A high-throughput assay to quantify protein hydrolysis in aerobic and anaerobic wastewater treatment processes

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Abstract

Proteins, an important fraction of the organic matter in wastewater, typically enter a treatment facility as high molecular weight components. These components are degraded by extracellular protein hydrolytic enzymes, denoted as proteases. Adequate protein hydrolysis monitoring is crucial, since protein hydrolysis is often a rate-limiting step in wastewater treatment. However, current monitoring tools lack a high sample throughput and reliable quantification. Here, we present an improved assay for high-throughput protein hydrolysis rate measurements in wastewater treatment applications. A BODIPY FL casein model substrate was implemented in a microplate format for continuous fluorescent quantification. Case studies on a conventional and a high-rate aerobic municipal wastewater treatment plant and a lab-scale, two-stage, anaerobic reactor provided proof-of-concept. The assay presented in this study can help to obtain monitoring-based process insights, which will in turn allow improving biological performance of wastewater treatment installations in the future.

Key points

- Protein hydrolysis is a crucial step in biological wastewater treatment
- Quantification of the protein hydrolysis rate enables in-depth process knowledge
- BODIPY FL casein is a suitable model substrate for a protein hydrolysis assay
- High sample throughput was obtained with fluorescent hydrolysis quantification

Keywords

Protein - hydrolysis - enzyme activity - fluorescence - wastewater treatment

Introduction

Biological conversion of organic matter is the cornerstone of all aerobic and anaerobic wastewater treatment facilities. This conversion process relies on the coordinated action of a mixed microbial community to metabolize the protein, carbohydrate and lipid fractions in the organic matter. This organic matter typically enters the treatment systems as high molecular weight (MW) components ($MW > 10\,000$ Da) or particulates in the micro- and millimeter range, while only molecules in the nanometer range ($MW < 1000$) can be taken up directly through the microbial cell membrane (Cadoret et al., 2002; Hashimoto et al., 2005). Therefore, the microbial community actively excretes substrate-specific, hydrolytic enzymes to break down the organics in the extracellular environment. This hydrolysis of particulates is often cited as the overall rate-limiting step for the conversion of organic matter in wastewater treatment (Burgess, 2008). A significant fraction of the particulates consists of proteins, up to 30% of the total incoming chemical oxygen demand (COD) (Raunkjær et al., 1994), which is hydrolyzed by protein hydrolytic enzymes, denoted as proteases.

Adequate protein hydrolysis monitoring is crucial in any system aiming at understanding and improving protein hydrolysis. Hydrolytic activity assays should mimic the full-scale situation as closely as possible with careful selection of model substrates. Casein has been widely acknowledged as a suitable protease model substrate because it has (*i*) a relatively open,

globular secondary structure with limited α -helices and β -sheets (Haugland and Zhou, 1998), (ii) a diverse amino acid composition (Holt, 2016), and (iii) a high solubility in water including amphiphilic behavior with other hydrophobic molecules (Fox and Brodkorb, 2008).

The most common colorimetric assay applied in wastewater treatment research is the azocasein assay, originally described by Charney & Tomarelli (1947). However, this assay has two main limitations. First, the chromogenic azocasein substrate and colored products have to be separated by precipitation with trichloroacetic acid. Precipitated azocasein substrate is then removed by centrifugation, to obtain a clear supernatant for spectrophotometric determination of product formation. This additional separation step inherently limits the sampling frequency and reduces the sample throughput, as the method is labor-intensive. Second, spectrophotometric methods have been recently criticized as they are highly susceptible to interference by components in the complex sludge matrix (Felz et al., 2019; Le et al., 2016) and have a low sensitivity (Nybroe, 1992). Fluorogenic casein substrates have been proposed as a robust and sensitive alternative for azocasein. The labelling degree with the fluorophore in these substrates is such that fluorescence is quenched intramolecular, but is released upon digestion of casein by proteases. Two examples of commercially available fluorogenic casein substrates are fluorescein isothiocyanate (FITC)-casein (Twining, 1984) and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY FL) casein (Jones et al., 1997). The BODIPY FL spectral characteristics are similar to those of fluorescein, but the fluorescent properties are stable over a wider pH range (Schade et al., 1996). Neither the use of FITC-casein, nor BODIPY FL casein has found application in wastewater treatment research so far with one exception. Xia, Kong, & Nielsen (2007) combined BODIPY FL casein staining with fluorescence in-situ hybridization (FISH) on samples from full-scale aerobic wastewater treatment plants, to identify protein hydrolyzing organisms in the mixed community. This microscopy-based method was later on extended to microbial communities involved in anaerobic digestion (Xia et al., 2015, 2012, 2011) and in colonizing the cow digestive tract (Xia et al., 2016). However, no studies have been published that convert this qualitative BODIPY FL casein staining into a fully developed protein hydrolysis assay.

This study aims to advance and provide proof-of-concept of the BODIPY FL casein protein hydrolysis assay as a full-fledged, high-throughput assay for application in wastewater treatment. Proof-of-concept for aerobic and anaerobic wastewater treatment systems will be provided through case studies on samples of a conventional municipal wastewater treatment plant (MWWTP Leuven), a high-rate municipal wastewater treatment plant (MWWTP Nieuwveer) and a lab-scale, two-stage, anaerobic reactor treating synthetic dairy wastewater.

Materials and methods

Protein hydrolysis assay

Protein hydrolysis was measured with the BODIPY FL casein substrate (EnzChek kit E6638, Thermofisher scientific, Waltham, Massachusetts, USA). The powder was resuspended in phosphate buffered saline (0.0017 M KH_2PO_4 , 0.005 M Na_2HPO_4 , 0.150 M NaCl, pH 7.4) at a

concentration of 1 g/L and stored as stock solution at -20 °C in 20 µL aliquots. A BODIPY FL casein substrate working solution was prepared by diluting the stock solution to 10 mg/L with digestion buffer (10 mM Tris-HCl, pH 7.8 and 0.1 mM sodium azide) just before use. Sludge samples, less than one week old unless stated otherwise, were mixed and diluted to 1 g total solids (TS)/L with 10 mM Tris-HCl, pH 7.8. A Tris-HCl buffer at 50 mM for improved pH control, was applied for MWWTP Nieuwveer samples only. Diluted sludge and the BODIPY FL casein working solution were incubated in triplicate or quadruplicate in a 96-well plate (art. no. 655090, Greiner bio one international GmbH, Kremsmünster, Austria) with a 100 µL: 50 µL ratio, resulting in a final biomass-to-substrate ratio of 200:1 gTS/g. The plates were incubated at ambient temperature (23-27 °C) with moderate, orbital, on-line mixing at 6 mm amplitude in an Infinite M200 pro microplate reader (Tecan trading AG, Männedorf, Switzerland). The temperature exceeded 27 °C on some occasions during summer, and annotated correspondingly in Results. Fluorescence intensity (arbitrary unit U) was determined every 10 minutes for at least 4 hours, with excitation/emission at 485/520 nm. Further increases in the measuring frequency caused excessive BODIPY FL casein bleaching (data not shown). The first measurement was obtained after an initial mixing period of 10 minutes. All assays included *substrate only* and *sludge only* blanks in triplicate or quadruplicate. The full protocol for this assay is provided in Online resource 1, Protocol S1.

Sludge inactivation with a steam autoclave (Laboklav SHP 160 MSL, SHP steriltechnik AG, Detzel Schloss, Germany) was performed with MWWTP Leuven samples of December 2, 2019. To this end, a 150 mL sludge sample was autoclaved for 20 minutes at 121 °C and subsequently cooled down to room temperature. The autoclaved samples were stored at 4 °C and analyzed the day after. Sludge inactivation by freeze-thaw cycles was performed with the MWWTP Leuven sample of September 26, 2019. To this end, a 50 mL sludge sample was frozen at either -20°C or -80°C for at least 24 h. Afterwards, samples were defrosted slowly at 4 °C for at least 12 h prior to analysis. A second freeze-thaw cycle on the same samples was performed with the same method.

