

Lipid hydrolysis monitoring in wastewater treatment: proof-of-concept for a high throughput vegetable oil emulsion based assay

P. Van Gaelen^{IWA^a}, D. Springael^{IWA^b} and I. Smets^{IWA^{a,*}}

^a Bio- and Chemical Systems Technology, Reactor Engineering and Safety (CREaS), Department of Chemical Engineering, KU Leuven, Celestijnenlaan 200F box 2424, B-3001 Leuven, Belgium

^b Division of Soil and Water Management, Department of Earth and Environmental Sciences, KU Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium

*Corresponding author. E-mail: ilse.smets@kuleuven.be

Abstract

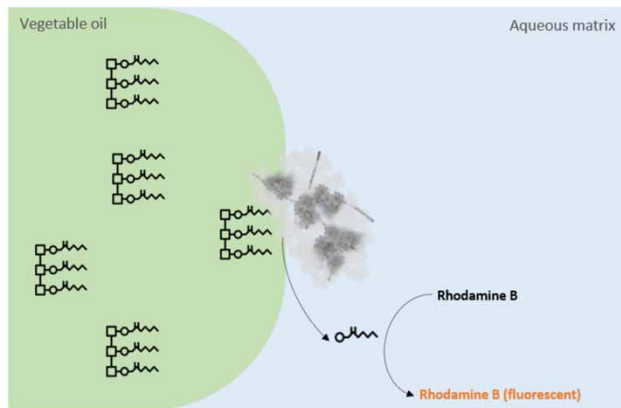
Lipids are one of the main organic components in industrial and municipal wastewaters. Lipid hydrolysis is the first step in the biological conversion process and requires a close contact between lipid emulsion droplets and microbial hydrolytic enzymes. Adequate lipid hydrolysis monitoring is crucial to obtain mechanistic knowledge on lipid hydrolysis in response to changes in the process conditions and to improve the overall lipid conversion efficiency in aerobic and anaerobic wastewater treatment systems. We set out to develop a high-throughput lipid hydrolysis monitoring method based on vegetable oil model substrates and fluorescent quantification of product formation by exploiting the interaction with Rhodamine B. Olive oil and soybean oil emulsions were prepared with a high interfacial area and acceptable emulsion stability. The method was easy to apply and allowed to obtain detailed kinetic data over a time course of several hours for up to 16 samples in parallel. A proof-of-concept was obtained with a commercial enzyme, Amano lipase, but remains to be provided for wastewater treatment sludge samples. The findings of this study pave the way for further method development in lipid hydrolysis monitoring.

Key words: biological wastewater treatment, enzyme activity, hydrolysis, lipids, Rhodamine B

Highlights

- Lipid hydrolysis monitoring is essential to optimize (an)aerobic wastewater treatment.
- High-throughput monitoring methods are required.
- The established p-nitrophenol palmitate method is not adequate.
- A lipid assay model substrate should resemble the actual lipids present in wastewater.
- Fluorescent lipid hydrolysis quantification with Rhodamine B is a promising approach for high-throughput monitoring.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Licence (CC BY-NC-ND 4.0), which permits copying and redistribution for non-commercial purposes with no derivatives, provided the original work is properly cited (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Graphical Abstract

INTRODUCTION

Lipids, or triacylglycerols, are one of the main organic components in industrial wastewaters such as dairy wastewater and vegetable oil processing wastewater. Similarly, they comprise up to one third of the total organic matter in municipal wastewater (MWW), with concentrations varying between 40 and 100 mg L⁻¹ (Quéméneur & Marty 1994).

Lipids are subjected to physico-chemical and microbiological transformation as soon as they are discharged. Enzymatic hydrolysis of the lipids into glycerol and long-chain fatty acids mediated by microbial lipase enzymes (triacylglycerol hydrolases, EC 3.1.1.3) can already occur in the sewer system or buffer tanks in industrial installations (Franke *et al.* 2011). Miron *et al.* (2000) for instance, noted that 40 percent of the lipid fraction in primary settled municipal wastewater solids were free long-chain fatty acids. Additional hydrolysis and biological conversion proceeds in the biological wastewater treatment system, either aerobically or anaerobically.

Lipids can be a benefit and a nuisance in MWW treatment. On the one hand, they have a high energy content for microbial growth and anaerobic conversion to methane (Alves *et al.* 2009; Diamantis *et al.* 2021), and they can serve as feedstock for biodiesel production. On the other hand, lipid metabolism and intracellular storage has been associated with sludge flotation in aerobic MWW treatment (Fan *et al.* 2020), and long-chain fatty acid inhibition in anaerobic systems (Sousa *et al.* 2009).

Reliable lipid hydrolysis monitoring is crucial to determine and to develop strategies to improve the lipid conversion efficiency (Palacios *et al.* 2014). A lipid hydrolysis assay aims to quantify the enzymatic lipid hydrolysis rate of a certain wastewater treatment sludge sample with a high sensitivity and experimental reproducibility, while mimicking the real substrate with a model substrate. A high sample throughput is typically required when screening multiple wastewater treatment systems simultaneously or a given treatment system at a high sampling frequency, such as hourly sampling.

Spectrophotometric monitoring methods, such as the *p*-nitrophenol palmitate method, have been reported most often in wastewater treatment research (Gessesse *et al.* 2003; Calderón *et al.* 2013; Ferrer-polonio *et al.* 2018; Petropoulos *et al.* 2018). The *p*-nitrophenol palmitate method involves incubation of a small portion of sludge with the *p*-nitrophenol palmitate model substrate under controlled temperature and pH conditions. A colored lipid hydrolysis product, *p*-nitrophenol, is produced during the incubation period and is quantified by spectrophotometry at several, predetermined time points. This method has some methodological limitations. First, *p*-nitrophenol palmitate is a poor model substrate, since the hydrolysis product is a secondary alcohol (Figure 1). In contrast, most

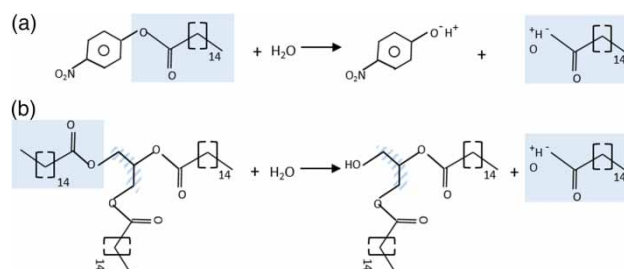


Figure 1 | Comparison of the *p*-nitrophenol palmitate reaction mechanism with hydrolysis of a triacylglycerol lipid. (a) Hydrolysis of *p*-nitrophenol palmitate to *p*-nitrophenolate and palmitic acid, (b) hydrolysis of a model triacylglycerol lipid substrate, 1,2,3-propanetriyl trihexadecanoate, to produce a diacylglycerol, 1,2-bis(hexadecanoate)-3-propanol, and palmitic acid. The first hydrolytic reaction of a triacylglycerol typically occurs at one of the outer glycerol ester bonds, as shown here. Palmitic acid moieties are indicated with shaded rectangles, glycerol moieties are indicated with dashed curved shapes.

natural lipase enzymes release primary alcohols upon hydrolysis of triacylglycerols. Similarly, the nitrobenzene moiety is a poor approximation of a bulky diacylglycerol moiety (Figure 1). Second, *p*-nitrophenol esters may be hydrolyzed by other components in a sludge sample such as esterases, non-enzymatic proteins, protein hydrolyzing enzymes and non-catalytic regions of lipases (Gargouri *et al.* 1984; de Caro *et al.* 1986). Esterases are enzymes that do not hydrolyze triacylglycerol lipids with long-chain fatty acids but instead hydrolyze ester bonds in triacylglycerols with short-chain fatty acids (less than 8 carbon atoms) and in other molecules (Romano *et al.* 2015). Third, the detergents that assist in emulsification of the water-insoluble *p*-nitrophenol palmitate substrate may cause non-specific inhibition of lipases or may compete with *p*-nitrophenol palmitate as substrate analogue (Palacios *et al.* 2014). Finally, *p*-nitrophenol assays are restricted to pH neutral reaction conditions, since only dissociated *p*-nitrophenol ($\text{pK}_a = 7.15$) can be quantified by absorbance measurement (Gilham & Lehner 2005).

