Anaerobic Conversion of Proteins in Aerobic Granular Sludge

By

Samyuktha Sathish

in partial fulfilment of the requirements for the degree of

Master of Science

in Environmental Engineering

at the Delft University of Technology,

Committee :	Prof. Dr. Ir. Merle de Kreuk,	TU Delft
	Prof. Dr. Ir. Jules van Lier,	TU Delft
	Mario Pronk	TU Delft, RHDHV
Daily Supervisor:	Ir. Sara Toja Ortega,	TU Delft

TU Delft

Acknowledgements

As I stand here at the final stage of this research, all that I feel for this journey is a deep sense of gratitude. This has been an incredible learning experience that extended much beyond just academics and I owe it to those who guided me, supported me and most of all, believed in me.

I am immensely grateful to my daily supervisor - Sara Toja Ortega, for her knowledge, patience, time, encouragement and her enthusiasm. She has always been available to mentor me during the research phase and she has been a huge support during the very unsettling COVID lockdown period.

I would also like to thank the chair of my committee, Prof. Dr. Ir. Merle de Kreuk, for her valuable supervision and guidance throughout the thesis. I have always felt comfortable approaching her and she has never turned me down. She made me believe in hard-work and good faith and she is going to be an inspiration, for years to come. I would also like to thank Prof. Dr. Ir. Jules van Lier and Mario Pronk for their recommendations and for agreeing to be a part of my thesis committee.

A big thank you to my friends here and across the ocean, for undermining all my successes and my failures, ensuring that they don't get to my head. This would not be complete without a special mention to Narayanee- my roommate and best friend who played a pivotal role in my well-being.

To my little sister - you are a blessing to me and seeing you smile is my biggest motivation. To my mother, father, aunt, uncle and my grandmother – I am immensely grateful for your unconditional love and you are my biggest strength. We as family are a crazy bunch and I wouldn't have it any other way!

Abstract

In an aerobic granular sludge (AGS) reactor treating urban wastewater, nutrient removal depends on the availability of carbon source. Domestic wastewater consists of 40-60% of slowly biodegradable complex substrates, out of which proteins form a major fraction. Despite this, little is known about the mechanisms of protein degradation in AGS. This research assessed the anaerobic availability of protein substrates for enhanced biological phosphorous removal (EBPR) in an aerobic granular sludge reactor. Proteins have to be first hydrolysed before being assimilated by the bacteria, and nutrient removal is often limited by the rate of hydrolysis. Therefore, a major part of this thesis attempted to look into the mechanisms of proteolysis in AGS. Next, the utilization of amino acids - the hydrolysis product of proteins - by PAO was explored, based on critical evaluation of available literature. Firstly, it is proposed that the important aspect likely to govern the hydrolysis of proteins in AGS is the substrate-granule interaction, taking into account the diffusion limitation of particulate substrates within the granules and the probable presence of hydrolytic enzymes on the granular surface. Further, it is seen that the amino acids may be utilized by the polyphosphate accumulating organisms (PAOs) either directly or after the anaerobic degradation of amino acids to simple VFAs (volatile fatty acids), which are then taken up by the PAOs. The anaerobic degradation or fermentation of amino acids may occur via two well-known pathways- Stickland pathway and the non-Stickland pathway. Non-Stickland reaction requires syntrophy with hydrogen consuming bacteria whose presence in AGS is questionable. The bacteria responsible for Stickland reaction are obligate anaerobes belonging to the genus Clostridium which has not been found in aerobic granular sludge. Thus, it seems more likely that the amino acids are directly taken up by the PAOs in an AGS reactor. However, the direct uptake of amino acids by the PAOs has been reported only for eleven amino acids in total. More research on the likely fate of the remaining amino acids is recommended, considering that very little is known about the fate of amino acids in AGS. Few laboratory experiments were also conducted to study the effect of substrate size and granule size on the rate of hydrolysis of proteins. From preliminary experiments, it was seen that aerobic granular sludge exhibited significant anaerobic phosphate-release activity when casein (protein) was the only available carbon source. In the lab experiment carried out with different sizes of protein substrates, the observed anaerobic phosphate-release activity was used to obtain the rate of hydrolysis of different sizes of casein. Based on the hydrolysis rate obtained, it is seen that in an AGS reactor with a typical sludge concentration of 8g/l, up to 90% of the proteins present in domestic wastewater influent could be potentially taken up by the PAOs, provided that the proteins are completely dissolved. It was also seen that 60-80% of the particulate casein COD (>0.45um) was hydrolysed within 24 hours of the assay. In another lab experiment, fluorescent protease assay was performed to assess the effect of granule size on the rate of hydrolysis. A significant decrease (by at least 2 times) in the specific rate of protein hydrolysis was observed when the aerobic granule size was increased from 1-2mm to 3.15-4mm. This may be significant especially when the stratification of granules and plug-flow feeding in an AGS reactor is taken into account. Further research is recommended to analyse the relative effect of substrate size and granule size on the rate of hydrolysis of proteins in AGS.

List of Abbreviations

AGS: Aerobic Granular Sludge

ASM: Activated Sludge Model

ATP: Adenosine Triphosphate

COD: Chemical Oxygen Demand

EBPR: Enhanced Biological Phosphorous Removal

EPS: Extracellular Polymeric Substances

NAD^+/ NADH: Nicotinamide Adenine Dinucleotide (Hydrogen)

PAO: Polyphosphate Accumulating Organisms

PBM: Particle Break-up Model

PHA: Poly-hydroxy Alkanoates

PHV: Poly-hydroxy Valerate

SA: Surface Area

SBK: Surface-Based Kinetics

VFA: Volatile Fatty Acids

VS: Volatile Solids

WWTP: Wastewater Treatment Plant

Contents

List of Tables

List of Figures

Chapter 1 Introduction

Clean and safe water is essential to life. Urbanization, industrialization, population growth etc. have led to the production of huge amounts of wastewater which if left untreated, can contain toxic pollutants that pose serious risk. According to the United Nations report 2018, over 2 million people live in countries experiencing water stress and two-thirds of the world's population experience severe water scarcity during at least one month of a year. Adequate treatment of wastewater not only ensures safety but also contributes to reducing water stress by enabling reuse and recycle.

Biological treatment of wastewater such as the activated sludge technology, the anaerobic digestion processes and the aerobic granular sludge technology rely on a complex ecosystem of microorganisms that decompose organic pollutants. Activated Sludge is a conventional process that is being employed for more than a century in wastewater treatment plants (WWTPs) across the globe [1]. The reason why activated sludge process is so widely applied is that it can effectively treat a variety of wastewater contaminants at high rates and can meet the good effluent standards. But, drawbacks of this conventional method include the requirement of large land foot-print and high energy demands, to name a few [1].

Aerobic Granular Sludge (AGS) technology is a rapidly emerging alternative to activated sludge systems for sustainable biological treatment of wastewater. Aerobic granular sludge has been extensively studied in lab-scale and has been effectively cultivated in full scale with both municipal wastewater and industrial wastewater [2] [3] [4]. Compared to the age-old suspended activated sludge process, the AGS technology is promising due to its lower foot print facilitated by its compact and fast-settling granules that enable smaller bio-reactor volume, lower energy costs, efficient nutrient removal, ability to withstand shock loading among other advantages.

1.1 Background

1.1.1 Aerobic Granular sludge

Aerobic granular sludge is characterized by the compact fast-settling granules that have a layered structure. Under suitable operating conditions, each granule will have an aerobic outer layer, followed by an anoxic middle layer and a subsequent anaerobic core. This structure of the granule facilitates simultaneous removal of nitrogen, phosphorus and COD. The full-scale AGS reactors (i.e Nereda) are operated through simultaneous feeding and effluent discharge phase (otherwise called the feeding phase), followed by a reaction period and a sludge settling period called the idle period. The reaction period and the idle period together make-up the famine phase. Typically, an AGS reactor is operated with an anaerobic feeding phase and an aerobic famine phase.

The presence of nutrients such as nitrogen and phosphorus in water leads to eutrophication. Treated wastewater and agricultural run-off are the potential sources of phosphorus in surface water. Hence, the removal of phosphorus in treatment plants is important. Enhanced Biological Phosphorus Removal (EBPR) is a biological process that ensures the removal and possible recovery of phosphorus in wastewater which also occurs in AGS systems [5].

1.1.2 Enhanced Biological Phosphorus Removal

Enhanced Biological Phosphorus Removal (EBPR) is commonly cited as one of the most sustainable and economical processes to remove phosphorus from wastewater [6]. The microorganisms that enable the EBPR process are called Polyphosphate Accumulating Organisms or PAOs. Anaerobically, the PAOs take up carbon substrates such as simple volatile fatty acids (VFAs) and store them as polyhydroxy-alkanoates (PHAs) [7]. The energy (in terms of ATP) for the uptake of the VFAs is obtained from the hydrolysis of intracellular polyphosphate (Poly-P) resulting in ortho-phosphate (P) release. The conversion of VFAs to PHA is facilitated by the reduction equivalents (such as NADH and NAD+) that are generated due to intracellular glycolysis. Glycogen is thus a key compound in the metabolism of PAOs. In the following aerobic phase, phosphorus is accumulated in order to restore the poly-P reserves of the PAO. The energy for the uptake of P is obtained from the stored PHA. The amount of aerobic P-uptake is greater than the amount of anaerobic P-release, leading to net P-removal. The efficiency of the EBPR process relies on the availability of appropriate carbon sources that the PAOs can assimilate and store [8]. P removal will depend on these stored C-sources which are the energy sources for aerobic P-uptake.

1.1.3 Knowledge gaps in AGS technology

The first-principles of aerobic granular sludge process are predominantly obtained from laboratory studies that deal with synthetic wastewater containing dissolved, readily biodegradable, substrates such as the simple VFAs and low molecular weight substrates (such as glucose, carbohydrates) that are metabolized easily [9] [10] [11] . Undoubtedly, such studies contribute significantly to the current understanding of various aspects of the aerobic granular sludge processes such as the microbial characterization, the formation of granules and the nutrient removal efficiency which forms the foundation for further research. Nonetheless, the above-mentioned synthetic wastewater is a simplification which does not encompass the full complexity of real municipal wastewater which will typically contain polymeric substrates and a higher concentration of particulates [12].

Urban wastewater contains slowly degradable, complex substrates such as proteins, lipids, polysaccharides, humic substances, nucleic acids and other heteropolymers [13]. The fraction of such particulate organic matter in pre-settled domestic wastewater usually accounts for 40-60% of the total organic matter [10]. These substrates not only have limited diffusibility across biofilms [14], they also have to be first hydrolyzed before being consumed by bacteria [15] [16]. Even though successful granulation of AGS treating real municipal wastewater in full-scale plants has been reported previously [3] [17], the number of studies that deal with the fate and the influence of particulate, polymeric substrates in AGS is limited [9] [18] [19].
The insights gained from studies dealing with AGS treating synthetic wastewater cannot be directly translated to AGS treating real wastewater containing complex, polymeric substrates. While it is evident that the characteristics of the AGS vary when the type of influent changes from simple synthetic wastewater to real wastewater [10] [20] [21]; the fate of the individual polymeric, complex substrates in AGS and the ways in which they influence AGS process is yet to be well researched.

Municipal wastewater typically contains 40-60% proteins, 25-50% carbohydrates and 10% lipids [22]. Even though it is site-specific, typically, proteins seem to form a major fraction of the municipal wastewater. Despite this, studies on the degradation of proteins in AGS is very

sparse [23] [24].Taking into account the importance of EBPR process and the predominance of proteins in municipal wastewater, this study aims to deal with how the proteins may contribute to EBPR process in AGS. As mentioned above, the efficiency of the EBPR process relies on the availability of appropriate carbon sources. Thus, this study will limit itself to certain aspects of anaerobic degradation of proteins to products assimilable by PAOs, in AGS.

1.2 Research scope

This research takes a closer look into the protein degradation processes namely: hydrolysis and fermentation, during the anaerobic phase of an AGS reactor. Typically, protein hydrolysates include both amino acids & peptides and consequently, amino acids may be fermented to a variety of end products. However, this study deals with hydrolysis of proteins to amino acids (which may be taken up by the PAOs) and the subsequent fermentation of amino acids to simple VFAs that PAOs can assimilate anaerobically. The evaluation of all probable anaerobic degradation pathways of amino acids is beyond the scope of this study and hence is limited to the anaerobic amino acid degradation pathways that lead to uptake by PAOs.
As mentioned above, research on anaerobic degradation of proteins that are present in the influent, in an AGS environment is minimal or even nil. Thus, past studies on anaerobic degradation of proteins and amino acids in other WWTP technologies are critically evaluated and translated to what may possibly occur in an AGS environment, along with a few primary experiments which can lay the foundation for future research.

This research is conducted by answering the questions below which are categorized as conceptual (critical literature analysis) and experimental, based on how they are dealt with. Under the conceptual category, questions pertaining to both hydrolysis and fermentation are dealt with. The experimental category is limited to questions on hydrolysis alone.

Conceptual

1) What are the factors regulating the proteolysis site in an AGS granule?
2) Is it probable that the amino acids are fermented to simple VFAs in AGS before being taken up by PAOs?

Experimental

3) How does the size of protein substrates affect the hydrolytic kinetics? (Experimental)
   It is hypothesised that as the substrate size increases, the rate of hydrolysis decreases.
4) How does the size of AGS granules affect the hydrolytic kinetics of proteins? (Experimental)
   It is hypothesised that as the granule size increases, the rate of hydrolysis decreases.

1.3 Thesis Outline

Chapter 2 analyses the various hydrolysis kinetics models available in literature to further evaluate the factors that may affect the anaerobic hydrolysis of complex substrates in AGS. **Chapter 3** deals with the utilization of amino acids by PAOs under anaerobic conditions. This chapter dives into the typical anaerobic amino acid fermentation to simple VFA pathways and evaluates the probability of occurrence of the discussed pathways in AGS. Later on, the direct uptake of amino acids by PAOs is dealt with in this chapter. **Chapter 4** is based on the laboratory experiments conducted to understand the influence of substrate size and granule size on the kinetics of anaerobic hydrolysis in AGS. Chapter 2 and Chapter 3 answers the conceptual research questions while Chapter 4 answers the experimental research questions.

Chapter 5 includes the major conclusions of this report along with its' limitations and the recommendations for future research.

Chapter 2 Hydrolysis of Proteins to amino acids during anaerobic feeding phase in an AGS reactor

During biological treatment of wastewater, bacteria cannot take up particulate, complex substrates directly. In activated sludge systems, the particulate substrates are instantaneously enmeshed into the sludge flocs and hydrolysed by microorganisms [25]. These heterotrophic microorganisms release extracellular enzymes which depolymerize the complex particulate substrates to soluble forms making them available for utilization. The rate of conversion of particle to soluble form is slower than the rate of assimilation of soluble particles by bacteria [13]. This implies that there is no accumulation of hydrolysis intermediates in the bulk [22].

Hydrolysis is often considered as the rate limiting step and many processes are modelled based on this assumption [26] [27] [28] [29]. In an activated sludge system, under anaerobic conditions, it was seen that the time required for amino acid removal was less than the time required for peptone removal in a mixture containing proteins and amino acids. Since hydrolysis is the slower reaction, it was considered to be rate-limiting [30] [26]. In wastewater treatment plants, when the influent consists of a high percentage of particulate substrates which cannot be taken up by the bacteria, the availability of carbon source for further processes will depend on the rate of hydrolysis of the complex substrates. The particulate organic fraction and the associated hydrolysis rate has a direct impact on the volume of the nutrient removing treatment activated sludge plant [31]. Hence, studying the kinetics of hydrolysis of particulate substrates may aid in improving the overall performance of a wastewater treatment plant.

Overall hydrolysis process can be studied based on the bulk parameters or the substrate oxygen utilization rate (SOUR), among other methods. In order to study the hydrolysis mechanisms specific to the substrates, the enzymes involved in the specific substrate depolymerization process [32] and the hydrolysis intermediates formed have to be looked into [31]. Even though such studies are not directly representative of complex wastewaters such as the municipal wastewater wherein a mixture of substrates is present in varying proportions, this approach can be used to understand how each of the substrate groups degrades and affects the overall biological treatment process. This study specifically deals with kinetics of protein hydrolysis which is one of the major complex substrates in domestic wastewater.

2.1 Hydrolysis Kinetics

The hydrolysis process of particulate organic matter is characterized by both surface-based and transport phenomena [28] . The colonization of the particulate substrates by the hydrolytic enzymes or biomass is a surface-based phenomenon, while the transport of enzymes from the bulk liquid or the reaction products from the surface to deeper layers in the granule is a transport phenomenon, both of which govern the hydrolysis of complex substrates.

2.1.1 Hydrolytic Kinetic Models

The rate of hydrolysis depends on various parameters such as temperature, pH, presence of humic acids, the type of substrates, the substrate concentration, the particle size and the microbial biomass responsible for enzymatic activity [26]. Hydrolysis kinetic models help to quantitatively explain the behavior of the hydrolysis process which in turn can be used in the design of reactors. Different mathematical models used to define the kinetics of hydrolysis process can be categorized broadly into empirical models and mechanistic models.

Empirical models: Traditionally, the rate of hydrolysis is empirically defined as first order kinetics at constant temperature and pH with respect to the remaining concentration of degradable particulate COD. According to first-order hydrolysis, the rate of the reaction is directly proportional to the substrate concentration and the proportionality constant (k_h) is defined in terms of 1/day as

$$\frac{dF}{dT} = -K(h).F \qquad \text{Equation 1}$$

where F is the substrate concentration in Kg/m^3 [33].

The literature published up to 1990 was reviewed by [33] [33] and indicated that all the works to date had used first order models to describe anaerobic hydrolysis of particulate waste. The first order kinetics is a cumulative representation of all the physical aspects of the particles and the enzymatic activity of the sludge, lumped together to give a simple relation for a complex, heterogenous system [26]. However, (Eastman and Ferguson, 1981)[26] did mention that the hydrolysis rate of different complex substrates such as starch and protein would be different [26]. (Hobson, 1987) reported that the particle size and sphericity have an effect on the rate of microbial degradation of substrates such as cellulose[34]. For particles of different sizes, different first order hydrolysis coefficients are also reported in literature [35]. Thus, there is a need for models that better correlate the characteristics of the particles and the enzymatic activity with the rate of hydrolysis. Such models, which aid in better understanding of the overall hydrolysis process, are dealt with in the following section.

Mechanistic models: Mechanistic models to describe hydrolysis kinetics more intrinsically are largely categorized into two: growth-based models and surface-based models. The growth-based models assume deficiency in enzyme activity whereas the surface-based models assume excess enzyme activity.

<u>Growth based hydrolysis model</u>

The growth-based models relate the substrate utilization to microbial growth. Michaelis-Menten kinetics is a widely used kinetic model based on experimentally determined coefficient values. There is a reduction in substrate mass with time as the substrate is being utilized by the microorganisms for growth and this dynamic relation can be represented using the equation: (Metcalf, 2003)

$$r(su) = -\left(\frac{kXS}{K(s) + X}\right) \qquad \text{Equation 2}$$

r(su) = rate of change of substrate concentration due to utilization ($g/m^{3.}$ day)

k = maximum specific substrate utilization rate in g substrate/g microorganisms

X = biomass concentration in g/m^3

S = growth limiting substrate concentration in g/m^3.

(Goel et al., 1998a) used dissolved starch in batch experiments with activated sludge and proposed that Michaelis-Menten type of kinetics is suited for defining the hydrolysis of dissolved starch [36]. (Sanders, Zeeman and Lettinga, 2002) [37] conducted experiments similar to (Goel et al., 1998a) [36] with dissolved gelatin substrate and found similar relation between substrate concentration and biomass concentration.
This model is better suited for soluble substrates wherein the substrate surface available for enzymatic hydrolysis is not a limitation, unlike with particulate substrates. That brings us to the surface-based model.