Reactor description and sampling

Samples were obtained from two full-scale MWWTPs and a lab-scale, two-stage, anaerobic reactor. A schematic of the full-scale plants and an overview of the operational performance at the time of sampling are provided in Fig. 1 and Online resource 1, Table S1, respectively.

MWWTP Leuven is operated by Aquafin (Belgium) and has a design capacity of 135 000 population equivalents (P.E., 60 gBOD₅/P.E./d). The incoming wastewater has a domestic origin with a minor fraction of industrial discharge (8% in 2018). The plant layout includes primary treatment (6 mm screens, oil and grease removal, sand trap), secondary treatment with anaerobic, aerobic and anoxic zones and secondary sedimentation. 0.5 L grab samples were collected at six different locations in the morning of August 6, September 26, October 31 and December 2, 2019, transported to the lab within one hour, and stored at 4 °C until the

time of analysis. Samples were analyzed the next day for all samples, except for the October 31, 2019 sample (stored for 4 days).

MWWTP Nieuwveer is operated by Waterschap Brabantse Delta (the Netherlands) and functions as a two-stage, high-rate, activated sludge process, also denoted as an AB system. It has a design capacity of 362 000 P.E. with one P.E. defined as 150 gTOD/d. TOD takes into account oxygen demand for carbon and nitrogen removal ($C_{TOD}=C_{BOD5} + 4.18C_{NH4-N}$, Clifford & McGaughey, 1982). This facility treats wastewater of domestic origin with a minor fraction of industrial discharge (<10%). Primary treatment consists of coarse screens, oil and grease removal and a sand trap. The two-stage process consists of a high-rate tank (3500 m³) with a desired hydraulic- (HRT) and sludge retention time (SRT) of 25 minutes and 0.6 day, respectively. Afterwards, sludge is allowed to settle in an intermediate settling tank with partial sludge recycle to the high-rate tank. The effluent from the intermediate settling is treated further in four parallel low-rate treatment tanks (three tanks 5400 m³ and one tank 12 000 m³). The low-rate tanks are comparable to a conventional activated sludge system and are followed by secondary settling. Settled sludge is recycled to the inlet of the low-rate tanks. Both the high-rate tank and low-rate tanks consist of a sequence of anoxic, facultative oxic and oxic zones. More information on the plant layout and operational performance can be found in De Graaff et al. (2016) and Meerburg et al. (2016). 0.5 L grab samples were collected at twenty different locations in the morning of January 14, 2020, transported to the lab within two hours, and stored at 4 °C until analyzed the day after.

The parallel, lab-scale, two-stage, anaerobic reactors were operated by Veolia Water Technologies Techno Center B.V. (Biothane, the Netherlands) for 200 days. The parallel reactors were fed with the same synthetic dairy wastewater, high in suspended solids, containing full fat milk (48 mL/L), granulated sugar (3.8 g/L), freeze dried blood (1.9 g/L), sodium hydrogen carbonate (NaHCO₃, 50 mM) and trace metals. Approximately 50% of the feed COD was non-soluble and it contained 3.7 g/L suspended solids. The parallel setups consisted of (i) an acidification tank (1 to 4.5 L, installed on day 72 in parallel setup 2) for partial hydrolysis of the feed and conversion of the soluble monomers to volatile organic acids, (ii) the reactor tank for additional hydrolysis, acidification and conversion to biogas (22 L), and (iii) the gravitational settling tank for biomass retention and recycling to the reactor tank. The reactor tank was inoculated with sludge from a full-scale anaerobic flocculent reactor system, while no inoculum was added to the acidification tank. Temperature control at 37 °C was applied for the reactor tank, while the acidification tank was operated at ambient conditions (23-26 °C). The first phase of the reactor experiment (day 0-50) was implemented to investigate the loading limits of a system with or without acidification tank, followed by a stabilization and recovery period from the high loading phase (day 50-100). From day 100 onwards, the focus was shifted to the study of an optimal HRT in the acidification tank. The biomass of the parallel acidification tank and reactor tanks was completely mixed and redistributed over the parallel setups on day 98 to ensure similar microbial communities at

the start of this phase. Complete mixing and redistribution was repeated on day 114 for the acidification tank only. Fresh samples were shipped cooled, not frozen, and arrived within 24 h. Samples were stored at 4 °C until the time of analysis. All samples were analyzed within 6 days after sampling, except for samples on day 0 (11 days after sampling) and day 30 (13 days after sampling).

All environmental and operational data for the different reactor systems were obtained from the system operators and were determined according to standard methods (Greenberg et al., 1992).

Data analysis

Raw fluorescence intensities for the individual replicate wells (arbitrary unit U) were corrected with the average intensity of the *substrate only* and the average intensity of the *sludge only* blanks for every time point. Time was recalculated with t=0 min as the moment of substrate addition to the first well in the plate. Manual filling of a full plate took approximately 3 minutes. A linear fit was calculated through the first 18 measurements, i.e., t=0 to t=200 min, with least squares regression in MS ExcelTM. Linear fit values were calculated for every replicate separately and normalized on a volatile solids (VS) basis. The coefficient of variation (CV) was calculated as the ratio of the sample standard deviation of the replicates over the mean of the replicates, expressed in percent values.

Results

Protein hydrolysis assay validation

Two experiments were conducted to test the assay validity. First, confirmation that measurements of protein hydrolysis rates are related to a *biological* action on the labelled BODIPY FL casein substrate, was obtained in a validation experiment with samples of municipal wastewater treatment plant (MWWTP) Leuven. Steam autoclaving eliminated almost all proteolytic activity (Table 1). Values for the effluent sample were somewhat inflated through volatile solids (VS) normalization. Freeze-thaw cycles with freezing at -20 °C or -80 °C did not have a similar reduction effect. The protein hydrolysis rate never declined to less than sixty percent of the original value (Table 2).

Second, possible effects of sample storage at 4 °C were evaluated in an experiment with samples of MWWTP Leuven, obtained on August 6, 2019 (Fig. 2). Protein hydrolysis rates decline in an approximately linear fashion for the first three weeks of storage. This linear trend was confirmed through additional storage experiments over a one-week period with samples obtained on September 26, 2019 and October 31, 2019. The average rate of decline was -1 percent of the initial activity per additional day of storage in the first week for these additional samples (minimum +0.5 percent, maximum -2.1 percent, N=19).

Distribution of protein hydrolysis over space and time

One of the main application niches of protein hydrolysis assays is the determination of the activity profile over a treatment installation, i.e., spatial profiling, and follow-up of protein

hydrolysis rates in a certain treatment zone over time. The latter is typically applied in research on factors influencing the metabolic activity of the microbial community, such as influent composition and load, temperature and pH. Proof-of-concept for the BODIPY FL casein assay is provided in this section with three different case studies aiming at profiling in different settings.

Full-scale municipal wastewater treatment

Protein hydrolysis rates were measured four times over the course of summer and autumn 2019 at MWWTP Leuven. Normalized values for protein hydrolysis rates of all replicates are shown in Fig. 3. Protein degradation was not limited to the secondary treatment stages, and already occurred in the raw influent and in the influent after screening. The protein hydrolysis rate in the effluent was low for all samples and varied from six to eight percent of the average obtained for the three secondary treatment samples. The data showed no clear seasonal trend in protein degradation, although water temperatures in the biological treatment tank did decrease over time from 21.8 ± 1.1 °C on average in August to 14.2 ± 1.2 °C on average in December.

