/v) ethanol followed by vortexing and 15 min precipitation period. The samples were then spun at 14000 rcf for 20 min and supernatant was removed. After addition of 160 μL fresh 70% ethanol, samples were spun at 14000 rcf for 10 min and supernatant discarded. This step was repeated with addition of 80 μL 70% ethanol. Sanger Sequencing was conducted by the Ramaciotti Centre for Gene Function and Analysis at the University of NSW, using standard ABI protocols.

2.4. Lipase assays

The lipase assay was adapted from Christensen et al [12]. Sludge samples (0.5 mL) were centrifuged at 16,100 rcf for 5 min. The supernatant was removed and filter sterilised (0.22 μm Millipore filter) to test for suspended extracellular lipase. The pellet was re-suspended via vortexing in 0.5 mL autoclaved MilliQ water with the addition of 0.1 mL Zirconium beads. The samples were bead beaten for three 45 sec cycles and centrifuged at 16.1 rcf for 5 min. The supernatant was removed and filter sterilised (0.22 μm Millipore filter) to test for EPS associated lipase. The pellet was re-suspended via vortexing in 0.5 mL autoclaved MilliQ water to test for membrane bound lipase. Subsamples of each fraction (100 μL) were incubated with 0.9 mL of p-nitrophenyl palmitate (p-npp) containing substrate solution for one hour before reaction termination by alkaline pH inactivation of lipase with 1 mL 1 M sodium carbonate. Cleaved p-nitrophenyl groups were quantified by spectrophotometry at an absorbance of 410 nm.

2.5. AHL assays

Four bacterial isolates associated with GT spheres were grown in Luria Bertani broth overnight at 37 ˚C with shaking at 140 rpm in the presence or absence of 1% (v/v) GT. Ethyl acetate extracts (20 mL) of culture supernatants were prepared and concentrated to 100 μL by evaporation. Extracts were assessed for the presence of AHL like activity using an *Escherichia coli* based AHL monitor strain harbouring the plasmid pJBA357 encoding the *luxR* gene and a *gfp*(ASV) reporter gene fused to the AHL-LuxR dependent *Photobacterium fischeri luxI* promoter [13]. The fluorescent output was monitored by excitation at 485 nm and emission at 535 nm.

3. Results

3.1. Glyceryl trioleate sphere formation and biomass association in activated sludge

To establish an experimental model of floc formation based on interactions between lipids and bacteria in activated sludge, glyceryl trioleate (GT) was added to activated sludge samples at 1% v/v. On addition GT formed a transparent and hydrophobic layer on the surface of the sludge before white GT spheres formed suspended below the culture surface within 7 days (Figure 1). The GT spheres varied in size but decreased in diameter until they were indistinguishable from the sludge after 21 days.

To examine the spatial relationships between sludge biomass and the GT spheres, samples were stained with a fluorescent DNA binding compound before imaging with epifluorescence microscopy. The fluorescent stain emits green light upon DNA binding and excitation while GT appears yellow and water appears black (Figure 2). Biomass associated preferentially with the GT spheres and was firmly attached as evidenced by the washing procedure applied prior to staining. This spatial distribution and cell density is reminiscent of surface associated biofilms and the lipid droplets with associated biomass could be considered prototype or juvenile flocculates.

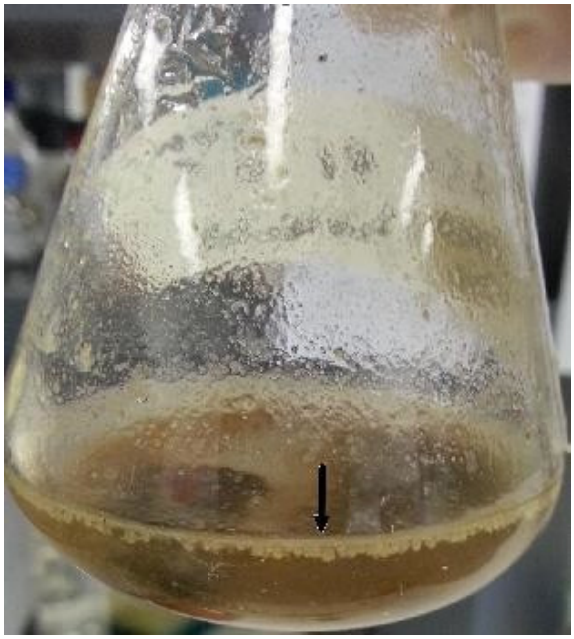

Figure 1. Spontaneous formation of glycerol trioleate (1% v/v) spheres upon addition to activated sludge. This image taken after 7 days aerobic incubation at 37 ˚C with shaking at 140 rpm. The lipid spheres decreased in size over time and were undetectable after 25 days.