Surface-based hydrolysis model

The surface-based hydrolysis model assumes that the enzyme activity is present in excess and that the rate of hydrolysis depends on the amount of surface available for the hydrolytic enzymes. As the particle size increases, the surface area per unit volume decreases which invariably has an effect on the surface available for hydrolysis. The deterministic Surface Based Kinetics (SBK) takes the surface area of the particle into account and states that the rate of change of mass of the substrate is directly proportional to the decrease in the surface area of the particulate available for hydrolysis. The hydrolysis constant of proportionality (k_{sbk}), in this case, is defined in terms of kg m^{-2} d^{-1} as,

$$\frac{dM}{dT} = -K(sbk) * A \qquad \text{Equation 3}$$

SBK is based on the assumption that the hydrolysable particulate substrate is fully covered with bacteria that excrete exoenzymes. (Sanders et al., 2000) verified the SBK model for the particulate substrate starch, and found the hydrolysis constant per unit area available for starch in (lab-scale) activated sludge batch reactors [35].

SBK model is completely valid only in the case of spherical particulates that are not susceptible to breaking but degraded by the continuous reduction of particulate diameter [35]. This is because it has been proved empirically, several times, that prolonged digestion decreases the rate of hydrolysis as a result of the decrease in the substrate concentration and available surface area [38] [39]. However, according to the SBK model, theoretically, the breaking of particles which will invariably increase the available surface area will also increase the rate of particulate hydrolysis. Although this is contradictory to what is usually seen during prolonged digestion, the phenomenon has been reported by (Aldin, 2010) [40] in anaerobic digestion and by (Dimock and Morgenroth, 2006) [41] in activated sludge, on the addition of slowly biodegradable COD fraction. (Sanders et al., 2000 pointed out that the total surface area of the particle is not equal to the surface area available for hydrolysis which could imply that the finer particles that “break off” from larger particles may not be fully biodegradable [35]. This is however not seen in starch , as proved by (Sanders et al., 2000) [35]. But other complex substrates may exhibit this phenomenon.

The applicability of the SBK model to the anaerobic hydrolysis of proteins in AGS reactors depends on whether the two assumptions mentioned above are met. The two assumptions are as follows:

1) The hydrolysable substrate is fully covered by bacteria that secrete exo-enzymes.
2) The particles are spherical and are not susceptible to breakage.

(Hobson, 1987) found that particulates in continuous digestors were fully covered with bacteria, which validates the above assumption [34]. However, whether this is also true in the case of batch-fed reactors should be validated. Although (Sanders et al., 2000) [35] conducted experiments in anaerobic granular sludge batch reactors and through microscopic observations found that particulates become fully covered with bacteria during the long anaerobic periods, in full- scale AGS reactors treating complex wastewater, it is unlikely that all the particles are fully covered by the granules. The anaerobic feeding phase in an AGS reactor is typically between 1-2 hours thus limiting the time available for entrapment of the particulates under anaerobic conditions, in addition to the limited attachment of the particulates to the granule surface as discussed later in the substrate-granule interaction section. Besides, the cycle time of an AGS reactor treating municipal wastewater is typically between 4-6 hours with selective removal of particulates [3] [10] and so the overall retention time of the particulates in the sludge is very low, further limiting its' hydrolysis. This is different from anaerobic digestors where all wastewater fractions have a longer retention time [42] [43].

The second assumption of the SBK model is based on the substrate characteristics. Unlike starch, which was used as the substrate in many SBK model studies, protein could be susceptible to breakage and may not be spherical. This will depend on the type of protein. Polymeric substrates such as proteins can be in the particulate or in the dissolved form. Besides, in terms of molecular structure, proteins can be broadly categorized as fibroid and globular protein. Fibroid proteins, like collagen, are water insoluble due to their structure and biological function. Globular proteins such as gelatine, on the other hand, are water soluble and are considered to be dissolved polymers [44]. All these possible structures suggest a more complex hydrolysis process than in carbohydrates and make it difficult to accept the assumptions needed for the SBK model.

2.1.2 Proteins

Comparing and contrasting the different ways used to represent hydrolytic kinetics discussed above, it is evident that the surface-based hydrolysis kinetics model is suitable for particulate substrates that degrade at a constant radius per unit time and the Michaelis-Menten kinetics is suited for dissolved complex substrates that are limited by enzymatic activity. As discussed above, the complexity of protein substrates with respect to structure and solubility extends beyond just the two broad categories. Fibroid proteins for example, which are in the insoluble particulate form do not conform to the surface based kinetic model because the fibroid structure does not allow equal enzyme access to all points. But, even the hydrolysis of globular proteins that have folded chains and are hence considered roughly spherical, cannot be strictly represented by surface-based kinetics. For example, (Dimock and Morgenroth, 2006) studied the influence of particle size on the enzymatic hydrolysis of proteins in activated sludge [41]. Egg albumin was used, which is a classic example of globular proteins. Hydrolysis of large protein particles results not only in the increase of specific surface area available for hydrolysis but also in the production of finer soluble substrates that break-off from the larger particles. (Dimock and Morgenroth, 2006) proposed a particle break-up model that takes into account the increase in the surface area as the hydrolysis progresses [41].

The particle break-up model (PBM) considers a variable surface-volume ratio that increases as a result of particle break-up during hydrolysis. The rate of particle break-up is also coupled to an overall hydrolysis rate.

$$\frac{dX}{dt} = -k'(h).f(av).X(s) = \rho(PBM) \quad \text{Equation 4}$$

$$\frac{df(av)}{dt} = c(av).\rho(PBM) \quad \text{Equation 5}$$

X- particulate substrate
$-k'_h$ = modified rate of hydrolysis in m/s
f_{av} = surface to volume ratio (m^{-1})
$\boldsymbol{\rho}_{PBM}$ = process hydrolysis rate
C_{av} = constant that relates the particle break-up rate to overall hydrolysis rate. (m^2 kg^{-1}).

The particle break-up model correlates the hydrolysis rate constant, initial specific surface area and the rate constant for increase in specific area [41].

The particle break-up model requires a thorough analysis of particle size distribution. In complex wastewater such as the municipal wastewater that has varying compositions, such detailed analysis of particle size distribution is not advocated when more simple, straightforward models such as the ASM3 model will also provide adequate information for design purposes.
However, such intrinsic models will aid in explaining and understanding the incomplete hydrolysis of substrates when the influent consists of greater fractions of proteins that behave as described above. When the hydraulic retention time of systems is designed based on the overall hydrolysis kinetics, some of the insoluble particulate substrates that degrade as explained above by (Dimock and Morgenroth, 2006) [41] may only be partially hydrolysed because of the insufficient hydrolysis time.

Effect of protein size on hydrolytic kinetics

The slowly biodegradable complex substrates such as proteins can be present either in the particulate form or in the dissolved form. The rate of hydrolysis between particulate protein and dissolved protein is significantly different. Dissolved proteins are hydrolyzed faster than particulate proteins [44], in accordance with the mechanisms explained above. At 35 degree Celsius and pH 7, it was observed by (Sanders, 2001) [45] that the complete hydrolysis of gelatin, a dissolved protein substrate, took 0.5 hours, while the complete hydrolysis of particulate protein took several days [46].

To demonstrate the significance of particle size on the rate of hydrolysis, (Aldin, 2010) reported that for a 10-fold decrease in particle size (from 500 µm to 50 µm), the hydrolysis rate coefficient increased 7- fold (0.034 per day to 0.298 per day) during batch anaerobic degradation of Casein protein [40]. (Dimock and Morgenroth, 2006) reported hydrolysis rate coefficient of egg white protein in activated sludge systems to vary between 0.038 and 0.24 per

day for particles of 60 µm chord length and 0.019 and 0.98 per day for particles of 390 µm chord length [41]. It can be seen that the reported values for the different sizes varies over a wide range. In fact, the upper limit of hydrolysis rate coefficient values reported for 390 um particles is higher than that reported for particles that are 6 times smaller. On the other hand, the hydrolysis rate constant used in activated sludge models (ASM 2d) typically is 2 per day at 10 degree Celsius and 3 per day at 20 degree Celsius [47]. There are significant differences in the values reported. Such huge variation in the reported values reiterate the importance of more detailed studies on the kinetics of protein hydrolysis.

So far, the hydrolytic kinetics of proteins has been held with in general. The factors affecting the rate of hydrolysis of proteins to amino acids, specifically in an AGS environment, will be held with, henceforth.

2.2 Protein hydrolysis in AGS

The rate of anaerobic hydrolysis limits the availability of particulate COD for uptake by PAOs [48]. Generally, factors such as the temperature, pH, and initial substrate concentration affect the rate of hydrolysis of complex substrates in all systems [26]. In AGS and other biofilm systems, another additional factor affects hydrolysis – the interaction between the surface of the granule and the substrate to be hydrolyzed.

Aerobic granular sludge is characterized by compact fast-settling granules with a layered structure. The occurrence of the layered structure originates from the diffusion limitation in the dense granules that lead to a low diffusion of the nutrients into the granule and back diffusion of metabolites, leading to a substrate concentration gradient from the surface to the core of the granule [49]. This phenomenon is held with, in the following section.

2.2.1 Diffusion limitation

In activated sludge plants, the particulates are instantaneously enmeshed into sludge flocs [25]. This is not the case in aerobic granular sludge. The aerobic granular sludge is characterized by well-settling granules composed of microbial aggregates that are glued together due to the extracellular polymeric substances (EPS matrix) excreted by the microorganisms. Only certain monomers or oligomers measuring a few hundred Daltons can be directly assimilated by bacteria. According to Fick's first law, the rate of transfer of mass to cells in a quiescent fluid is directly proportional to diffusivity [22]. Low diffusivity implies that the substrates will not be dispersed well within the biofilms, which will negatively affect their degradation. Diffusion or mass transfer occurs via the channels or pores in the granules. Logically, the pore size has to be wider than the atomic radius of the substrate that has to diffuse. In the case of AGS granules, these may be covered by the viscous EPS that holds the granules together, further affecting the mass transfer [50]. So apart from diffusion limitation, the contact of the influent proteins with the biomass at the centre of the granules might be affected by size exclusion. Studies report that substrate masses of less than 1000 Da can only be assimilated by bacteria[22] [13] [31]. Thus, complex substrates which have higher substrate masses have to be depolymerized to smaller units not only in order to be assimilable by bacteria but also for diffusion into biofilms. For illustration, Bovine serum albumin, with a molecular weight of 65000 Da has a diffusivity of 68 x 10^{-8} cm^2 s^{-1} in biofilms compared to the amino acid leucine which has a mass of 131 Da and a diffusivity of 874 x 10^{-8} cm^2 s^{-1} [22]. Here, the penetration

of leucine into the EPS matrix or its assimilation by bacterial cells is more likely than that of BSA. Limited data exists, with respect to the quantification of diffusivity in relation to the substrate atomic mass under different biofilm types such as fluffy biofilms versus smooth and compact granules where penetration might be further compromised.

In aerobic granular sludge, where the diffusivity of complex substrates seems to be a limiting factor, the polymers may deposit on the surface of the granule where they are hydrolysed before they penetrate the granular structure. In suspended cultures or continuous flow reactors, the substrate is fully covered by hydrolytic bacteria (excess enzyme activity) and the substrate surface available influences the hydrolysis process, at constant temperature and pH. Similarly, in biofilm systems such as AGS the particulate substrate interaction with the granular surface is important for hydrolysis. As the size of the granule increases, the diffusion depth is compromised leading to insufficient substrates in the deeper granular layers which would in turn lead to inactive biomass in the core of the granule [24]. The granular surface is likely to have hydrolytic bacteria which will break down the polymers to oligomers/monomers that further interact with bacteria in deeper layers of the granule.

2.2.2 Location of the hydrolytic enzymes

One important characteristic of AGS is the selection of quickly settling granules implying smaller HRT when compared to activated sludge systems. The location of hydrolytic enzyme activity becomes important because if the enzyme activity is in the bulk phase, the hydrolysis intermediates or the enzyme themselves could be potentially washed-out at very low HRTs [31]. On the other hand, if the enzymes are in the sludge instead of the bulk liquid, even though better retention of the enzymes is possible, their contact with substrates will be compromised due to diffusion limitation (2.2.1).

Extracellular enzymes can either be dissolved in the bulk liquid, associated with the cell surface of the hydrolytic bacteria, or immobilized in the EPS matrix/loosely associated with the cell or entrapped within the floc/EPS [51] [52]. The depolymerases, those enzymes acting on larger polymers, are located at or outside the cell wall while the oligomerases, which act on depolymerase products may be located in the periplasmic space, or even in the cytosol [31].In pure cultures, the hydrolytic enzyme activity is associated with the bulk-liquid [52] whereas in activated sludge systems the hydrolytic enzyme activity is mainly associated with the biomass. (Goel et al., 1998a) [36] and (Confer and Logan, 1997) [22] used bovine serum albumin, a water-soluble model substrate, and found that the protein hydrolytic activity in batch and continuous flow suspended cultures, as well as in attached biofilms, is cell associated. This was also confirmed by (Confer and Logan, 1998) wherein the location of leucine amino-peptidase activity (an enzyme that liberates amino acids from the N-terminal of proteins) was found to be cell-surface bound in suspended culture and biofilm reactors [53]. A model for cell-bound hydrolysis and release of hydrolytic fragments was proposed wherein the macromolecules diffused to the surface of the cell in a biofilm or aggregate, the macromolecules are hydrolysed and the incomplete depolymerized fragments hence produced diffuse back to the bulk-liquid or to other cells. This process repeats until the fragments are small enough to be directly assimilated by bacteria [22] [54]. (Goel et al., 1998b) also found protease activity to be loosely bounded to cell or entrapped within flocs [52]. (Goel et al., 1998a) [36] and (Goel et al., 1997) [55] studied the location of extracellular activity in activated sludge and found that it was associated with the sludge. [32] also had similar observations with negligible bulk liquid

enzymatic activity of amino peptidase enzyme respectively[32]. (Guellil et al. (2001) extracted EPS from activated sludge and compared enzymatic activity in the bulk water and in the EPS extract [13]. They found that protein hydrolysis mainly resulted from the enzymatic activity of EPS, in activated sludge systems. Thus, many studies suggest that the extracellular enzymes responsible for hydrolysis are present in the EPS matrix which is likely to be true in AGS as well.

Immobilization of extracellular polymers [51] is advantageous as microorganisms need not waste energy to replenish the enzyme pool continuously [52], and other advantages include higher operational stability and easy access to co-enzymes and substrate. As discussed in the diffusion section, it is considered that particulate substrates above a certain size range can neither penetrate the granule nor be assimilated by bacteria before being hydrolysed. In such a case, if the bacteria in the deeper layers of the aerobic granular sludge have to have access to C-source, presence of hydrolytic bacteria on the outer surface of the granule seems more favourable. The surface hydrolysis of non-diffusible particulate substrates could allow the diffusion of hydrolytic products to deeper layers, thus ensuring that the bacteria in the deeper layers have access to C-source.

2.2.3 Substrate-granule interaction

Taking into account the diffusion limitation of complex protein substrates into aerobic granules and the prospect that the presence of hydrolytic bacteria is mainly on the surface of the granule, the important aspect that is likely to determine whether the particulates are being hydrolysed or not is the substrate-granular surface interaction. In other words, the particulate proteins have to first attach or adsorb to the surface of the granule before they can be hydrolysed and broken down. This contact between the particulate protein and the granular surface is referred to as *substrate-granule interaction* in this study.

During the feeding phase of AGS reactors, a slow anaerobic plug-flow is employed to facilitate diffusion of the substrate through the entire granule by ensuring high substrate concentrations near the surface [56] and also to facilitate the hydrolysis of particulate substrates [3].
Very recently, some studies looking into understanding the mechanism of the transport of particulate substrate through a granular sludge bed reveal that only a minor fraction of the particulates attach to the granular surface during the anaerobic feeding phase[57] [58] [21]. Magnetic Resonance Imaging (MRI) results from the studies show that the particulate substrates retention is governed by sedimentation and surface filtration mechanisms. The particles seem to accumulate in the voids between the granules, in the bottom of the reactor indicating that their attachment/adsorption to the granule surface is limited. These studies indicate that the particulates resuspend during the subsequent aeration phase and become available for attachment. It is speculated that the resuspended particles preferentially attach to the flocs, since they have higher specific surface area [57] [58].
The particulate retention and availability for attachment during anaerobic plug flow is said to improve at increased influent particulate concentration and lower up-flow velocity [57]. Introducing an anaerobic slow-mixing phase right after plug-flow feeding is suggested to allow resuspension of the particulates and improve contact with the granules, consequently improving anaerobic hydrolysis of particulates [57].
It is worthwhile to note that flocs may play a significant role in the hydrolysis of particulate substrates and in fact may have a competitive advantage over granules during the mixed-

aeration phase [21] [57]. However, this study only focuses on the anaerobic degradation of proteins in AGS reactors so that they can serve as C-source for PAOs. During the anaerobic feeding phase in an AGS reactor, the influent substrates may not come in contact with the flocs due to the heterogenous distribution of biomass over the length of the reactor, wherein the large granules are most often present at the bottom of the reactor and the flocs at the top [59]. Also, in an AGS reactor, it is seen that the relatively slow growing microorganisms such as the PAOs are more enriched in the granules than in the flocs [59] [60]. Hence the report limits itself to suggestions for improved anaerobic interaction between particles and granules, which will ensure the availability of particulate COD for EBPR process.

Thus, the models generally used to describe hydrolysis of particulate substrates in activated sludge systems, such as the surface-based kinetics (SBK) model and the particle break-up model (PBM) may not fully describe the hydrolysis of proteins in AGS where the substrate-granule interaction is an additional limitation. Introduction of a slow anaerobic mixing phase in an AGS reactor, right after anaerobic plug-flow feeding may improve the substrate-granule interaction. This may result in improved anaerobic hydrolysis of particulate substrates such as proteins which may in-turn lead to an increase in the anaerobic carbon-availability for nutrient removal processes. In the next chapter, the fate of protein hydrolysates in relation to the EBPR process, is dealt with.

Chapter 3 Utilization of Amino acids by PAOs during the anaerobic phase in an AGS reactor

This chapter will deal with the fate of the protein hydrolysates- the amino acids, in relation to their contribution to the enhanced biological phosphorous removal process in AGS systems. The fermentation of amino acids in anaerobic digestion is very well-studied in the literature. However, studies on the degradation of amino acids in an AGS reactor is limited.
Based on critical evaluation of available literature, this chapter aims to delve into the possible amino acid degradation pathways that may lead to their uptake by PAOs under anaerobic conditions in an AGS reactor.

3.1 The intricacies of amino acids

Amino acids form the functional group of proteins and all amino acids contain a central carbon, a carboxyl group, an amino group, hydrogen and a side-chain R-group [12]. Amino acids can be classified in many ways based on their polarity, C-chain, end products etc., Based on their end products, they can be classified as ketogenic, glucogenic or both. Based on their C-chain, they can be classified as straight chain or branched chain amino acids. Except glycine, all amino acids have two isomers, D- and L-. which are mirror images of each other. Usually the L-form is the naturally occurring isomeride of amino acids. All these different characteristics increase the complexity of studying amino acids because they influence the metabolism including the enzymes that will be involved and the part of the amino acid structure that the enzyme may attack first.
Amino acids are the major protein hydrolysates and this chapter deals with the fate of amino acids during the anaerobic phase in an AGS reactor for efficient EBPR process. For this purpose, a brief overview of the EBPR process pertaining to the metabolism of PAOs is dealt with first.

3.2 Metabolism of Polyphosphate Accumulating Organisms

The EBPR process is facilitated by the metabolism of PAOs. The two main PAOs commonly found in AGS wastewater treatment plants are *Candidatus Accumulibacter* & *Tetrasphaera* [61]. While both these PAOs share the metabolic ability to accumulate phosphorous, they have different metabolisms and are phylogenetically diverse. The *Candidatus Accumulibacter* is a *Rhodocyclus*-related bacterium belonging to phylum *Bacteroidetes* whereas *Tetrasphaera* belong to phylum *Actinobacteria* [62].

