Enhanced digestion and alginate-like-exopolysaccharides extraction from Nereda sludge

Anthonie Hogendoorn

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Enhanced digestion and alginate-like-exopolysaccharides extraction from Nereda sludge

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Abstract

Aerobic granular sludge/Nereda technology has proven to be more beneficial compared to activated sludge systems. Until now not much research has been spend on treatment of Nereda excess sludge (NES). In this study the biodegradability of NES is investigated, combined with thermal pressure hydrolysis (TPH) as pre-treatment method and the application of an up-flow digester concept. The maximal biodegradability is estimated at 50% volatile solids (VS) (VS content of raw sludge is 80%). In a conventional CSTR digester 42% VS is converted, whereas TPH increased this to 48% (HRT=20 days). The up-flow digester performed slightly better than the conventional system: 43% VS is converted. Modelling of the CSTR systems showed to yield well fitting results; modelling of up-flow digesters yielded results which deviated a lot from the measured values.

Besides anaerobic digestion, the extraction procedure of alginate-like-exopolysaccharides (ALE) is optimized and the mutual influence of digestion and ALE extraction is researched. Extraction of ALE can be done with a lower amount of chemicals as the initial procedure. ALE seems to be slowly biodegraded during anaerobic digestion, although the amount of ALE recovered from digested sludge did not differ from undigested sludge. ALE extraction applied prior to digestion could be a synergistic hybrid, as the extraction procedure can act as a pre-treatment method to reduce sludge volume and increase biodegradability rate. The optimal combination is however not yet found.

Finally the influence of ALE-extraction method and protein content is investigated. ALE seems to contain a significant fraction of protein (20-40%), although large differences were observed between protein detection methods and ALE extraction methods.

Preface

This thesis is the result of my graduation research project, performed at the Sanitary Engineering Section at the faculty of Civil Engineering and Geosciences of Delft University of Technology and in the laboratory of RoyalHaskoningDHV in Amersfoort. The subjects of this study are various aspects of Nereda excess sludge treatment.

Deze studie is uitgevoerd met hulp van veel personen, waarvan ik er een aantal in het bijzonder wil noemen. Allereerst Eddie Koornneef: als hoofdbegeleider in Amersfoort was jij degene op wie ik kon terugvallen met alle vragen. Je klus-skill kwamen ook vaak goed van pas voor de typische lab problemen. Dank voor de mogelijkheden die je me bood om het onderzoek ook binnen zeker kaders zelf vorm te geven. David Berkhof wil ik bedanken voor de prettige en constructieve samenwerking. Viviane Miska: dank voor je aandacht voor de 'vrouwelijke' kant van het werk. Een speciaal dankwoord voor Alexander Hendriks. Je bent een voorbeeld voor me wat betreft kritische houding, nieuwsgierigheid buiten het vakgebied en scherpe blik voor details. Succes met je promotiestudie en wie weet tot ziens in de Delftse onderzoeksomgeving. Helle van der Roest is degene die me overgehaald heeft bij RHDHV af te studeren. Ik bewonder je passie en oneindige gedrevenheid voor het werk. Ik hoop dat mensen mij later zullen karakteriseren als iemand die (wat dat betreft) een tik van jou heeft meegekregen. Ook wil ik Dennis Heijkoop bedanken voor de prettige tijd onder zijn bewind.

Dank ben ik ook verschuldigd aan Jules van Lier, Merle de Kreuk en Mark van Loosdrecht. Dank voor de kritische vragen en doordenking van mijn werk en resultaten. Ik heb het als een eer beschouwd te mogen afstuderen onder leiding van professionals als jullie.

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Ook de leden en bestuur van Dispuut Watermanagement mogen niet onvermeld blijven. Dank voor de plezierige omgang in de afgelopen 2 jaar. Jullie hebben me er snel van kunnen overtuigen dat TUD toch minstens zo'n goede sfeer kent als de Wageningse variant. A special word of thanks to Luis Seminario Ruiz: Thank you for the nice cooperation in the first year. I have learned a lot from the great accuracy you performed your work with.

Deze scriptie is niet alleen het einde van mijn onderzoeksproject, maar ook van mijn studie. Ik wil graag mijn ouders (en bredere familie kring) bedanken voor de support die jullie al zo lang op diverse manieren hebben geboden. Zonder jullie had ik deze mijlpaal niet bereikt. Ook Elza wil ik bedanken voor de steun en vrijheid van de afgelopen tijd.

Soli Deo Gloria

Anthonie Hogendoorn
Zwijndrecht, juli 2013

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Lists of abbreviations

AGS	*Aerobic granular sludge*
ALE	*Alginate like exopolysaccharides*
BMP	*Biological methane potential*
BSA	*Bovine serum albumin*
CAS	*Conventional activated sludge*
CSTR	*Continuous stirred tank reactor*
DCB	*Divalent cation bridging theory*
DLVO	*Derjaguin and Landau, Verwey and Overbeek theory*
DS	*Dry solids*
EPS	*Extracellular polymeric susbstances / exo polysaccharides*
GG	*Polyguluronate*
HRT	*Hydraulic retention time*
kJ-N	*Kjeldahl-nitrogen (organic nitrogen)*
MG	*Heteropolymeric sequence of both mannuronate and guluronate*
MM	*Polymannuronate*
NES	*Nereda excess sludge*
NMR	*Nuclear magnetic resonance*
PF	*Plug flow reactor*
SBR	*Sequencing batch reactor*
SRT	*Sludge retention time*
SU	*Schwarting-Uhde*
TPH	*Thermal pressure hydrolysis*
UFD	*Up-flow digester*
VS	*Volatile solids*
WAS	*Waste activated sludge*
WWTP	*Waste water treatment plant*

1 Introduction

In 2010 the foundation applied research water management (STOWA) published the roadmap for the wastewater treatment plant (WWTP) of 2030. Instead of solely reducing the amount of pollutants to the receiving water, a WWTP could function as a resource factory; e.g. recover water, energy and nutrients from wastewater. One of the strategies to achieve this goal is by optimizing sludge treatment, which is the objective of this thesis. More specifically the focus is on two aspects: enhanced digestion of Nereda excess sludge (NES) and the extraction of alginate like exopolysacharides (ALE) of the same sludge. First the enhanced digestion is discussed, followed by the issues ongoing in ALE extraction, where after the research questions for this thesis are presented.

1.1 Sludge digestion

One of the major cost factors in waste water treatment is sludge treatment, accounting for 20-30% of the total costs (Dutch situation) (UVW et al., 2009). If primary clarification is applied at a WWTP, two types of sludge are produced. Primary sludge, which is composed of solids present in the wastewater and secondary sludge, consisting of bacterial cell mass produced during biological wastewater treatment. The objective of sludge stabilization is organic solids removal and improved dewaterability properties; anaerobic digestion is a widely applied method for this purpose. Digestion results in a reduction of organic content, increased dewaterability and production of biogas (Appels et al., 2008).