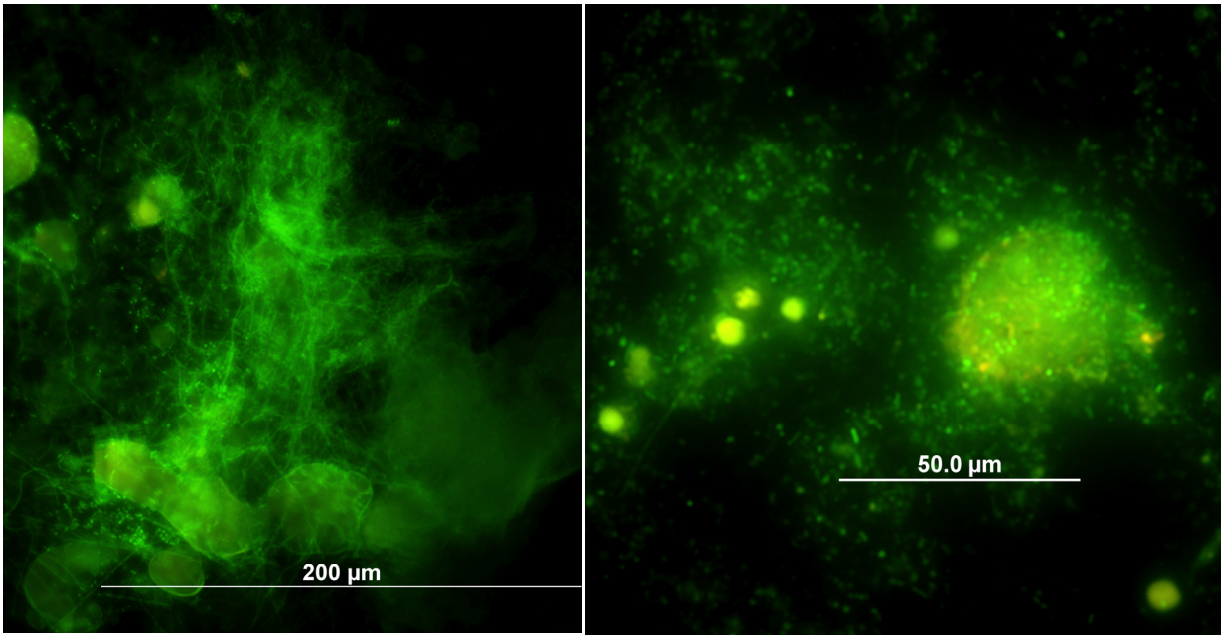

Figure 2. Epifluorescence microscopy images of sybr green stained DNA (biomass in green) associated with microscopic glycerol trioleate spheres (yellow) using 10× (A) and 40× (B) objective lenses. Filamentous, coccoid and rod shaped bacteria (red arrows) are visible as the activated sludge biomass adsorbs to the lipid droplets (blue arrows) forming 'juvenile' flocculates.

3.2. Upregulation of extracellular lipase activity in response to glyceryl trioleate

Lipase activity was monitored in the sludge supernatant, in EPS and in membrane associated fractions after GT addition (Figure 3). Lipase activity was highest in membrane-associated fractions over the course of the incubation but there was no apparent impact of GT addition. Lipase activity in EPS and supernatant fractions was more variable across replicates but there was a significant elevation in lipase activity in response to GT addition in these fractions ($p < 0.05$). These data suggest the presence of the GT spheres causes an increase in extracellular lipase production.