Anaerobically, *Candidatus Accumulibacter* take up small molecular weight compounds such as VFAs and store them as PHA. The ATP obtained from the hydrolysis of poly-P and the NADH obtained from glycolysis and/or anaerobic operation of TCA cycle facilitates this process [63].Under aerobic conditions, the stored PHAs generate energy for growth and for the uptake of P which results in the replenishment of the poly-P reserves. This model provides a basis for analysis of transcription of genes, which is now used in studies to identify other probable metabolic pathways. *Candidatus Halomonas phosphatis*, which has also been found in full-scale EBPR plants have a similar metabolic model as described above [64].

Tetrasphaera-related organisms can take up other C-sources such as sugar and amino acids. When the C-source is glucose, it has been reported that the *Tetrasphaera* takes up the glucose under anaerobic conditions and ferments it to succinate and other components. Unlike *Accumulibacter* where glycolysis occurs, *Tetrasphaera* does not have the ability to produce PHA but synthesizes glycogen as the storage polymer. The energy for this is obtained from the hydrolysis of poly-P and from substrate fermentation [63]. Aerobically, the intracellularly synthesized glycogen is catabolized to provide energy for Poly-P accumulation and for growth. Thus, an overview of the metabolic pathway of *Accumulibacter* and *Tetrasphaera* is given above in general, to mark that different PAOs have distinct cell-physiology, thereby enabling them to preferably take up different carbon substrates.

3.3 Amino acids as the C-source for PAOs

The substrate of interest for this study is the amino acids, which are products of protein hydrolysis. Amino acids aid the EBPR process by being a potential C-source for the PAOs. There are two probable ways in which the amino acids can be the substrates for PAOs

1) The amino acids may be degraded anaerobically to simple VFAs which will subsequently be taken-up by the PAOs (or)
2) The amino acids may also be taken up directly by the PAOs

3.3.1 Anaerobic degradation of amino acids to simple VFAs

Degradation of amino acids in anaerobic digestion has been extensively studied in the past years [65] [66]. Anaerobic digestion typically consists of four steps: hydrolysis, acetogenesis, acidogenesis and methanogenesis. The end byproducts of anaerobic digestion are carbon dioxide and methane gas. On the other hand, aerobic granular sludge reactors are operated with an anaerobic feeding phase, followed by an aerobic famine phase. During anaerobic phase, many complex substrates are broken down, similarly to in anaerobic digestion, going through hydrolysis and fermentation. The subsequent processes that are driven by strict anaerobes in anaerobic digestion, are unlikely to take place in the AGS systems. Anaerobic degradation of amino acids in aerobic granular sludge and in anaerobic digestors themselves might differ because the microbial matrix and hence the catalytic enzymes of an AGS reactor and an anaerobic digestor would be different. AGS microbial matrix will not constitute of sufficient number of organisms that are highly sensitive to oxygen whereas anaerobic digestors would not contain obligate aerobes. This would probably influence the rate of degradation, the intermediate products, the end products, the inhibitory factors etc., of anaerobic amino acid degradation in AGS reactors and anaerobic digestors.

Amino acid degradation in anaerobic digestors by obligate anaerobes has been extensively studied, in the past. In anaerobic digestion, oxidative deamination, transamination and alpha-keto acid oxidation commonly occur in anaerobic amino acid degradation, but oxygenation reactions and fatty acid oxidations are excluded [65]. However, amino acid fermentation by facultative bacteria is yet to be explored. This study will rest on previously explored, well-established findings on anaerobic amino acid fermentation in activated sludge and anaerobic digestors. Extrapolating the earlier findings on anaerobic amino acid degradation to AGS reactor conditions (during the anaerobic phase) might help in forming a basis for detailed analysis of amino acid fermentation by facultative bacteria in anaerobic conditions. Shedding a light on the complexity of amino acid degradation would stress upon the importance of

furthering and continuing the already existing amino acid degradation studies to more specific conditions.

Anaerobic degradation pathways

Amino acid degradation pathways can be roughly classified in two types: through Stickland reactions and Non-Stickland reactions. Stickland reaction is the degradation of pairs of amino acids through a redox reaction where the amino acid with longer carbon chain acts as the electron donor and the one with fewer carbons acts as the electron acceptor [67]. Non-Stickland reaction is basically the fermentation of single amino-acids. They are the reactions wherein single amino acids are fermented in syntrophy with hydrogen utilizing bacteria [29].

In comparison to the degradation of single amino acids via Non-Stickland reactions, the fermentation of amino acids in pairs a.k.a via the Stickland reactions is said to be the dominant reaction [67], the simplest way to ferment amino acids [29] and is said to occur rapidly when compared to uncoupled amino acid degradation [68]. (Ian R. Ramsay & Pratap, 2002) also reported that when protein concentration in the feed or the feed flow rate is doubled, the amino acids predominantly degrade via the Stickland pathway during anaerobic treatment [29]. Also, as mentioned above, Non-Stickland reactions occur in syntrophy with hydrogen consuming bacteria. Methanogens, which are one of the main group of microorganisms in anaerobic digestors, are also hydrogen consumers. Similarly, sulphate reducing bacteria also consume hydrogen. However, both methanogens and sulphate reducing bacteria are obligate anaerobes and thus their presence in an AGS matrix seems unlikely and has not been reported so far [69]. Thus, the occurrence of Non-Stickland pathway in an AGS reactor seems rather unlikely.

The other typical amino acid degradation pathway that is often discussed in anaerobic digestion is the Stickland pathway. Way back in 1943, L.H Stickland, after whom the pathway was named, extensively studied the metabolism of genus *Clostridium*. The bacteria under this genus are obligate anaerobes and they are able to only grow on proteins or amino acids. Even though the bacteria do ferment carbohydrates, they are neither essential nor sufficient for the growth of the genus. Inability of the bacteria to grow under appreciable concentrations of oxygen may be attributed to its' need for low oxidation-reduction potential or even the potential formation of toxic hydrogen peroxide [70].

In order to assess the possibility of the occurrence of the Stickland pathway in AGS, it is necessary to take a brief look into the metabolic reactions that may occur. In Stickland pathway, certain bacteria belonging to genus *Clostridium* such as the *Clostridium sporogenes* catalyze the redox reactions between pairs of amino acids, wherein one of them acts as an electron donor(oxidation) and the other acts as an electron acceptor(reduction). Donor amino acid is deaminated to alpha keto acid. Alpha keto acid is further decarboxylated oxidatively, which together produces four hydrogen, ammonia, carbon dioxide and fatty acids. This process is diagrammatically represented in Figure 1.

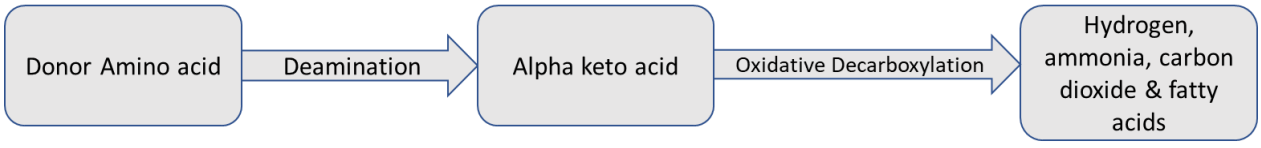

Figure 1 Amino acid oxidation process in Stickland reaction

The hydrogen formed during the oxidative decarboxylation of the donor amino acid is taken up by an acceptor amino acid, thus forming a redox reaction. The ultimate reduction products also include a variety of short-chain fatty acids [65].The Stickland reaction is predominantly carried out by certain bacteria belonging to genus *Clostridium* which are obligate anaerobes. The examples for amino acids that serve as electron donors include D-alanine, D-valine and L-leucine and the ones found to serve as electron acceptors include L-proline, L-hydroxyproline and glycine. Serine and tyrosine are the only amino acids that liberated ammonia when incubated alone. All the other amino acids have to be incubated in pairs, one donor and one acceptor, in order to be deaminated [70].

There are about 20 different amino acids and every amino acid (except glycine) has isomers. It is beyond the scope of this report to study the metabolic degradation pathway of every single amino acid. For example, glutamate alone has nine different pathways through which it is degraded. However, to demonstrate the complexity of amino acid degradation, the metabolic pathway of a very well-known Stickland pair is studied.

Alanine & glycine [71]

LH Stickland dealt with pairs of amino acids and found that when alanine and glycine were incubated together, Clostridia sporogenes completely deaminated alanine (hydrogen donator) and glycine (hydrogen acceptor). One molecule of alanine was seen to reduce two molecule of glycine [70]

Alanine and glycine coupled deamination by *Clostridium* sporogenes is:

$$CH3.CH(NH2).COOH + 2H20 \rightarrow 4H + NH3 + CO2 + CH3 - COOH$$ Equation 6 (Oxidation of alanine)

$$2CH2NH2 - COOH + 4H \rightarrow 2NH3 + 2CH3 - COOH$$ Equation 7 (Reduction of glycine)

The complete redox reaction between d-alanine and glycine is as follows:

$$CH3.CH(NH2).COOH + 2CH(NH2).COOH + 2H2O \rightarrow 3CH3C00H + 3NH3 + CO2$$ Equation 8

In 1987, J Winter[71] and his associates studied the fermentation of alanine alone, glycine alone and alanine along with glycine, in pure *Clostridium sporogenes* and syntrophic cultures with hydrogen and sulfate reducing bacteria. Alanine degradation alone in pure *Clostridium sporogenes* culture saw accumulation of H2 gas which resulted in poor growth. Alanine fermentation was observed to stop when hydrogen partial pressure exceeded 5.3×10^{-2} atm in the gas phase. In syntrophic cultures, there was no hydrogen accumulation. Instead, hydrogen was used by methanogens to reduce CO2 to methane. Alanine was degraded to acetate, CO2, H2 and NH3 by *Clostridium sporogenes* in syntrophic cultures. Glycine, when introduced alone in pure *Clostridium sporogenes* was degraded by reductive deamination to acetate and

oxidation to CO2, H2 and ammonia. In syntrophic culture with methanogens, less glycine seemed to be reduced and more of it oxidized, resulting in increased H2 production which means increased CO2 reduction to methane. This means that there is a need for cooperation of Clostridia with hydrogen consuming bacteria when degrading individual amino acids. The co-operation between the groups of micro-organisms via interspecies hydrogen transfer ensures that toxic hydrogen gas is not accumulated [71]. This need is not completely mitigated when the amino acids are degraded in pairs via the Stickland reaction. Even when alanine and glycine were introduced together to pure *Clostridium sporogenes* culture, though most of the H2 from alanine degradation was used for glycine reduction, there was still some hydrogen gas. Typically, a 10% dearth in the Stickland acceptors is seen in a classic amino acid mix [75], such as casein hydrolysate for example which could lead to accumulation of hydrogen. Thus, there is potential for hydrogen production even when the amino acids are degraded via the dominant [67] Stickland pathway.

It seems that for the Stickland reaction to occur in an AGS, the syntrophy with hydrogen consuming bacteria may be necessary. Besides, the microbial community responsible for the degradation of amino acids via Stickland reaction are obligate anaerobes. Currently, only *Clostridial* species are mainly known to possess the Stickland pathway [74] [73]. The *Clostridia* are a class of anaerobic bacteria belonging to phylum Firmicutes, including genus *Clostridium.* The presence of this genus in AGS is questionable due to the fact that they are strict anaerobes. However, AGS granules have an anaerobic core under suitable operating conditions, wherein these bacteria may be present. For stronger evidence, the data obtained from the microbial community analysis of AGS reported by (Ali et al., 2019b) [69] is processed to obtain the relative abundance of *Clostridium* in AGS. The AGS granules used in that study were from Garmerwolde, The Netherlands. The method employed to process this data is attached in the appendix (A.1). The relative abundance of genus *Clostridium* in the influent, in the small and big AGS granules and in flocs is represented in the figure below Figure 2 Relative abundance of Clostridium genera in GarmerwoldeFigure 2).

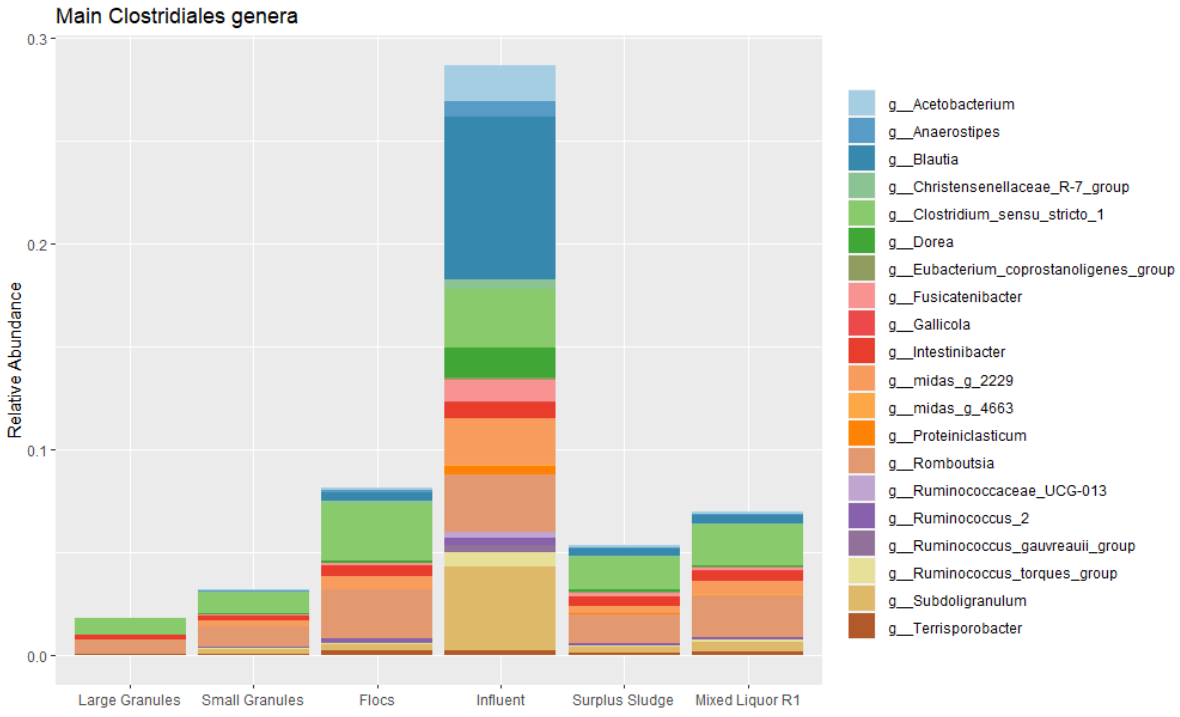

Figure 2 Relative abundance of Clostridium genera in Garmerwolde

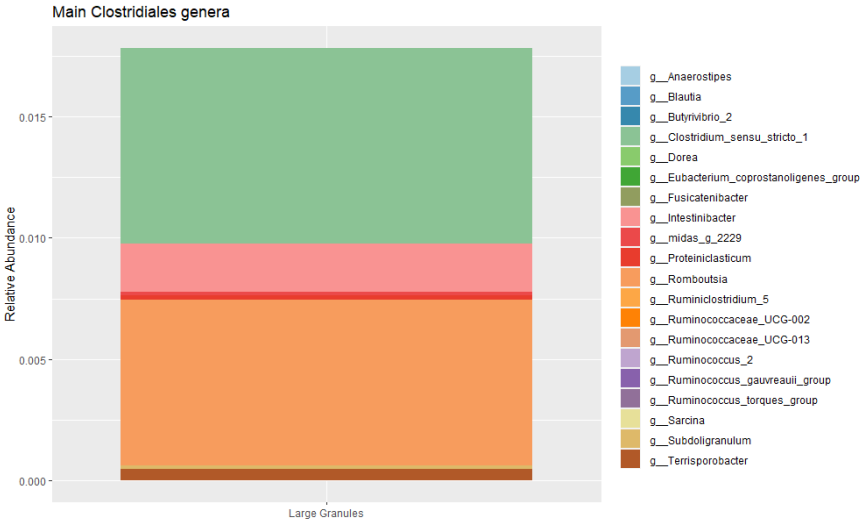

Figure 3 Relative abundance of Clostridium genera in large granules in Garmerwolde

It seems that genus *Clostridium* is present in the granules albeit at a very low abundance. It is also seen that the relative abundance of genus *Clostridium* progressively decreased with increase in granule size to become very rare in large granules (relative abundance of <0.02, Figure 3). (Ali et al., 2019b) [69] reported a net negative growth rate (<0 d^{-1}) of these bacteria in AGS. This can also be observed when taking a look at the relative abundance of the family of bacteria in the influent wastewater vs that in the granules. This may imply that the bacteria are not active in AGS which could also be hypothetically attributed to its' lack of access to the products of protein hydrolysis which is likely to occur in the granular surface (2.2.3). From (Figure 2), it is seen that the flocs have a higher percentage of the *Clostridiale* bacteria despite the fact that they are potentially fully exposed to oxygen under aerobic conditions unlike granules which may have an anaerobic core where the bacteria could potentially not be inhibited.

Thus, the occurrence of either of these amino acid degradation pathways in AGS seems rather unlikely. If amino acids are not being degraded to simple VFAs in AGS, then another potential pathway that allows amino acids to contribute to EBPR process may be its' direct uptake by PAOs.

3.3.2 Direct uptake of amino acids by the PAOs

Amino acids may be directly taken up by the PAOs, both by *Accumulibacter* and *Tetrasphaera*. The following section deals with the similarities and differences in the uptake of amino acids by the different PAOs.

Amino acid metabolism by Tetrasphaera vs Accumulibacter

Tetrasphaera was first found to be the dominant PAO that accumulates amino acids anaerobically which is then used for aerobic P-uptake [76]. It is observed that when casamino acids are the C-source for *Tetrasphaera*, under anaerobic conditions glycolysis occurs while the glycogen is restored under aerobic conditions [77]. This is similar to what is observed in *Accumulibacter* on the uptake of acetate [6] but contradictory to what (Kristiansen et al., 2013) [63] reported in *Tetrasphaera* on the uptake of glucose. *Accumulibacter* on the other hand, is

very well known to take up simple VFAs. In fact, in mixed cultures, it has been often speculated that while *Tetrasphaera* took up amino acids as the C-source, *Accumulibacter* depended on the fermentation products of these amino acids for carbon-source [77]. Even though *Accumulibacter* metabolic studies are mostly confined to its VFA uptake, there is evidence that certain *Accumulibacter* clades take up amino acids directly, under anaerobic conditions with concomitant P-release [78].

When compared to VFA uptake and metabolism by *Accumulibacter*, the uptake of amino acids by certain clades of *Accumulibacter* is relatively new-found knowledge and still requires extensive research. It is likely that the metabolism of amino acids is different in different PAOs and may also vary depending on the type of amino acid. Also, not all the amino acids can be taken up by the PAOs. Studies reveal that out of 20 amino acids that are usually tested, both *Accumulibacter* & *Tetrasphaera* only have the ability to take up 11 of them[78] [62] (List of amino acids included in the Appendix - Table S 1). The amino acids resulting in the highest anaerobic P-release are different forboth *Accumulibacter* and *Tetrasphaera*. In the case of *Accumulibacter*, the highest P-release is observed for aspartate, glutamate, asparagine and glutamine [78] while in the case of *Tetrasphaera*, the highest P-release is observed for glycine, aspartate, cysteine and alanine [62]. However, in both cases, the amino acids histidine, arginine, lysine, methionine, tryptophan, phenylalanine, proline and valine do not result in P-release [78] [62]. This could probably mean that these amino acids cannot be taken up directly by the PAOs and have to be degraded first. In terms of storage compounds, like mentioned earlier, *Accumulibacter* produces the storage polymers in the form of PHA whereas *Tetrasphaera* does not have the ability to produce PHA.