Several alternative lipid hydrolysis quantification principles have been reported in other niches of applied microbiology. A recent method reported by Zottig *et al.* (2016) based on the work of Jette and Ziomek (1994) employed a representative model substrate (olive oil), had a high sample throughput and allowed measurements to be obtained at a high frequency in a 96-well plate format. The quantification of lipid hydrolysis for this method was based on Rhodamine B, a fluorescent dye. When Rhodamine B is excited with UV light at 350 nm, it emits orange light (emission maximum at 580 nm) with an intensity proportional to the amount of free fatty acids that is released from the olive oil substrate. Rhodamine B visualization of long-chain fatty acid production has been applied extensively in *qualitative* screening of bacterial cultures for lipid hydrolytic activity on agar plates amended with vegetable oil and Rhodamine B. In contrast, Zottig *et al.* (2016) were the first to report on *quantitative* and high-throughput lipid hydrolysis screening in liquid media; that is, olive oil emulsions. To date, the vegetable oil – Rhodamine B emulsion approach has been applied only to purified lipase enzymes and in screening of mutagenetic libraries for lipase expression (Zottig *et al.* 2016, 2017; Quaglia *et al.* 2017), but not to samples with mixed microbial communities. Wastewater treatment sludges are complex matrices that might pose additional challenges to the quantification of lipid hydrolysis.

Therefore, we set out to develop an alternative, high-throughput lipid hydrolysis monitoring approach for wastewater treatment systems, based on the vegetable oil – Rhodamine B method. Furthermore, we aimed to extend the applicability of the vegetable oil – Rhodamine B method to other lipid substrates that are relevant for wastewater treatment processes, including soybean oil emulsions. Soybean oil emulsions are a relevant model substrate to resemble wastewaters high in linoleic acid lipids, as soybean oil contains 53 percent of linoleic acid ($\text{C}_{18:2}$ [*cis*-9,12]). In contrast, olive oil is a model substrate for wastewaters high in oleic acid lipids (Dubois *et al.* 2007).

METHODS

Sludge samples and commercial lipase

A commercial lipase was applied for initial testing. An Amano lipase from *Pseudomonas fluorescens* was purchased from Sigma-Aldrich (art. no 534730-10G) and dissolved in a buffer appropriate for the lipid hydrolysis assay at concentrations of 1 g L^{-1} or lower. All lipase preparations were prepared on the day of the experiment and shaken on an orbital shaker to improve dissolution of the enzyme (Grant bio PTR60, 10 minutes at 100 rpm).

Lipases from *Pseudomonas fluorescens* have been previously shown to hydrolyze different substrates, including triolein, tributyrin, *p*-nitrophenol butyrate and *p*-nitrophenol palmitate (Vorderwulbecke *et al.* 1992). It should be noted that this type of commercial lipase is a partially characterized mixture of enzymes and other proteins, lipids, salts and cell debris remaining from the microbial cultivation and downstream processing of the enzyme extract.

Four different sludge types were obtained from lab-scale and full-scale wastewater treatment installations. First, a sample from a lab-scale, anaerobic CSTR treating synthetic municipal wastewater, was obtained on March 9, 2018.

Second, a sludge sample from a full-scale sequencing batch reactor was obtained on June 15, 2020. The sequencing batch reactor treated wastewater from a tank-cleaning company and contained lipid residues from transport of various food-related products, such as vegetable oil. The reactor operational regime incorporated both aerobic and anaerobic phases.

Third, three samples were obtained from a full-scale, aerobic municipal wastewater treatment plant operated by Aquafin (Leuven, Belgium) on June 17, 2020. This conventional activated sludge system treated municipal wastewater with a minor fraction of industrial discharge. The plant layout consists of primary treatment (6 mm screens, oil and grease removal, sand trap), secondary treatment with anaerobic, aerobic and anoxic zones, and secondary sedimentation. Samples were obtained from the raw incoming wastewater, the sludge in the anaerobic secondary treatment zone, and the sludge in the aerobic secondary treatment zone.

Fourth, two samples were obtained from a full-scale, anaerobic reactor on June 11 and June 23, 2020. This reactor treated waste streams from ice cream production; that is, wasted ice cream and process wastewater.

Sludge samples were transported to the laboratory under cooled conditions ($4 \text{ }^\circ\text{C}$). Subsequently, they were stored at $4 \text{ }^\circ\text{C}$ prior to analysis. The total storage time amounted to maximum 13, 5, 2, 20 and 8 days for the lab-scale anaerobic reactor, full-scale sequencing batch reactor, full-scale municipal wastewater treatment system and the samples from the anaerobic reactor, respectively. Both samples from the full-scale anaerobic reactor were transported frozen and allowed to defrost at room temperature upon arrival in the laboratory.

The total solids (TS) and volatile solids (VS) concentrations were determined for all sludge samples as a proxy for biomass concentration. The determination proceeded according to standard methods (APHA 2012). Various sludge pretreatment methods were implemented (Table 1).

p-nitrophenol palmitate lipid hydrolysis monitoring

A modified *p*-nitrophenol palmitate assay was implemented based on the protocol by Gessesse *et al.* (2003). *p*-nitrophenol palmitate in isopropanol (8 mM) was emulsified in a Tris-HCl buffer solution (50 mM, pH 8) with Arabic gum (1 g L^{-1}) and Triton X-100 (4 g L^{-1}) in a 1:9 volumetric ratio to obtain a substrate emulsion. Next, sludge was added to an aliquot of the substrate emulsion with a 1:9 sludge:substrate volumetric ratio and incubated in an orbital shaker at 30 rpm for 60 minutes. The starting time of the assay was defined as the moment that the sludge was added to

Table 1 | Sludge pretreatment methods

Fraction name	Disintegration	Fractionation	Fraction separation	Filtration
Negative control	Heating (1 h, 120 °C)	–	–	–
Thickened	–	Sedimentation	Supernatant removal	–
SN	–	Sedimentation	Supernatant collection	–
SN F0.6	–	Sedimentation	Supernatant collection	Glass fibre ^a (0.6 µm)
Cfg	–	Centrifugation (15,000 × g, 5 min)	Supernatant collection	–
Cfg F0.6	–	Centrifugation (15,000 × g, 5 min)	Supernatant collection	Glass fibre ^a (0.6 µm)
BB	Bead beating ^b (5 min, 2,850 rpm)	–	–	–
UT	Ultraturrax ^c (10 min, 10,000 rpm)	–	–	–
BB + cfg	Bead beating ^b (5 min, 2,850 rpm)	Centrifugation (15,000 × g, 5 min)	Supernatant collection	–
UT + cfg	Ultraturrax ^c (10 min, 10,000 rpm)	Centrifugation (15,000 × g, 5 min)	Supernatant collection	–

^aFilter by Macherey Nagel, MN GF3.

^bBead beating on a Vortex genie digital 2 with 12 mL sample and 1 mL 1 mm Biospec zirconia/silica beads.

^cIKA T25 digital, dispersion tool S25N-18G.

the substrate emulsion. Subsamples (1.5 mL) were obtained every 15 minutes and centrifuged at 15,000 × g for 5 minutes prior to absorbance quantification of *p*-nitrophenol at 410 nm in analytical triplicate. The raw absorbance values were corrected with a substrate-only and sludge-only blank, recalculated to *p*-nitrophenol concentrations with a linear calibration curve (extinction coefficient 12.663 cm⁻¹ mM⁻¹) and normalized to sludge volatile solids (VS).