A lot of organic material is however not degraded during digestion. The biodegradability of secondary sludge is lower (45%) compared to primary sludge (60%) (STOWA, 2011). Various strategies can be applied to enhance the biodegradation of (secondary) sludge. In this thesis two methods are used to increase the conversion of organic matter: thermal pressure hydrolysis and the use of a modified version of the Schwarting-Uhde concept. The process of thermal pressure hydrolysis can be summarized as a pre-treatment step in which sludge is heated to a high temperature at a high pressure for 0.3-1.5 hours, resp. 140 – 170 ^{0}C and 6-8 bars. Pre-treatment allows for a faster biodegradation, resulting in a shorter retention time required and subsequently to a compacter digester system. The Schwarting-Uhde concept stands for vertical up-flow digestion (UFD), in which sedimentation of solids is realized. In Chapter 2 these technologies are discussed more extensively.

1.2 Alginate like exopolysacharides

A special characteristic of the aerobic granular sludge (AGS) is the presence of alginate like exopolysaccharides (ALE) in higher concentrations and with different properties compared to conventional activated sludge (Lin et al., 2012). AGS produces a component with similar characteristics as alginate, which is a polymer normally harvested from brown seaweed (Lin et al., 2010). ALE can be harvested and used for several purposes (de Bruin et al., 2012). Alginate serves as a gelling agent in textile printing, food preparation and paper industry (McHugh, 1987). It is expected that more WWTP's will be equipped with aerobic granular sludge technology (van der Roest et al., 2012), enabling ALE extraction at larger scale from the excess sludge of the AGS/Nereda process. To make the extraction process beneficial, optimization is required. Literature on alginate extraction from seaweed reported several extraction parameters (pH, extraction time, temperature) influencing the amount and characteristics of extracted alginate (Hernández-Carmona et al., 1999). These parameters will be investigated in this research.

It is yet not fully clear what the exact composition is of ALE. Lin et al. (2010) found a carbohydrate content of 47% in ALE with sodium alginate used as standard. This finding suggests that other components are present, although no protein was detected. Previous research noted that the protein content of extracellular polymeric substances of AGS is higher than activated sludge (Adav and Lee, 2008, McSwain et al., 2005). In this research the possible protein content of ALE is further analyzed.

1.3 Research questions and research set-up

It is expected that in the future both digestion and ALE extraction is applied on NES. Knowledge is lacking on how both processes influence each other. The processes can also be optimized: digestion performance can be increased by sludge disintegration methods or application of a different reactor concept, ALE extraction can probably be done with less/other chemicals or less/cheaper separation equipment. Also the possible protein content of ALE is unclear, as well as how the extraction method influences the possible protein contamination.

The research problem for this thesis is therefore stated as follows: *'previous research does not provide a sufficient basis for the set-up of a process scheme for a sludge treatment plant for Nereda excess sludge, including both the process of (enhanced) sludge digestion and the mutual influence of optimized ALE-extraction and digestion. Also the relation of ALE extraction method and possible protein contamination is unclear'.*

Following from the problem statement two main research questions are formulated:

- *'Under which process conditions can both ALE-extraction and enhanced digestion, by means of an UFD or pre-treatment with TPH, be applied on Nereda excess sludge?'*
- *'To what extent is ALE contaminated with protein and how can the extraction method be changed to reduce the extent of contamination?'.*

The following sub-questions are distinguished:

- To what extent is the digestibility of Nereda excess sludge increased due to high temperature pre-treatment with an HRT of 20 and 12 days[1]?
- To what extent is the digestibility increased due to the application of an UFD instead of a CSTR with an HRT of 20 and 12 days?
- How is the ALE extraction yield affected after digestion, also when thermal pre-treatment or an UFD is applied?
- How can the ALE extraction procedure be optimized by lower chemical dosing and different/adapted separation methods?
- How is the possible protein content and intercellular protein contamination of ALE influenced by the alkali and/or temperature applied in the ALE extraction process?

To answer the above listed questions, the following research methods are applied:

- Literature study on sludge treatment and sludge disintegration;
- Experimental research on digestion performance and ALE-yield;
- Experimental research on protein content of ALE in relation to extraction method;
- Application of modelling methods in combination with data analysis to find optimal process settings.

1.4 Thesis outline

Chapter 2 provides background information on the various topics discussed in this thesis. Chapter 3 describes the applied materials and methods. The results of the experiments and modelling are presented in chapter 4, where after these results are synthesized in the discussion (chapter 5). Chapter 6 summarizes the main findings and contains the answers to the research questions.

[1] The choice for 12 days is made in line with STOWA 2012. *Thermische Slibontsluiting - Pilot-onderzoek naar de mogelijkheden en randvoorwaarden,* Amersfoort.

2 Theoretical background

The main purpose of this chapter is to provide the required background information to understand the content of this thesis. After a general introduction to wastewater treatment and aerobic granular sludge technology (section 2.1), the theory of bioflocculation, granulation and exopolysacharides (EPS) is discussed (2.2). In the subsequent section alginate and its biodegradability is discussed (2.3), followed by information on sludge digestion (2.4) and an investigation of thermal pre-treatment methods (2.5). Finally, information is provided on the Schwarting-Uhde concept (up-flow digester) (2.6).

2.1 Wastewater treatment

Wastewater treatment is applied in Western countries for over one century for health and environmental reasons. Starting with the removal of solid and colloidal material, later on soluble organic components and nutrients were also removed in the treatment process (Metcalf and Eddy, 2004).

2.1.1 Activated sludge systems

Currently, activated sludge systems are the most used systems for wastewater treatment. In some cases a primary clarifier is applied prior to the activated sludge process, to remove part of the solid material. In activated sludge systems a mixed culture of biomass is growing and converting organic carbon and nutrients from the influent.

The system consists of two treatment steps: a (partly) aerated tank in which biochemical conversions occur and a clarifier in which the biomass is separated from the effluent. The biochemical stage can be separated in aerobic tanks (oxidation of organic carbon and ammonium), anoxic tanks (denitrification for nitrate removal) and anaerobic zones (to select for phosphorus accumulating organisms) (Seviour et al., 2003). In the clarifier aggregated biomass settles and is separated from the effluent. This settling process is relatively slow (velocity < 1m/h), requiring a long hydraulic retention time (HRT) of the water in the clarifier. Part of the separated sludge is fed back to the biochemical stage to inoculate the treatment process; the remaining part is thickened, treated (an)aerobically and disposed or disposed directly.

2.1.2 Compact wastewater systems

As indicated above, activated sludge systems require large surface areas for clarification. WWTP's are often located in human settlements hindering expansion. Compact systems are developed during last decades as a result. These developments comprise membrane bioreactors, biofilm systems and aerobic granular sludge. As this thesis is limited to latter one, for the first two developments reference is made to Judd (2010), Yang et al. (2006) and Nicolella et al. (2000) respectively.