Similarly, protein hydrolysis was determined for samples collected on January 14, 2020 at the high-rate MWWTP Nieuwveer. Normalized values for protein hydrolysis rates of all replicates are presented in Table 3. On average, protein hydrolysis rates in the high-rate stage were considerably lower than the values recorded for both low-rate stages (667 ± 70 versus 1248 ± 34 and 1215 ± 55 , $N=9$). The low-rate stage sludge recycle for tank 2 had a lower biomass concentration than the corresponding low-rate biological tank. This lower concentration was most probably related to sedimentation in the recycle ditch. The flow rate at the time of sampling was moderate, which could have allowed partial sludge settling. Furthermore, one outlier value was detected for the secondary settling tank connected to tank 4 ($1168 \text{ U min}^{-1} \text{ mg VS}^{-1}$). This outlier was most probably related to washout sludge in the upper layers of the settling tank.

Lab-scale, two-stage anaerobic reactor

Protein hydrolysis rates were determined at nine time points throughout an experimental trial with two parallel two-stage, lab-scale, anaerobic reactors (Fig. 4). From day 100 onwards, one of the setups was subjected to variable hydraulic retention times in the acidification tank, by adapting the volume of the acidification tank, but not the volume of the reactor tank, while keeping the influent flow constant. Remarkably, the proportion of the total protein hydrolysis rate in the acidification tank increased significantly between day 107 and day 133 for the setup subjected to variable hydraulic loading.

Discussion

Protein hydrolysis assay validation

Any enzyme assay should fulfill five fundamental requirements, being (i) a close approximation of reality through selection of the model substrate (ii) stability of the substrate

and the enzyme prior to and throughout the assay, (iii) close contact between substrate and enzyme under the assay conditions, (iv) reproducible, sensitive measurement of product formation over time and (v) adequate data normalization relevant for the research question (German et al., 2011).

First, it is widely acknowledged that casein is a suitable model substrate to measure the diversity of proteases in biological systems, as mentioned in the introduction. The hydrolytic action of different protease subtypes on BODIPY FL casein specifically, has been established previously with protease preparations from different origins, such as snake venom (Price, 2015), bacterial and fungal proteases (Ha et al., 2012) and yeast proteases (Hutter et al., 2005).

Second, substrate stability is ensured for BODIPY FL based substrates as the BODIPY moiety has low fluorescence bleaching rates and is stable over a pH range from 2 to 11 (Schade et al., 1996). All BODIPY FL casein substrates in powder form or as concentrated stock were stored at -20 °C according to the manufacturer's instructions. *Substrate only* control samples did not show significant storage-induced changes in fluorescence (Online resource 1, Table S3). In contrast, sludge stability might differ depending on the sample origin. Protein hydrolysis rates declined slowly, in an approximately linear fashion in this study (Fig. 2). Proteases are floc-associated, either linked to the microbial cell membrane or encapsulated in the extracellular polymeric matrix (Frolund et al., 1995; Gessesse et al., 2003; Ni et al., 2017). This association stabilizes proteases to a large extent and most probably protects against fast degradation by proteolysis, as previously described in lake aquatic ecology (Kiersztyn et al., 2012). Analysis of fresh samples should remain the standard, as not all samples have a fully developed floc structure or the floc morphology is unknown beforehand.

Temperature and pH should remain constant as well throughout one assay, and should be reproducible throughout a series of assays. Protein hydrolysis assays are typically performed with a buffered medium in wastewater treatment research (pH 7.5-8 with phosphate or Tris-HCl buffers), to enable comparison over different experiments and to avoid (i) issues with substrate aggregation, (ii) inhibitory effects of acidic conditions on the enzyme activity and (iii) a mismatch between the assay pH and the actual process conditions, the latter often close to neutral pH (Cadoret et al., 2002; German et al., 2011; Gessesse et al., 2003). Moreover, buffer molarity should be adapted to the sample in question, as sludge samples can have a large natural buffering capacity. The 10 mM Tris-HCl buffer, suggested by Xia et al. (2007), did not provide a diluted sample pH close to 7.8 for all samples. Therefore, the buffer molarity was increased to 50 mM in the experiments for MWWTP Nieuwveer, with good results in terms of diluted sample pH (Online resource 1, Table S4). The assay temperature typically varies from ambient temperature to 37 °C in wastewater treatment research on proteases. Also here, the assay temperature should be adapted to resemble the process conditions (German et al., 2011). A distinct difference should be noted for aerobic treatment processes often operated at ambient temperature and anaerobic processes functioning optimally at 37 °C. Temperature control could not be applied in the experiments presented here. Therefore, results should be analyzed with this practical limitation in mind.

Third, a close contact between the BODIPY FL casein substrate and the enzymes encapsulated in the sludge was provided through moderate mixing in the intervals between fluorescence measurements. The rate of substrate transport towards the enzymes is determined by two opposing factors. On the one hand, diffusion limitations can slow down substrate transport, as casein is a stable micelle with a diameter of 50 to 500 nm (Fox and Brodkorb, 2008). Cadoret et al. (2002) observed that azocasein degradation did not increase in activated sludge by prior floc dispersion with ultrasound or cation exchange resin. This observation led the authors to conclude that there was no diffusion limitation for the azocasein substrate in that case. On the other hand, the matrix of extracellular polymeric substances (EPS) in the floc can also serve as a substrate trap by adhesion forces, hindering the release of the substrate back into solution, which in turn leads to a driving force of substrate transport into the EPS matrix (Sivadon et al., 2019). Therefore, this assay incorporates not only the rate of substrate degradation but also the rate of substrate transport towards the protease enzyme for a given sludge sample.

Fourth, the measurement of product formation over time can be affected by fluorescence quenching in the sludge matrix and by fluorescent signal development derived from physico-chemical action on the substrate. Quenching through sludge-BODIPY interactions was not evaluated in this study, but could be studied in more detail with purified BODIPY FL labelled peptides or tailor-made labelled substrate analogs (Rezende et al., 2013). Excessive bleaching should be avoided by tailoring the measurement frequency to the fluorophore. Measurements at 5-minute intervals, for example, were found to cause excessive bleaching with BODIPY FL casein in this study.

The measurement reproducibility can be analyzed with the coefficient of variation (CV) for the replicates. The CV ranged from 2 to 8.5% for most full-scale MWWTP samples, with five outliers in the dataset of 42 fresh samples shown in Fig. 3 and Table 3. These outliers included three of the effluent samples for MWWTP Leuven with CV 12.6%, 22.9% and 89.7% for sampling on October 31, 2019, August 6, 2019, and December 2, 2019, respectively. The other outliers were a sample for one of the secondary settling tanks at MWWTP Breda, experiencing sludge washout (CV 75.1%) and the influent sample after screening, sand and grease removal at MWWTP Breda (CV 17.2%). A similar range was obtained for the lab-scale, two-stage anaerobic reactors (Q1-Q3 3-7%, N=30), with five outliers. The reproducibility of the results presented here, is in line with the study by Kreutz et al. (2016) with an L-alanine-aminopeptidase assay on a full-scale MWWTP. These authors defined an acceptable cutoff of 20% for the coefficient of variation of experimental quadruplicates. The sample heterogeneity is the main cause for outliers in reproducibility in this study, and not measurement variability. The sample heterogeneity cannot be reduced without introducing new possible errors. Sample homogenization prior to the assay, with ultrasound or high-speed mixing for example, drastically changes the original floc morphology and can cause cell lysis.

Fifth, data normalization is often performed in previous research on a volumetric basis [$\text{product units time}^{-1} \text{ sample volume}^{-1}$] and sometimes correlated later on with the sludge biomass concentration. A further complication is the measurement of product units, as these

are defined differently for every assay substrate. In this study, data were normalized directly to the closest available approximation of active biomass, i.e., volatile solids [product units time^{-1} volatile solids $^{-1}$]. Product units are direct measurements of fluorescence intensity (arbitrary unit U).