Vegetable oil – Rhodamine B lipid hydrolysis monitoring

The protocol for the vegetable oil – Rhodamine B lipid hydrolysis assay was based on the work of Jette & Ziomek (1994) and Zottig *et al.* (2016), with modifications to incorporate the analysis of sludge samples in a high-throughput format. Different combinations of the lipase sources with olive oil – Rhodamine B or soybean oil – Rhodamine B emulsions were evaluated for their lipid hydrolysis behavior in kinetic experiments. First, exploratory experiments were conducted with the Amano lipase from *Pseudomonas fluorescens*, in order to establish the optimal detection settings and to reproduce the results by Zottig *et al.* (2016). Next, the four different sludge samples were screened for their lipid hydrolysis activity.

The starting solution for emulsion preparation was a 50 mM potassium phosphate buffer (KH₂PO₄/K₂HPO₄) to which Arabic gum powder was added to a final concentration of 50 g L⁻¹. Arabic gum was heated for at least three hours at approximately 50 °C while stirring to improve dissolution and afterwards stirred overnight at ambient temperature. If applicable, β-cyclodextrin was added at a final concentration of 3 g L⁻¹. When needed, undissolved Arabic gum was filtered out of the solution with a glass fibre filter (Macherey Nagel, MN GF3) with pore size 0.6 µm.

Olive oil or soybean oil was added to the Arabic gum solution to a final concentration of 25 mL L⁻¹ while stirring. The olive oil was either commercial grade (extra virgin olive oil, Terra Creta, Greece) obtained at a local supermarket or analytical grade (Acros organics, art. no. 416530250). The soybean oil was of analytical grade (Fisher Scientific, art. no. J61399). The oil in the Arabic gum solution was then emulsified by Ultraturrax treatment for 10 minutes at 10,000 rpm (IKA T25 digital, dispersion tool S25N-18G), the pH was corrected to pH 7 with 1 M sodium hydroxide and 1 mL of a 1 g L⁻¹ concentrated Rhodamine B stock was added (final emulsion concentration 10 mg L⁻¹). The emulsion was stored at 4 °C while stirring on a magnetic stirring plate. All prepared emulsions were used within two days and protected from light during storage.

The emulsions were characterized with an inverted, LED-illuminated microscope (Olympus, IX83). Images were acquired with bright field observation and the diameter of individual droplets was determined with the '3 point circumference' measurement function of the microscope software (Olympus CellSens dimension).

The lipid hydrolysis assay consisted of four steps. First, the enzyme sources, either a commercial lipase or a sludge sample, were diluted to the desired concentration with the Arabic gum solution. Note that the previously mentioned *diluted* sludge concentrations are reported in this study and not the final sludge concentration in the well after addition of the substrate emulsion. Second, 100 μL aliquots of all enzyme sources were added to a 96-well plate suitable for fluorescence measurements (Greiner bio one, art. no. 655090) in triplicate. Third, the microplate reader (Tecan, infinite M200 pro) was set up to perform a kinetic cycle of at least 4 hours with continuous orbital shaking at 6 mm amplitude, interrupted every 10 minutes for fluorescence measurements of the wells with excitation/emission at 350/580 nm and fluorescence gain 100. Finally, 50 μL aliquots of the substrate emulsion were added to the well just before analysis. The plates were incubated at ambient temperature (23–30 °C) and the first measurement performed after an initial mixing period of 10 minutes. All assays included substrate-only, sample-only, sample with Rhodamine B and Rhodamine B-only blanks in triplicate with identical sample and substrate volumes.

The raw sample fluorescence intensities (arbitrary unit U) were corrected with the sample and Rhodamine B-only blank measurements for individual wells and for every measurement separately. This correction aimed to account for changes in the Rhodamine B signal due to interactions with the sludge that were not related to lipid hydrolysis and for changes in the signal due to photobleaching. The measurement time was recalculated with $t = 0$ minutes as the moment of substrate addition to the first well in the plate. Manual filling of a full plate took approximately 4 minutes. Next, a linear fit was calculated through the first 10 measurements; that is, $t = 14$ to $t = 120$ min approximately, with least squares regression in MS ExcelTM assuming zero order kinetics. Linear fit values were calculated for every replicate separately and normalized on a volatile solids (VS) basis, if applicable. Data for analytical replicates were shown as [minimum-maximum] to indicate the variability of the analytical results due to sample heterogeneity, sample preparation and data fitting. The coefficient of determination was used in this study to assess the goodness of fit of the linear approximation. Values below 0.8 were denoted 'low confidence' linear approximations and annotated as such in the results section.

RESULTS AND DISCUSSION

Lipid hydrolysis monitoring with *p*-nitrophenol palmitate quantification

An experiment was conducted with the current state-of-the-art method in wastewater treatment applications; that is, the *p*-nitrophenol palmitate assay, to determine the advantages and potential limitations of the method in practice. The results for a sample from an anaerobic, lab-scale reactor treating synthetic municipal wastewater at 30 °C are shown in [Figure 2](#).

Three conclusions can be deduced from this figure. First, lipid hydrolysis in the sample was primarily associated with the sludge solid fraction and not in the sludge supernatant after centrifugation. Second, the initial absorbance signal for the heat-inactivated sludge (120 °C, 1 hour) was not zero, likely related to interactions between the sludge matrix and the *p*-nitrophenol palmitate emulsion that could not be accounted for through the sludge-only and emulsion-only blanks. Third, and most importantly, the absorbance of the subsamples derived from the sludge incubation with *p*-nitrophenol palmitate appeared to increase even after all sludge solids were separated from the *p*-nitrophenol products by centrifugation (see the crosses and dashed lines in [Figure 2](#)). It is unclear if this is related to a true biological activity in the aqueous phase or an absorbance measurement artifact. Additional

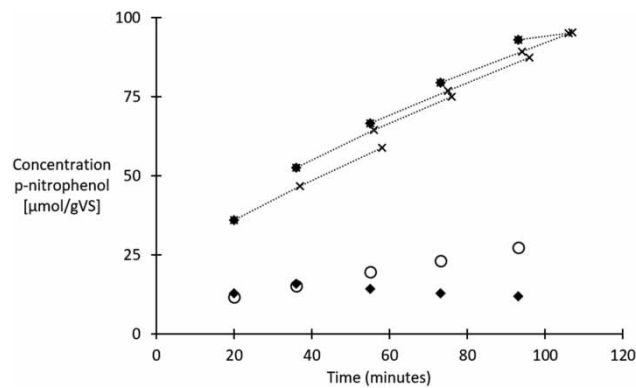


Figure 2 | Lipid hydrolysis quantification with the *p*-nitrophenol palmitate lipid hydrolysis assay on a sample of an anaerobic lab-scale reactor treating synthetic municipal wastewater. Full circle: undiluted sludge sample, open circle: sludge supernatant after centrifugation (15,000xg), diamond: heat-inactivated sludge (120 °C, 1 h). Crosses and dashed lines: follow-up measurements of *p*-nitrophenol cuvettes that were not incubated further. Subsamples were collected after 0, 15, 30, 45 and 60 minutes of incubation in an orbital shaker. Time zero was defined as the moment of sludge addition to the substrate emulsion.

experiments with an adapted protocol including freezing, filtration on a 0.2 μm membrane filter or addition of ethanol to terminate the enzymatic reaction did not provide satisfactory results (data not shown). Similar issues with reaction termination have been reported previously in studies with purified lipase solutions (Palacios *et al.* 2014).

In conclusion, the *p*-nitrophenol palmitate assay, as applied here, did not accurately detect lipid hydrolysis activity in an anaerobic sludge sample. Some practical shortcomings, such as a lack of activity termination, absorbance interferences and the requirement for individual subsampling over time were observed. In addition, the *p*-nitrophenol palmitate method also has some conceptual limitations, as outlined in the introduction.