In the case of acetate as the C-source, *Accumulibacter* takes up acetate and converts it to Poly-hydroxy butyrate (PHB). In the case of amino acid as the C-source, different amino acids are stored differently. Out of the four amino acids that result in the highest anaerobic P-release, aspartate and asparagine, like propionate, are stored as Poly-hydroxy valerate (PHV). However, glutamate and glutamine seem to be stored as free amino acids and are not converted to storage polymers [79] [78]. In the case of amino acid uptake by *Tetrasphaera* it has been observed that, anaerobically, glycine amino acid induces the most P-release [62]. On the uptake of glycine, part of it seems to be stored in *Tetrasphaera* which is then used up aerobically for P-uptake and part of the glycine is fermented and the fermentation products such as acetate, succinate and alanine, are excreted to the bulk medium [62]. It was seen that on the uptake of glycine, glycogen was consumed anaerobically as was seen in the case of casamino acid mixture. However, it was seen that glutamate and aspartate amino acids uptake lead to anaerobic glycogen production, as was seen in the case of glucose uptake [77].

With the limited number of studies, it is not yet possible to have a central metabolic model for amino acid uptake by *Accumulibacter* or *Tetrasphaera*. However, it can be seen that one of the important fates of amino acids in the anaerobic phase of an AGS reactor is its ability to act as the C-source for the EBPR process. It is also interesting to note that amino acids as carbon sources for PAOs may have an advantage over simple VFAs. It is consistently reported that the rate of anaerobic P-release in the case of amino acid uptake is lower when compared to the P-release rate during acetate uptake [62] [78]. Lower anaerobic P-release means reduced net phosphorous that has to be taken up aerobically. In fact, (Marques et al., 2017) reported anaerobic P-uptake as opposed to P-release, during the metabolism of the amino acids glycine, aspartate and glutamate individually through energy generated by fermentation of the

respective amino acids [77]. During the metabolism of amino acid mixture, this could also result in reduced net Pi that has to be taken up during the aerobic phase.

Though *Accumulibacter* was predominantly assumed to take up only simple VFAs such as acetate and propionate, now studies reveal that certain clades can take up both simple VFAs and amino acids. Unlike the uptake of acetate and propionate wherein the presence of the latter inhibits the uptake of the former, Candidatus *Accumulibacter* may be able to perform simultaneous uptake of amino acids and simple VFAs. While acetate and propionate uptake is achieved by an acetate proton symporter, glutamate and aspartate uptake is achieved by glutamate/aspartate proton symporter, both driven by proton motive force. Since the transporters are different, both types of substrates can be taken up simultaneously without affecting the rate of uptake of either [78].

3.4 Summary of the chapter

In complex wastewater, proteins are a major component. While proteins are too large and complex to be taken up by bacteria, they are hydrolyzed and these protein hydrolysates are either directly taken up by bacteria or may be further degraded to simpler forms before being taken up. Amino acids are major protein hydrolysates. While the fate of amino acids can be many, this study focuses on the probable use of amino acids as C-source by PAOs for EBPR process. In the EBPR process, PAOs take up carbon source while releasing phosphorus, anaerobically. This carbon source serves as energy during the aerobic phase when the PAOs take up phosphorus leading to P-removal in treated water.

Amino acids are either degraded to simple VFAs which are then taken up by the PAOs. In some cases, amino acids are also directly taken up by the PAOs. These two probable scenarios are dealt with, in detail.

3.4.1 Scenario 1: Amino acids degraded to simple VFAs which are then taken up by the PAOs.

PAOs such as *Candidatus Accumulibacter* readily takes up simple VFAs such as acetate and propionate. In this case, it is important that the amino acids are degraded to simpler VFAs first, before they can become suitable substrates for PAOs. Studies that deal with anaerobic digestion show that amino acids are degraded to simple VFAs via two major pathways- the Stickland reaction and the Non-Stickland reaction. In Stickland reaction, the amino acids are degraded in pairs via a redox reaction where one amino acid acts as electron donor and another amino acid acts as the electron acceptor. In the Non-Stickland pathway, individual amino acids are degraded leading to production of hydrogen. While in anaerobic digestion this hydrogen is taken up by hydrogen consuming bacteria such as the methanogens and the sulphate reducing bacteria, such hydrogen consumers has not be observed in an AGS matrix, so far to the best of my knowledge [59]. Also, even in anaerobic digestors, the Stickland reactions seems to be the dominant pathway through which amino acids are degraded and hence this chapter gives an overview of the Stickland pathway. However, it is seen that a typical amino acid mix has a 10% dearth in Stickland acceptors which will lead to hydrogen production. Besides, AGS does not seem to have *Clostridium* bacteria which are responsible for the Stickland reaction. Thus, the occurrence of either of these pathways in AGS seems rather unlikely.

3.4.2 Scenario 2: Amino acid directly taken up by PAOs

PAOs such as *Tetrasphaera* and certain clades of *Accumulibacter* can directly take up amino acids as C-source. However, studies so far reveal that not all amino acids are taken up and only 11 out of the 20 usually studied are taken up. Uptake of amino acids by PAOs have certain advantages such as the lower P-release to C-uptake ratio in comparison to the uptake of VFA. Lower anaerobic P-release means less net phosphorous that will have to be taken up aerobically by PAOs. Although the direct uptake of amino acids may seem more plausible when compared to the degradation of amino acids to simple VFAs and the subsequent uptake of these simple VFAs by the PAOs in AGS system, experimental evidence is still lacking. The direct uptake of amino acids by PAOs is a novel finding and the metabolism of all amino acids that can be taken up by the PAOs is rather complex and is not fully-known.

In the next chapter, laboratory experiments conducted to further investigate the possibility of the contribution of proteins to EBPR process in an AGS system is discussed.

Chapter 4 Laboratory experiments

The second part of the research consisted of laboratory experiments to understand the influence of particle size and granule size on the rate of hydrolysis of protein substrates and their potential contribution to the enhanced biological phosphorous removal process in AGS. Experiments were conducted with casein as a substrate and AGS sludge collected from the Nereda plant in Utrecht, The Netherlands. The laboratory experiments were arranged in two phases. Phase 1 consisted of experiments performed with different sizes of AGS granules in a 96-well plate incubated with labelled casein. The differences in rate of hydrolysis were analyzed based on fluorescence. Phase 2 comprised of batch experiments with different casein substrate sizes. The rate of hydrolysis was analyzed based on anaerobic P-release, in this case.

The procedure and the results of each of these experiments is held with hereon.

4.1 Phase 1: Studying the effect of granule size on hydrolysis

The objective of the experiment is to analyze how granule size affects the rate of hydrolysis of proteins in AGS. The collected sludge is sieved into four fractions composed of three size ranges of granules of diameters 1-2mm, 2-3.15mm & 3.15-4mm and flocs of size (<0.5mm).
Molecular Probes' EnzChek® Protease Assay kit containing casein derivates heavily labelled with red fluorescent BODIPY TR-X (E6639) dye is used in the experiment. The principle behind the fluorescent protease assay is that, upon hydrolysis, the highly fluorescent BODIPY TR-X dye–labelled peptides will be released. The resulting increase in fluorescence is measured using a microplate reader. The rate of increase in fluorescence is directly proportional to the rate of hydrolysis of the labelled casein.
For each type of sludge studied, three blanks (with no substrate), three autoclaved sludge blanks (with substrate), two 10ug/ml standards and two 5 ug/ml standards are included. The blanks with no substrates account for possible increase in absorbance due to compounds released from the sludge while the autoclaved blanks account for the background fluorescence of the non-hydrolysed substrate. The standards are prepared by hydrolysing a known amount of substrate with commercial enzyme.

4.1.1 Method

First, the granules are sieved into the different size fractions. A part of each of the size fractions, except the flocs, are crushed using a tissue grinder. The volatile solids (VS) concentration is measured in mg/granule for each of the intact fractions and in mg/l for the crushed fractions and the flocs. For the VS measurement of intact granules, 20 number of granules per size fraction are taken while for the crushed fractions and the flocs, 30 ml each is taken. The volatile solids concentration is obtained by measuring the weight difference after first heating the samples in a 105-degree Celsius oven, followed by a 550-degree Celsius oven.

The fluorescent protease assay is performed in a 96-well plate and monitored on a fluorometer. To each well, 100ul of 1g/l sludge solution is added prepared with 10mM TRIS solution in the case of flocs and the crushed fractions. In the case of the intact granules, 100ul of the digestion buffer solution is added followed by an individual granule to each well. 50 ul of 10ug/ml substrate solution is added to each cell. In the samples of blank 50 ul of digestion buffer is added instead of substrate. In standard samples, 50 ul of the corresponding standard are added to the well, instead of substrate. The plate is then inserted into the reader and the experiment is run for two hours with samples being taken at a time interval of 5 minutes. The experiments

were performed at 20 degrees Celsius and pH 7.8. At the end of the experimental run, the actual granule diameter was measured using a digital microscope, from which the surface area and the volume of the granule is calculated.

4.1.2 Results

The fluorescence assay was performed with three different granule fractions in their intact and crushed forms, along with flocs. The characteristics of each of the fractions are presented in Table 1. The sphere equivalent surface area is calculated from the measured granule diameter

Granule form	**Size range [mm]**	**Volatile Solids**	**Unit of VS**	**Average SA per granule [mm^2]**	**Surface area/volume [mm^2/mm^3]**	**Granule Density [mg VS/mm^3]**	**Surface Area/VS [mm^2/mg VS]**
Intact granule	1-2	0.65±0.1	mg / granule	15±4	2.8±0.3	0.13±0.05	23±6
	2-3.15	0.75±0.1		29.5±5	2 ±0.3	0.05±0.02	39±7
	3.15-4	1.75±0.5		44±5	1.6±0.1	0.07±0.01	25±3
Crushed	1-2	4.53±0.4	mg / litre				
	2-3.15	3.30±0.3					
	3.15-4	2.73±0.1					
Flocs	<0.5	5.93±0.3	mg / litre				

Table 1 Characteristics of different granule fractions used in the fluorescent assay

The protein hydrolysis activity measured in terms of per gVS and per surface area (SA) for all the replicates and the measured surface area of all granule fractions and their replicates is given in the appendix Table S 2. The average protein hydrolysis activity per gVS of both the intact & crushed fraction and the average protein hydrolysis activity per SA of the intact fractions is presented in the Table 2.

	Unit of measurement	**3.15-4mm**	**2-3.15 mm**	**1-2mm**	**<0.5mm**
Activity (per gVS) of intact fraction	mg protein/g VS/hour	0.07 ± 0.01	0.19 ±0.04	0.21 ±0.1	
Activity (per gVS) of crushed fraction	mg protein/g VS/hour	1.34 ±0.08	1.30 ±0.3	1.26 ±0.2	4.08 ±0.5
Activity (per SA) of intact fraction	mg Protein hydrolysed/SA of granule/hour	3.19E-06 ±7.3E-07	4.88E-06 ±1.4E-06	9.56E-06 ±5.5E-06	
Ratio of Activity (per gVS) of Crushed granules/Intact granules	-	17.92	6.98	6.04	

Table 2 Average proteolytic activity per g VS and per SA

The measured protein hydrolysis activities in terms of mg Protein hydrolyzed/gVS/h of the different intact granular fractions and along with their replicates is represented in Figure 4. It is seen that the biggest size fraction has the lowest proteolytic activity per g VS.

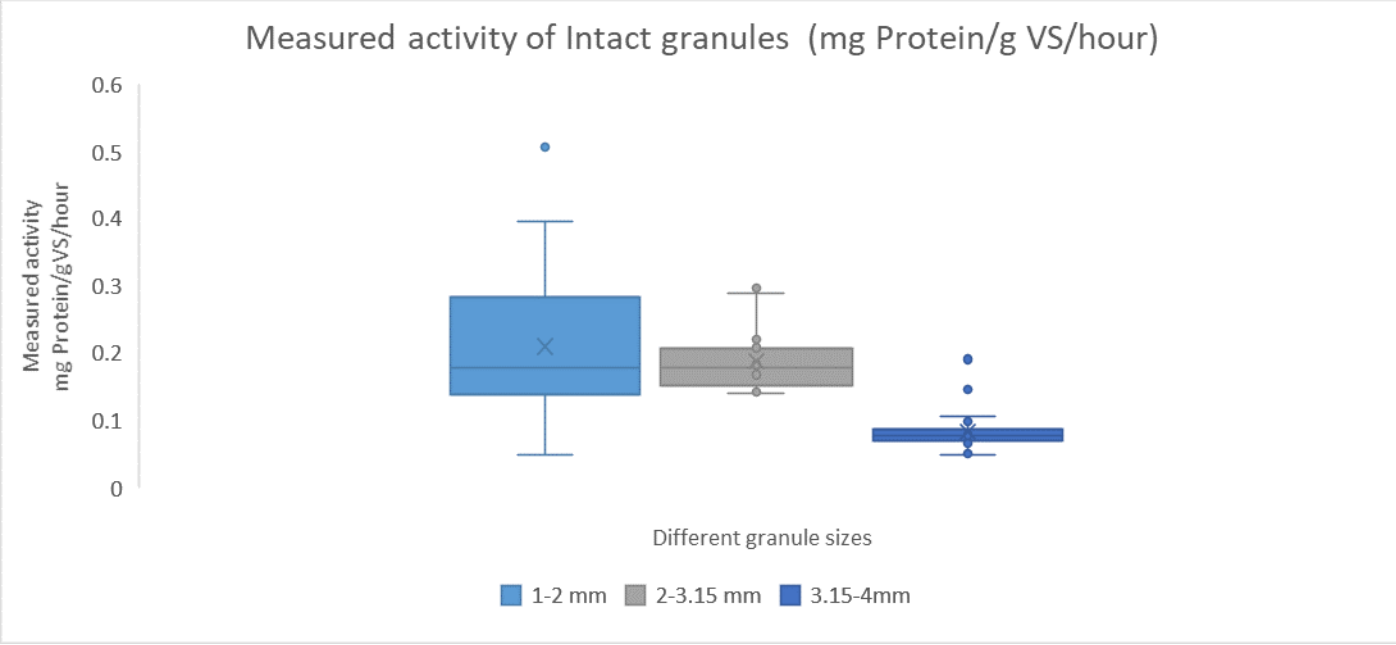

Figure 4 Measured proteolytic activities of three different sizes of intact granules (per gVS)

The measured protein hydrolysis activities in terms of mg Protein/SA/hour of all the intact granular fractions and their replicates is presented in Figure 5. The average proteolysis activity of the 1-2mm(smallest), 2-3.15 mm(middle) and 3.15-4 mm(biggest) fraction in mgProtein/SA/hour is 9.563E-06, 4.987E-06 and 3.263E-06 respectively. It is seen that the protein hydrolysis activity measured per surface area decreases as the size increases with 3.15-4 mm granules having the least proteolytic activity per surface area, followed by 2-3.15mm fraction. However, difference in the proteolytic activity between smallest fraction and the middle fraction was found to be insignificant.

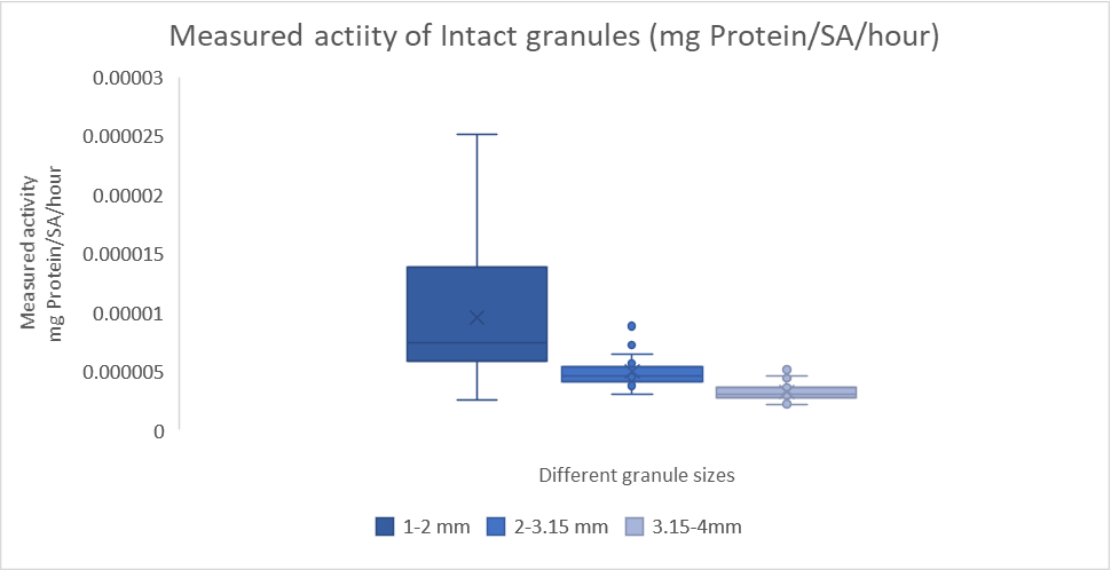

Figure 5 Measured proteolytic activities of three different sizes of intact granules (per SA)

The measured protein hydrolysis activities in terms of mg Protein/g VS/hour of three intact granule fractions and their corresponding crushed fractions is presented in Figure 6. Here, it is seen that the crushed fractions have a higher proteolytic activity than their intact counterparts. While the activity of the smallest and the middle fraction increases 6 times on crushing, the proteolytic activity of the biggest fraction (3.15-4mm) increases 16 times. However, all the crushed fractions seem to have similar proteolytic activity.

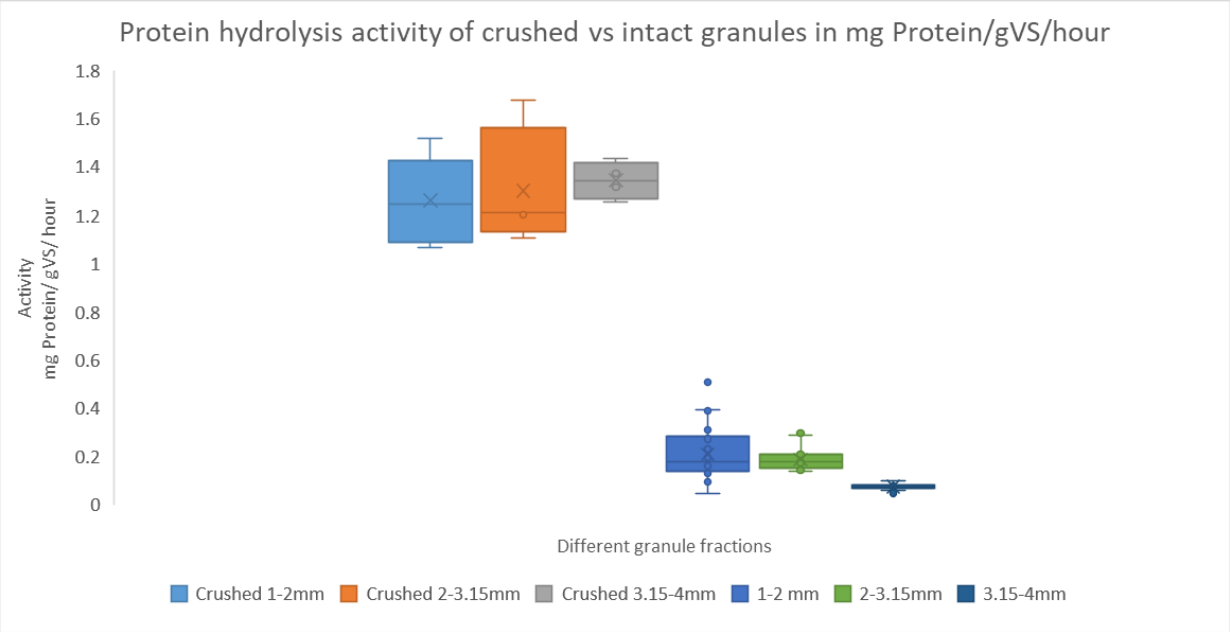

Figure 6 Measured proteolytic activities of intact granules vs their crushed fraction (per gVS)

The measured protein hydrolysis activities of the different crushed fractions is compared to the measured protein hydrolysis activity of flocs, in terms of mg Protein/g VS/hour, as presented in Figure 7. It is seen that the proteolytic activity of flocs per g VS is higher than that of crushed granules.