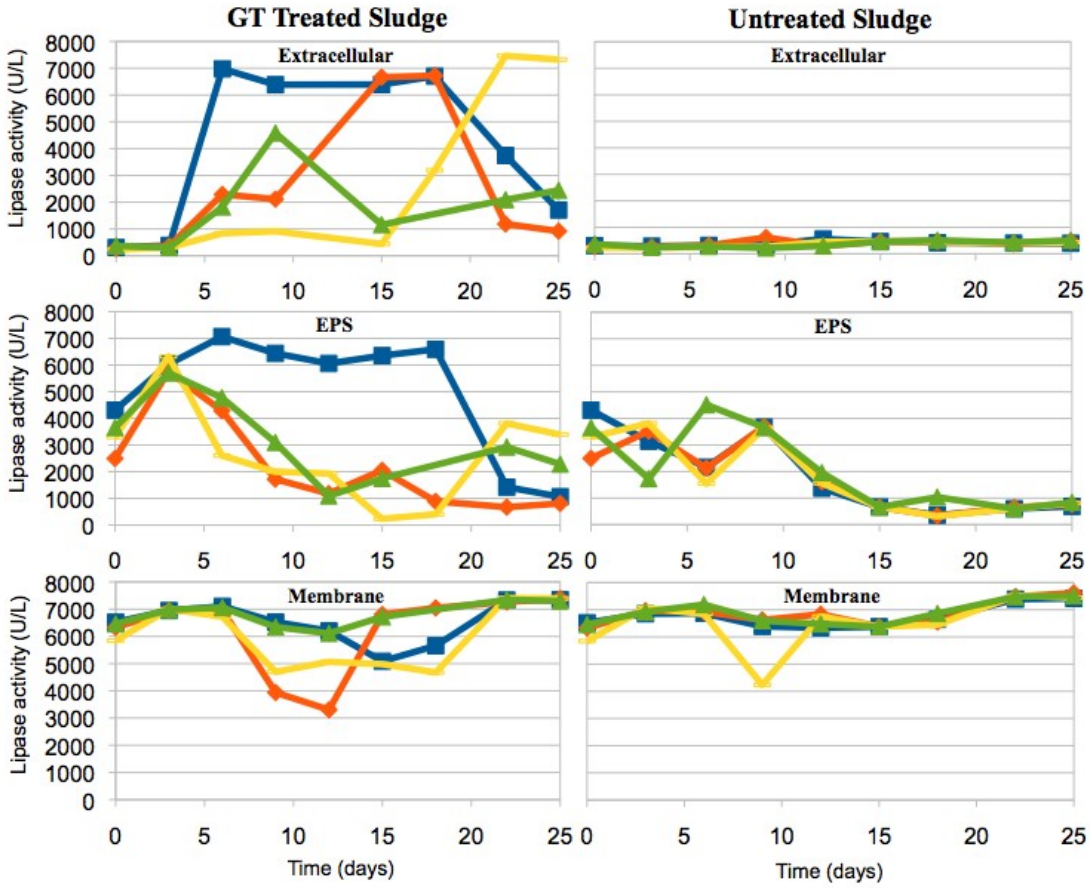

Figure 3. Lipase activity in quadruplicate cultures (blue, green, yellow and orange) of glycerol trioleate treated activated sludge (left panels) and untreated activated sludge (right panels) over 25 days incubation. Sludge samples were separated into extracellular (top panels), extracellular polymeric substance (EPS) (middle panels) and membrane (bottom panels) fractions before quantifying lipase activity. Whilst significant variation is observed between biological replicates with respect to timing of emergence of lipase activity, clear evidence of upregulation of activity is observed in the extracellular fraction in response to glycerol trioleate addition.

3.3. Microbial community changes in the presence of GT spheres

Figure 4 shows community fingerprints of GT amended and unamended control cultures over 25

days incubation. A clear shift in bacterial community composition is apparent in response to GT addition. Bands were excised, sequenced and matched with existing bacterial sequences in the NCBI database (Table 1). Initially abundant *Bacillus*, *Dechloromonas* and *Azospira* lineages were replaced with *Novosphingobium*, *Sphingomonas* and *Roseomonas* lineages. This represented a general shift from Betaproteobacteria to Alphaproteobacteria in response to GT amendment.