Characterization of a vegetable oil – Rhodamine B system

A representative assay involving lipids requires a stable contact between the lipase enzyme and the lipid substrate at the emulsion interface. Therefore, this section aims to characterize the emulsion in terms of droplet size, general appearance and stability. The images showed green droplets of olive oil surrounded by a halo of Rhodamine B, as expected (Supplementary material, Figure S1). Rhodamine B is water soluble but has an apolar xanthene core, which would promote interaction with the apolar lipid surface (Beija *et al.* 2009). The droplet size varied over a wide range from close to 1 μm diameter to up to 30 μm and more if droplets coalesced (Figure S1, image (c)). The size of 228 droplets was determined in the CellSens imaging software. This analysis resulted in a median droplet diameter of 6 μm, with an interquartile range of 2.5 to 6.2 μm. These results were in line with the specifications of the Ultraturax device (IKA T25 digital) and the corresponding dispersion tool S25N-18G that were employed to disperse the olive oil in the Arabic gum matrix. The manufacturer of the dispersion tool specifies that droplet diameters in the range of 1 to 10 μm can be obtained.

The Arabic gum at a final concentration of 50 g L⁻¹, as suggested by Zottig *et al.* (2016), was not completely soluble in the phosphate buffer, as was observed visually during the emulsion preparation protocol. Some solid crystals were observed in the microscopic images (Figure S1, image (c)). Therefore, undissolved Arabic gum was filtered out of the Arabic gum solution prior to preparation of the final emulsion in later experiments.

The stability of the vegetable oil emulsion over the time course of a lipid hydrolysis assay was determined by incorporating substrate only samples; that is, 150 μL Arabic gum solution with 50 μL substrate emulsion in all experiments. In general, a decrease in fluorescence was observed throughout the assay (Supplementary material, Figure S2). This trend was also observed for blanks containing

only Arabic gum and Rhodamine B, indicating that the vegetable oil was not responsible for the decrease in fluorescence intensity (data not shown). Slightly lower fluorescence intensity values were observed in one of three identical experiments, which was likely linked to the temperature-dependent fluorescence behavior of Rhodamine B (Beija *et al.* 2009). This experiment was conducted at a higher operational temperature between 28.3 and 29.4 °C in the measurement device, while the temperature in the other two experiments rose steadily from 22.4 and 21.9 °C to 28.8 and 29 °C. In conclusion, the overall decrease in signal for the substrate-only samples is most probably related to a combined effect of photo bleaching and temperature-dependent behavior of the Rhodamine dye. These effects were incorporated in experiments with enzyme solutions by correcting the raw enzyme emission values with a blank containing all components but the vegetable oil substrate.

The emulsion stability during storage was not assessed in this work. However, the emulsions were used within two days after preparation in all experiments. Emulsions were stored at 4 °C with continuous stirring to avoid coalescence. A slight pH drop from 7 to 6.8 was observed in the emulsions during storage but not corrected.

In conclusion, the droplet size distribution of the vegetable oil emulsion was a reasonable approximation of lipids in wastewater, and the emulsions appeared stable over the timespan of a lipid hydrolysis assay.

Lipid hydrolysis monitoring with a vegetable oil – Rhodamine B system

First, excitation and emission spectra were measured for an olive oil emulsion incubated with Amano lipase (1 g L⁻¹) in Arabic gum (50 g L⁻¹) or with an Arabic gum blank (50 g L⁻¹). The excitation/emission couple of 350/580 nm suggested by Zottig *et al.* (2016) was appropriate to detect lipid hydrolysis, as shown in Figure 3.

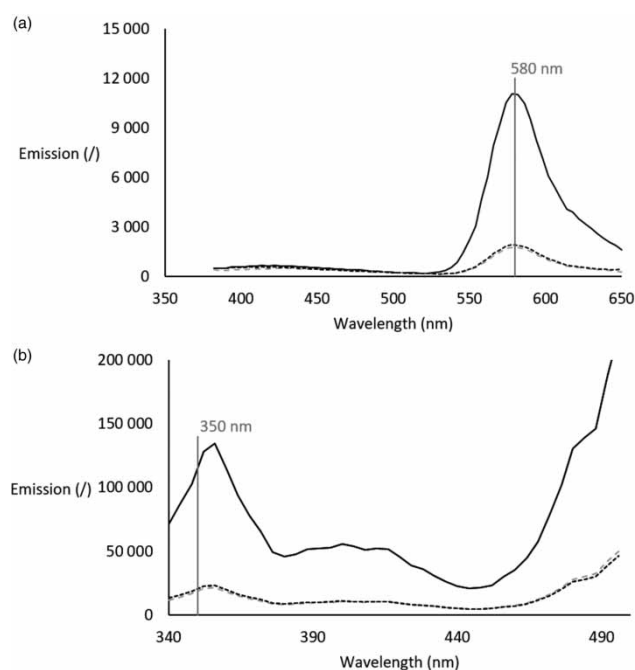


Figure 3 | Emission and excitation spectra for an Amano lipase solution. (a) Emission spectrum with excitation at 350 nm, (b) excitation spectrum with emission at 580 nm. Solid line: Olive oil emulsion (commercial grade) incubated with Amano lipase (1 g L⁻¹), dashed line: Olive oil emulsion (commercial grade) incubated with an Arabic gum blank.

Second, kinetic experiments were conducted with serially diluted Amano lipase solutions incubated with olive oil and soybean oil emulsions (Figure 4). The enzyme concentration varied from 0.03125 to 1 g L⁻¹, which corresponds to an enzyme mass per well of 4.7 and 150 µg, respectively. The total

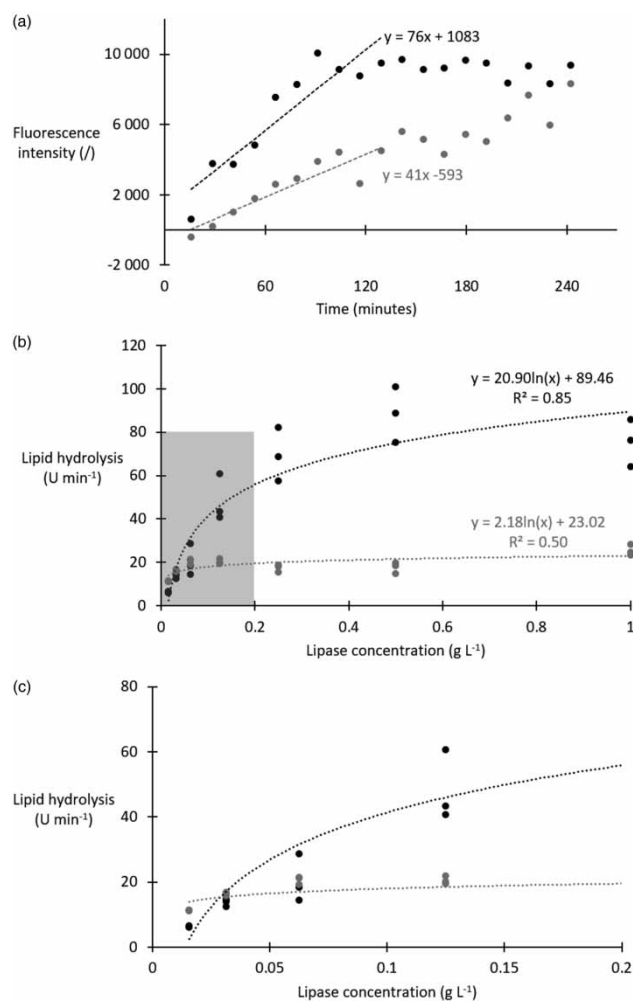


Figure 4 | Lipid hydrolysis rates for Amano lipase solutions at different concentrations. (a) Time profile of fluorescence intensity for an individual replicate of a 1 g L^{-1} Amano lipase solution (black dots) and a 0.125 g L^{-1} Amano lipase solution (grey dots) incubated with an olive oil emulsion (commercial grade). Dashed lines are linear trend lines for the first ten observations, i.e., approximately 2 hours incubation time. The fluorescence intensity was corrected for the *Rhodamine B* only fluorescence. (b) Slopes of the linear trend lines, i.e. lipid hydrolysis activity in arbitrary units U per minute, for different Amano lipase concentrations and individual replicates. An olive oil emulsion (commercial grade, black circles) and soybean oil emulsion (analytical grade, grey dots) were applied. (c) Zoom of shaded area in plot (b).

experimental time was approximately four hours, with continuous orbital shaking in the measurement device and a fluorescence measurement every 10 minutes. Lipid hydrolysis activity could be confirmed visually as an orange color in the wells after several hours of incubation (Supplementary material, Figure S3).