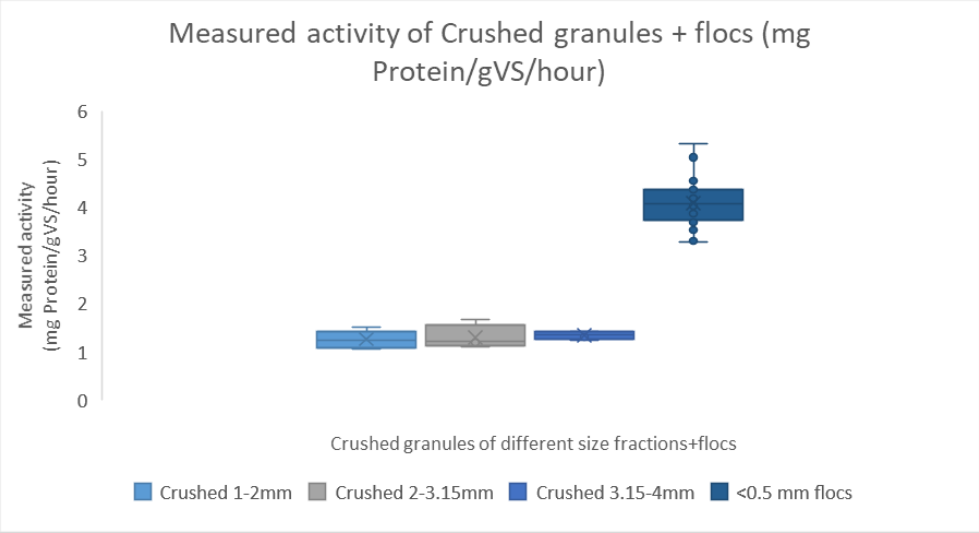

Figure 7 Measured proteolytic activities of crushed granules vs flocs (per g VS)

4.1.3 Discussion

The AGS reactors are composed of granules of different size ranges with the average size varying between 0.2 mm and 5 mm [17] [80]. In the past, studies have dealt with the effect of

granule diameter on the efficiency of nutrient removal attributing it to limited mass transfer [50], [24],[81]. This study will deal with the effect of granule diameter on the rate of hydrolysis of casein substrate. As discussed earlier (Section 2.2.3), the rate of hydrolysis of complex substrates is limited by the interaction between the substrate and the granular surface.

As the size of the granule increases, the surface area to volume ratio decreases. Thus, the smallest granules have the highest surface area to volume ratio. Considering that hydrolysis occurs at the surface of the granule in AGS (Section 2.2.3), it is hypothesized that, if all the granule fractions have the same organic matter (g VS) per unit volume; then the smaller granules with more available contact area will enable more amount of substrate to interact with the granule surface. Thus, the specific hydrolytic activity may decrease as the granule size increases.

In this experiment it is seen that the biomass density varies among the different granule sizes. The smallest granule with the highest specific surface area also has the highest weight to volume ratio. Thus, if the activity per volume is deduced, the above hypothesis may hold true. The specific proteolysis activity of the 3.15-4mm granules is the lowest, followed by the 2-3.15mm granules (Figure 4). But, on comparing the protein hydrolysis activity per gVS of the middle fraction (2-3.15 mm) with the smallest fraction (1-2mm), the difference is insignificant. This could be attributed to the difference in biomass density between the two fractions. Even though the 1-2mm fraction has higher specific surface area than the middle fraction, the middle fraction (2-3.15mm) has lower weight per volume than the 1-2mm fraction and therefore has higher surface area per g VS. Thus, the activity per g VS of 1-2mm fraction is lower than expected.

If all the granule sizes had the same organic matter (g VS) per volume, then the smallest granules with higher specific surface area will also have the highest surface area per g VS and therefore it is expected to also have the highest proteolytic activity per g VS. In this experiment however, it is seen that the organic matter varies with granule size (Table 1).

Although a clear trend in the size of the granules affecting the specific protein hydrolysis rate is not obtained, it is seen that the specific protein hydrolytic activity observed in the smallest granule(1-2mm) is at least 2 times higher than that observed in the biggest granule (3.15-4mm).

On comparing the specific protein hydrolysis activity per unit time of each intact granule size fraction and their corresponding crushed fraction (Figure 6), it is seen that the crushed fraction has a higher activity than the intact fractions. It seems that on crushing the granules, more volume of granule is exposed to the substrate, thereby enabling higher specific proteolytic activity. This probably means that there is a certain percentage of granule that has proteolytic activity but is not exposed to the substrate when the granule is intact. When the ratio of crushed proteolytic activity to intact proteolytic activity of each of the size fractions is compared, it is seen that the biggest size fraction has approximately 20 times more activity when crushed. However, for both 2-3.15 mm and 1-2 mm fractions, the increase in activity when crushed is very similar (approximately 10 times) (from Table 2). The higher increase in specific proteolytic activity in the biggest granules when compared to the smaller two fractions can be attributed to the increasing surface area to volume ratio with decreasing size (Table 1). The biggest granules have the smallest surface area to volume ratio and hence they have more volume that is inaccessible to the complex substrates. Therefore, when the biggest granules are crushed, the increase in specific protein hydrolysis activity is higher than that observed in the

smallest granules. When intact, the smallest granules have lesser volume that is inaccessible to the complex substrates, owing to their higher surface area to volume ratio. Thus, crushing the smallest granules does not increase its' specific hydrolytic activity that much. On the other hand, the increase in activity when crushed is similar for medium and small granules probably because even though the 1-2mm granules are smaller in size, they have higher biomass density (Table 1).

It was expected that the rate of hydrolysis per surface area of all the granules will be similar. However, there is a significant difference in the protein hydrolysis rate per SA of each of the granules. The increase in granule size is leading to a decrease in proteolytic activity per SA (Figure 5).One of the reasons for this could be that the surface area characteristics such as the microbial composition of different size ranges of granules may be different [69]. The microbial composition of different granules might be different, depending on their position in the reactor and their exposure to particulate substrates. Depending on the level of exposure to the substrates, the enzymatic production between various granule sizes may also vary. The difference in microbial compositions with the variation in size, is not explored and is beyond the scope of this research.
Apart from this hypothesis, it is difficult to explain why the biggest granules has less proteolytic activity (per SA) when compared to middle fraction.

The specific proteolytic activity of the crushed granules is compared to that of the flocs in Figure 7. It is seen that flocs have a much higher activity than the crushed granules. The structure of the flocs or lack there-off, provides it different enhanced capabilities to capture the particulate substrates [82]. Thus, justifying the fact that the protein hydrolysis activity is the highest in flocs.
Granules, on crushing, lose their structure as well and more percentage of the granule is exposed to the substrate, making it more similar to the flocs. Even then, the activity of the crushed granules is much less than the activity of the flocs. This means that the ability of the flocs to capture more substrates is not the only factor governing higher specific proteolytic activity. When the granules are intact, the microorganisms on the insides of the granules may not see particulate protein substrates and hence, they may not produce proteolytic enzymes. Therefore, when these granules are crushed, even though more of it is exposed to substrate, the potential proteolytic activity may remain the same and is lower than that of the flocs which often see such substrates in a reactor and consequently may be enriched with such enzymes [21] [57].

It is important to note that the overall proteolytic activity rates obtained in the fluorescent assay are less than what is seen in the following experiment. This is because substrate to sludge ratio (mg substrate available for hydrolysis/gVS) employed in this assay (9e-04) is at least 1000 times less than the substrate to sludge ratio employed in the following experiment (0.64), discussed below with casein dissolved particles. In a typical AGS reactor, with a sludge concentration of 8g/l and a domestic wastewater influent COD of 150 mg/l per reactor [56] at 30% exchange ratio, the substrate to sludge ratio is 0.02. The overall specific hydrolysis rate is underestimated in this assay and over-estimated in the next. Thus, the overall specific proteolytic rates in this assay are not representative of what is typically seen. This study only

analyzes the pattern or the shift in the proteolytic activity rates with changes in the size of the granule.

4.1.4 Summary

The specific proteolytic activity for three different granule sizes (3.15-4.mm, 2-3.15mm and 1-2mm) both in their intact and crushed forms and the specific protein hydrolysis of flocs was measured in a fluorescent assay. In an AGS system, considering that the hydrolysis is limited by substrate-surface interaction (Section 2.2.3), it was hypothesized that an increase in the granule size will lead to a decrease in the rate of hydrolysis. From the experiment, it is seen that the biggest fraction has the lowest specific hydrolysis rate and smallest granule fraction has the highest specific hydrolysis rate. However, the difference in the specific rate of protein hydrolysis between the middle (2-3.15mm) fraction and the smallest (1-2mm) fraction is insignificant. If the different granule sizes have the same biomass density, then the specific surface area and thus the specific hydrolysis activity is expected to increase with decrease in granule size, as can be seen in this experiment when the biggest and the smallest granules are compared. In this experiment, the biomass density is not obtained directly. In order to correlate the granule size with hydrolytic activity, the biomass density of the granule is an important factor. Thus, it is recommended that the granule density be measured using more accurate and direct methods, enabling to better correlate granule size to hydrolytic activity in AGS.

Earlier studies have confirmed the effect of the granule size on nutrient removal rates, owing it to increased mass transfer limitations with increase in granule size [24], [49]. Reduced particulate removal rates by biofilm systems is linked to limited active adsorption sites [83]. Mature AGS granules also have a smooth surface which does not offer many locations for attachment of particles in comparison to irregular fluffy biofilms or flocs [57]. Thus, limited surface-substrate interaction is observed in AGS granules which will potentially affect the rate of hydrolysis. This is further enhanced by the lack of ability of the granules to capture the particulates, as discussed in the previous chapter (Section 2.2.3).

In this study, the effect of granule size on the rate of hydrolysis of casein is observed. On comparing the proteolytic rates between the biggest and the smallest fraction, it is observed that there is a significant decrease in the rate of hydrolysis when the size range increases from 1-2mm to 3-4.15mm. However, the significance of such a change between closely related granular sizes is not completely evident in this study.

4.2 Phase 2: Studying the effect of protein substrate size on hydrolysis

The objective of this experiment is to understand how the rate of hydrolysis in aerobic granular sludge is affected by the size of protein substrate. The protein substrate used is casein. The casein substrate is divided into three different size fractions as casein big (CB), casein medium (CM) and casein small (CS). The different size ranges are described in the Table 3.

Fraction	Size range
Casein Big (CB)	>100 um
Casein Medium (CM)	100 um-0.45 um
Casein Small (CS)	<0.45 um (dissolved)

Table 3 Different substrate size ranges

In the experiment, the rate of hydrolysis is linked to the rate of anaerobic P-release. The anaerobic uptake of C-source by the PAOs occurs concomitantly with anaerobic P-release (as discussed under EBPR in the introduction). The proteins have to be first degraded before being taken up by the PAOs. This study focusses on the contribution of proteins to anaerobic EBPR process and using the P-release as an indicator for rate of hydrolysis of proteins will aid in indicating the fraction of protein being utilized by the PAOs alone. The experiment is designed such that the only C-source present will be casein. This casein has to be hydrolyzed first before the PAOs can take it up and release P (as discussed in the hydrolysis introduction section). Thus, the rate of anaerobic P-release observed in this experiment can be associated with the rate of hydrolysis of the casein substrate used. A blank assay under anaerobic conditions with aerobic granular sludge and tap water, without any other substrate, was used to take into account the residual P release in the sludge.

It is known that PAOs can easily assimilate simple VFAs such as acetate. Thereby, acetate induces the highest P-release among other substrates and the P release is reported to be approximately between 0.3-0.7 mmol P/g VSS [84] with molar P-release to C-uptake ratio for acetate reported to be approximately between 0.4 and 0.6 [78] [79]. Acetate is used as a positive control in this experiment to ensure that it induces sufficient anaerobic P-release in the sludge and also to be able to compare the activities of the sludge, when needed.

P-release obtained from casein hydrolysate will be analogous to that observed in the casein assays and hence casein hydrolysate is used as a second control. Since casein has to be first hydrolyzed before being taken up, the rate of P-release obtained directly from hydrolyzed casein will be higher than that obtained from the casein assay. The reported molar P-release to C-uptake ratio for casein hydrolysate is 0.35 [79]. The COD uptake by the sludge and the associated P-release obtained from the fully soluble casein hydrolysate assay is used to calculate the theoretical COD uptake in the casein assay, based on the observed anaerobic P-release.

4.2.1 Method

Preparation of the sludge, substrate solutions and the vials
The collected sludge is stored under anaerobic conditions (closed bottle). On the day of the experiment, we aerate the sludge for 30-40 minutes before the beginning of the assay.

The acetate assay, the casein hydrolysate assay and the three casein assays (for three different size ranges) are all done in triplicates amounting to a total of 11 vials (duplicate blanks included). All the assays are performed on the same day with the same sludge, in two sets. The granular sludge is sieved to achieve a granule size of 1-2mm. Known wet weight of granular sludge is added to each vial.

The three different size fractions of casein are achieved by stirring the casein substrate for different time intervals followed by sieving. A known amount of casein is first added to tap water and is subjected to stirring. After 10 minutes of stirring, the casein solution is filtered through a 100um sieve. The particles retained on the 100um sieve is the casein big (CB) fraction. The filtrate is collected and then stirred again for another 15 minutes and is filtered again through a 100um sieve and a 0.45um sieve kept in sequence. The particles retained on the 0.45um sieve is collected as the casein medium (CM) fraction. The casein small fraction (CS) is prepared by dissolving the casein particles by adding 5 g/l of sodium bicarbonate in tap

water. This is stirred for more than 60 minutes or until the particles become invisible to naked eyes. Each of the substrate solutions is buffered with TRIS-HCl at pH 7.8. Several trials were conducted in the lab to achieve the desired substrate sizes.

The initial measured COD of each of the triplicate substrate solutions is mentioned in Table 4 along with the volatile solids' concentration of the aerobic granular sludge in each of the vials.

Fraction	Influent COD (in mg/l)	VS (in g/l)
Acetate	580.3	5.4
Casein hydrolysate	517.0	4.7
Casein Big (CB)	2329.3	3.6
Casein Medium (CM)	1661.0	3.6
Casein Small (CS)	2328.7	3.4

Table 4 Influent characteristics

Each of the size ranges contained particles sizes beyond their strict limits. For example, the biggest fraction (CB) also contained particles smaller than 100um while fully dissolved CS fraction contained particles >5um as well. The COD of other size fractions within each size range is presented in Table 5.

Size fraction	COD in mg/l	% w.r.t the average total COD
Casein Big		
CB > 25 um	2162.3	92.8
CB 25-5um	53.0	2.3
CB 5-0.1 um	69.0	3.0
CB < 0.1 um	45.0	1.9
Casein Medium		
CM>25 um	675.0	40.6
CM 25- 5 um	357.0	21.5
CM 5-1 um	298.0	17.9
CM 1-0.1um	299.0	18.0
CM <0.1 um	32.0	1.9
Casein Small		
CS > 5um	564.7	24.2
CS 5-.1um	891.0	38.3
CS < 0.1 um	873.0	37.5

Table 5 COD of different size fractions within each size range

Once the required amount of sludge is weighed and added to each vial, the substrate solution is added one after the other. The sludge to substrate ratio in each vial is 1:3. The vial is closed. Bottles are flushed with N2 via the sampling tube for two minutes and the vial is kept on a shaker. This is subsequently done for all the vials. In the required time interval, samples are

taken using a syringe. The samples are filtered through a 0.45 um sieve during the sampling and they are collected in the 200 ul Eppendorf's. The phosphate concentrations and the COD concentrations in the samples are measured. The COD concentrations were measured using Hach Lange kit and the orthophosphate concentrations were measured in discrete analyzer. The anaerobic P-release activity calculated is obtained by taking the slope of all the linear points in the rate of P-release plot (Figure 8).

4.2.2 Results

Blanks & controls

The rate of P-release observed in the blank assay, in the acetate assay and in the casein hydrolysate assay is observed and presented in the appendix (Figure S 1). Two of the three casein hydrolysate vials were observed for close to 4 hours while one of them was observed for more than 24 hours. The average specific P-release activity observed in the acetate assay is 6.31 mg P/g VS/hour and that observed in the casein hydrolysate assay is 5.31 mg P/g VS/hour. The average P-release to COD uptake ratio obtained from the casein hydrolysate assay is 0.34 mgP/mgCOD.

Casein assay results

The increase in average P-release concentration with respect to time, in the vials containing the big(>100um), medium(100-0.45um) and small (dissolved, <0.45um) casein fractions is presented in Figure 8. It is seen that rate of P-release varies among the different fractions and it is seen that the smallest fraction has induced the most anaerobic P-release.

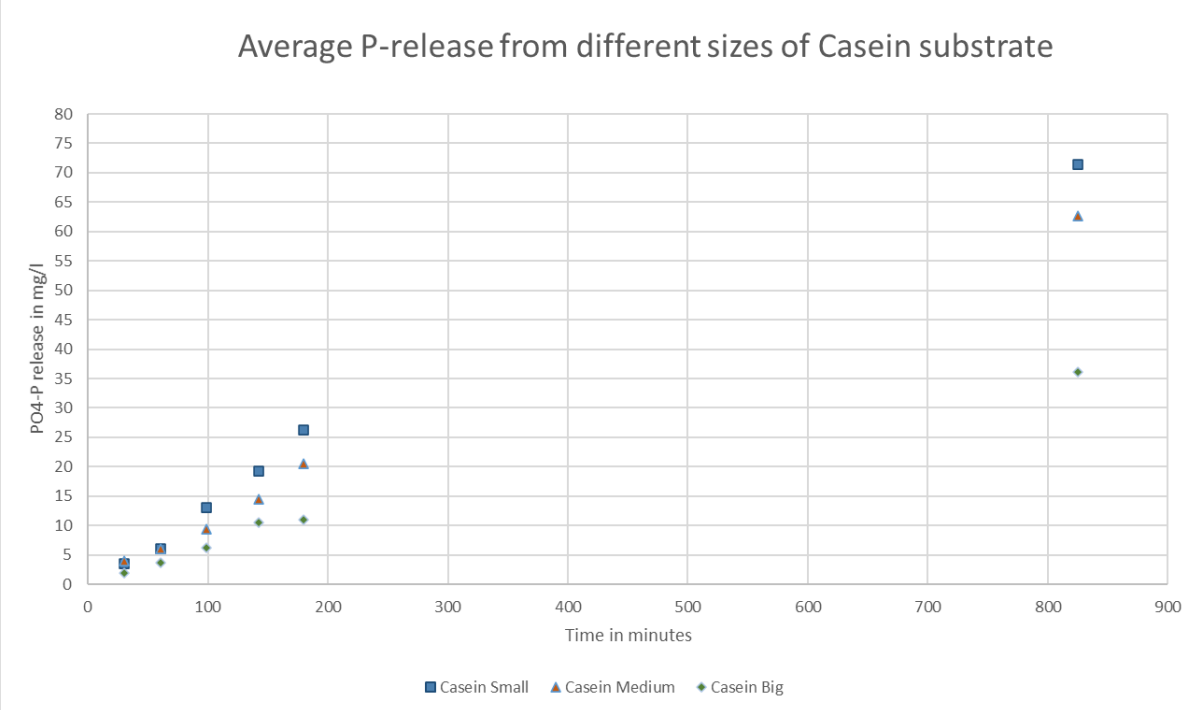

Figure 8 Rate of P-release due to different sizes of casein substrate.