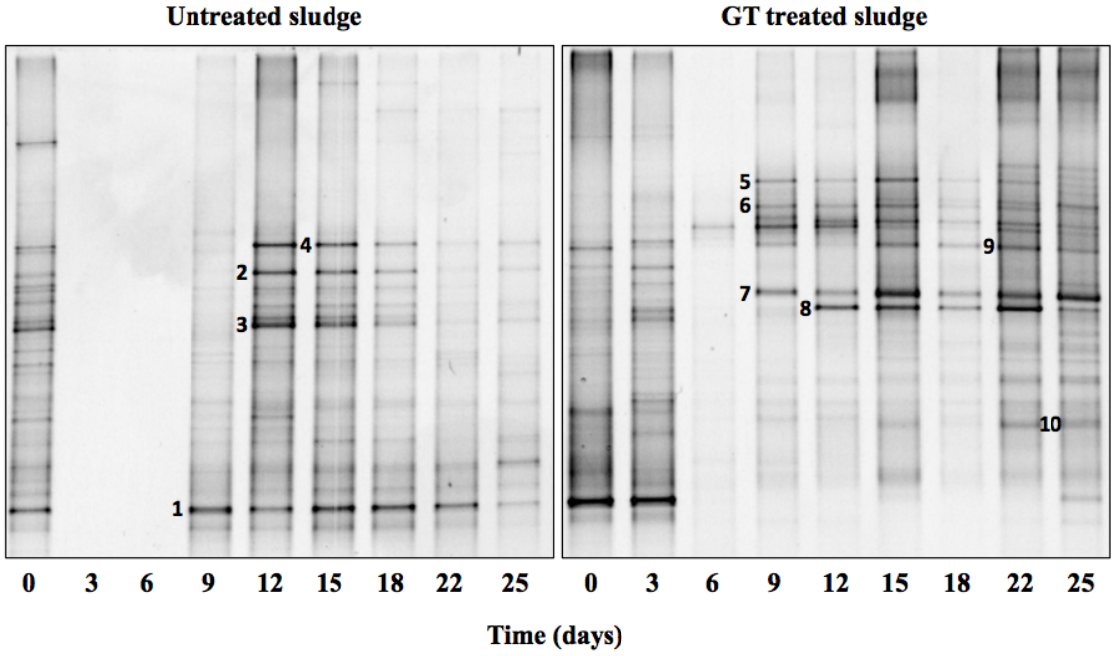

Figure 4. Denaturing gradient gel electrophoresis based bacterial community analysis of untreated sludge (left panel) and glycerol trioleate treated sludge (right panel) over a 25 day incubation. A shift in bacterial community composition was observed in glycerol trioleate amended cultures. Numbered bands were sequenced (Table 1) revealing a shift from Betaproteobacteria to Alphaproteobacteria. Sphingomonas and Novosphingomonas species dominated in response to glycerol trioleate addition.

Table 1. Closest relatives to sequences retrieved from community fingerprints (Figure 4).

Band Identity	Class	Closest relative (Acc. No.)
1	*Bacilli*	*Bacillus* sp. (GU271888.1)
2	Betaproteobacteria	Uncultured *Dechloromonas* sp.
3	Betaproteobacteria	*Azospira oryzae* (KF260987)
4	Betaproteobacteria	Uncultured *Dechloromonas* sp.
5	Alphaproteobacteria	*Novosphingobium* sp. (KF544940.1)
6	Alphaproteobacteria	*Novosphingobium* sp. (KF544932.1)
7	Alphaproteobacteria	*Sphingomonas* sp. (AY521009.2)
8	Alphaproteobacteria	*Sphingomonas suberifaciens*
9	Alphaproteobacteria	*Sphingomonas* sp. (JQ928361.1)
10	Alphaproteobacteria	*Roseomonas* sp. (KF254767.1)

3.4. Isolation of GT degrading bacteria

GT spheres were washed and plated on solid media supplemented with GT. Colonies were subcultured to isolate bacteria that use the lipid as a carbon and energy source. Four isolates were subsequently shown to utilise GT in liquid media as evidenced by growth in the presence of GT but not in its absence. SSU rRNA gene sequencing revealed the four GT sphere colonising isolates as Betaproteobacteria *Pandoraea* (KF378759.1) and *Achromobacter* (KF378759.1) and Gammaproteobacteria *Enterobacter* (KF411353.1) and *Pseudomonas* (KC822768.1) species. Unfortunately, none of the alphaproteobacterial *Novosphingobium* or *Sphingomonas* lineages observed in community fingerprints (Figure 4) were isolated.