2.1.3 Aerobic granules

The subject of this thesis encompasses treatment of aerobic granular sludge (AGS). This technology is recently applied at full scale at WWTP Epe. Heijnen and van Loosdrecht (1998) discovered the conditions under which AGS develops. Beun (2001) and de Kreuk (2006) developed this technology further to a full scale wastewater treatment plant (WWTP) for domestic sewage. Aerobic granular sludge has various benefits compared to activated sludge: among others a 80% smaller footprint (de Bruin et al., 2004), a 30% lower aeration requirement and no need for sludge recycle flows (de Kreuk and van Loosdrecht, 2012).

For aerobic granular sludge, a Sequencing Batch Reactor (SBR) is required, which is fed discontinuously (Morgenroth et al., 1997). A typical cycle consists of a filling, reaction, settling and withdrawal phase, see Figure 2.1. By applying an anaerobic feeding phase, followed by an aeration phase, both COD, nitrogen and phosphorus are successfully removed from the wastewater (Van Loosdrecht and De Kreuk, 2004). The SBR operation allows for high loading rates (Van Loosdrecht et al., 1995, Beun et al., 2002). Combined with a high shear stress, dense and compact granules are obtained (Kwok et al., 1998).

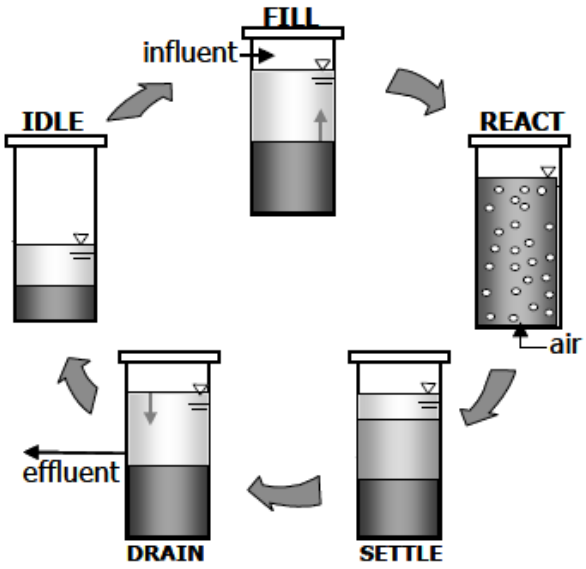

Figure 2.1. Typical cycle of a sequencing batch reactor (figure from de Kreuk (2006)).

2.2 Sludge bioflocculation and granulation

Since the removal efficiency of a WWTP is directly related to the settling performance of sludge in the final clarifier, bioflocculation is one of the key parameters for the final effluent quality (Higgins et al., 2004, Murthy and Novak, 2001). Activated sludge flocs consist of microbial aggregates, filamentous organisms, organic and inorganic particles and extracellular polymeric substances (EPS) (Murthy, 1998). EPS has two different origins: from metabolism or lysis of microorganisms (proteins, polysaccharides and lipids) and from the wastewater itself (cellulose and humic acids) (Urbain et al., 1993). This EPS forms a gel-like, hydrated and often charged matrix, in which micro-organisms grow and get more or less immobilized (Flemming et al., 2007). The EPS functions as a protective barrier against biotic and abiotic influences from the environment and provides micro organisms the opportunity to form a stable aggregate of different cells (Wingender et al., 1999, Wilkinson, 1958). EPS are known for their property to form gels with cations (Sutherland, 2001).

2.2.1 Role of cations in flocculation

Three theories have been proposed to explain the mechanism by which cations influence flocculation(Sobeck and Higgins, 2002):

1. **Double layer theory** (also named DLVO theory). This theory describes charged particles with a double layer of counterions surrounding the particle. The concentration of ions in the double layer decreases in proportion with the distance from the particle, until the concentration of ions is equal of that of the bulk solution. This cloud of ions around a particle results in repulsion of adjacent particles and limits aggregation. An increase in ionic strength will reduce the double layer size and enhance aggregation (Zita and Hermansson, 1994, Hermansson, 1999).
2. **Divalent Cation Bridging theory (DCB).** This theory states that the overall floc structure is negatively charged and is the result of physic-chemical interactions between microorganisms (predominantly bacteria), inorganic particles, extracellular polymeric substances (EPS) and multivalent cations (Urbain et al., 1993). Sobeck and Higgins (2002) stated that the negative charge of the flocs is mainly caused by proteins and uronic acids present in the EPS. Due to the negative charge, divalent cations (e.g. Mg^{2+}, Ca^{2+}) are of crucial importance for microbial flocculation to occur (Endo et al., 1976, Eriksson and Alm, 1991) and hence for good settling and dewatering properties (Higgins and Novak, 1997b). Hence the name 'Divalent Cation Bridging Theory' (Tezuka, 1969). Higgins and Novak (1997a) reported that settling and dewatering was deteriorated when the monovalent to divalent cation ratio exceeded 2 to 1, expressed on equivalent basis. The specifity of components in the EPS against the available divalent cations is not often studied; only Urbain et al. (1993) found a higher affinity of Mg^{2+} for DNA and Ca^{2+} for protein in the EPS.
3. **Alginate theory.** This theory can be considered as a variation of the DCB theory. Bruus et al. (1992) showed that addition of monovalent cations to sludge led to a smaller particle size. They argued that with help of Ca^{2+} an alginate-like-gel is formed, acting as a backbone of the floc structure. Alginate forms a gel by complexing with divalent cations as Ca^{2+}, Ba^{2+} and Sr^{2+} (Smidsrød and Skjaak-Braek, 1990). Side by side aligning of two ployguluronate (GG) blocks results in diamond shaped holes, which are the location for cross-links. This is called the 'egg-box model', see Figure 2.2 (Grant et al., 1973, Morris et al., 1978, Li et al., 2007, Sikorski et al., 2007). More information regarding alginate can be found in section 2.3.

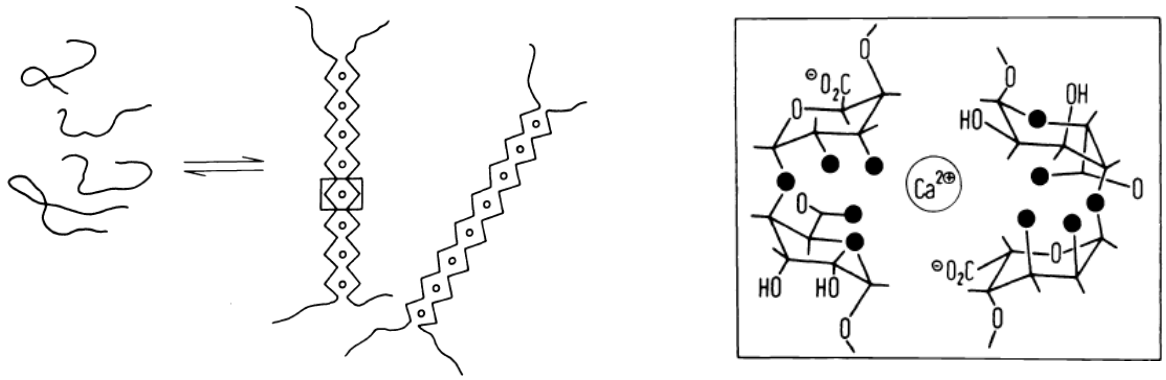

Figure 2.2. Schematic representation of the egg-box mechanism for alginate gelation. At the left the conversion of random coils to buckled ribbons is depicted, containing arrays of Ca^{2+} ions. At the right the stereochemistry of Ca^{2+} ion complexation is shown, with the involved oxygen atoms indicated as filled circles. (Figure from Rees (1981))