The average P-release activity (in terms of mg P/ g VS/ hour) corresponding to different size ranges of casein substrate used, is presented in Figure 9. It is seen that the smallest casein

fraction has the highest specific activity, corresponding to the rate of P-release observed in Figure 9.

The comparison of the average P-release activity (in terms of mg P/ g VS/ hour) between each of the casein size ranges, acetate and casein hydrolysate assays, is presented in Figure 9. It is seen that among the casein assays, the smallest casein fraction has the highest specific activity, corresponding to the rate of P-release observed in Figure 9.

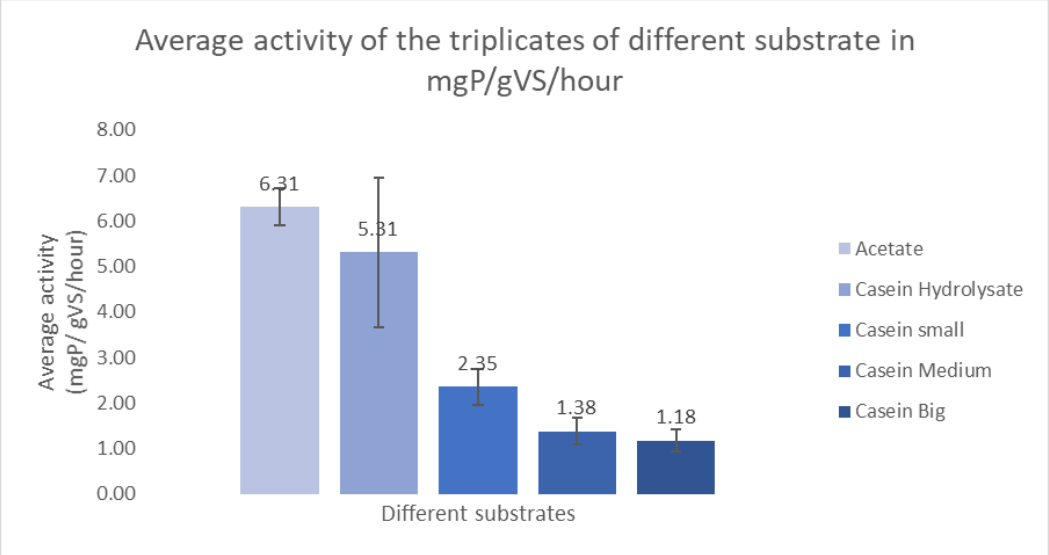

Figure 9 Comparison of P-release activity between different casein size ranges, casein hydrolysate and acetate.

As seen in Table 5, casein big and medium fractions also contained soluble casein. The soluble COD concentration was measured in the beginning of the assay, at 30 minutes, 1 hour 30 minutes and at 24 hours after the start of the assay. The average sCOD of CB, CM and CS fraction at each of the sampling points is presented in the Figure 10

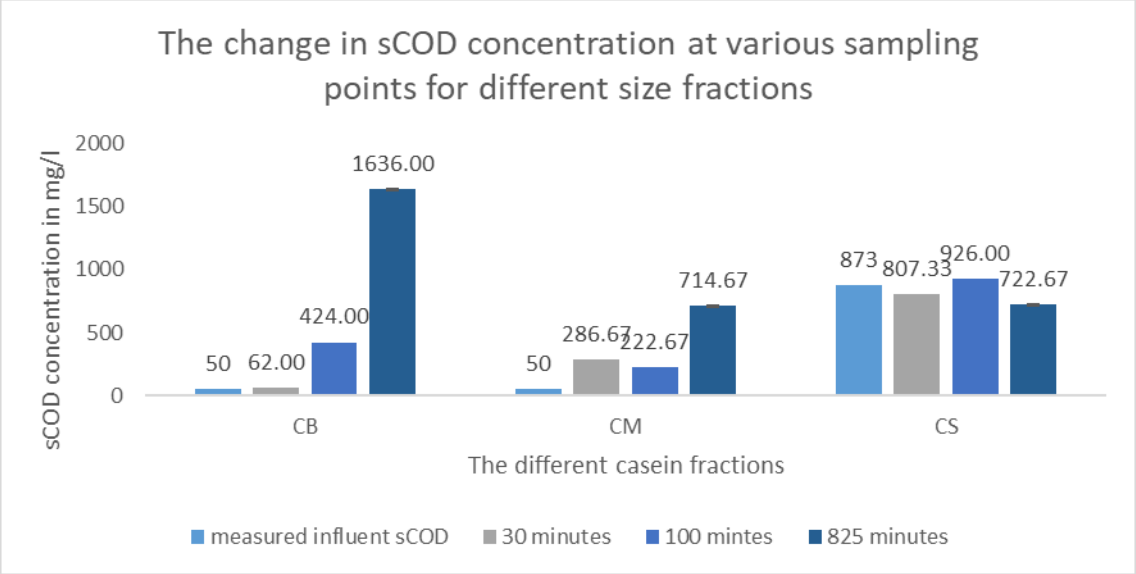

Figure 10 Change in sCOD concentration with time

The casein small fraction is also composed of 40% of particulates. Based on average P-release to COD uptake ratio observed in casein hydrolysate, the COD expected to be taken for the

maximum P-release observed in the casein small fractions is calculated in Table 6. This is compared to the actual soluble COD concentration that was measured at the point when there was maximum P-release. From the difference between the measured sCOD and the sCOD expected to be taken up (calculated based on the P:C ratio observed in the casein hydrolysate assay) it seems that there may have been more soluble COD uptake than what was actually measured.

	P release (mg/l) measured	Calculated(hypothetical) COD uptake in mg/l	Calculated(hypothetical) COD uptake in mgCOD/gVS	Measured sCOD uptake (mg/l)	Measured COD uptake in mgCOD/gVS
CS1 t6	74.13	175.5	54	214	61.5
CS2 t6	72.442	164	47	140	38
CS3 t6	67.776	161.2	47	136	38

Table 6 sCOD uptake- hypothetical vs measured.

4.2.3 Discussion

It is hypothesized that the rate of hydrolysis of particulate protein substrate decreases as the size of the substrate increases. In aerobic granular sludge reactors, one of the limitations of the hydrolysis of particulate substrates is the contact between the particulates and the surface of the granule. Thus, under limited substrate-surface contact, the rate of hydrolysis of particulate protein under anaerobic conditions is studied here. Further, the rate of hydrolysis of particulate protein affects the availability of substrate for PAOs which in turn affects the rate of anaerobic P-release. Thus, the rate of hydrolysis is linked to the rate of anaerobic P-release. The effect of particulate protein size on the rate of hydrolysis in AGS is studied in this section.

The concentration of P in the blanks was observed to be less than 1mg/l after more than 3 hours (Figure S 1)This ensures that the sludge used in the experiment, which was also acclimatized beforehand, did not have residual P-release.

Variation of P-release rates with respect to substrate size

When the rate of P-release in different size fractions of casein is compared, the rate of P-release from the biggest casein fraction (CB) is the slowest. On comparing the P-release after 24 hours from each of the size fractions, it is seen that the P concentration in the CS fraction is the highest (Figure 8). CS fraction also has higher specific P-release activity overall when compared to the other two fractions (Figure 8).

Thus, the smallest casein fraction induces higher P-release than the casein medium and casein big fractions at a specific rate close to 0.5 times higher than the other two. This means that the smallest fraction of casein is being hydrolyzed faster than the casein medium and the casein big fractions. The probable reason for this could be that the casein smallest fraction is mostly composed of <0.45 um size range, implying that they are mostly dissolved. In the case of dissolved substrates, the surface area of the substrate available for hydrolysis is not limited as opposed to the particulate fraction.

Occurrence of hydrolysis of particulates

In the case of particulate fraction, the surface of the substrate available for hydrolysis is limited. To assess the influence of this on the rate of hydrolysis, the activity of casein big and medium fractions composed of more than 60% of particulate substrates (Table 5), is compared. In this experiment, the difference in activities between the medium and the big fractions don't seem to be significant (Figure 9). For example, the P-release activity in CM3 is the same as that in CB3. However, because the influent COD concentration is very high in both cases, a clear conclusion cannot be drawn from this data. To elaborate, from Figure 10 we see that both CM and CB have close to 50 mg/l of dissolved casein COD. As seen earlier, the dissolved casein will be hydrolyzed faster than the particulate fraction and will become available for uptake by PAOs. It is difficult to categorize whether the P-release observed was induced by the uptake of hydrolysis products of the dissolved fraction or the particulate fraction. Thus, the effect of particle size on the rate of hydrolysis and subsequent uptake by PAOs cannot be deduced by comparing the results obtained from the big and medium fractions used in this experiment.

However, from the changes observed in the soluble COD concentrations (Figure 10), it is possible to deduce if the particulate proteins are being hydrolyzed or not. The change in the COD fractionation before the start of the assay and at the end of the assay for both casein small and medium fractions is represented in Figure 11. The COD taken up is calculated from the P release to COD uptake ratio observed in casein hydrolysate assay. It is seen that at the end of the assay, some of the COD is being taken up for P-release and that there is an increase in the soluble COD fraction in both the casein small and casein medium assays.

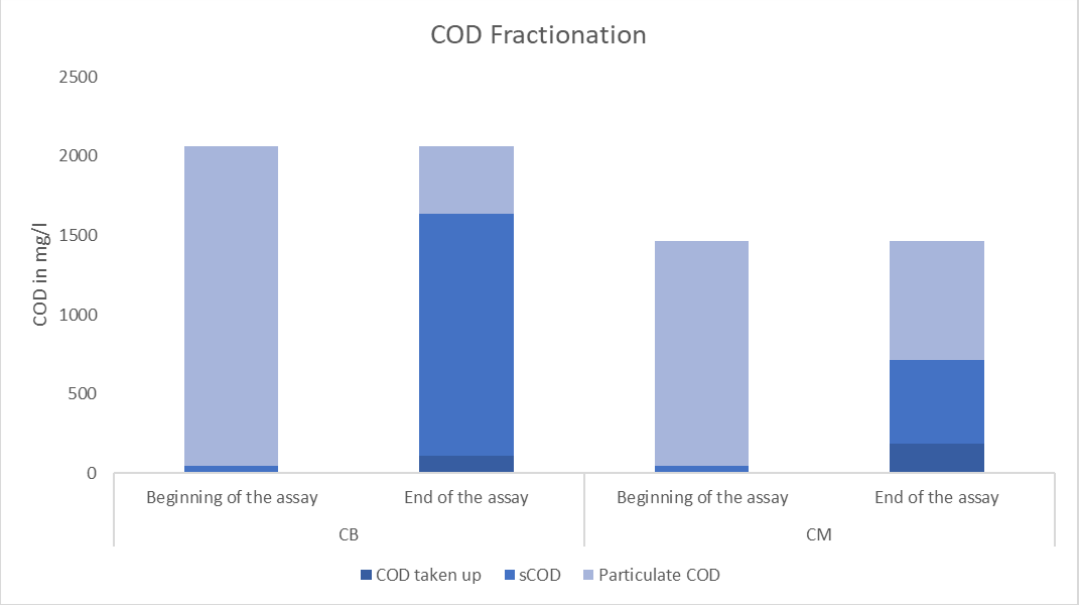

Figure 11 COD fractionation in CB and CM assays

The soluble COD concentration in the casein big fraction increases with time from 50mg/l in the beginning to 1636mg/l at the end of 24 hours. The substrate solution made of casein big fraction consisted of 2012 mgCOD/l of > 0.45um particles. Increase in sCOD concentration at the end of 24 hours implies that close to 80% of the casein particles have been hydrolyzed.

In the casein medium fraction, there is an overall increase in the sCOD concentration, even though the increase is not continuous. The sCOD concentration increases in the first 30 minutes and then decreases and then again increases at the end of 24 hours. The overall increase in sCOD from 50 mg/l to 714 mg/l can be attributed to hydrolysis of the particulates. It is seen that close to 60% of the particles >0.45 present in the casein medium fraction is being hydrolyzed. With the limited data, it is difficult to explain the decrease in sCOD after 30 minutes.

In the casein small fractions, it is seen that at the end of 24 hours, for the P-release observed, the sCOD concentrations expected to have been taken up by the PAOs is greater than the sCOD concentration that was actually measured. In other words, for the sCOD concentration measured, less P-release would have occurred than what is observed (Table 6). This implies that the sCOD taken up was more than what was measured. This excess sCOD had to come from the hydrolysis of the particulate fraction present in the casein small substrate solution. The sCOD concentration remained constant while there was C-uptake (P release), so exactly what was produced in hydrolysis seems to be taken up by the sludge. This could imply that the rate of hydrolysis and rate of C-uptake is more or less the same.

From closely analyzing the variation in the soluble COD concentration in the casein big, medium and small substrate assays, it is evident that the hydrolysis of particulate substrates in AGS does occur in 24 hours. However, the anaerobic feeding time in an AGS reactor is typically between 1-2 hours. Improvements to the experimental procedure that may aid in better deducing the rate of particulate hydrolysis is provided under the recommendations section. The changes in the sCOD concentration with time may probably indicate that the presence of significant amounts of easily hydrolysable dissolved fraction does not inhibit the hydrolysis of the particulate fraction.

The average specific P-release activity observed in the casein small fraction consisting of mostly dissolved casein substrates is 2.4 mgP/gVS/hour (Figure 9). The anaerobic P-release rates observed in full scale biological nutrient removal plants is typically observed to be between 4.4 and 18.8 mgP/gVS/hour [8]. The average P-release to COD uptake ratio for casein hydrolysate observed in this experiment is 0.345 mgP/mgCOD which is in accordance with what was observed by [77]. Based on this ratio, the COD uptake rate would be 6.82 mgCOD/gVS/hour. Based on this, in a reactor with 8g/l sludge concentration, the soluble casein COD taken up potentially in one hour would be around 55 mgCOD/l. The average influent COD concentration in domestic wastewater is 500mg/l [56]. If an exchange ratio of 30% is taken into account, the influent COD per reactor would be 150 mg/l out of which 40% would be composed of proteins [85]. Thus around 90% of the influent proteins COD, can be potentially be taken up by the PAOs for P-removal in a one-hour anaerobic feeding time.

4.2.4 Summary

The effect of the size of casein substrate on the rate of hydrolysis in AGS is analyzed here. The rate of hydrolysis is associated with the specific anaerobic P-release activity because the main substrate solution in the assays is casein and for the PAOs to produce P anaerobically, these casein substrates have to be hydrolyzed and then taken up. It was seen that both acetate assays and casein hydrolysate assays induced higher specific P-release activity than casein Figure 9,

proving that the rate of hydrolysis limits the availability of casein substrate for uptake by PAOs. It was seen that the smallest casein fraction, which was composed mostly of <0.45 um particles, induced higher specific P-release activity when compared to the big casein and medium casein fractions. The high soluble COD concentrations in the substrate solutions used in this experiment did not allow the analysis of the effect of particle size on the rate of hydrolysis of proteins in AGS. However, based on the accumulation of soluble COD in the casein small and big fractions, it seems that the presence of soluble COD does not inhibit the hydrolysis of particulate fraction. Based on the specific P-release activity obtained in this experiment, it is seen that around 90% of the influent protein COD may be potentially taken up by the PAOs under anaerobic condition, provided that they are fully dissolved.

Chapter 4 Conclusions & Recommendations

This study assessed the anaerobic availability of protein substrates for enhanced biological phosphorous removal in AGS, identifying key factors governing hydrolysis and amino acid uptake on AGS. The main conclusions of the study were

1) Anaerobic P-release is observed in AGS when proteins are the only available C-source, implying that proteins are potential PAO substrates. As expected, the anaerobic P-release activity (measured in mgP/gVS/hour) is lower in casein when compared to acetate.
2) Hydrolysis of proteins in AGS

- The literature study performed in this thesis, as well as the laboratory experiments conducted indicate that the hydrolysis rate is affected by the size of the particulate substrates. In anaerobic digestion, decrease in particulate substrate size from 500 um to 50 um leading to a seven-fold increase in the rate of hydrolysis has been reported in literature. The laboratory experiments performed with aerobic granular sludge also show that out of the three casein size ranges used, the smallest casein fraction induced the highest specific P-release activity implying that the rate of hydrolysis of proteins in AGS is affected by the size of the substrate.
- At the end of 24 hours, it was seen that close to 86% of the particulates in the casein big fraction (3.15-4mm) and close to 60% of the particulates in the casein medium fraction (2-3.15mm) was hydrolysed. The accumulation of soluble COD at the end of 24 hours in the casein assays implies that the hydrolysis of particulate substrates in AGS does occur. The anaerobic feeding time in an AGS reactor is typically 1-2 hours and to understand if hydrolysis of particulate proteins occurs within this time, improvements in the experimental procedure are recommended.
- When the specific P-release activity and the specific COD uptake rate observed for dissolved casein used in this experiment is compared to a reactor treating domestic wastewater with a sludge concentration of 8g/l, it is seen that close to 90% of the influent protein COD can be potentially taken up by the PAOs for P-removal in a one-hour feeding time, provided that the protein COD is fully dissolved. This implies that proteins may potentially be a promising substrate for EBPR process. However, hydrolysis of bigger particulate substrates may take longer and thus if the influent wastewater contains significant amount of particulate proteins, introducing a longer anaerobic phase in an AGS reactor may ensure increased contribution of proteins to the EBPR process.
- The effect of granule size on the rate of hydrolysis is observed and it is seen that there is a significant decrease in the rate of hydrolysis of casein when the size of the granule increases from 1-2mm to 3-4.15mm. However, the significance of such a change between closely related granular sizes is not completely evident in this study.
- It was expected that all the granules will have similar hydrolytic activity per surface area. However, it was seen that the biggest granule had the least surface-area related hydrolysis activity. It is hypothesized that the observed difference in activity per surface area may be attributed to the difference in microbial composition of the different granule sizes.

- It is seen that the crushed granules have higher specific proteolysis activity than their corresponding intact granules. While all the crushed fractions have similar proteolytic activity, it is seen that the increase in specific hydrolysis activity when crushed is the highest for the biggest granule. This may imply that the large granules have more volume that is inaccessible to the protein substrates which leads to a higher increase in activity when the granules are crushed. Thus, the diffusion limitation is more pronounced in a reactor with large granules where the hydrolytic activity may be compromised due to inaccessibility of the complex substrates into the deeper layers of the granule. It could also mean that in an AGS reactor with mixed granule sizes, the protein degradation may occur more in some places in the reactor than others leading to localization of proteolysis within the granular sludge bed
- It is seen that the flocs have the highest specific proteolytic activity despite the fact that both the flocs and the crushed granules have enhanced capabilities to capture particulates owing to increased exposure to substrate due to their loose structure. This can be attributed to the fact that the flocs which often see complex substrates in a reactor may consequently be enriched with hydrolytic enzymes when compared to granules whose deeper layers does not often interact with complex substrates due to diffusion limitation. Thus, on crushing the granules, even though more of the granule is exposed to the substrate, the potential proteolytic activity may remain the same and is lower than that of the flocs which often see such substrates in a reactor.
- Based on the critical evaluation of literature, it seems factors such as the type of protein substrates, the diffusion limitation of the granule and the location of hydrolytic enzymes in a granule regulate the site of protein hydrolysis in aerobic granular sludge. Thus, the hydrolysis of proteins in AGS is governed by substrate-granule interaction. However, the comparative effect of the granule size and the substrate size on the rate of hydrolysis remains to be studied. The data obtained from the experiments is insufficient to analyse whether the granule size or the substrate size has a higher effect on the hydrolysis rate in AGS. This may be achieved with some improvements in the experimental procedure.
- Models generally used to describe hydrolysis of particulate substrates in activated sludge systems, such as the surface-based kinetics (SBK) model and the particle break-up model (PBM) assume that the particulates are completely covered by hydrolytic microorganisms. However, this assumption may not hold true in an AGS system wherein additional factors have to be taken into account than in the other treatment technologies because of the limited substrate-sludge contact. Thus, neither of these models may fully be able to describe the hydrolysis process in an AGS system.