Distribution of protein hydrolysis over space and time

Full-scale municipal wastewater treatment

Three common trends were observed in the results for MWWTP Leuven and MWWTP Nieuwveer. First, protein hydrolysis rates were similar in the aerated and anoxic zones of a biological treatment tank. This observation is somewhat surprising, as the electron acceptor conditions are different in anoxic and aerobic zones. However, a similar result was obtained with an azocasein assay on a lab-scale sequencing batch reactor with anaerobic-aerobic process conditions by Goel et al. (1998) and later confirmed with an azocasein and L-alanine aminopeptidase assay in a full-scale membrane bioreactor and a full-scale conventional activated sludge system by Gómez-Silván et al. (2013) and Kreutz et al. (2016), respectively. Sludge recirculation and hydrodynamic mixing appear to distribute the protein hydrolysis process evenly over interconnected reactor zones.

Second, the majority of the incoming protein was indeed hydrolyzed in the secondary treatment stage, as expected. The low-rate stage showed higher activities than the high-rate stage. To the authors' knowledge, no previous studies have been performed on protein hydrolysis in full-scale, two-stage systems. Additional data are required to confirm this as a recurring observation.

Third, the specific, volatile solids normalized activity in the raw influent was at least 70% of the average activity in the biological treatment stages and even exceeded that activity in two sampling moments at MWWTP Leuven. The influent was often not analyzed in previous research, with two exceptions. Fischer et al. (2013) and Wiegand-Rosinus et al. (1995) report a tenfold lower volumetric activity in the influent compared to the biological treatment stage. Both authors obtained these values with an L-alanine aminopeptidase assay. When recalculating values to a volumetric activity, by multiplying the normalized values with the volatile solids concentration for influent and reactor samples, similar ratios of ten to twelve percent for the influent versus biological treatment samples are obtained, except for the October 31, 2019 sample at MWWTP Leuven with a higher value (45%). This sample had a high influent solids concentration, suggesting a high influent organic load, for example due to diurnal patterns in emission of organic contaminants. A similar peak in hydrolytic activity related to a high influent organic load, measured as influent COD, was also observed by Fischer et al. (2013). The importance of the influent as a continuous source of active biomass immigrating into the wastewater treatment facility has only been recognized in recent years. The sewer system receives constant microbial inputs from domestic discharge and groundwater infiltration, which results in a highly dynamic sewer ecosystem in space and time (Frigon and Wells, 2019). Although the net effect of immigration on the full microbial

community in the treatment facility might be modest (Saunders et al., 2016), there is increasing evidence that immigration from the sewer system should not be neglected in specific cases such as nitrification at low temperatures (Jauffur et al., 2014). Therefore, it is suggested to incorporate influent samples in any future research on hydrolysis, especially for systems operated near their operational limits, e.g., high-rate activated sludge or low-temperature biological treatment.

Lab-scale, two-stage anaerobic reactor

The specific protein hydrolysis activities obtained in this trial, are lower than the values obtained for full-scale aerobic MWW treatment facilities in this study, although the synthetic wastewater for the two-stage anaerobic reactors contained full fat milk as one of the main carbon sources. At present, it is unclear if this observation is related to a difference in overall microbial activity under aerobic and anaerobic conditions or related to other factors (Nybroe, 1992).

The results for this trial should be analyzed with care, as a two-stage anaerobic reactor is dynamic in terms of biomass composition and metabolic activity. The complete lack of biomass retention and low hydraulic retention times in the acidification tank may result in rapid changes in protein hydrolysis rates. The results for day 107 indeed confirm this, with almost non-detectable (system 1) versus considerable activity (system 2) in the acidification tank, even though the parallel acidification tank and reactor tank contents were mixed and redistributed over system 1 and system 2 on day 98. The period of low activity for system 1 coincided with a low pH in the acidification tank, below 5.2 from day 102 to 120, and a steep increase in the levels of propionic acid, from 140 mg/L on day 1 to 1675 mg/L on day 12.

Nevertheless, it can be concluded that the operating conditions of the reactor systems can be adapted to increase the protein hydrolysis rate. On day 133, system 1 had developed a protein hydrolysis metabolic activity also in the acidification tank, through (i) complete mixing and redistribution of acidification tank contents over both parallel systems on day 114 and (ii) a decrease in the hydraulic retention time (HRT) for the acidification tank from day 114 onwards. The latter decrease in HRT also enabled a decrease in the concentrations of organic acids (5.3; 0.7; 0.4 gTVFA-COD/L on day 107; 128; 136). High levels of organic acids can cause a pH drop, even when carbonate buffering is applied in the incoming wastewater, i.e., 50 mM NaHCO₃ in this trial. A low pH most probably affects protein hydrolysis through physico-chemical effects. Milk casein is known to aggregate and sediment below a pH of 5.2 (Dalglish and Corredig, 2012), and therefore becomes unavailable for microbial hydrolysis in suspension.

In conclusion, the time course of protein hydrolysis in the two-stage anaerobic reactors indicates that a two-stage reactor setup is highly dynamic. A balance between hydraulic regime, buffering capacity, microbial activity and operational pH should be maintained to ensure good performance in these anaerobic systems.

This study demonstrates that the BODIPY FL casein substrate can be applied successfully for fast and accurate measurement of protein hydrolysis in wastewater treatment systems. However, the current assay focuses only on proteins, while carbohydrates and lipids also comprise a large fraction of the incoming organic load in municipal wastewater treatment and in various industrial waste streams. Therefore, additional fluorescent substrates should be identified and integrated with the current approach. BODIPY FL DQ starch is a promising substrate for amylase activity and has been applied previously for microscopic studies of activated sludge (Xia et al., 2008) and high-throughput screening of fungal strains for amylase activity (Beneyton et al., 2016). Improvements in the throughput of this method could be obtained by automating not only the measurement, but also the sample processing steps such as sample dilution and mixing. Such a fully automated technique could then be integrated with other techniques such as BIOLOG physiological profiling (Button et al., 2016) or integrated multi-omics sample processing (Roume et al., 2013).

The ultimate goal of enzymatic screening efforts in wastewater treatment systems is to develop a mechanistic understanding of the dynamics in enzymatic activity in response to changing process conditions (Gómez-Silvan et al., 2013; Hassard et al., 2020; Nybroe, 1992). A mechanistic understanding would then lead to improved process control and biological performance. This goal can only be obtained through long-term measuring campaigns with a high sampling frequency, i.e., at least daily, in parallel with in-depth monitoring of relevant process parameters such as operating temperature, influent flow, influent composition and load, redox potential, pH and hydraulic and solids retention times in the system. Such a sampling campaign can only be executed if the throughput of the protein hydrolysis assay is sufficiently high.

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Author contribution statement

PVG conceived, designed and conducted experiments, performed data analysis and wrote the first draft of the manuscript. DS and IS supervised the project, reviewed the manuscript and acquired funding. All authors read and approved the final manuscript.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

Fig. 1 Schematic overview of the (a) aerobic conventional municipal wastewater treatment plant Leuven and (b) aerobic high-rate municipal wastewater treatment plant Nieuwveer. Numbers in the bottom right corner indicate the number of tanks. Green diamonds indicate sampling points. Intermediate settling tanks and secondary settling tanks were either rectangular, as depicted below (6 intermediate, 6 secondary) or circular (1 intermediate, 3 secondary). Anaer = anaerobic, Anox = anoxic. *after the anaerobic zone, flows are diverted towards two carroussels, each with two subzones that can be aerated or anoxic

Fig. 2 Evolution of protein hydrolysis rates during storage at 4 °C for raw influent (a), secondary treatment anaerobic zone (b), secondary treatment aerobic zone (c) and distribution point to secondary settling (d) at MWWTP Leuven, sampled on August 6, 2019. Protein hydrolysis rates are volatile solids normalized data rescaled to the average of the first measurement (value 100). Data points are individual values for all replicates. The sample collection date is day 0. Temperature exceeded 27 °C during protein hydrolysis rate determination in the first three experiments for every sample ($T_{\max}$ =29.9; 28.7; 32.1/30.7 on day 1; 2; 22/23)