Conflicting research results regarding the different flocculation mechanisms have led to the situation that the three models are still used, suggesting that the influence of cations on flocculation properties is sludge specific (Sobeck and Higgins, 2002). The same applies for the constituents of the EPS involved (DCB and alginate theory) in the flocculation process. Dignac et al. (1998) found proteins as the main constituent of EPS from activated sludge and argued that proteins rather than sugars are involved in electrostatic bonds with multivalent cations. Proteins exhibit also hydrophobic properties. The same conclusions were drawn by Higgins and Novak (1997a), stating that lectin-like proteins are the main binding agents between polysaccharides and divalent cations. On the other hand Lin et al. (2010) suggested the high amount of GG-blocks in alginate-like-exopolysaccharides responsible for the hydrophobicity of AGS.

2.2.2 Role of EPS in aerobic granulation

The crucial role of EPS in aerobic granulation is recognized by several researchers (Weber et al., 2007, Seviour et al., 2009a, Seviour et al., 2009b). Also the necessity of divalent cations for granulation has been observed (Cui et al., 2013, Jiang et al., 2003). Ren et al. (2008) reported a higher granule strength at elevated Ca^{2+} concentrations. An increase in granule size resulted in $CaCO_3$ precipitation within the granule and reduced bio activity. Saline conditions or high ammonium concentrations result in ion-exchange with multivalent cations and subsequently in washout of EPS and worsened granulation (Pronk et al., 2013, Yang et al., 2004). In this respect aerobic granular sludge behaves similar to anaerobic granular sludge, see e.g. Yu et al. (2001), De Graaff et al. (2011), Van Der Star et al. (2008), Ismail et al. (2010) and Mahoney et al. (1987).

Although a lot of research has already been published on the role of EPS in granulation, there is no consensus about the primarily involved polysaccharide in the granulation process. Seviour et al. (2010b) proposed a novel complex heteropolysacharide (named *'Granulan'*) as key component, whereas Lin et al. (2010) argued that alginate-like-exopolysacharides (ALE) are mainly involved. Both theories are discussed by Seviour et al. (2012). In this thesis the ALE-theory is used as a starting point.

2.3 Alginate

This section contains information about alginate, which is considered as useful for the scope of this thesis. In the last part of this section the information is synthesized with regard to alginate-like-exopolysacharides.

'Alginate' is a term which refers to the salts of alginic acid. These alginate polymers are present in the cell walls of brown algae as the calcium, magnesium and sodium salts of alginic acid (McHugh, 1987). Alginic acid is a linear copolymer of mannuronic acid and guluronic acid (Haug et al., 1966). The residues are organised in blocks of polymannuronate (MM) and polyguluronate (GG), as well as heteropolymeric sequences of both acids (MG) (Haug et al., 1974). The different groups are depicted in Figure 2.3. Note that the shape of a G-block is axial, so a chain of G-blocks is buckled. Latter aspect is of importance for the egg-box model, discussed in section 2.2. The stiffness of alginate gels is directly related to the amount of crosslink points (Lin et al., 2012), i.e. the higher the content of GG-blocks, the higher the stiffness of the gel. Although it is argued that Mg^{2+} can also serve as an ion inducing chelation (Fang et al., 2007); in other research it is shown that Mg^{2+} did not result in an increase of viscosity and thus no gel was formed (Haug and Smidsrod, 1965, Lattner et al., 2003). Smidsrod and Haug (1972) showed that alginate had a higher selectivity coefficient for Ca^{2+} rather than Mg^{2+} and the selectivity coefficient increased with the Ca^{2+} equivalent fraction in the polymer phase (till a fraction of around 0.5). Latter results indicate a gel reinforcing mechanism; e.g. a higher Ca^{2+} concentration leads to a stronger gel and subsequently to a stronger affinity for Ca^{2+}. Although alginate is produced by seaweed, bacterial alginate production is known from the human disease cystic fibrosis (Gacesa, 1998). Mannuronate is produced by several bacterial species; guluronate is derived from mannuronate by epimerase (Franklin et al., 1994, Remminghorst and Rehm, 2006).

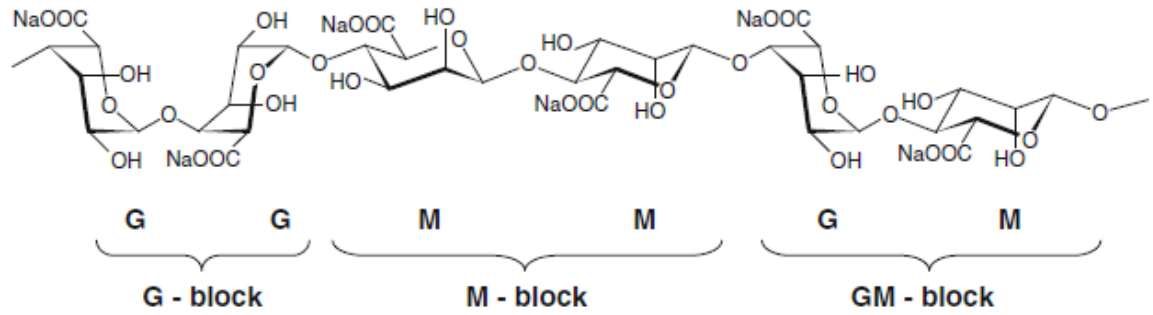

Figure 2.3. Chemical block composition of alginate with G-blocks, M-Blocks and MG-blocks after Donati and Paoletti (2009).

2.3.1 Alginate sources and uses

Alginate is mainly harvested from brown seaweed. Alginate composition is dependent on the species used for alginate extraction. The most important seaweed species *are Laminaria, Macrocystis* (Kelp) and *Ascophyllum* (McHugh, 1987).

Alginate is used in the food industry as stabiliser and moisture retention agent. Furthermore it is used as emulsifier in the cosmetical industry and as a thickener of paints and paper (Dhargalkar and Pereira, 2005). Lin (2013) reported that ALE extracted from aerobic granular sludge showed similar sizing effects with commercial sizing chemicals. For a full overview of the uses of alginates, reference is made to McHugh (1987).