3) <u>Utilization of amino acids by PAOs</u>

- Based on critical evaluation of literature, it is seen that there are two ways in which protein hydrolysates – amino acids, may be used as an anaerobic C-source for EBPR process. They are either directly taken up by the PAOs or they are first degraded to simple VFAs which are in turn taken up by the PAOs.
- It is seen that both *Tetrasphaera* and certain clades of *Accumulibacter* PAOs have the ability to directly take up a few amino acids. *Tetrasphaera* PAOs have the additional ability to ferment the amino acids that they assimilate. Direct uptake of amino acids by PAOs seems to yield lower P-release to C-uptake ratio in comparison to the uptake of simple VFA which in turn means less net phosphorous that will have to be taken up

aerobically by PAOs. However, the uptake rate of amino acids by PAOs is lower than the rate of uptake of acetate. Thus, even though PAOs can directly assimilate amino acids and result in increased aerobic P-removal, the limited uptake rate of amino acids by PAOs may result in the accumulation of amino acids in the anaerobic phase of an AGS reactor. The fate of the excess amino acids is not exactly known; they may be taken up by the GAOs or they may be degraded to simpler VFAs.

- The anaerobic degradation of amino acids may occur via two very well-known pathways- the Stickland and the non-Stickland pathway. These two reactions are well-established in degradation of amino acids in anaerobic digestion. The degradation of amino acids via both these pathways seems less likely to occur in AGS considering that the bacteria responsible (*Clostridium)* are obligate anaerobes and if at all present, they are likely to be at the core of the granule whereas the hydrolysis of proteins is likely to occur at the granule surface. Besides, the relative abundance of *Clostridial* bacteria in AGS granules is very little (<0.02) and thus, it seems even less likely to occur in aerobic granular sludge.

Bibliography

[1] Y. V Nancharaiah, M. Sarvajith, and T. V. K. Mohan, "Aerobic granular sludge : the future of wastewater treatment."

[2] M. Pronk, M. K. de Kreuk, B. de Bruin, P. Kamminga, R. Kleerebezem, and M. C. M. van Loosdrecht, "Full scale performance of the aerobic granular sludge process for sewage treatment," *Water Res.*, vol. 84, no. JULY, pp. 207–217, 2015, doi: 10.1016/j.watres.2015.07.011.

[3] S. Bengtsson *et al.*, "Technology Treatment of municipal wastewater with aerobic granular sludge Treatment of municipal wastewater with aerobic granular sludge," vol. 3389, 2018, doi: 10.1080/10643389.2018.1439653.

[4] A. Mosquera-corral and J. M. Garrido, "Aerobic granulation with industrial wastewater in sequencing batch reactors," vol. 38, pp. 3389–3399, 2004, doi: 10.1016/j.watres.2004.05.002.

[5] S. M. L. Stubbé and D. Millman, "The Fate of Phosphate in Full-scale Aerobic Granular Sludge Systems."

[6] C. Feng, L. Welles, X. Zhang, M. Pronk, D. de Graaff, and M. van Loosdrecht, "Stress-induced assays for polyphosphate quantification by uncoupling acetic acid uptake and anaerobic phosphorus release," *Water Res.*, vol. 169, p. 115228, 2020, doi: 10.1016/j.watres.2019.115228.

[7] T. Mino, M. C. M. V. A. N. Loosdrecht, and J. J. Heijnen, "Microbiology and Biochemistry of Enhanced Biological Phosphate Removal Process," vol. 32, no. 11, 1998.

[8] J. Drewnowski and J. Makinia, "The role of biodegradable particulate and colloidal organic compounds in biological nutrient removal activated sludge systems," *Int. J. Environ. Sci. Technol.*, vol. 11, no. 7, pp. 1973–1988, 2014, doi: 10.1007/s13762-013-0402-1.

[9] N. Schwarzenbeck, R. Erley, and P. A. Wilderer, "Aerobic granular sludge in an SBR-system treating wastewater rich in particulate matter," *Water Sci. Technol.*, vol. 49, no. 11–12, pp. 41–46, 2004, doi: 10.2166/wst.2004.0799.

[10] J. Wagner, D. G. Weissbrodt, V. Manguin, R. H. Ribeiro da Costa, E. Morgenroth, and N. Derlon, "Effect of particulate organic substrate on aerobic granulation and operating conditions of sequencing batch reactors," *Water Res.*, vol. 85, pp. 158–166, 2015, doi: 10.1016/j.watres.2015.08.030.

[11] N. Schwarzenbeck, J. M. Borges, and P. A. Wilderer, "Treatment of dairy effluents in an aerobic granular sludge sequencing batch reactor," *Appl. Microbiol. Biotechnol.*, vol. 66, no. 6, pp. 711–718, 2005, doi: 10.1007/s00253-004-1748-6.

[12] Y. Yao and A. Science, "Use of Carbohydrate , Protein and Fat to Characterise Wastewater in Terms of its Major Elemental Constituents and Energy," 2014.

[13] A. Guellil, M. Boualam, H. Quiquampoix, P. Ginestet, J. M. Audic, and J. C. Block, "Hydrolysis of wastewater colloidal organic matter by extracellular enzymes extracted from activated sludge flocs," in *Water Science and Technology*, 2001, vol. 43, no. 6, pp. 33–40, doi: 10.2166/wst.2001.0334.

[14] X. L. T. KF Janning, “Hydrolysis of Organic Wastewate particles in laboratory scale and pilot scale biofilm reactors under anoxic and aerobic conditions,” *Elsevier*, 1998.

[15] A. D. Levine, G. Tchobanoglous, and T. Asano, “Size distributions of particulate contaminants in wastewater and their impact on treatability,” *Water Res.*, vol. 25, no. 8, pp. 911–922, 1991, doi: 10.1016/0043-1354(91)90138-G.

[16] P. H. Nielsen *et al.*, “A conceptual ecosystem model of microbial communities in enhanced biological phosphorus removal plants,” vol. 4, 2010, doi: 10.1016/j.watres.2010.07.036.

[17] N. Derlon, J. Wagner, R. Helena, and E. Morgenroth, “Formation of aerobic granules for the treatment of real and low-strength municipal wastewater using a sequencing batch reactor operated at constant volume,” *Water Res.*, vol. 105, pp. 341–350, 2016, doi: 10.1016/j.watres.2016.09.007.

[18] M. K. de Kreuk, N. Kishida, S. Tsuneda, and M. C. M. van Loosdrecht, “Behavior of polymeric substrates in an aerobic granular sludge system,” *Water Res.*, vol. 44, no. 20, pp. 5929–5938, 2010, doi: 10.1016/j.watres.2010.07.033.

[19] E. Dulekgurgen, S. Ovez, N. Artan, and D. Orhon, “Enhanced Biological Phosphorus Removal by Granular Sludge in a Sequencing Batch Reactor Enhanced biological phosphate removal by granular sludge in a,” no. June, 2003, doi: 10.1023/A.

[20] Q. Wang *et al.*, “Bioresource Technology Aerobic granules cultivated with simultaneous feeding / draw mode and low- strength wastewater : Performance and bacterial community analysis,” *Bioresour. Technol.*, vol. 261, no. March, pp. 232–239, 2018, doi: 10.1016/j.biortech.2018.04.002.

[21] M. Layer, A. Adler, E. Reynaert, A. Hernandez, M. Pagni, and E. Morgenroth, “Organic substrate diffusibility governs microbial community composition , nutrient removal performance and kinetics of granulation of aerobic granular sludge,” *Water Res. X*, vol. 4, p. 100033, 2019, doi: 10.1016/j.wroa.2019.100033.

[22] D. R. Confer and B. E. Logan, “Molecular weight distribution of hydrolysis products during the biodegradation of model macromolecules in suspended and biofilm cultures. II. Dextran and dextrin,” *Water Res.*, vol. 31, no. 9, pp. 2137–2145, 1997, doi: 10.1016/S0043-1354(97)00050-X.

[23] S. S. Adav, D. Lee, and J. Lai, “Proteolytic activity in stored aerobic granular sludge and structural integrity,” vol. 100, pp. 68–73, 2009, doi: 10.1016/j.biortech.2008.05.045.

[24] Y. Liu, Y. Liu, and J. Tay, “Relationship between size and mass transfer resistance in aerobic granules,” pp. 312–315, 2005, doi: 10.1111/j.1472-765X.2005.01695.x.

[25] J. A. Jimenez, E. J. La Motta, D. S. Parker, J. A. Jimenez, E. J. La Motta, and D. S. Parker, “Kinetics Chemical of Removal Oxygen of Particulate in the Demand Process,” vol. 77, no. 5, pp. 437–446, 2015, doi: 10.2175/106143005X67340.

[26] J. a Eastman and J. F. Ferguson, “Solubilization organic phase of of carbon anaerobic particulate during the digestion acid,” *J. (Water Pollut. Control Fed.*, vol. 53, no. 3, pp. 352–366, 1981.

[27] T. Mino, “Estimation of rate of SBCOD hydrolysis under different e acceptor conditions,” 1995.

[28] V. A. Vavilin, B. Fernandez, J. Palatsi, and X. Flotats, “Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview,” *Waste Manag.*, vol. 28, no.

6, pp. 939–951, 2008, doi: 10.1016/j.wasman.2007.03.028.

[29] 2 Ian R. Ramsay1 & Pratap C. Pullammanappallil∗, 1, "Protein degradation during anaerobic wastewater treatment: derivation of stoichiometry," *Entomol. Exp. Appl.*, vol. 103, no. 3, pp. 239–248, 2002, doi: 10.1023/A.

[30] Y. Ubukata, "KINETICS AND FUNDAMENTAL MECHANISMS OF PROTEIN REMOVAL BY ACTIVATED SLUDGE : HYDROLYSIS OF PEPTONE TO AMINO ACIDS IS THE RATE-DETERMINING STEP," *Water Sci. Technol.*, vol. 38, no. 8–9, pp. 121–128, 1998, doi: 10.1016/S0273-1223(98)00685-4.

[31] E. Morgenroth, R. Kommedal, and P. Harremoës, "Processes and modeling of hydrolysis of particulate organic matter in aerobic wastewater treatment - A review," in *Water Science and Technology*, 2002, vol. 45, no. 6, pp. 25–40, doi: 10.2166/wst.2002.0091.

[32] B. A. Boczar, W. M. Begley, R. J. Larson, B. A. Boczar, W. M. Begley, and R. J. Larson, "Characterization of enzyme activity in activated sludge using rapid analyses for specific hydrolases," vol. 64, no. 6, pp. 792–797, 1992.

[33] E. Giraldo-Gomez, "Kinetics of anaerobic treatment: A critical review," *Crit. Rev. Environ. Control*, vol. 21, no. 5–6, pp. 411–490, 1991, doi: 10.1080/10643389109388424.

[34] P. N. Hobson, "A model of some aspects of microbial degradation of particulate substrates," *J. Ferment. Technol.*, vol. 65, no. 4, pp. 431–439, 1987, doi: 10.1016/0385-6380(87)90140-3.

[35] W. T. M. Sanders, M. Geerink, G. Zeeman, and G. Lettinga, "Anaerobic hydrolysis kinetics of particulate substrates," *Water Sci. Technol.*, vol. 41, no. 3, pp. 17–24, 2000, doi: 10.2166/wst.2000.0051.

[36] R. Goel, T. Mino, H. Satoh, and T. Matsuo, "Comparison of hydrolytic enzyme systems in pure culture and activated sludge under different electron acceptor conditions," in *Water Science and Technology*, 1998, doi: 10.1016/S0273-1223(98)00126-7.

[37] W. T. Sanders, G. Zeeman, and G. Lettinga, "Hydrolysis kinetics of dissolved polymer substrates.," *Water Sci. Technol.*, vol. 45, no. 10, pp. 99–104, 2002, doi: 10.2166/wst.2002.0301.

[38] D. Okutman, S. Övez, and D. Orhon, "Hydrolysis of settleable substrate in domestic sewage," *Biotechnol. Lett.*, vol. 23, no. 23, pp. 1907–1914, 2001, doi: 10.1023/A:1013737901624.

[39] D. Orhon, D. Okutman, and G. Insel, "Characterisation and biodegradation of settleable organic matter for domestic wastewater," *Water SA*, vol. 28, no. 3, pp. 299–305, 2002, doi: 10.4314/wsa.v28i3.4898.

[40] S. Aldin, "The effect of particle size on hydrolysis and modeling of anaerobic digestion," no. Electronic Thesis and Dissertation Repository, p. 60, 2010.

[41] R. Dimock and E. Morgenroth, "The influence of particle size on microbial hydrolysis of protein particles in activated sludge," *Water Res.*, vol. 40, no. 10, pp. 2064–2074, 2006, doi: 10.1016/j.watres.2006.03.011.

[42] B. Kayranli and A. Ugurlu, "Effects of temperature and biomass concentration on the performance of anaerobic sequencing batch reactor treating low strength wastewater," *Desalination*, vol. 278, no. 1–3, pp. 77–83, 2011, doi: 10.1016/j.desal.2011.05.011.

[43] S. Uemura and H. Harada, “Treatment of sewage by a UASB reactor under moderate to low temperature conditions,” *Bioresour. Technol.*, vol. 72, no. 3, pp. 275–282, 2000, doi: 10.1016/S0960-8524(99)00118-2.

[44] W. Sanders, *Anaerobic hydrolysis digestion of complex substrates*. 2001.

[45] A. M. Breure, J. G. van Andel, T. Burger-Wiersma, J. Guijt, and J. Verkuijlen, “Hydrolysis and acidogenic fermentation of gelatin under anaerobic conditions in a laboratory scale upflow reactor,” *Appl. Microbiol. Biotechnol.*, vol. 21, no. 1–2, pp. 50–54, 1985, doi: 10.1007/BF00252361.

[46] A. Palenzuela Rollón, “Anaerobic Digestion of Fish Processing Wastewater with Special Emphasis on Hydrolysis of Suspended Solids,” p. 123, 1999.

[47] M. Henze, W. Gujer, T. Mino, and M. van Loosedrecht, “Activated Sludge Models ASM1, ASM2, ASM2d and ASM3,” *Water Intell. Online*, vol. 5, no. 0, pp. 9781780402369–9781780402369, 2015, doi: 10.2166/9781780402369.

[48] P. Jabari, Q. Yuan, and J. A. Oleszkiewicz, “Potential of Hydrolysis of Particulate COD in Extended Anaerobic Conditions to Enhance Biological Phosphorous Removal,” doi: 10.1002/bit.25999.

[49] J. Tay, S. T. Tay, V. Ivanov, S. Pan, H. Jiang, and Q. Liu, “Biomass and porosity profiles in microbial granules used for aerobic wastewater treatment,” pp. 297–301, 2003.

[50] C. Wu, Y. Peng, S. Wang, and Y. Ma, “Enhanced biological phosphorus removal by granular sludge : From macro- to micro-scale,” *Water Res.*, vol. 44, no. 3, pp. 807–814, 2010, doi: 10.1016/j.watres.2009.10.028.

[51] Fr¢lund, T. Griebe, and P. H. Nielsen, “Enzymatic activity in the activated-sludge floc matrix,” no. January, pp. 755–761, 1995.

[52] R. Goel, T. Mino, H. Satoh, and T. Matsuo, “Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor,” *Water Res.*, vol. 32, no. 7, pp. 2081–2088, 1998, doi: 10.1016/S0043-1354(97)00425-9.

[53] D. R. Confer and B. E. Logan, “Location of protein and polysaccharide hydrolytic activity in suspended and biofilm wastewater cultures,” *Water Res.*, vol. 32, no. 1, pp. 31–38, 1998, doi: 10.1016/S0043-1354(97)00194-2.

[54] Confer & Logan, “A conceptual model describing macromolecule degradation by suspended cultures and biofilms.,” 1998.

[55] R. Goel, T. Mino, H. Satoh, and T. Matsuo, “EFFECT OF ELECTRON ACCEPTOR CONDITIONS ON HYDROLYTIC ENZYME SYNTHESIS IN BACTERIAL CULTURES,” vol. 31, no. 10, pp. 2597–2603, 1997.

[56] M. Pronk, M. K. de Kreuk, B. de Bruin, P. Kamminga, R. Kleerebezem, and M. C. M. van Loosdrecht, “Full scale performance of the aerobic granular sludge process for sewage treatment,” *Water Res.*, vol. 84, pp. 207–217, 2015, doi: 10.1016/j.watres.2015.07.011.

[57] M. Layer, K. Bock, F. Ranzinger, H. Horn, E. Morgenroth, and N. Derlon, “Particulate substrate retention in plug-flow and fully-mixed conditions during operation of aerobic granular sludge systems,” *Water Res. X*, vol. 9, p. 100075, 2020, doi: 10.1016/j.wroa.2020.100075.

[58] F. Ranzinger *et al.*, “Transport and retention of arti fi cial and real wastewater particles inside a bed of settled aerobic granular sludge assessed applying magnetic resonance

imaging," *Water Res. X*, vol. 7, p. 100050, 2020, doi: 10.1016/j.wroa.2020.100050.

[59] M. Ali *et al.*, "Importance of species sorting and immigration on the bacterial assembly of different-sized aggregates in a full-scale Aerobic granular sludge plant," *Environ. Sci. Technol.*, vol. 53, no. 14, pp. 8291–8301, 2019, doi: 10.1021/acs.est.8b07303.

[60] A. Adler and C. Holliger, "Multistability and Reversibility of Aerobic Granular Sludge Microbial Communities Upon Changes From Simple to Complex Synthetic Wastewater and Back," *Front. Microbiol.*, vol. 11, no. November, pp. 1–20, 2020, doi: 10.3389/fmicb.2020.574361.

[61] A. Muszyński and A. Miłobędzka, "The effects of carbon/phosphorus ratio on polyphosphate- and glycogen-accumulating organisms in aerobic granular sludge," *Int. J. Environ. Sci. Technol.*, vol. 12, no. 9, pp. 3053–3060, 2015, doi: 10.1007/s13762-015-0828-8.

[62] H. T. T. Nguyen, R. Kristiansen, M. Vestergaard, R. Wimmer, and P. H. Nielsen, "Intracellular accumulation of glycine in polyphosphate-accumulating organisms in activated sludge, a novel storage mechanism under dynamic anaerobic-aerobic conditions," *Appl. Environ. Microbiol.*, vol. 81, no. 14, pp. 4809–4818, 2015, doi: 10.1128/AEM.01012-15.

[63] R. Kristiansen *et al.*, "A metabolic model for members of the genus Tetrasphaera involved in enhanced biological phosphorus removal," *ISME J.*, vol. 7, no. 3, pp. 543–554, 2013, doi: 10.1038/ismej.2012.136.

[64] A. C. Mchardy *et al.*, "Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities," vol. 24, no. 10, pp. 1263–1269, 2006, doi: 10.1038/nbt1247.

[65] H. A. Barker, "Amino acid degradation by anaerobic bacteria," pp. 23–40, 1981.

[66] E. Doktorgrades, "Enzymes of two clostridial amino-acid fermentation pathways," 2008.

[67] J. Park, S. Park, and M. Kim, "Anaerobic degradation of amino acids generated from the hydrolysis of sewage sludge," *Environ. Technol. (United Kingdom)*, vol. 35, no. 9, pp. 1133–1139, 2014, doi: 10.1080/09593330.2013.863951.

[68] Barker HA, "Fermentation of nitrogenous organic compounds.," *Bact. Acad. Press. New York, Gansalus IC Stanier RY*, vol. Vol. 2, p. (pp 151– 207)., 1961.

[69] M. Ali *et al.*, "Importance of species sorting and immigration on the bacterial assembly of different-sized aggregates in a full-scale Aerobic granular sludge plant," *Environ. Sci. Technol.*, vol. 53, no. 14, pp. 8291–8301, 2019, doi: 10.1021/acs.est.8b07303.