Fig. 3 Protein hydrolysis rates for MWWTP Leuven, sampled throughout summer and autumn in 2019. Data points are individual values for all replicates ($\text{U min}^{-1} \text{mg VS}^{-1}$). $\circ$: August 6, 2019 ($N=6$), $\blacklozenge$: September 26, 2019 ($N=6$), $\bullet$: October 31, 2019 ($N=6$), $\diamond$: December 2, 2019 ($N=4$). None of the influent or effluent samples was diluted, except for the raw influent and the influent after screening for October 31, 2019. Temperature exceeded 27°C ($T_{\min}=21^\circ\text{C}$, $T_{\max}=30^\circ\text{C}$) during protein hydrolysis rate determination for the August 6, 2019 sample. Raw data for this figure are provided in Online resource 1, Table S2

Fig. 4 Protein hydrolysis rates for two parallel anaerobic reactor setups. a) two-stage reactor with variable HRT in the acidification tank and b) two-stage reactor with stable HRT in the acidification tank. Protein hydrolysis rates are averages over the replicates. AT = acidification tank, RT = reactor tank, *no acidification tank was installed, NA indicates that no sample was available. HRT values are presented as [HRT acidification tank; HRT reactor tank]. All samples were analyzed within 6 days after sampling, except for samples on day 0 (11 days after sampling) and day 30 (13 days after sampling). Temperature exceeded 27 °C during protein hydrolysis rate determination for samples on day 107 ($T_{\max}$ = 30 °C) and day 133 ($T_{\max}$ = 28.2 °C)

Table 1 Protein hydrolysis rate for MWWTP Leuven, sampled on December 2, 2019, before and after steam autoclaving. Values are shown as [minimum-maximum] based on individual values for replicates. <0 indicates values below zero.

Sampling location	Autoclaved	TS (g/L)	VS (g/L)	Protein hydrolysis rate (U min ⁻¹ mg VS ⁻¹)
Influent, raw	No	1.03	0.36	1886 ^a – 1993 ^a
	Yes	1.05	0.37	2.9 ^a - 3.0 ^a
Secondary treatment, anaerobic zone	No	7.81	5.53	1294 - 1375
	Yes	8.15	5.77	<0
Secondary treatment, aerated zone	No	6.30	4.34	1210 - 1236
	Yes	7.06	4.86	<0
Effluent	No	0.63	0.15	102 ^a – 609 ^{a,b}
	Yes	0.66	0.17	14.5 ^a - 16.1 ^a

^asample not diluted in protein hydrolysis rate determination, ^boutlier value (102; 192; 609).

Table 2 Protein hydrolysis rate for MWWTP Leuven, sampled on September 26, 2019, before and after one and two cycles of freeze-thawing at -20 °C and -80 °C.

Sampling location	TS (g/L)	VS (g/L)	Freeze-Thaw	Protein hydrolysis rate (U min ⁻¹ mgVS ⁻¹) ¹	
Secondary treatment, anaerobic zone	5.91	4.05	No	1032 - 1109	100% ²
			1x -20 °C	596 - 749	64% ²
			1x -80 °C	712 - 723	67% ²
			2x -20 °C	706 - 808	71% ²
			2x -80 °C	697 - 737	67% ²
Secondary treatment, aerated zone	7.62	5.27	No	1003 - 1043	100% ²
			1x -20 °C	688 - 770	71% ²
			1x -80 °C	703 - 705	69% ²
			2x -20 °C	702 - 773	73% ²
			2x -80 °C	701 - 731	70% ²

¹values are shown as [minimum-maximum] based on individual values for replicates,

²percentages are calculated with averages of replicates.

TS = total solids, VS = volatile solids.

Table 3 Protein hydrolysis rate for MWWTP Nieuwveer, sampled January 14, 2020. Values are shown as [minimum-maximum] based on individual values for replicates.

Sampling location	TS (g/L)	VS (g/L)	Protein hydrolysis rate (U min ⁻¹ mg VS ⁻¹)
Influent, raw	0.55 ^a	0.49	736 - 915
Influent, after primary treatment	0.44 ^a	0.42	1068 - 1465
High-rate stage, zone I, anoxic	3.41	2.66	656 - 782
High-rate stage, zone II, aerated	3.59	2.97	567 - 626
High-rate stage, zone III, aerated	3.60	2.94	632 - 695
Sludge recycle from intermediate settling	13.00	10.50	489 - 532
Intermediate settling, tank 4, start ^c	1.71	1.37	664 - 690
Intermediate settling, tank 4, end ^c	0.37 ^a	0.26	974 - 1119
Intermediate settling, tank 7 ^d	0.37 ^a	0.24	1576 - 1633
Low-rate stage, parallel tank 2, anoxic zone	4.03	3.09	1266 - 1289
Low-rate stage, parallel tank 2, aerated zone 1	4.04	3.32	1186 - 1255
Low-rate stage, parallel tank 2, aerated zone 2	2.96	2.34	1232 - 1268
Sludge recycle, parallel tank 2	1.63	1.2	1415 - 1526
Secondary settling, after parallel tank 2, start ^c	3.10	2.38	1234 - 1370
Secondary settling, after parallel tank 2, end ^c	0.54 ^a	0.25	150 - 172
Low-rate stage, parallel tank 4, anoxic zone	3.46	2.79	1146 - 1168
Low-rate stage, parallel tank 4, aerated zone 1	3.31	2.66	1215 - 1282
Low-rate stage, parallel tank 4, aerated zone 2	3.39	2.70	1197 - 1296
Secondary settling, after parallel tank 4 ^d	0.59 ^a	0.22	304 – 1168 ^b
Effluent	0.50 ^a	0.14	158 - 161

^asample not diluted in protein hydrolysis rate determination, ^boutlier value (304; 410; 1168),

^crectangular settling tank, ^dcircular settling tank.

Supplementary material

A high-throughput assay to quantify protein hydrolysis in aerobic and anaerobic wastewater treatment processes

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Table S1 An overview of the plant operational conditions and performance for the full-scale municipal wastewater treatment plants Nieuwveer and Leuven

		MWWTP Nieuwveer high-rate stage ⁵	N	MWWTP Nieuwveer low-rate stage ⁵	N	MWWTP Leuven	N
Sampling period	-	14.01.2020				06.08.2019 - 02.12.2019	
Season	-	Winter				Summer - Autumn	
Operational data period	-	01.01.2020 - 31.01.2020				31.07.2019 - 31.12.2019	
Temperature reactor tank (°C)	Min - Max	10.2 - 13.5			31	11.8 - 24.0	154
Influent flow (m ³ /d)	Min - Med - Max	47 100 - 57 700 - 169 800				21 300 - 27 100 - 92 800	154
Capacity (P.E.)	-	362 000 ²				135 000 ³	-
COD influent (mgO ₂ /L)	Min - Med - Max	501 - 576 - 743			5	296 - 408 - 548	10
BOD ₅ influent (mgO ₂ /L)	Min - Med - Max	126 - 216 - 261			5	117 - 153 - 216	10
TSS influent (mg/L)	Min - Med - Max	249 - 343 - 472			5	118 - 164 - 288	10
COD removal efficiency (%)	Avg ± St.dev	54 ± 11 ¹	37	70 ± 7 ¹	37	93 ± 1	10
N _{total} influent (mg/L)	Min - Med - Max	31 - 39 - 60			5	53 - 59 - 66	9
Kjeldahl N influent (mg/L)	Min - Med - Max	31 - 39 - 60			5	53 - 59 - 66	9
NH ₄ ⁺ influent (mg/L)	Min - Med - Max	NA			-	14 - 44 - 53	10
N removal efficiency (%)	Avg ± St.dev	33 ± 11 ¹ (as KjN)	37	52 ± 12 ¹ (as KjN)	37	93 ± 4 (as N _{total})	9
P _{total} influent (mg/L)	Min - Med - Max	4.8 - 6.2 - 7.1			5	4.7 - 6.8 - 8.3	10
Orthophosphate influent (mg/L)	Min - Med - Max	NA			-	1.5 - 3.9 - 4.5	10
P _{total} removal efficiency (%)	Avg ± St.dev	47 ± 18 ¹	37	45 ± 16 ¹	37	87 ± 4	10
HRT (h)	Avg ± St.dev	0.6 ± 0.3 ¹	38	4.5 ± 2.4 ¹	38	19.5 ± 6.1 ⁴	365
SRT (d)	Avg ± St.dev	1.7 ± 0.5 ¹	37	34.4 ± 28.8 ¹	37	12.5 ± 5.2 ⁴	41