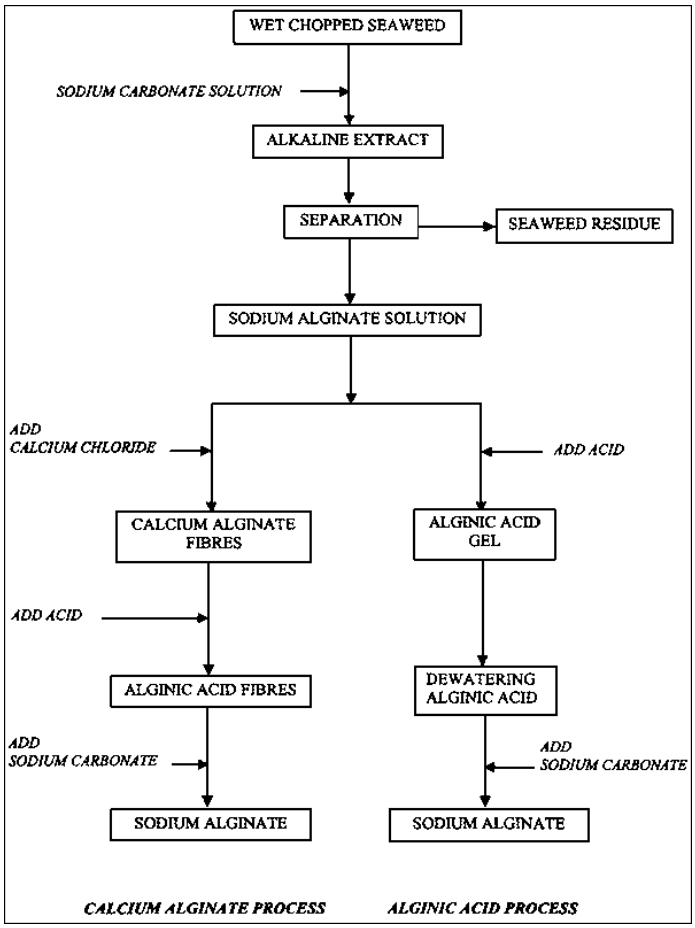

Figure 2.4. Process scheme of the extraction of alginate from seaweed by means of the calcium alginate process and sodium alginate process (figure from McHugh (2003)).

2.3.2 Alginate extraction

The extraction of alginate from seaweed is a relatively simple process. A process scheme is depicted in Figure 2.4. This section is based on McHugh (1987).

Raw seaweed is chopped into small pieces (5-10 mm) to increase the penetration depth of following treatment with chemicals. After size reduction, an ion exchange process is applied to solubilise the alginate. Alginate is present in brown seaweed as the calcium salt of alginic acid, during ion exchange the Ca^{2+} ions are replaced with Na^{+} ions, destroying the egg-box-model gel complex. Usually sodium carbonate is used for its low cost, although also sodium hydroxide is used. The extraction takes place at 50-95 ^{0}C for 1-2 hours. (Davis et al., 2004).

Smidsrod and Haug (1968) indicated that the required alkali dosage can be reduced by treating the seaweed with acid prior to the alkali treatment.

After separation of the seaweed residues by flotation, filtration or centrifugation, the sodium alginate solution is obtained. Since this solution is too diluted, a concentration step is required. Two methods are used: the calcium alginate process and the alginic acid process. In some cases a bleaching step with sodium hypochlorite is included before the concentration phase.

Calcium alginate process

By addition of the sodium alginate to a calcium chloride solution, calcium alginate will precipitate in the form of fibres. These fibres can be separated by sieving. After addition of acid, the Ca^{2+} ions are exchanged for H^{+} ions and fibrous alginic acid is formed. This alginic acid can be dewatered using a filter press. The concentrated alginic acid is mixed with solid alkali (usually sodium carbonate), resulting in a heavy paste. The paste is extruded and chopped into pellets which are dried, after which they are milled into smaller particles. Although this process involves more process steps and chemicals compared to the alginic acid process, the fibrous alginate makes dewatering much easier.

Alginic acid process

The sodium alginate solution is treated with HCl or H_2SO_4, giving a gelatinous precipitate, which can be separated by centrifugation or flotation. To reach a solid concentration of 25%, several repetitive steps are required. After concentration, the alginic acid is converted to sodium alginate, similar to the calcium alginate process.

2.3.3 Alginate stability

To know how alginate-like-exopolysacharides will behave during digestion, the available literature is summarized in this section.

Leenen (1996) used Ba-Ca-alginate as support material for immobilization of nitrifying biomass and concluded that this alginate was not biodegradable at all in an aerobic environment. Sodium alginate on the contrary is biodegradable (Sawabe et al., 1995, Doubet and Quatrano, 1982), which indicates that multivalent ion cross-links limit biological degradation. The extent of this limitation is not clear as research of Boyd and Chakrabarty (1994) & Boyd and Chakrabarty (1995) detected alginate lyase activity on Ca-alginate gels. Moen et al. (1997b) found Ca-crosslinks limiting for biodegradation under anaerobic conditions, whereas this was not observed for aerobic conditions. This contradicts however to findings of Leenen (1996).

Østgaard et al. (1993a) showed 70% reduction of sodium alginate by alginate lyases in batch and semi-continuous reactors fed with innoculum from kelp fermenters. These enzymes catalyze a β-elimination reaction, cleaving the chain and leaving an unsaturated uronic acid residue.

Alginate lyase for seaweed alginate is reduced by several environmental conditions and molecular conformations:

- Presence of polyphenols, originating from the seaweed cell tissue: Moen et al. (1997a), Moen et al. (1997c) found that Na-alginate lyase is reduced both under aerobic and anaerobic conditions due to the presence of polyphenols;
- Acetyl-group presence: deacitilized substrates are preferred by alginate lyases: Kennedy et al. (1992) and Nguyen and Schiller (1989) observed higher enzymatic activity on non-acetylated substrates with a high mannuronic acid content compared to substrates with a higher acetyl content;
- Block distribution: lyases have different specifities against the different block structures of the alginate polymer; guluronate lyase have the highest activity, although enzymatic access to Ca-crosslinked guluronate residues is restricted (Østgaard et al., 1993b, Moen and Ostgaard, 1997);
- Presence of divalent cations: Vogelsang and Østgaard (1996) reported a higher stability for Ba-alginate gels than Ca-gels, since Ba-gels form a stronger gel complex (Bajpai and Sharma, 2004).

2.3.4 Synthesis: alginate-like-exopolysacharides

In chapter 1 it was already mentioned that EPS extracted from aerobic granular sludge can be characterized as 'alginate-like- exopolysacharides' (ALE). The background information of alginate is in this section applied to ALE.

ALE-characteristics

Lin et al. (2008) compared the behaviour of EPS from aerobic flocculent and granular sludge. The EPS was extracted according to the method used in alginate extraction from seaweed (described in the previous section). Hence the exctracted EPS is named: Alginate-like-exopolysacharides (ALE). Dripping the ALE from granular sludge into a $CaCl_2$ solution resulted in granular shaped particles, whereas the ALE from flocculent sludge resulted in small sized flocs. This finding indicated a higher GG-content in the ALE extracted from granular sludge compared to ALE from flocculent sludge. In later research, the exact ratio was determined: ALE from granular sludge consisted for 69% of GG-blocks, whereas flocculent sludge contained only 35% (Lin et al., 2012). The different rheological behaviour of EPS extracted from flocculent and granular sludge is also confirmed by Seviour et al. (2009a). MALDI-TOF and UV-visible spectra of ALE and sodium alginate showed big similarities (Lin et al., 2010).