Figure 4(a) shows that the fluorescence measurements were noisy, especially towards the end of the experiment. In addition, the variation between replicate wells was large for experiments with the olive oil emulsion (Figure 4(b)). The coefficient of determination for the linear approximation based on the ten first measurement values was 0.93 ± 0.08 (42 values) due to the measurement noise. A total incubation time of approximately 120 minutes corresponded to the linear part of the curve for all lipase solutions. Note that the slope of the linear approximation; that is, the lipid hydrolysis activity (arbitrary unit U min^{-1}) was sometimes over- or underestimated due to the measurement noise.

Measurement noise and experimental variability were expected beforehand, because (i) the substrate emulsion was heterogeneous, (ii) the lipase enzyme solution was heterogeneous, since the powder was only partly purified, and (iii) lipid hydrolysis required interfacial binding of the lipase enzyme (Jette & Ziomek 1994). Interfacial binding could have been disturbed during the assay due

to turbulence. In addition, emulsion droplets might have coalesced during the assay, an inevitable process for thermodynamically unstable emulsions. The mixing mode during the assay, orbital mixing of the 96-well plate with 6 mm amplitude, could have been insufficient to prevent coalescence completely. A sufficient number of analytical replicates and experiment repetitions should be considered for further research to evaluate and address the experimental variability.

Figure 4(b) and 4(c) show an initial increase of the hydrolysis activity with increasing lipase concentration, while concentrations above 0.2 g L^{-1} ($30 \mu\text{g}$ lipase per well) did not result in a proportional increase in the lipid hydrolysis activity. This behavior indicates that the substrate excess was insufficient at these high enzyme concentrations. Furthermore, Amano lipase had a higher activity towards the olive oil substrate than towards soybean oil (Figure 4(b) and 4(c)). Olive oil is mainly composed of lipids with oleic acid chains (C18:1 [*cis*-9]), while more than 50 percent of the long-chain fatty acids in soybean oil is linoleic acid (C18:2 [*cis*-9,12]) (Dubois *et al.* 2007). Although the cause of the preference towards olive oil cannot be determined from these data, it is generally accepted that lipases have several types of substrate selectivity, such as regioselectivity, based on the position of the fatty acid on the glycerol backbone, fatty acid type selectivity, and stereoselectivity.

In conclusion, these exploratory experiments indicate that quantification of lipid hydrolysis by fluorescence (excitation/emission 350/580) is feasible for a partially purified commercial lipase. The enzyme-to-substrate ratio should be tailored to ensure a linear correlation between the amount of enzyme and the measured activity; that is, well below 0.2 g L^{-1} for the Amano Lipase in this study. Furthermore, an experimental time of 120 min is sufficient to observe lipid hydrolytic behavior and to obtain sufficient fluorimetric data for quantification. Finally, analytical replicates and experiment repetitions are crucial to obtain reliable data in the presence of considerable measurement noise.

Next, three different sludge types from aerobic and anaerobic wastewater treatment systems were analyzed, taking into account the findings of the exploratory experiments with commercial lipase enzyme preparations. Considerable lipid hydrolysis activity ($>10 \text{ U min}^{-1}$) was expected for all sludge types, since the incoming wastewater contained lipids from food transport tank cleaning (sequencing batch reactor), household activities (municipal wastewater treatment plant) and ice cream production (anaerobic reactor).

First, the sludge samples were tested in different sample dilutions to determine if there was lipid hydrolytic activity present. Two different lipid substrates; that is, olive oil and soybean oil, were tested. The volatile solids normalized results ($\text{U min}^{-1} \text{ mg VS}^{-1}$) are summarized in Table 2 and were low for all samples. It should be noted that both samples from the anaerobic reactor treating ice cream wastes were frozen during transport, which could have negatively affected the lipid hydrolysis activity. The *absolute* lipid hydrolysis activity values (U min^{-1}) were below three for all samples, and often close to zero, see Figure 4 to compare with the values obtained for the commercial lipase enzyme.

The hydrolytic activity with a soybean oil emulsion was slightly higher than the activity for an olive emulsion for two samples, pointing towards a substrate preference. Additional experiments are required to provide further evidence for this hypothesis.

Second, several sludge fractions were separated from the original sludge to determine if the low absolute lipid hydrolysis activity values could be due to interference of the sludge aggregates with the lipid emulsion (Tables 3 and 4). Unfortunately, none of the sludge fractions showed significant lipid hydrolysis activity, similar to the case for the original samples.

Third, the sequencing batch reactor sample treating tank-cleaning wastewater was spiked with Amano lipase in three different concentrations to evaluate if the sludge sample contained inhibitory components. The concentration of lipase in the sludge sample varied between 0.17 and 0.5 g L^{-1} . The values obtained for the spiked sludge sample were very similar to the lipase solution alone, indicating that the sludge matrix was not inhibitory for lipase enzymes (Table 5).

Finally, it was attempted to mobilize the long-chain fatty acid hydrolysis products into the aqueous phase during the assay with a mobilizer, β -cyclodextrin. Cyclodextrins are circular oligosaccharides

Table 2 | Volatile solids normalized lipid hydrolysis rates ($\text{U min}^{-1} \text{mg VS}^{-1}$) for the municipal wastewater treatment plant (influent only), the sequencing batch reactor sample treating tank cleaning wastewater and the anaerobic reactor treating ice cream wastes

Reactor:	MWWTP – influent			Tank cleaning			Ice cream			Ice cream		
Sampling date:	June 17, 2020			June 15, 2020			June 11, 2020			June 23, 2020		
Substrate:	OO ^a	SO		OO ^a	SO		OO	SO		OO	SO	
	g VS L^{-1}	$\text{U min}^{-1} \text{mg VS}^{-1}$		g VS L^{-1}	$\text{U min}^{-1} \text{mg VS}^{-1}$		g VS L^{-1}	$\text{U min}^{-1} \text{mg VS}^{-1}$		g VS L^{-1}	$\text{U min}^{-1} \text{mg VS}^{-1}$	
							7.5	0–1	0–1	7.2	<0	<0
							6.3	0–1	1–2	6.0	<0	<0
							5.0	0–2	1–3	4.8	<0	<0
				3.9	2–4	4–5	3.8	1–3	0–3	3.6	<0	<0
							2.5	1–3	0–5	2.4	<0	0–0.7
				1.1	1–3	7–12	1.3	4–7	0–10	1.2	<0	0–2.7
				0.6	7–10	6–15						
	0.37	3–26 ^b	28–38 ^b									

Several sample dilutions were tested. The lipid hydrolysis values are represented as [minimum – maximum] for the analytical triplicates. The blue shaded values were obtained with low confidence, i.e., a coefficient of determination of the linear approximation lower than 0.8. The results of the anaerobic zone and the aerobic zone in the secondary treatment phase of the municipal wastewater treatment plant were not shown here but were <0 for olive oil and soybean oil emulsions. The amount of biomass was calculated based on the 150 μL sample volume. MWWTP: municipal wastewater treatment plant, OO: Olive oil emulsion, SO: Soybean oil emulsion.

^aCommercial grade oil.

^bValues are inflated by normalization to a low biomass concentration.