¹literature values for October 2013-July 2014 (Meerburg et al., 2016. *High-rate activated sludge communities have a distinctly different structure compared to low-rate sludge communities, and are less sensitive towards environmental and operational variables. Water Res. 100, 137–145*), ²one P.E. defined as 150 gTOD/d, ³one P.E. defined as 60 gBOD/d, ⁴yearly average for 2019, ⁵total removal efficiencies for MWWTP Nieuwveer in January 2020 amounted to 93±1, 61±10 and 70±10 percent for COD, N_{total} and P_{total}, respectively (N=5).

P.E = person equivalent, TOD = total oxygen demand, measure for oxygen demand to convert carbonaceous and nitrogenous components, COD = chemical oxygen demand, BOD₅ = 5-day biological oxygen demand, TSS = total suspended solids, HRT = hydraulic residence time, SRT = solids retention time, KjN = Kjeldahl nitrogen Min = minimum, Med = median, Max = maximum, Avg = average, St.dev = standard deviation, N = number of samples.

Table S2 Protein hydrolysis rate for MWWTP Leuven, sampled throughout summer and autumn in 2019

Sample collection date	Sampling location	TS (g/L)	VS (g/L)	Protein hydrolysis rate (U min ⁻¹ mgVS ⁻¹) ³
Aug 6, 2019	Influent, raw	0.87 ¹	0.25	2950 ² - 3106 ²
	Influent, after screening	0.90 ¹	0.27	2352 ² - 2481 ²
	Secondary treatment, anaerobic zone	5.60	3.29	1888 ² - 1893 ² a
	Secondary treatment, aerated zone	6.50	3.95	1747 ² - 1890 ² a
	Distribution point to secondary settling	6.42	3.88	1784 ² - 1896 ² a
	Effluent	0.73 ¹	0.18	104 ² - 166 ²
Sep 26, 2019	Influent, raw	1.03 ¹	0.57	967 - 1009 b
	Influent, after screening	1.07 ¹	0.68	954 - 1022 b
	Secondary treatment, anaerobic zone	5.91	4.05	1094 - 1105 c
	Secondary treatment, aerated zone	7.62	5.27	1056 - 1140 c
	Distribution point to secondary settling	7.93	5.4	1077 - 1184 c
	Effluent	0.71 ¹	0.50	63 - 73
Oct 31, 2019	Influent, raw	3.01	1.76	1587 - 1663 d
	Influent, after screening	2.91	1.66	1585 - 1635 d
	Secondary treatment, anaerobic zone	6.51	4.24	1465 - 1607 de
	Secondary treatment, aerated zone	6.13	3.35	1852 - 1923
	Distribution point to secondary settling	6.37	4.15	1402 - 1538 e
	Effluent	0.78 ¹	0.17	108 - 139
Dec 2, 2019	Influent, raw	1.03 ¹	0.36	1886 - 1993
	Influent, after screening	NA	NA	NA
	Secondary treatment, anaerobic zone	7.81	5.53	1294 - 1375 f
	Secondary treatment, aerated zone	6.30	4.54	1210 - 1236 f
	Distribution point to secondary settling	NA	NA	NA
	Effluent	0.63 ¹	0.15	102 - 609 ⁴

¹sample not diluted in protein hydrolysis rate determination, ²temperature exceeded 27 °C during protein hydrolysis rate determination (T_{min}= 21 °C, T_{max}= 30 °C), ³activity values are shown as [minimum-maximum] based on individual values for replicates, ⁴outlier value (102; 192; 609).

NA not applicable, TS = total solids, VS = volatile solids

a – f samples are not significantly different ($\alpha=0.05$). Multiple pairwise comparisons were made for every sample collection date separately, with two-sided t-tests in Rstudio (version 1.2.5033, R version 3.6.2). Adjusted p-values were calculated with the Benjamini-Hochberg method (p.adjust, stats package) to correct for multiple testing.

Table S3 Fluorescence intensity measurements for *BODIPY FL casein only* wells during the protein hydrolysis assay

Experiment date	Test kit ⁵	Powder vial ⁵	Replicate 1 (/) ⁴	Replicate 2 (/) ⁴	Replicate 3 (/) ⁴
18.04.2019 ¹	1	1	949±15	949±19	970±20
20.05.2019 ¹	1	1	1075±16	1138±11	1189±17
14.06.2019 ¹	1	2	882±27	907±21	934±22
04.07.2019 ¹	1	2	901±16	904±12	937±14
29.07.2019 ¹	1	2	938±14	967±10	1013±18
07.08.2019 ²	1	3	998±24	998±25	1007±25
23.08.2019 ¹	1	3.1	825±17	843±15	844±16
23.08.2019 ¹	1	3.2	853±15	861±15	902±25
19.09.2019 ¹	1	4	778±18	828±26	943±49
27.09.2019 ²	1	5	991±16	1083±32	1439±92
09.10.2019 ¹	2	1.1	999±19	1018±17	1090±40
09.10.2019 ¹	2	1.2	928±18	978±12	984±14
04.11.2019 ²	2	2	841±16	859±26	915±23
03.12.2019 ²	2	3	751±31	764±26	773±29
15.01.2020 ³	2	4.1	912±27	912±30	938±42
15.01.2020 ³	2	4.2	875±30	888±29	888±30
16.01.2020 ³	2	4.3	663±18	673±16	682±15
16.01.2020 ³	2	4.4	730±7	897±9	1152±27

¹protein hydrolysis assay for two-stage anaerobic reactor, ²protein hydrolysis assay for MWWTP Leuven, ³protein hydrolysis assay for MWWTP Nieuwveer, ⁴values are represented as average ± standard deviation for the replicates (N=18), ⁵test kit and powder vial numbers are tracking numbers to indicate which test kit and which of the five powder vials in the test kit was utilized for the experiment, multiple working solutions per experiment are indicated in the column powder vial (x.1, x.2).

Table S4 pH values for original and diluted sludge samples of municipal wastewater treatment plant Leuven and the two-stage anaerobic reactor

Sample collection date	Sampling location	TS (g/L)	pH original ²	pH diluted ³
Aug 6, 2019	Influent, raw	0.87 ¹	7.71	
	Influent, after screening	0.90 ¹	7.73	
	Secondary treatment, anaerobic zone	5.60	7.34	ND
	Secondary treatment, aerated zone	6.50	7.09	ND
	Distribution point to secondary settling	6.42	7.11	ND
	Effluent	0.73 ¹	7.49	
Sep 26, 2019	Influent, raw	1.03 ¹	7.76	
	Influent, after screening	1.07 ¹	7.85	
	Secondary treatment, anaerobic zone	5.91	7.01	7.81
	Secondary treatment, aerated zone	7.62	6.77	7.8
	Distribution point to secondary settling	7.93	6.77	7.77
	Effluent	0.71 ¹	7.41	
Oct 31, 2019	Influent, raw	3.01	7.71	8.04
	Influent, after screening	2.91	7.53	7.98
	Secondary treatment, anaerobic zone	6.51	7.11	7.73
	Secondary treatment, aerated zone	6.13	6.98	7.66
	Distribution point to secondary settling	6.37	6.99	7.59
	Effluent	0.78 ¹	7.98	
Dec 2, 2019	Influent, raw	1.03 ¹	7.73	
	Influent, after screening	NA	NA	NA
	Secondary treatment, anaerobic zone	7.81	6.82	7.19*
	Secondary treatment, aerated zone	6.30	6.56	7.11*
	Distribution point to secondary settling	NA	NA	NA
	Effluent	0.63 ¹	7.25	
Day 133	Anaerobic system 1 - acidification tank	11.20	ND	7.56*
	Anaerobic system 1 - reactor tank	10.50	ND	7.83
	Anaerobic system 2 - acidification tank	9.80	ND	7.35*
	Anaerobic system 2 - reactor tank	9.90	ND	7.84
Day 161	Anaerobic system 1 - acidification tank	9.76	5.81	7.48*
	Anaerobic system 1 - reactor tank	12.33	7.91	7.91
	Anaerobic system 2 - acidification tank	9.11	5.43	7.16*
	Anaerobic system 2 - reactor tank	7.48	7.91	7.95
Day 182	Anaerobic system 1 - acidification tank	10.84	6.74	7.8
	Anaerobic system 1 - reactor tank	11.68	8.06	8
	Anaerobic system 2 - acidification tank	11.51	6.36	7.81
	Anaerobic system 2 - reactor tank	7.81	8.21	8.03