ALE-constituents

The constituents of the EPS of AGS are still under discussion. McSwain et al. (2005), Adav et al. (2007a) and Adav and Lee (2008) found that the EPS (extracted with 8 different methods) of aerobic granular sludge contains more protein rather than saccharides, whereas in flocculent sludge the opposite was observed. Zhang et al. (2007) found that the polysaccharide content in the EPS increased at higher influent COD loads, whereas the protein content decreased. Lin et al. (2010) reported a protein content below the detection limit of the assay used in the research of ALE-characterization. On the other hand: a polysaccharide content in ALE of around 50% was measured, suggesting that besides saccharides also other components are present. In the discussion of this thesis the protein content of ALE from granular sludge is discussed more extensively.

ALE-biodegradability

The exact behaviour of ALE during digestion cannot be predicted fully based on earlier research. A few things are clear: sodium alginate is easily biodegradable, whereas cross-linked alginate (e.g. Ca-alginate, Ba-alginate etc.) seems to have limited biodegradability. The extent to which cross-links limit biodegradation is not known, both for aerobic and anaerobic conditions. Based on the research of Leenen (1996) biodegradability of ALE can be neglected, although other authors mention that EPS is (partly) biodegradable (Li et al., 2006, Zhang and Bishop, 2003, Wang et al., 2007, Nielsen et al., 1996).

2.4 Sludge production & digestion

In biological treatment processes, cell growth occurs together with the oxidation of organic components. The ratio of the amount of biomass produced to the amount of substrate consumed is defined as the biomass yield. The yield can be estimated by several methods (stoichiometry, bioenergetics) and can be measured. Generally a value of 0.4 gr VSS/gr COD removed is applied for activated sludge systems without nutrient removal (Metcalf and Eddy, 2004). With regard to aerobic granular biomass it is not clear what the exact yield value is. Both de Kreuk et al. (2005b), Mosquera-Corral et al. (2005) and Liu et al. (2005) estimated the yield at 0.2-0.25 gr VSS/gr COD removed. In all these tests synthetic wastewater was used with acetate as sole carbon source. Liu et al. (2005) reported that the amount of CO_2 produced/gr COD removed was higher compared to activated sludge, indicating a lower sludge yield. Smolders et al. (1994) showed a 13% biomass yield reduction by biological phosphorus removal organisms in comparison with biological treatment systems without bio-P removal. When full nitrogen removal is also incorporated, the yield is reduced with 20-30% compared to systems without nutrient removal (Kuba et al., 1994 according to Oehmen et al., 1997). This observation can be explained by the increase of energy costs involved in the metabolic cycles (Atkinson, 1977). Still, a significant fraction of the organic components in the influent is converted into bacterial biomass, which is often stabilized for economical reasons. Anaerobic digestion is the most widely applied method to reduce the amount of sludge and decrease the net energy consumption for waste water treatment (STOWA, 2010b).

2.4.1 Sludge digestion

Sludge stabilization is required for pathogen removal, volume reduction, prevention of putrefaction and increased dewaterability, depending on the way of disposal. Anaerobic digestion is most often applied to accomplish this. As agricultural use of (stabilized) sludge is forbidden in the Netherlands (LNV/VROM, 1998), incineration is required.

Most anaerobic digestion systems operate at mesophilic conditions, i.e. 36 ^{0}C, although systems operated at thermophilic conditions (56 ^{0}C) also exists. The main difference between both processes is the rate of conversion, which follows the Arrhenius equation (Veeken and Hamelers, 1999). In anaerobic digestion biodegradable organic compounds are converted into CH_4 and CO_2. The process consists of 4 steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis and are depicted in Figure 2.5. All steps will be discussed shortly.

During **hydrolysis** enzymes, excreted from acidogenic bacteria, catalyse biochemical conversion reactions of complex, insoluble material into simpler, solube materials which are able to pass cell walls and membranes of fermentative bacteria (Vasconcelos Fernandes, 2010). These smaller components can subsequently be taken up and converted by micro-organisms. Hydrolysis is considered as the rate limiting step under stable digestion conditions (Eastman and Ferguson, 1981). More specifically: the accessible surface for enzymes of substrates is limited, making the hydrolysis rate particle size dependent (Vavilin et al., 1996, Sanders, 2001).

Acidogenesis is the subsequent step in the digestion process. In this phase dissolved compounds (amino acids, simple sugars, long chain fatty acids) are converted into simple compounds, like volatile fatty acids (VFA's), lactic acid, alcohols, CO_2 and H_2. This step is the most rapid conversion step in the anaerobic digestion process. The Gibbs free energy involved is the highest, resulting in a high growth rate of acidogenic bacteria. As acid is produced during this reaction, a sudden increase in substrate availability will result in a high acid production and possibly a pH drop, if the buffering/hydrogen scavenging capacity is low. Methanogenic activity is inhibited by a low pH, which leads to an even quicker pH drop (van Lier et al., 2008). To prevent this, enough methanogenic bacteria should be present to scavenge the hydrogen produced. An hydraulic retention time (HRT) of 20 days is generally applied in digester treating WWTP sludge, to assure stable process performance (STOWA, 2011).

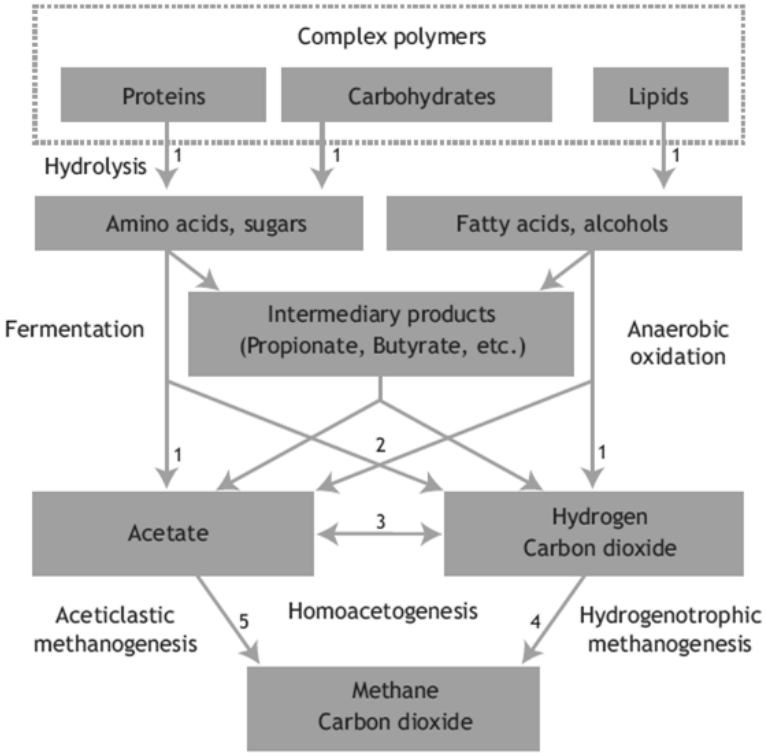

Figure 2.5. Scheme of reactions involved in anaerobic digestion of polymeric materials. Numbers indicate the specific groups of bacteria involved: 1. Hydrolytic and fermentative bacteria, 2. Acetogenic bacteria, 3. Homo-acetogenic bacteria, 4. Hydrogenotrophic methanogens, 5. Aceticlastic methanogens (figure from van Lier et al. (2008)).