The four GT sphere colonising isolates were tested for the production of Acylated Homoserine Lactones (AHLs). AHL mediated gene expression has been linked to lipase activity in Proteobacteria [14] and therefore may play a role in lipid based floc formation. A LuxR based bioassay in which expression of gfp is upregulated in the presence of AHLs was used for the detection of AHLs in culture supernatants. The *Achromobacter* and *Enterobacter* species gave strong positive responses to the LuxR based bioassay whilst the *Pandoraea* and *Pseudomonas* sp. gave a negative response. Interestingly the positive AHL bioassay responses elicited by *Achromobacter* and *Enterobacter* were enhanced in the presence of GT and whilst the negative AHL bioassay result for *Pandoraea* was unchanged by GT addition, this isolate generated a pink pigment in the presence of GT but not in its absence.

4. Discussion

Activated sludge flocculates underpin the global wastewater treatment industry but the design, production and deployment of flocs to date has involved a top-down engineering approach focused on function rather than underlying structure. Here we have produced an experimental foundation for a bottom-up scientific approach to the rationale construction of wastewater treatment flocs. Specifically, five aims were addressed relating to spatial aspects of lipid partitioning and colonisation, impacts on bacterial community composition, isolation of lipid degrading bacteria and testing for AHL production.

The physical partitioning behaviour of lipids upon introduction to activated sludge is poorly described. As a starting point it was unclear whether the addition of GT to activated sludge would result in the formation of free phase lipid or whether existing sludge flocs would be coated in lipid with an absence of free phase or if multiple small lipid droplets or an emulsion would form. This lack of information on the physical partitioning behaviour in activated sludge hampers development of an experimental model for lipid dependent floc formation. To this end, GT was incubated in the presence of activated sludge and direct observations were made.

During the enrichment on GT, multiple spheres formed that were suspended throughout the sludge. Objects that resemble GT spheres in oleic acid challenged anaerobic sludge have been reported previously [15]. It stands to reason that such lipid droplets represent an attractive surface substratum for colonisation by activated sludge bacteria enabling evasion of predation and ready access to a carbon and energy source. Epifluorescence microscopy was used to observe physical interactions (spatial relationships) between the activated sludge biomass and the GT spheres. The microscopy images obtained showcase the close association of biomass with the lipids. Whether the dense mass

of cells observed is surrounding and growing on the lipid or whether it was embedded within the lipid phase is unclear from the microscopy images generated. Whilst the aggregation around the droplets resembles biofilm formation, it is not clear if the physicochemical properties of cells and lipid droplets drive the association or if active energy dependent processes result in microbial colonisation of the lipid droplets. Cells embedded in the lipid, with no access to water, are unlikely to proliferate. Biofilm formation on lipid droplets would ultimately generate aggregated biomass resembling a floc. The result was encouraging in that it suggested lipid colonisation represents a realistic model system for floc formation and development.

Lipid droplet size was observed to decrease over time until they were no longer visible. This is consistent with microbes colonising the droplets and degrading the surface substratum as a carbon and energy source. Lipase activity was also observed to increase in activated sludge samples amended with GT. An increase in lipase activity was observed in the EPS fraction over the first three days of incubation but the greatest fold increase in lipase activity was observed in the culture supernatant after three days of incubation as the EPS based lipase activity decreased. It has previously been shown that lipases are weakly bound to the EPS of flocs through hydrogen bonding [16]. The sequential appearance of lipase first in the EPS and then in the supernatant fraction is likely due to release of lipases from the EPS.

While lipase activity was observed in the membrane fraction, there was little difference in activity when comparing GT amended treatments and unamended controls. The unamended controls displayed a high background level of lipase activity presumably owing to the presence of background levels of lipid in activated sludge. The decline in lipase activity observed in the EPS fraction in unamended controls is concordant with this, with the background lipid component being consumed.