*substantial deviations between theoretical (7.8) and actual pH for diluted sludge sample, ¹sample not diluted for protein hydrolysis rate determination, ²sample pH measured on the day of the protein hydrolysis rate measurement, ³dilution with a 10 mM Tris-HCl pH 7.8 buffer. NA = not applicable, ND = not determined, TS = total solids.

Protocol S1 Protein hydrolysis assay with BODIPY FL casein

This document is designed as a step-by-step protocol to measure microbial protein hydrolytic activity in wastewater treatment research. It can be applied to different types of wastewater treatment systems, at lab-, pilot- or full-scale, provided that the incoming wastewater contains a source of proteins. Protein hydrolysis and further degradation is mediated by mixed microbial communities in wastewater treatment systems. This mixed microbial community including the native liquid matrix is typically denoted as 'sludge sample'.

The assay principle is based on a commercially available protein model substrate, BODIPY FL casein. Quantification of protein hydrolytic activity is based on fluorescence measurements of the protein hydrolytic products over time. Fluorescence is measured with a microplate reader in a 96-well plate format.

Possible applications of the protein hydrolysis assay include (i) resolving the distribution of protein hydrolysis in a given wastewater treatment system over space and time (ii) screening of seed sludge (synonyms: inoculum, start-up community) and (iii) research aiming at improved protein hydrolysis by changing the reactor design and/or adapting process and environmental conditions.

Consumables

- Sample containers for sample dilution
 - Sample containers for preparation of pure enzyme solution (optional)
 - Sample containers (protected from light) for fluorescein external standard (optional)
 - Eppendorfs for BODIPY FL casein stock- and working solution

 - Microplate, Greiner bio, ref. 655090 (polystyrene, black wells, flat µClear bottom, chimney wells, for fluorescence) or equivalent microplate
 - Microplate seal for microplate transport or storage (optional)

 - Micropipette tips
 - Laboratory gloves

 - EnzChek™ Protease Assay Kit, green fluorescence, 100-1000 assays, art. no. E6638
- This kit contains: 5 vials of lyophilized BODIPY FL casein powder (200µg each) and 13 mL of a 20x concentration digestion buffer solution. Store all kit contents at -20 °C, and protect from light. Stable for 6 to 12 months.

Hardware

- Microplate reader
- Necessary functions: fluorescence measurement function (excitation/emission 485/530 nm), sample holder and read-out of 96-well plates.
- Optional functions: on-line mixing, temperature control

Stock solutions

- Hydrogen chloride, 3 M (for pH corrections Tris-HCl buffer)
- Sodium hydroxide, 0.1 M (optional, for fluorescein external standard)

- Tris-HCl buffer, buffer molarity 10-50 mM, pH 7.8

This buffer is prepared with Trizma base (CAS 77-86-1) and adapted to pH 7.8 with concentrated HCl. A low molarity buffer (10 mM) is sufficient for most cases, but molarity could be increased if diluted sludge samples have pH values considerably lower than 7.8.

- Phosphate buffered saline (PBS, to prepare BODIPY FL casein stock solution)

Typical composition of PBS: 1.7 mM KH_2PO_4 , 5 mM Na_2HPO_4 , 150 mM NaCl, pH 7.4

- BODIPY FL casein stock solution, 1 g/L, 10x20 μL

This stock solution is prepared with the lyophilized BODIPY FL casein powder vial (200 μg). 200 μL of phosphate buffered saline (PBS) is added and mixed properly, e.g. 10 minutes at 100rpm in an orbital shaker. 20 μL aliquots are distributed over 10 sample containers, and frozen at -20 °C or -80 °C (if possible). Protect all solutions from light. This solution is stable for several weeks. Only thaw aliquots right before the protein hydrolysis assay, cf. below, and avoid repeated freeze-thawing.

- Digestion buffer, 10 mM Tris-HCl (pH 7.8) with 0.1 mM sodium azide

Prepare a 20x diluted solution with the concentrated stock of digestion buffer supplied in the EnzChek™ protease assay kit. Allow the stock to defrost at room temperature, do not heat up.

- Fluorescein external standard in 0.1 M NaOH, 1 mM (optional)

Prepare a 1mM fluorescein stock from analytical-grade fluorescein powder. Dissolve the powder in 0.1M NaOH. Fluorescein is poorly water soluble at neutral conditions. Fluorescence of fluorescein is strongly pH dependent below pH 8, and maximal in alkaline solutions above pH 9. Therefore, a 0.1 M NaOH dilution medium is preferred. Protect fluorescein solutions from light at all times.

Protein hydrolysis assay

General precautions:

- a. Adhere to the guidelines in your institution concerning good laboratory practices. As a basic minimum: wear gloves, goggles and a lab coat, if applicable.
- b. Always use fresh sludge samples, if possible. Store samples at 4 °C if the protein hydrolysis assay cannot be performed immediately.

These items should be checked the day beforehand:

- a. Sample biomass concentration is determined. At least total solids (TS) should be known, volatile solids (VS) will be required for further data analysis. Please refer to standard methods for determination of total- and volatile solids (Greenberg et al., 1992).
- b. All necessary consumables are present, cf. above.
- c. All necessary stock solutions are prepared, cf. above.
- d. Reserve necessary equipment, if applicable.
- e. Prepare the protein hydrolysis assay template, cf. below.

1. Optional: Prepare a small volume of fresh pure enzyme solution (trypsin,...), and allow to dissolve completely. This solution can serve as external standard in the microplate.

2. Optional: Prepare at least 2 mL of 0.1 µM fluorescein working solution from the 1mM fluorescein stock. Dilute with 0.1 M NaOH. Protect stock and working solution from light, either with aluminum foil or Falcons developed for fluorescence applications.

Side note: fluorescence intensities as measured on the microplate reader, can be influenced by several sample- and instrument related parameters. Therefore, a standard such as fluorescein can be incorporated to account for instrument-to-instrument and day-to-day variations in measurements

3. Prepare a fresh 10 mg/L BODIPY FL casein working solution. Add 1.98 mL of digestion buffer to a 20 µL aliquot of the 1 g/L BODIPY FL casein stock solution, previously frozen at -20 °C. Mix properly, e.g., 10 minutes at 100 rpm in an orbital shaker.

Side note: 12 samples can be analyzed in triplicate with one working solution

4. Prepare fresh diluted sludge samples. Mix original sample properly and allow to warm to room temperature, if applicable. Subsample a sufficient volume with a micropipette and add Tris-HCl buffer (pH 7.8) to obtain a 1 gTS/L diluted sludge sample.

Side note: highly concentrated or viscous samples can be diluted by weight.

Side note: additional sample homogenization steps can be implemented, but cell lysis should be avoided at all times.