During **acetogenesis** the acidogenic products are converted into acetate, H_2 and CO_2. Acetogenic reactions will not occur under standard conditions, as the Gibb's free energy is negative. Under stable digestion conditions, with properly functioning methane producing bacteria, the H_2 partial pressure is so low that the reactions becomes exergonic and will yield energy for the acetogens (Zeeman, 2010).

In the **methanogenic** phase acetate, hydrogen, carbonate, formate and methanol are converted into CH_4 and CO_2. The presence of other electron acceptors (oxygen, nitrate, sulphate) will result in a lower specific gas production (vol CH_4/mass $COD_{degraded}$).

Since only enzymes are directly involved in the first step (hydrolysis), no cell growth takes place during this phase. In the other steps new cell material is formed, although the amount of cell material is low compared to aerobic systems (yield value of 0.03 – 0.18 gr VSS/gr COD removed) (van Lier et al., 2008).

2.5 Thermal pre-treatment of sludge

A strategy to increase the hydrolysis rate and/or extent of biodegradability is to pre-treat sludge prior to digestion (Van Lier et al., 2001). The pre-treatment methods cause a. o. (and depending on the specific method) cell lysis or disintegration of bacterial cells and make the intercellular matter available to anaerobic micro-organisms. Particle size decreases, increasing the available surface, resulting in enhanced hydrolysis rates. Besides that, a major part of proteins will solubilize and since the cell structure is destroyed, internal cell water and capillary water is released, resulting in a better dewaterability (Pinnekamp, 1989, Neyens and Baeyens, 2003).

The following pre-treatment methods are distinguished (STOWA, 2005, Carrère et al., 2010):

- Biological pre-treatment (see e.g. Tamis et al. (2011) & Yang et al. (2010));
- Chemical pre-treatment (see e.g. Lin et al. (1997) & Penaud et al. (1999));
- Mechanical pre-treatment (see e.g. Chu et al. (2002) & Tiehm et al. (2001));
- Thermal pre-treatment.

Obviously, also combinations of above listed methods can be applied.
This thesis is limited to thermal pre-treatment. For a review of the other methods, reference is made to Ødegaard (2004), Weemaes and Verstraete (1998) and Müller (2001).
Two types of thermal pre-treatment can be distinguished: high temperature pressure hydrolysis and low temperature hydrolysis. In latter process the temperature applied is below boiling point, omitting the necessity of a pressurized system. Both processes are discussed below.

2.5.1 High temperature sludge pre-treatment

Stuckey and McCarty (1984) investigated the effect of pre-treatment temperature on the anaerobic bioconvertibility of waste activated sludge. They concluded that solubilisation of organics and bioconvertibility takes place, until a maximum of 175^0C. At higher temperatures the bioconvertibility decreased sharply, attributed to the thermal degradation of soluble organics to insoluble, refractionary components (Stuckey and McCarty, 1978). This process can even occur at temperatures of 130 ^{0}C, depending on differences in sludge composition (Climent et al., 2007). Solubilization of COD is enhanced at higher pH values; although the anaerobic biodegradability is not necessarily directly influenced (Penaud et al., 2000b). Hodge (1953) indicated that during thermal pre-treatment melanoidins (brown nitrogen copolymers) and humic acids are formed, which are known to be refractionary (Dwyer et al., 2008). Melanoidins are formed in the final stage of the Maillard reaction (Penaud et al., 2000a), which is a known reaction in food chemistry (Mauron, 1981). The primary mechanism of the formation of brown colour is polymerisation of low molecular weight intermediates, such as carbohydrates and amino compounds. Cross-linking of proteins and reactive carbohydrates is also reported as Maillard reaction (Hofmann, 1998). Melanoidin formation is considered as the responsible mechanism for the difference in COD solubilisation during pre-treatment and the lower corresponding methane yield (Penaud et al., 2000b).

Haug et al. (1978) reported that an increase in degredation rate is only obtained for activated sludge rather than for primary sludge, although other researchers report opposite results (Pinnekamp, 1989).

2.5.2 Low temperature sludge pre-treatment

Although the major part of thermal pre-treatment of sludge deals with temperatures around 160 ^{0}C (see e.g. Neyens and Baeyens (2003)), also pre-treatment at temperature below 100 ^{0}C is applied. This process is mentioned in literature as part of the *'temperature phased anaerobic digestion'* process (see e.g. Ge et al. (2011b)) or as *'hyper thermophilic pre-digestion'* (Lu et al., 2008).

Mason (1986) reported that thermal treatment just above thermophilic temperature showed good results with regard to microbial solids destruction. At 60 ^{0}C cell lysis occurs, allowing for cryptic growth and omitting the earlier described problems of maillard reaction product formation (Ge et al., 2010). Nielsen et al. (2004) suggested that pre-treatment of cattle manure at 70 ^{0}C enhances biological activity of thermophilic bacteria and thus enhancing the degradation rate of organics. Similar results were found for primary (Ge et al., 2010), secondary (Ge et al., 2011a, Audrey et al., 2011) and mixed sludge from WWTP's (Ferrer et al., 2008). The main mechanism behind the process is attributed to both enhanced biological activity (enzymes) and physical/chemical processes (cell lysis); the main responsible mechanism is however not discovered yet.

2.6 Schwarting-Uhde concept

A different reactor concept can be used to increase the conversion efficiency of a digester as well instead of sludge pre-treatment methods. An example is the Schwarting-Uhde (SU) process. This concept can be considered as an extension of the two-stage digestion system proposed by Pohland and Ghosh (1971). For the SU process two up-flow reactors are placed in series. The first reactor operated at mesophilic conditions, the second one at thermophilic conditions. A figure of one of the reactors is shown in Figure 2.6. The system behaves as a plug-flow reactor, with as modifications that sludge is recycled from top to bottom and solids can settle at the bottom and plates, by which the SRT is uncoupled from the HRT.

The main purpose of the first reactor is hydrolysis and acidification, whereas the second one is used for methane production. Although acetate conversion of thermophilic methanogens is not optimal at 55 ^{o}C (van Lier, 1995), the second reactor combines rapid conversion with hygienisation of sludge (Gessler and Keller, 1995). The sludge is pumped upward through perforated plates, guaranteeing uniform movement and thus up-flow conditions. Short term mixing is ensured by a pulsating recycle pump. Recycling also provides addition of methanogenic bacteria to the new substrate to scavenge produced hydrogen and to add alkalinity to prevent possible pH drops. The conversion efficiency of the system is around 60% organic solids for a sludge mixture 30% primary and 70% secondary sludge (Zaher et al., 2007, Weemaes and Verstraete, 1998, Trösch and Niemann, 1999). Boersma (2013) used a modified version of the Schwarting-Uhde process for digestion of activated sludge. The main modification compared to the conventional Schwarting-Uhde process is the application of two reactors operated at the mesophilic temperature range. Latter modification in temperature is also applied in this study.