Table 3 | Absolute and volatile solids normalized lipid hydrolysis rates for different sludge fractions of the sequencing batch reactor sample treating tank cleaning wastewater

Sludge fraction	Original	Thickened	SN	SN F0.6	Cfg	BB + cfg	UT + cfg
Biomass (g TS L^{-1})	6.8	11.7	3.2	3.1	NA		
Biomass (g VS L^{-1})	3.9	8.5	0.4	0.4	NA		
Lipid hydrolysis (U min^{-1})	0.9–1.3	0–0.5	0.2–0.3	0.3–0.4	< 0		
Lipid hydrolysis ($\text{U min}^{-1} \text{mg VS}^{-1}$)	2	0–0.4	2–4	5–6	NA		

The original sample and the sludge fractions were tested without sample dilution and incubated with an olive oil emulsion prepared with commercial grade olive oil. The lipid hydrolysis values are represented as [minimum – maximum] for the analytical triplicates. The blue-shaded values were obtained with low confidence, i.e., a coefficient of determination of the linear approximation lower than 0.8 for at least one of the replicates. The amount of biomass was below 1 mg VS for all samples, except for the thickened sludge (150 μL sample volume). NA: not applicable.

and have a toroid molecular shape with a relatively hydrophobic molecular interior and a hydrophilic molecular exterior. Therefore, they can have a detergent-like action and help to solubilize long-chain fatty acids in aqueous solvents (Junior *et al.* 2014). No positive effects were observed of the addition of β -cyclodextrin in an experiment with sludge from the anaerobic reactor treating ice cream waste and an olive oil emulsion with analytical grade olive oil (Table 4). However, this sludge sample had been frozen and defrosted, which might have affected the lipid hydrolysis activity to some extent. Moreover, cyclodextrin oligosaccharides might be subject to microbial degradation during the lipid hydrolysis assay.

Several hypotheses can be formulated to explain the low to non-existent lipid hydrolysis in absolute terms (U min^{-1}) for the raw and pre-treated aerobic and anaerobic wastewater treatment samples with the vegetable oil – Rhodamine B system. The hypotheses are presented visually in Figure 5.

First, lipid hydrolysis requires a close contact between the lipase enzyme and the substrate at the oil – water interface (Figure 5(a)). Detergents or other unknown components with amphiphilic behavior in the sludge sample could have blocked the interface, resulting in a low activity (Figure 5(b)). The

Table 4 | Absolute and volatile solids normalized lipid hydrolysis rates for different sludge fractions of the anaerobic reactor treating ice cream wastes

β -cyclodextrin Sludge fraction	No					Yes						
	Original	BB	UT	BB + cfg	UT + cfg	Original	cfg	cfg F0.6	BB	UT	BB + cfg	UT + cfg
Sludge biomass (g TS L ⁻¹)	8.5			NA		8.5	NA		8.5	8.5	NA	
Sludge biomass (g VS L ⁻¹)	7.2			NA		7.2	NA		7.2	7.2	NA	
Lipid hydrolysis (U min ⁻¹)	0–3	<0	<0	1.9–2.3	2.1–2.6	0–1	1–3	2	0	0	3	3
Lipid hydrolysis (U min ⁻¹ mgVS ⁻¹)	0–2	<0	<0	NA	NA	0–1	NA	NA	0	0	NA	NA

The sample and the sludge fractions were tested without sample dilution and incubated with an olive oil emulsion prepared with analytical grade olive oil, without or with β -cyclodextrin at a final concentration of 3 g L⁻¹ in the emulsion. Undissolved Arabic gum was filtered out prior to preparation of the lipid emulsion. The lipid hydrolysis values are represented as [minimum – maximum] for the analytical triplicates. The blue-shaded values were obtained with low confidence, i.e., a coefficient of determination of the linear approximation lower than 0.8 for at least one of the replicates. The amount of biomass was 1.1 mg VS for all samples without centrifuge pretreatment (150 μ L sample volume). NA: not applicable.

Table 5 | Absolute and volatile solids normalized lipid hydrolysis rates for the sequencing batch reactor sample treating tank cleaning wastewater with different concentrations of spiked Amano lipase

Sludge fraction	Original	L0.17	L0.33	L0.5
Biomass (g TS L ⁻¹)	6.8	5.7	4.5	3.4
Biomass (g VS L ⁻¹)	3.9	3.2	2.6	1.9
Lipid hydrolysis (U min ⁻¹)	0–3	57–62	49–71	57–72
Lipid hydrolysis (U min ⁻¹ mgVS ⁻¹)	0–5	NA	NA	NA

Original/L0.17/L0.33/L0.5: 0/0.17/0.33/0.5 g L⁻¹ Amano lipase in the sludge sample. All samples were incubated with an olive oil emulsion (commercial grade oil). The lipid hydrolysis values are represented as [minimum – maximum] for the analytical triplicates. The blue-shaded value was obtained with low confidence, i.e., a coefficient of determination of the linear approximation lower than 0.8 for at least one of the replicates.

presence of detergents is a reasonable assumption for all sludge samples tested in this work. Both the sequencing batch reactor and the anaerobic reactor treated wastewaters derived from industrial *cleaning* activities in food truck transport or ice cream manufacturing, respectively. Furthermore, detergents are common components in municipal and industrial wastewater.

In addition, lipases are entrapped in the sludge aggregates, which might hinder a stable substrate-enzyme contact (Gessesse *et al.* (2003), Figure 5(c)). The experiment in this study with commercial lipase spiking in a sludge sample provides potential evidence for the lipase entrapment as bottleneck. The free commercial enzyme was not hindered by the sludge matrix and was able to hydrolyze lipids at the oil interface, even in the presence of the sludge aqueous matrix.

Second, a fluorescent signal can only be produced when long-chain fatty acids interact with Rhodamine B. Long-chain fatty acids might have remained in the lipid organic phase or remained entrapped on, or in, the sludge aggregates (Figure 5(d)). In this regard, it is surprising that β -cyclodextrin did not have any positive effect on mobilization of the long-chain fatty acids. Optimization of the optimal β -cyclodextrin concentration during the assay (3 g L⁻¹ in the emulsion for this work) might improve the method. In addition, long-chain fatty acids may have been consumed by the microbial community over the time course of the assay, allowing no time for interaction with Rhodamine B (Figure 5(e)). However, degradation of long-chain fatty acids is generally recognized as a slow process for *anaerobic* wastewater treatment systems, since it requires syntrophic β -oxidation.

Third, the lipase enzymes may have been inactivated or inhibited prior to or during the assay. Inactivation prior to the assay may have occurred due to general storage effects, or freezing and defrosting for the samples from an anaerobic reactor treating ice cream wastes (Figure 5(f)). In addition, product inhibition by long-chain fatty acids formed during sample storage or during the assay may have caused