Side note: Filtered sludge supernatant could be employed as alternative for the Tris-HCl buffer, if applicable for the research question. pH should be monitored closely in that case.

5. Homogenize the diluted sludge sample, e.g., 5 minutes at 50rpm in an orbital shaker.

6. Measure pH of original and diluted sludge samples.

7. Prepare the microplate. Fill the microplate with 100µL of all solutions in triplicate, but do not add the BODIPY FL casein working solution yet. See also the template below.

8. Prepare the microplate reader settings. These settings include online mixing, fluorescence measurement (excitation/emission, detector gain, top/bottom reading,...), temperature control if possible, and total experiment time (typically 3h or more). A typical excitation/emission setting is 485/530 nm for the BODIPY FL fluorophore. Allow the detector lamp to pre-heat, if applicable, and save the measurement settings in a measurement script. *Side note: A microplate cover during measurement is not preferred, as condensation on the cover inner surface might disturb the measurement.*

9. Start the experiment. Execute the following steps as fast as possible (i) add 50µL BODIPY FL casein working solution to all wells (ii) insert the microplate in the microplate reader (iii) close the microplate reader port (iv) start the mixing and measurement protocol. Write down the time of first addition of BODIPY FL casein substrate to a well (t=0 minutes).

Side note: Take note of the filling scheme if multiple BODIPY FL casein working solutions are required for one experiment. BODIPY FL casein background fluorescence should be accounted for in data analysis.

10. Stop the measurement sequence when sufficient data have been obtained. Save the measurement output in a general data format (.csv, .xlsx) and transfer to your personal device, if necessary.

11. Complete the protein hydrolysis assay template (cf. below).

12. Discard of chemical and biological wastes properly. All mixtures containing sodium azide, a component of the digestion buffer, are toxic to aquatic life and should be discarded as chemical waste.

13. Perform data analysis, with dedicated microplate reader software or classic data analysis software (MS Excel, MATLAB,...). The following steps should be considered:

- a. Rescaling the time axis with t=0 for addition of BODIPY FL casein to the first well, and conversion from seconds to minutes/hours, if applicable.
- b. Annotation of wells with sample ID.
- c. Correcting raw fluorescence measurement for sludge background fluorescence and BODIPY FL background fluorescence
- d. Data quality assessment, e.g., variability for experimental replicates, comparison with previous experiments and comparison with pure enzyme internal standard.
- e. Calculation of the linear increase in fluorescence signal.
- f. Normalization of the raw values by biomass quantity (total solids (TS) or volatile solids (VS)).
- g. Rescaling of the fluorescence intensity with a predetermined calibration curve, e.g., with a fluorescein standard (optional).
- h. Averaging over sample replicates, if applicable.

Protein hydrolysis assay with BODIPY FL casein

14. Update a metadata file containing sample IDs, relevant protocol information, and processed results for future reference.
15. Proceed with advanced data analysis depending on the research question. Statistical analysis can be performed in R or other equivalent statistical software.

Protein hydrolysis assay with BODIPY FL casein

Template

Experiment track #	
Experiment type	
Experiment date	
Experiment operator	
Samples <i>Reactor name</i> <i>Sampling date</i> <i>Biomass concentration</i> <i>pH original</i> <i>pH diluted</i>	A.
	B.
	C.
	D.
	E.
	F.
	G.
	H.
	I.
	J.
BODIPY FL casein working solution	Kit purchase date: Preparation date: Shaking: Buffer: Buffer preparation date: Buffer pH:
Sample dilution	10 mM 50 mM Tris-HCl pH 7.8 Buffer preparation date: Buffer pH check: Dilute to 1g TS/L Other: Sample pretreatment: No Yes: Sample mixing: No Yes rpm min
Pure enzyme control <i>Optional</i>	Desired concentration: Enzyme art. no.: Mass weighed: Buffer: Buffer preparation date: Sample mixing: No Yes rpm min
Fluorescein standard <i>Optional</i>	1 mM stock composition: 1 mM stock preparation date: Working solution concentration: μM Working solution preparation date:

Protein hydrolysis assay with BODIPY FL casein

Microplate type	Greiner 655090	Other:
Microplate reader settings	Plate definition file: Wells selected for measurement: Shaking: Shaking settings: Temperature control: Measurement frequency: Ex/Em wavelength: Detector gain: Top/bottom reading: Experiment duration:	
Substrate addition	Start time: Stop time:	
Data saving	Script file name(s): Raw data file name(s): Files transferred to:	

Protein hydrolysis assay with BODIPY FL casein

	1	2	3	4	5	6	7	8	9	10	11	12
Row A	A100 X50	A100 X50	A100 X50	B100 X50	B100 X50	B100 X50	C100 X50	C100 X50	C100 X50	D100 X50	D100 X50	D100 X50
Row B	A100 Y50	A100 Y50	A100 Y50	B100 Y50	B100 Y50	B100 Y50	C100 Y50	C100 Y50	C100 Y50	D100 Y50	D100 Y50	D100 Y50
Row C	E100 X50	E100 X50	E100 X50	F100 X50	F100 X50	F100 X50	G100 X50	G100 X50	G100 X50	H100 X50	H100 X50	H100 X50
Row D	E100 Y50	E100 Y50	E100 Y50	F100 Y50	F100 Y50	F100 Y50	G100 Y50	G100 Y50	G100 Y50	H100 Y50	H100 Y50	H100 Y50
Row E	I100 X50	I100 X50	I100 X50	J100 X50	J100 X50	J100 X50						
Row F	I100 Y50	I100 Y50	I100 Y50	J100 Y50	J100 Y50	J100 Y50						
Row G												
Row H	Y100 X50	Y100 X50	Y100 X50	T100 X50	T100 X50	T100 X50	T100 Y50	T100 Y50	T100 Y50	V150	V150	V150

All values in the table are volumes in μL . Legend to the symbols:

A-J = sludge samples, cf. table above

V = Fluorescein standard

X₁ X₂ = BODIPY-FL casein working solutions

T = Pure enzyme solution

Y = Buffer

Chemical substances

Component	Formula	CAS	MM (g/mol)
BODIPY FL casein	NA	NA	NA
Fluorescein (free acid)	C ₂₀ H ₁₂ O ₅	2321-07-5	332.31
Hydrogen chloride	HCl	7647-01-0	36.46
Potassium phosphate monobasic	KH ₂ PO ₄	7778-77-0	136.09
Sodium azide	NaN ₃	26628-22-8	65.01
Sodium chloride	NaCl	7647-14-5	58.44
Sodium hydroxide	NaOH	1310-73-2	40.00
Sodium phosphate dibasic	Na ₂ HPO ₄	7558-79-4	141.96
Sodium phosphate dibasic dihydrate	Na ₂ HPO ₄ .2H ₂ O	10028-24-7	177.99
Trisma base	NH ₂ C(CH ₂ OH) ₃	77-86-1	121.14

Hazards involved with these substances:

	H290	H300	H302	H312	H314	H315	H319	H332	H335	H410
Fluorescein			1	1				1		
HCl					1				1	
KH ₂ PO ₄										
NaN ₃		1								1
NaCl										
NaOH	1				1					
Na ₂ HPO ₄										
Trisma base						1	1		1	

Suggested reading

This protocol is loosely based on a microscope protocol with BODIPY FL casein, originally described by Xia et al. (2007) and Nielsen et al. (2010). Additional information can be found in the manual supplied with the EnzChek™ protease assay kit (Thermofisher scientific Inc., E6638, latest revision 3 November 2004). This manual is available on the supplier website.

The underlying mechanism of the BODIPY FL casein substrate is explained in more detail by Jones et al. (1997) and Schade et al. (1996).

Other applications of the BODIPY FL casein method are incorporated in the work by Goffredi & Orphan (2010), Stefanovic et al. (2017), Paul & Pohnert (2013) and Xia et al. (2016).

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