It is not known which bacteria are responsible for the degradation of lipids in activated sludge. This information is crucial in the development of an activated sludge floc formation model based on lipid colonisation. The response of bacteria in activated sludge to the addition of GT was assessed using DGGE. It is evident from the DGGE community analysis that lipids have a profound impact on the microbial community structure in activated sludge. Whilst bacterial lineages enriched in the presence of GT can be implicated in lipid degradation in activated sludge this does not represent unambiguous evidence of lipid biodegradation by these bacteria.

Based on band intensity the most abundant bacterial lineages in the untreated sludge samples belonged to the genera *Bacillus*, *Dechloromonas* and *Azospira*. *Bacillus* species have been observed in sludge previously and the sequence retrieved here was associated with lipolytic activity in olive mill wastewater [17]. *Bacillus* species have also been associated with bioflocculation in starch wastewater treatment [18].

Dechloromonas species have been observed in membrane fouling biofilms of municipal wastewater treatment plants in Japan, with over 30% of clones belonging to this genus [19]. *Azospira* are also common activated sludge occupants [20]. Overall, the DGGE profiles of the unamended sludge community were typical of activated sludge and did not differ greatly over the incubation period.

In sludge samples exposed to GT the DGGE profiles over time shifted dramatically. The most abundant bacteria observed in the unamended sludge were replaced within nine days of incubation with *Sphingomonas*, *Novosphingobium* and *Roseomonas* species. *Sphingomonas* species can be present at 5–10% relative abundance in sludge as shown by FISH [21] and play an important role in wastewater treatment. Members of this genus degrade testosterone and sterol hormones as well as the

pollutant nonylphenol [9]. *Novosphingobium* species have been shown to play an important role in wastewater remediation by degrading toxic dyes and estrogen [22,23]. *Roseomonas* species have been found at 5% relative abundance in activated sludge and are known to degrade organophosphate pesticides [24]. None of these lineages have previously been associated with lipid consumption in wastewater treatment plants. From the data generated here bacteria belonging to the *Sphingomonas*, *Novosphingobium* and *Roseomonas* genera can be considered candidates for inclusion in an experimental system for investigating lipid based floc formation.

Four activated sludge bacterial strains isolated directly from lipid droplets and shown to use GT as a sole carbon and energy source were identified and screened for AHL quorum sensing activity. All four isolates are Proteobacteria but only two (*Achromobacter* and *Enterobacter*) displayed AHL like activity in the LuxR based bioassay used. This was surprising given that the two isolates that gave negative responses (*Pandoraea* and *Pseudomonas*) have close relatives known to produce AHLs and that lipase activity has only been definitively linked with AHL mediated gene transcription in *Pseudomonas* species. A soil isolate belonging to the *Pandoraea* genus has been shown to secrete octanoyl homoserine lactone [25]. It is possible that a more comprehensive screen for AHLs using AHL bioassays with a variety of LuxR type receptors or a direct mass spectrometry based method would reveal the production of AHLs outside the response range of the LuxR protein. The role of AHLs or quorum sensing in the colonisation or biodegradation of GT spheres in activated sludge remains to be explored.

5. Conclusion

Bacterial floc formation is essential for the remediation of domestic wastewater. This study sought to supplement the limited information available about floc formation and the impact of lipid addition on activated sludge structure as well as microbial community composition. GT addition to sludge resulted in the formation of lipid spheres that were rapidly colonised and consumed over 25 days, which may reflect seeding and maturation phases of a flocculate "life-cycle". During this process extracellular lipase activity increased and *Sphingomonas* and *Novosphingomonas* species increased in relative abundance. Lipid degrading bacteria isolated from the lipid spheres produced quorum sensing signal activity. Deciphering the nature of colonisation in a biofilm-like manner around lipid spheres extends our understanding of the mechanism of floc formation.

To interrogate this newly developed experimental system for floc formation future research should focus on the isolation of lipid degrading *Sphingomonas* and *Novosphingomonas* species from activated sludge, on generating pure culture flocculates based on the colonisation of lipid spheres and on describing AHL mediated gene expression systems in lipid degrading isolates to assess whether quorum sensing regulates lipase activity in activated sludge.

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Conflict of interest

All authors declare no conflict of interest in this paper.

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