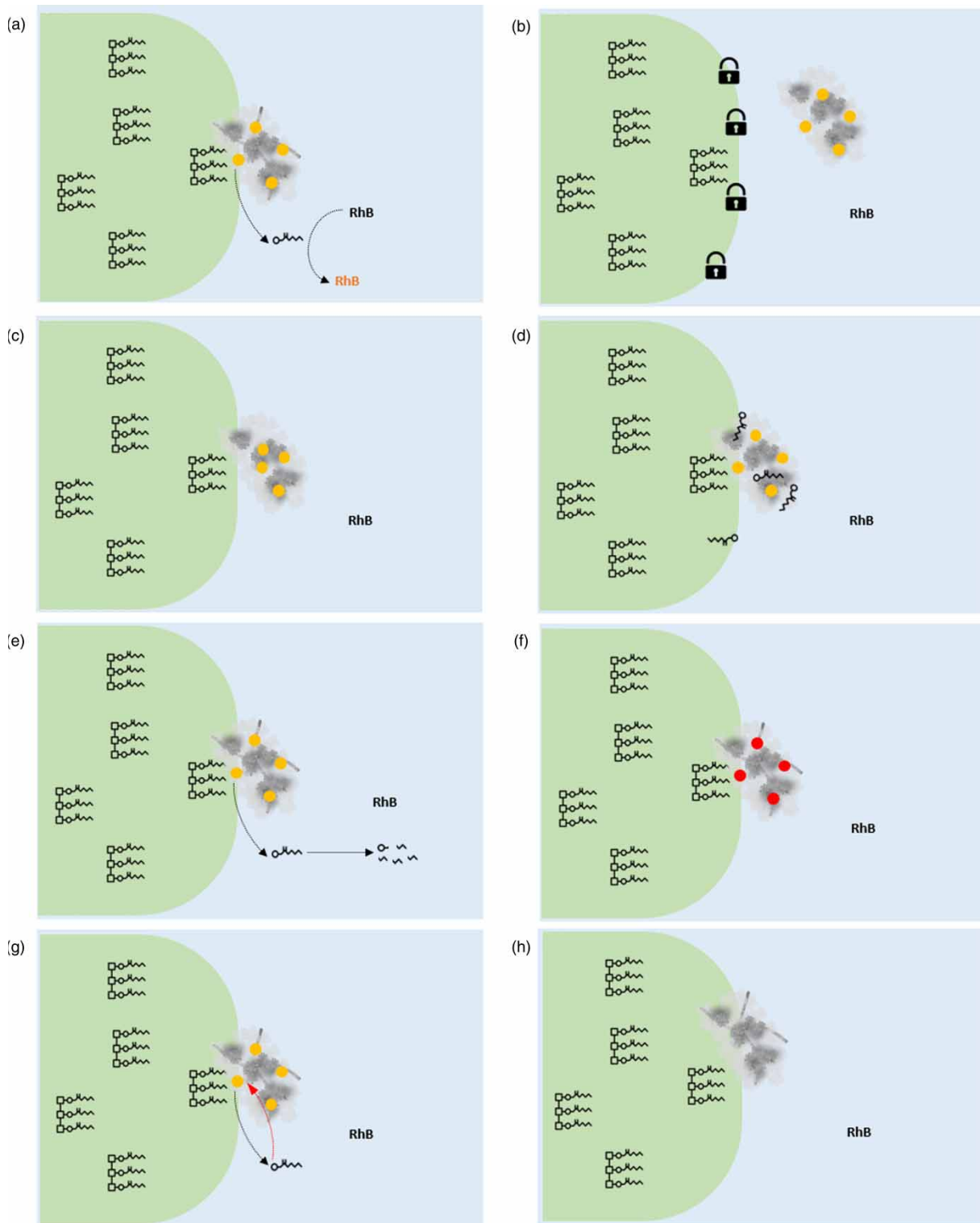


Figure 5 | Visual representation of the hypotheses explaining low to non-existent lipid hydrolysis activity in the aerobic and anaerobic sludge samples. Green shaded area: vegetable oil emulsion droplet with depiction of triacylglycerol lipid substrate, yellow circle: active lipase enzyme, red circle: inactivated lipase enzyme, grey cluster: microbial aggregate, RhB: Rhodamine B, either in the ground state (black) or in the excited state (orange). Panels: (a) normal activity, (b) blocking of the oil – water interface, (c) lipase enzymes enclosed in the sludge matrix, (d) long-chain fatty acid entrapped in the sludge matrix or the lipid phase, (e) degradation of long-chain fatty acids, (f) lipase inactivation, (g) product inhibition by long-chain fatty acids, (h) absence of lipase enzymes.

low lipase activities (Figure 5(g)). Similar to the previous hypothesis, it is reasonable to assume that β -cyclodextrin would alleviate effects of product inhibition, if applicable.

Finally, it is possible that the samples in this study did not contain any lipase enzymes (Figure 5(h)). The underlying assumption that lipid hydrolysis is mediated mostly by microbial enzymes, and not by physico-chemical processes in the wastewater treatment system, might be incorrect. However, an extensive body of research on aerobic municipal wastewater treatment (Molina-Muñoz *et al.* 2010; Cortés-lorenzo *et al.* 2012; Calderón *et al.* 2013; Ferrer-polonio *et al.* 2018), anaerobic municipal wastewater treatment (Petropoulos *et al.* 2018) and industrial wastewater treatment (Silva-bedoya *et al.* 2016; Luján-Facundo *et al.* 2018) has been published to corroborate the assumption of enzymatic lipid hydrolysis. It should be noted that this prior research was most often conducted with *p*-nitrophenol synthetic substrates, which target both true lipases, hydrolyzing triacylglycerol lipids, and esterases, hydrolyzing several types of ester bonds. The vegetable oil – Rhodamine B alternative system has been mostly applied in lipase screening experiments and isolation of pure cultures from mixed culture samples to date and has not been applied yet to wastewater treatment systems.

CONCLUSIONS

Further improvements in the treatment efficiency of lipid-containing wastewaters require mechanistic knowledge on lipid hydrolysis in response to changes in the operational temperature or other process conditions. We aimed to develop a high throughput lipid hydrolysis assay with lipid substrates representative for aerobic and anaerobic wastewater treatment systems. In addition, the envisaged assay should be versatile in terms of lipid substrate or sludge type and should guarantee a high sensitivity and experimental reproducibility.

These requirements were partially fulfilled for the vegetable oil – Rhodamine B assay. Olive oil and soybean oil emulsions were prepared with a high interfacial area; that is, small droplet size, and acceptable emulsion stability. The assay was easy to apply and obtained detailed kinetic data over a time course of several hours for up to 16 samples in parallel. A proof-of-concept, in the form of measurable and reproducible hydrolysis rates, is obtained with a commercial lipase preparation but remains to be provided for wastewater treatment sludge samples. Although all tested sludge samples were taken from treatment systems for which the lipid hydrolysis was deemed significantly high, the assay did not indicate any hydrolysis activity. Several hypotheses for this lack of validation are formulated but most probably narrow down to a physico-chemical reason, implying a lack of intensive contact between the enzymes and the (non-soluble) lipid molecules. In addition, the measurement noise should be reduced further.

These optimization challenges should be tackled in future experiments by (i) stabilizing the emulsion with additional or alternative stabilizing agents such as proteins or non-adsorbing polysaccharides, while avoiding potential interference of microbial degradation of the stabilizing agent during the assay, (ii) promoting the mobility of the long-chain fatty acid hydrolysis products with optimized addition of β -cyclodextrin, and (iii) ensuring a stable contact between the vegetable oil substrate and the lipase enzyme at the oil – water interface through selection of proper mixing conditions.

ACKNOWLEDGEMENTS

Pieter Van Gaelen was supported as doctoral candidate (aspirant) by the Research Foundation – Flanders (FWO – *Vlaanderen*; file number 28738). The research foundation Flanders did not participate in the creation of this study. We gratefully acknowledge Aquafin (Belgium) and the BioGEM research

group at the University of Antwerp (Belgium) for providing sludge samples. We thank Johan Martens and Tom Bosserez (KU Leuven) for providing access to the Infinite M200 pro microplate reader.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