[70] L. H. Stickland, "Studies in the metabolism of the strict anaerobes (genus Clostridium)," *Biochem. J.*, vol. 28, no. 5, pp. 1746–1759, 1934, doi: 10.1042/bj0281746.

[71] J. Winter, F. Schindler, and F. X. Wildenauer, "Fermentation of alanine and glycine by pure and syntrophic cultures of Clostridium sporogenes," vol. 45, pp. 153–161, 1987.

[72] N. Fonknechten *et al.*, "Clostridium sticklandii, a specialist in amino acid degradation:Revisiting its metabolism through its genome sequence," *BMC Genomics*, vol. 11, no. 1, 2010, doi: 10.1186/1471-2164-11-555.

[73] C. Lane and F. Nor, "The Amino Acid-fermenting Clostridia," vol. 2, pp. 47–56.

[74] B. Nisman, "THE STICKLAND REACTION," no. 10, pp. 16–42.

[75] T. Nagase, M; Matsuo, "Interactions between amino-acid-degrading bacteria and methanogenic bacteria in anaerobic digestion," *Biotechnol. Bioeng.; (United States)*, vol. Journal Vo, doi: https://doi.org/10.1002/bit.260241009.

[76] Y. Kong, J. L. Nielsen, and P. H. Nielsen, "Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants," *Appl. Environ. Microbiol.*, vol. 71, no. 7, pp. 4076–4085, 2005, doi: 10.1128/AEM.71.7.4076-4085.2005.

[77] R. Marques *et al.*, "Metabolism and ecological niche of Tetrasphaera and Ca. Accumulibacter in enhanced biological phosphorus removal," *Water Res.*, vol. 122, pp. 159–171, 2017, doi: 10.1016/j.watres.2017.04.072.

[78] G. Qiu *et al.*, "Metabolic Traits of Candidatus Accumulibacter clade IIF Strain SCELSE-1 Using Amino Acids As Carbon Sources for Enhanced Biological Phosphorus Removal," *Environ. Sci. Technol.*, vol. 54, no. 4, pp. 2448–2458, 2020, doi: 10.1021/acs.est.9b02901.

[79] G. Qiu, R. Zuniga-montanez, Y. Law, and S. Swa, "Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources," no. November, 2018, doi: 10.1016/j.watres.2018.11.011.

[80] X. Liu, H. Yu, B. Ni, and G. Sheng, "Characterization , Modeling and Application of Aerobic Granular Sludge for Wastewater," doi: 10.1007/10.

[81] Z. Li, Y. Zhu, Y. Zhang, Y. Zhang, and C. He, "Characterization of aerobic granular sludge of different sizes for nitrogen and phosphorus removal," *Environ. Technol.*, vol. 0, no. 0, pp. 1–27, 2018, doi: 10.1080/09593330.2018.1483971.

[82] Y. Liu and J. Tay, "The competition between flocculent sludge and aerobic granules during the long-term operation period of granular sludge sequencing batch reactor," vol. 3330, 2012, doi: 10.1080/09593330.2012.673011.

[83] J. P. Boltz and E. J. La Motta, "Kinetics of Particulate Organic Matter Removal as a Response to Bioflocculation in Aerobic Biofilm Reactors," no. December 2016, 2007, doi: 10.2175/106143007X156718.

[84] P. Wilinski, "Kinetics and stoichiometry of P-release with different carbon sources in the anaerobic phase of the biological phosphorus removal process in activated sludge wastewater treatment p ... Kinetics and stoichiometry of P-release with different carbon sources ," no. August 2009, 2016.

[85] E. A. Tepari, G. Nakhla, M. Idris, B. M. Haroun, and H. Hafez, "Stoichiometry of Anaerobic Protein Fermentation," *Biochem. Eng. J.*, vol. 158, no. October 2019, p. 107564, 2020, doi: 10.1016/j.bej.2020.107564.

4.1 Recommendations

The study combines experimental procedures with literature analysis to investigate the anaerobic degradation of proteins in AGS and their contribution to the EBPR process. Some recommendations to improve the experimental methods used in this study is provided below along with some knowledge gaps that could not be addressed in this research.

To compare the effects of granule size and substrate size on the rate of hydrolysis of proteins in aerobic granular sludge, it is important to obtain a factor that correlates each of them separately to the rate of hydrolysis. The data obtained from the experiments is insufficient to quantify the effect of each of the factors on the rate of hydrolysis which may be met by employing the following recommendations in the experimental procedure:

- In the experiment where the effect of the size of granule on the rate of protein hydrolysis is studied, it is seen that the density of the granule is an important parameter. In this study, the density of the granule is obtained by measuring the average volatile solids per granule and relating that to the average volume of the granule calculated from the measured radius of each granule. The drawback of this method is that the volume of the granule is not measured directly leading to accumulation of errors and reduced accuracy. Only the radius of the granule is measured directly and assuming that the granule is a perfect sphere, the volume is calculated. Also, determination of the organic content based on the oven drying method is subject to errors. Thus, it is recommended that standard methods be employed to measure the density of the granules.
- In the experiment where the effect of substrate size on the rate of hydrolysis of proteins in AGS is studied, the high substrate COD concentrations leading to the presence of significant amounts of easily hydrolysable soluble COD limits the possibility of studying the effect of particulate proteins alone on the rate of hydrolysis. In order to mitigate this limitation, it is recommended to reduce the overall influent substrate COD concentration and to thereby ensure that there is only insignificant concentration of soluble COD present so that the P-release observed can be attributed directly to the hydrolysis of particulate substrates.
- In the experiment where the effect of granule size on the rate of hydrolysis is studied, fluorescence protease assay is used wherein the average specific proteolytic activity obtained is less than what is typically seen due to less substrate available per sludge concentration. Experiments with more realistic substrate to sludge ratio may mitigate the uncertainties associated with the variability in substrate concentration.

4.2 Research Gaps for future study

- With respect to the utilization of amino acids by PAOs, the percentage of amino acids that would be degraded before being taken up by the PAOs vs the number of amino acids that would be directly taken up by the PAOs is not yet known. Also, the fate of the other nine out of the 20 amino acids that are not taken up by the PAOs is also not known. In the case of degradation to simple VFAs, whether the amino acids reach the core of an AGS granule where a small percentage of Clostridia will be present has to be studied.
- From literature study, it is seen that if the amino acids are degraded to simple VFAs before being taken up by the PAOs, then the degradation of amino acids via Stickland pathway also may lead to a slight production of hydrogen due to the 10% shortage in

the amino acids that are reduced. Studying the possible fate of this hydrogen or the concentration at which accumulation of hydrogen will lead to toxicity may allow more concrete conclusions on the occurrence of Stickland pathway in AGS.

Appendix

A.1 Method for processing microbial composition data to obtain the relative abundance of Clostridia in AGS.

The 16S DNA sequences used in this study were generated by (Ali el al., 2019). The raw sequencing data were retrieved from the National Center for Biotechnology (NCBI) Sequence Read Archive (SRA), accession number SRP115069. The raw sequences were processed to an OTU table, using the QIIME2 platform (Bolyen et al., 2019). In short, forward and reverse sequences were trimmed with the settings trunc-len-f and trunc-len r: 300). Forward and reverse reads were joined using the q2-vsearch plugin. All the positons of the joined reads had a quality score of over 35. The paired-end sequences were denoised using Deblur (Amnon et al, 2017). The setting trim-length 437 was used in Deblur. A phylogenetic tree was constructed to perform diversity analyses, using the q2-phylogeny plugin. Beta diversity metrics (Bray–Curtis and Unweighted Unifrac) were derived from the rarefied sequence table, and differences in beta diversity between = sludge types were analysed using PERMANOVA (Anderson, 2001). An OTU table was generated by aligning the sequences to the MiDAS 3.6 database (Nyerichlo et al., 2020). Sample sub-setting and visualization was performed in R, using the Phyloseq package (McMurdie and Holmes, 2013).

A.2 Amino acids tested for uptake by PAOs

The 20 amino acids tested for uptake by PAOs in [78], [62] is presented in the table below.

Amino acids that induced P-release in *Tetrasphaera* PAOs [62] and in Candidatus *Accumulibacter* clade IIF [78] is indicated with a ☑ mark and the amino acids that do not induce P-release in neither of the PAOs is indicated in red.

Amino Acids	**Uptake by *Tetrasphaera*** [62]	**Uptake by *Accumulibacter***[78]
Alanine	☑	☑
Arginine		
Asparagine	☑	☑
Aspartate	☑	☑
Cysteine	☑	☑
Glutamate	☑	☑
Glutamine	☑	☑
Glycine	☑	☑
Histidine	☑	
Isoleucine		
Leucine		
Lysine		
Methionine		
Phenyloalanine		
Proline		
Serine	☑	
Threonine	☑	☑
Tryptophan		
Tyrosine	☑	
Valine		

Table S 1 Amino acids inducing P-release in PAOs

A.3 Proteolysis activity of granules of different sizes

Fraction	mg Protein/gVS/hour	mg Protein/SA/hour	Surface Area in mm2
1-2 mm	0.207	1.38E-05	9.792
1-2 mm	0.278	7.43E-06	24.271
1-2 mm	0.185	5.82E-06	20.685
1-2 mm	0.094	3.51E-06	17.423
1-2 mm	0.319	1.50E-05	13.782
1-2 mm	0.162	7.11E-06	14.787
1-2 mm	0.195	7.32E-06	17.290
1-2 mm	0.273	8.72E-06	20.372
1-2 mm	0.389	2.08E-05	12.167
1-2 mm	0.116	7.70E-06	9.776
1-2 mm	0.137	9.01E-06	9.881
1-2 mm	0.142	5.91E-06	15.560
1-2 mm	0.248	7.20E-06	22.404
1-2 mm	0.170	6.76E-06	16.374
1-2 mm	0.150	5.69E-06	17.092
1-2 mm	0.141	5.53E-06	16.532
1-2 mm	0.309	1.59E-05	12.629
1-2 mm	0.301	1.61E-05	12.106
1-2 mm	0.138	6.88E-06	13.068
1-2 mm	0.300	1.40E-05	13.874
1-2 mm	0.178	9.34E-06	12.379
1-2 mm	0.161	9.53E-06	10.956
1-2 mm	0.098	4.65E-06	13.638
1-2 mm	0.049	2.59E-06	12.211
1-2 mm	0.179	6.26E-06	18.597
1-2 mm	0.230	8.20E-06	18.254
1-2 mm	0.131	6.47E-06	13.183
1-2 mm	0.105	5.23E-06	12.984
1-2 mm	0.507	2.51E-05	13.106
1-2 mm	0.395	1.93E-05	13.338
2-3.15mm	0.183	3.87E-06	35.478
2-3.15mm	0.141	6.45E-06	16.396
2-3.15mm	0.221	7.70E-06	21.499
2-3.15mm	0.208	4.76E-06	32.766
2-3.15mm	0.195	5.66E-06	25.796
2-3.15mm	0.289	7.25E-06	29.890
2-3.15mm	0.178	3.78E-06	35.351
2-3.15mm	0.145	4.26E-06	25.598
2-3.15mm	0.189	3.94E-06	36.040
2-3.15mm	0.152	4.64E-06	24.525
2-3.15mm	0.211	4.63E-06	34.233

2-3.15mm	0.177	4.52E-06	29.340
2-3.15mm	0.169	4.25E-06	29.793
2-3.15mm	0.296	8.82E-06	25.187
2-3.15mm	0.152	4.00E-06	28.416
2-3.15mm	0.191	4.55E-06	31.460
2-3.15mm	0.178	5.06E-06	26.330
2-3.15mm	0.208	4.61E-06	33.829
2-3.15mm	0.143	3.01E-06	35.700
2-3.15mm	0.168	4.61E-06	27.349
2-3.15mm	0.152	4.36E-06	26.157
2-3.15mm	0.191	4.13E-06	34.586
2-3.15mm	0.146	3.41E-06	32.150
3.15-4 mm	0.069	3.10E-06	40.919
3.15-4 mm	0.105	4.64E-06	41.819
3.15-4 mm	0.075	3.20E-06	43.160
3.15-4 mm	0.080	3.05E-06	48.103
3.15-4 mm	0.059	2.82E-06	38.430
3.15-4 mm	0.072	3.61E-06	36.993
3.15-4 mm	0.072	2.83E-06	47.076
3.15-4 mm	0.079	3.66E-06	39.916
3.15-4 mm	0.078	2.99E-06	48.300
3.15-4 mm	0.066	3.04E-06	40.140
3.15-4 mm	0.069	2.95E-06	43.265
3.15-4 mm	0.089	3.68E-06	44.675
3.15-4 mm	0.081	4.41E-06	33.860
3.15-4 mm	0.098	5.15E-06	34.962
3.15-4 mm	0.049	2.17E-06	41.511
3.15-4 mm	0.079	3.29E-06	44.049
3.15-4 mm	0.070	2.69E-06	48.140
3.15-4 mm	0.090	3.44E-06	48.250
3.15-4 mm	0.051	2.23E-06	42.233
3.15-4 mm	0.070	2.49E-06	51.657
3.15-4 mm	0.082	2.79E-06	54.341
3.15-4 mm	0.069	2.56E-06	49.814
3.15-4 mm	0.068	2.68E-06	46.651
3.15-4 mm	0.079	3.13E-06	46.409

Table S 2Proteolytic activity per gVS and per SA of the replicates of intact granules along with their surface areas

Fraction	mg Protein/gVS/hour
crushed 1-2 mm	1.174
crushed 1-2 mm	1.063
crushed 1-2 mm	1.096
crushed 1-2 mm	1.397
crushed 1-2 mm	1.321
crushed 1-2 mm	1.519

crushed 2-3.15	1.217
crushed 2-3.15	1.677
crushed 2-3.15	1.106
crushed 2-3.15	1.200
crushed 3.15-4 mm	1.253
crushed 3.15-4 mm	1.315
crushed 3.15-4 mm	1.374
crushed 3.15-4 mm	1.433
<0.5mm	4.023
<0.5mm	4.370
<0.5mm	3.784
<0.5mm	4.322
<0.5mm	4.049
<0.5mm	3.959
<0.5mm	3.744
<0.5mm	3.301
<0.5mm	3.990
<0.5mm	3.522
<0.5mm	4.258
<0.5mm	3.394
<0.5mm	4.413
<0.5mm	4.299
<0.5mm	3.684
<0.5mm	3.719
<0.5mm	4.199
<0.5mm	4.431
<0.5mm	4.190
<0.5mm	3.866
<0.5mm	4.543
<0.5mm	4.072
<0.5mm	4.326
<0.5mm	4.622
<0.5mm	3.567
<0.5mm	5.031
<0.5mm	5.305
<0.5mm	3.824
<0.5mm	4.368
<0.5mm	3.283

Table S 3 Proteolytic activity of the replicates of crushed fraction and flocs

A.4 Rate of P-release

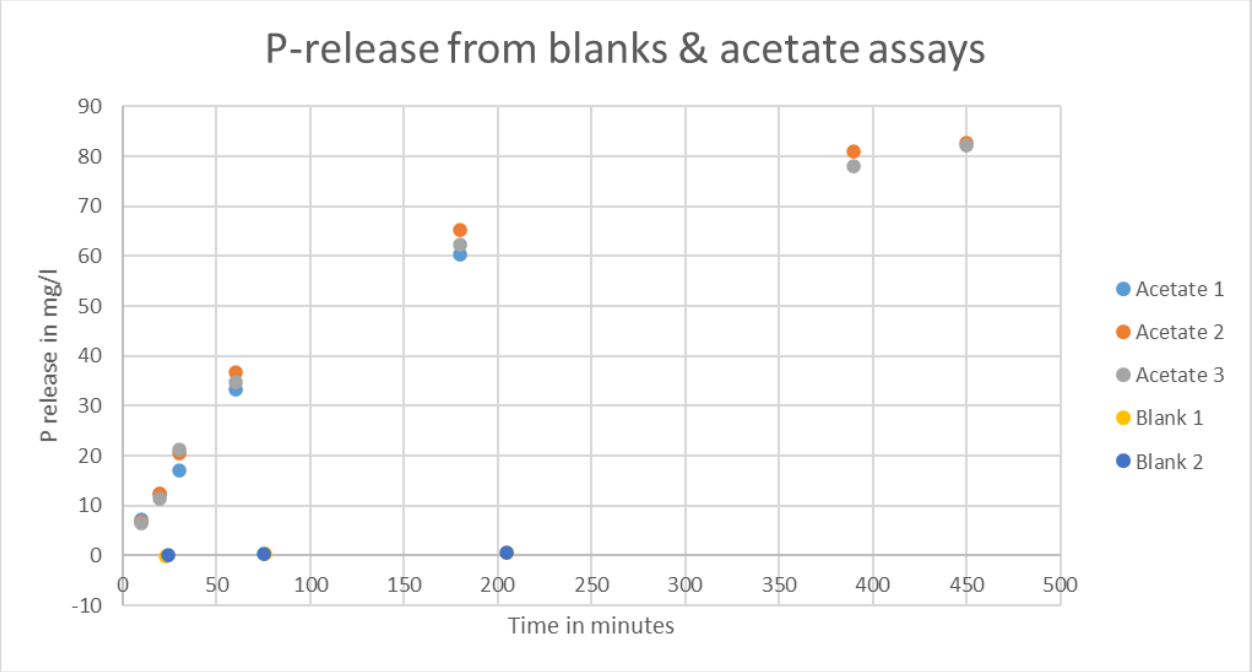

Figure S 1 Rate of P-release in Blank assay and acetate assay

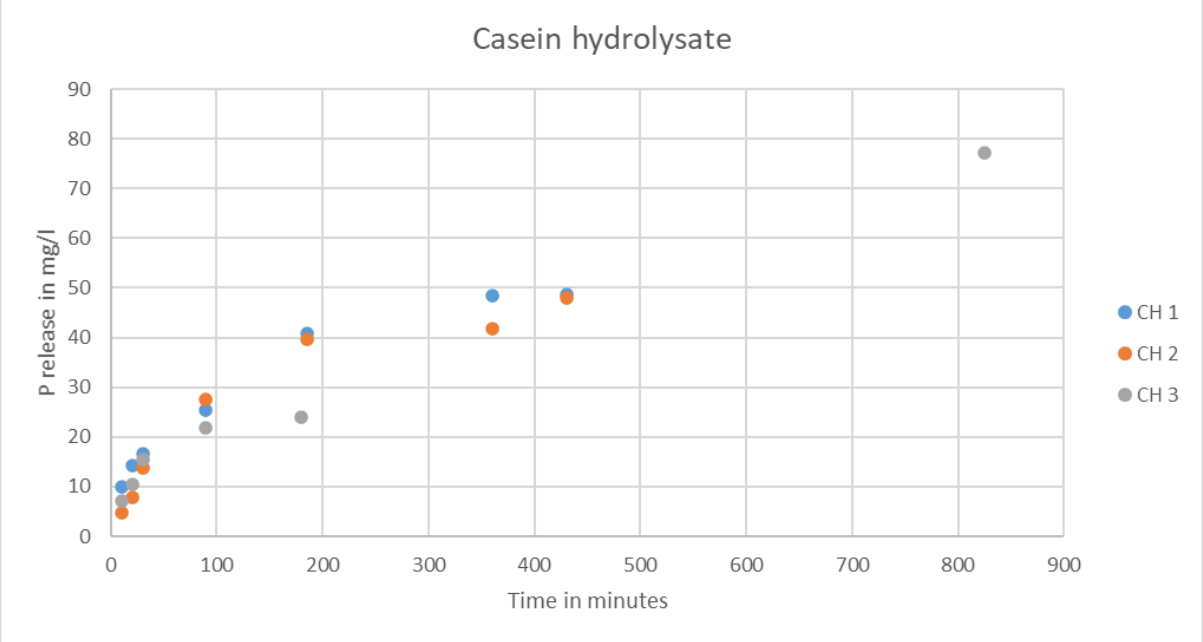

Figure S 2 Rate of P-release in Casein Hydrolysate assay