- Alves, M. M., Pereira, M. A., Sousa, D. Z., Cavaleiro, A. J., Picavet, M., Smidt, H. & Stams, A. J. M. 2009 **Waste lipids to energy: how to optimize methane production from long-chain fatty acids (LCFA)**. *Microb. Biotechnol.* **2**, 538–550. <https://doi.org/10.1111/j.1751-7915.2009.00100.x>.
- APHA 2012 *Standard Methods for the Examination of Water and Wastewater*, 22nd edn. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC, USA.
- Beija, M., Afonso, C. A. M. & Martinho, J. M. G. 2009 **Synthesis and applications of rhodamine derivatives as fluorescent probes**. *Chem. Soc. Rev.* **38**, 2410–2433. <https://doi.org/10.1039/b901612k>.
- Calderón, K., Reboleiro-rivas, P., Rodríguez, F. A., Poyatos, J. M., González-lópez, J. & Rodelas, B. 2013 **Comparative analysis of the enzyme activities and the bacterial community structure based on the aeration source supplied to an MBR to treat urban wastewater**. *J. Environ. Manage.* **128**, 471–479. <https://doi.org/10.1016/j.jenvman.2013.05.048>.
- Cortés-lorenzo, C., Rodríguez-díaz, M., López-lopez, C., Sánchez-peinado, M. & Rodelas, B. 2012 **Effect of salinity on enzymatic activities in a submerged fixed bed biofilm reactor for municipal sewage treatment**. *Bioresour. Technol.* **121**, 312–319. <https://doi.org/10.1016/j.biortech.2012.06.083>.
- de Caro, J. D., Rouimi, P. & Rovey, M. 1986 **Hydrolysis of p-nitrophenylacetate by the peptide chain fragment (336–449) of porcine pancreatic lipase**. *Eur. J. Biochem.* **158**, 601–607. <https://doi.org/10.1111/j.1432-1033.1986.tb09797.x>.
- Diamantis, V., Eftaxias, A., Stamatelatos, K., Noutsopoulos, C., Vlachokostas, C. & Aivasidis, A. 2021 **Bioenergy in the era of circular economy: anaerobic digestion technological solutions to produce biogas from lipid-rich wastes**. *Renew. Energy* **168**, 438–447. <https://doi.org/10.1016/j.renene.2020.12.034>.
- Dubois, V., Bretion, S., Linder, M., Fanni, J. & Parmentier, M. 2007 **Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential**. *Eur. J. Lipid Sci. Technol.* **109**, 710–732. <https://doi.org/10.1002/ejlt.200700040>.
- Fan, N. S., Qi, R., Huang, B. C., Jin, R. C. & Yang, M. 2020 **Factors influencing *Candidatus* *Microthrix parvicella* growth and specific filamentous bulking control: a review**. *Chemosphere* **244**, 125371. <https://doi.org/10.1016/j.chemosphere.2019.125371>.
- Ferrer-polonio, E., Fernández-navarro, J. & Alonso-molina, J. L. 2018 **Influence of organic matter type in wastewater on soluble microbial products production and on further ultrafiltration**. *J. Chem. Technol. Biotechnol.* **93**(11), 3284–3291. <https://doi.org/10.1002/jctb.5689>.
- Franke, W., Ettl, M., Roldan, D., Kuhn, G., Langholm, A. M. & Amar, M. 2011 **Fat, oil and grease – sewer contamination prevention strategies and double-dosage concept for fat traps and pressure mains**. *Water Pract. Technol.* **6**(2), wpt2011036. <https://doi.org/10.2166/wpt.2011.036>.
- Gargouri, Y., Julien, R., Sugihara, A., Verger, R. & Sarda, L. 1984 **Inhibition of pancreatic and microbial lipases by proteins**. *Biochim. Biophys. Acta* **795**, 326–331. [https://doi.org/10.1016/0005-2760\(84\)90082-1](https://doi.org/10.1016/0005-2760(84)90082-1).
- Gessesse, A., Dueholm, T., Petersen, S. B. & Nielsen, P. H. 2003 **Lipase and protease extraction from activated sludge**. *Water Res.* **37**, 3652–3657. [https://doi.org/10.1016/S0043-1354\(03\)00241-0](https://doi.org/10.1016/S0043-1354(03)00241-0).
- Gilham, D. & Lehner, R. 2005 **Techniques to measure lipase and esterase activity in vitro**. *Methods* **36**, 139–147. <https://doi.org/10.1016/j.jymeth.2004.11.003>.
- Jette, J. F. & Ziomek, E. 1994 **Determination of lipase activity by a Rhodamine-triglyceride-agarose assay**. *Anal. Biochem.* **219**, 256–260. <https://doi.org/10.1006/abio.1994.1265>.
- Junior, I. I., Gaudino, E. C., Martina, K., Cravotto, G., Luque, R. & De Souza, R. O. M. A. 2014 **Improving the esterification activity of *Pseudomonas fluorescens* and *Burkholderia cepacia* lipases via cross-linked cyclodextrin immobilization**. *RSC Adv.* **4**, 45772–45777. <https://doi.org/10.1039/C4RA03797A>.
- Luján-Facundo, M. J., Fernández-Navarro, J., Alonso-Molina, J. L., Amorós-Muñoz, I., Moreno, Y., Mendoza-Roca, J. A. & Pastor-Alcañiz, L. 2018 **The role of salinity on the changes of the biomass characteristics and on the performance of an OMBR treating tannery wastewater**. *Water Res.* **142**, 129–137. <https://doi.org/10.1016/j.watres.2018.05.046>.
- Miron, Y., Zeeman, G., van Lier, J. B. & Lettinga, G. 2000 **The role of sludge retention time in the hydrolysis and acidification of lipids, carbohydrates and proteins during digestion of primary sludge in CSTR systems**. *Water Res.* **34**, 1705–1713. [https://doi.org/10.1016/S0043-1354\(99\)00280-8](https://doi.org/10.1016/S0043-1354(99)00280-8).
- Molina-Muñoz, M., Poyatos, J. M., Rodelas, B., Pozo, C., Manzanera, M., Hontoria, E. & Gonzalez-Lopez, J. 2010 **Microbial enzymatic activities in a pilot-scale MBR experimental plant under different working conditions**. *Bioresour. Technol.* **101**, 696–704. <https://doi.org/10.1016/j.biortech.2009.08.071>.

- Palacios, D., Busto, M. D. & Ortega, N. 2014 Study of a new spectrophotometric end-point assay for lipase activity determination in aqueous media. *LWT – Food Sci. Technol.* **55**, 536–542. <https://doi.org/10.1016/j.lwt.2013.10.027>.
- Petropoulos, E., Dolfing, J., Yu, Y., Wade, M. J., Bowen, E. J., Davenport, R. J. & Curtis, T. P. 2018 Lipolysis of domestic wastewater in anaerobic reactors operating at low temperatures. *Environ. Sci. Water Res. Technol.* **4**, 1002–1013. <https://doi.org/10.1039/C8EW00156A>.
- Quaglia, D., Ebert, M. C. C. J. C., Mugford, P. F. & Pelletier, J. N. 2017 Enzyme engineering: a synthetic biology approach for more effective library generation and automated high-throughput screening. *PLoS One* **12**, 1–14. <https://doi.org/10.1371/journal.pone.0171741>.
- Quéméneur, M. & Marty, Y. 1994 Fatty acids and sterols in domestic wastewaters. *Water Res.* **28**, 1217–1226. [https://doi.org/10.1016/0043-1354\(94\)90210-0](https://doi.org/10.1016/0043-1354(94)90210-0).
- Romano, D., Bonomi, F., Carlos, M., Mattos, D., Sousa, T., De Ferreira, C., Oliveira, D. & Molinari, F. 2015 Esterases as stereoselective biocatalysts. *Biotechnol. Adv.* **33**, 547–565. <https://doi.org/10.1016/j.biotechadv.2015.01.006>.
- Silva-bedoya, L. M., Sánchez-pinzón, M. S., Cadavid-restrepo, G. E. & Moreno-herrera, C. X. 2016 Bacterial community analysis of an industrial wastewater treatment plant in Colombia with screening for lipid-degrading microorganisms. *Microbiol. Res.* **192**, 313–325. <https://doi.org/10.1016/j.micres.2016.08.006>.
- Sousa, D. Z., Smidt, H., Alves, M. M. & Stams, A. J. M. 2009 Ecophysiology of syntrophic communities that degrade saturated and unsaturated long-chain fatty acids. *FEMS Microbiol. Ecol.* **68**, 257–272. <https://doi.org/10.1111/j.1574-6941.2009.00680.x>.
- Vorderwulbecke, T., Kieslich, K. & Erdmann, H. 1992 Comparison of lipases by different assays. *Enzyme Microb. Technol.* **14**, 631–639. [https://doi.org/10.1016/0141-0229\(92\)90038-P](https://doi.org/10.1016/0141-0229(92)90038-P).
- Zottig, X., Meddeb-Mouelhi, F. & Beaugard, M. 2016 Development of a high-throughput liquid state assay for lipase activity using natural substrates and Rhodamine B. *Anal. Biochem.* **496**, 25–29. <https://doi.org/10.1016/j.ab.2015.11.020>.
- Zottig, X., Meddeb-Mouelhi, F., Charbonneau, D. M. & Beaugard, M. 2017 Characterization of a novel alkalophilic lipase from *aneuribacillus thermoaerophilus*: lid heterogeneity and assignment to family I.5. *Protein J* **36**, 478–488. <https://doi.org/10.1007/s10930-017-9743-9>.

First received 2 December 2020; accepted in revised form 19 February 2021. Available online 4 March 2021