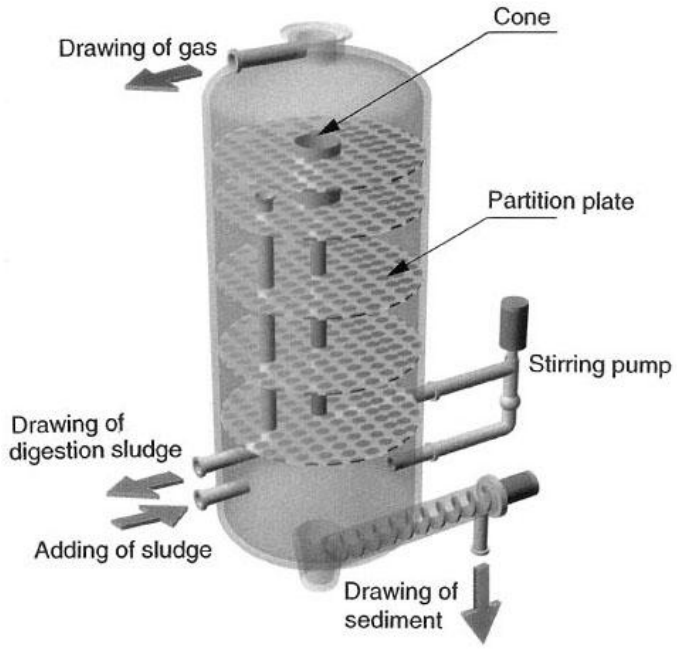

Figure 2.6. Schwarting-Uhde reactor with perforated plates and recycle pump (figure from JAEE (2002))

3 Materials and methods

3.1 Experimental set-up

3.1.1 Thickened sludge

Sludge was collected from WWTP Epe. The influent of this WWTP consists of domestic waste water and slaughterhouse waste water (±25% of influent). The treatment consist of sand/grease removal, biological treatment in Nereda tanks and sand filtration. Since the sludge of this WWTP still looks like activated sludge, it cannot be characterized as aerobic granular sludge. Therefore it is called Nereda excess sludge (NES) to discriminate between 'real' granular sludge (AGS). At WWTP Epe NES is thickened with a filter belt press. Thickened sludge is also composed of backwash water residue of the sand filters. With time intervals of 2 weeks new sludge is collected. The sludge is stored at < 4°C. Of every new batch of sludge the DS, VS, pH and conductivity is measured. Particle size distribution is measured once to check the risk of pore blockage in the UFD.

3.1.2 Continuously fed digester systems

For the continuous test 2 CSTRs (see Figure 3.1) and 3 (semi-) plug flow reactors are used. The CSTR's have a volume of 20 L (HLL DN200-20B). R1 is fed with raw, non-treated sludge. R2 is initially fed with untreated sludge. After two weeks of similar VS conversion, the reactor is fed with sludge which is treated with thermal pressure hydrolysis.

3 up-flow reactors are placed in series, resulting in 1 (semi-)plug flow system. The set-up is depicted in Figure 3.2. UFD 1 is recycled with a recycle: feed ratio of 2:1. UFD 2 & 3 are initially recycled; recycling is stopped when first UFD is able to neutralize the sludge. All three reactors have an effective volume of 8 L and are divided in 5 equal sized sections, separated by permeable disks (pore diameter: 5mm). The HRT of the individual UFD's is thus equal (1/3 of total HRT). A target dry solids concentration of the feed sludge of 4.5% is applied; if required the sludge is diluted with effluent from WWTP Epe. Although in practice generally a higher feed solids concentration is applied, the peristaltic pumps cannot deal with sludge with higher solids concentrations.

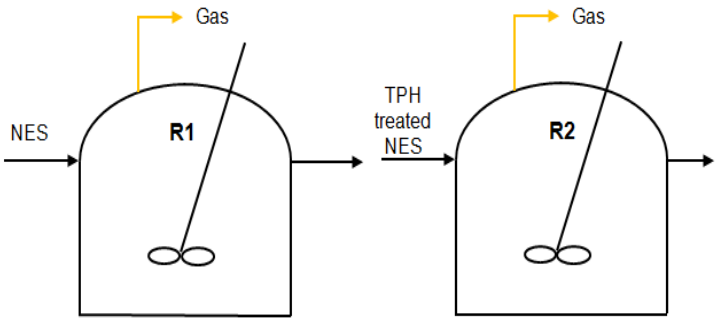

Figure 3.1. Drawing of 2 CSTR systems, fed with untreated, raw sludge and sludge treated with thermal pressure hydrolysis

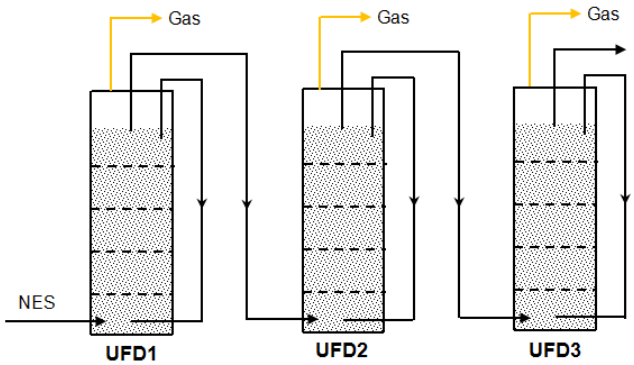

Figure 3.2. Drawing experimental set-up up-flow digesters.

The reactors are semi-continuously fed with peristaltic pumps (Watson Marlow 504U). Once every hour the pumps are automatically switched on and inject around 1/24 of the total day volume into the digester. An equal amount of sludge is withdrawn from the system. The total and organic solids content and biogas production is measured daily on working days.

(Volatile) solids removal is calculated on a daily basis by using both the mass balance equation (1) as the Van Kleeck equation (2). In latter equation the VS conversion is calculated based on the fractional increase of inorganic material of a digested sample (Switzenbaum et al., 2003).

$$VS\ destruction[\%] = \frac{VS_{in} - VS_{out}}{VS_{in}} \cdot 100 \quad (1)$$

$$VS\ destruction[\%] = \frac{VS_{frac,in} - VS_{frac,out}}{VS_{frac,in} - (VS_{frac,in} \times VS_{frac,out})} \cdot 100 \quad (2)$$

In which $VS_{in/out}$ is the concentration of volatile solids in a sample. $VS_{frac,in/out}$ is the volatile fraction of the solids.

VS destruction calculated with equation (1) encompasses both the processes of anaerobic conversion and storage in the system. By assuming that the settled material contains a comparable fraction of organic material as the surrounding sludge, Equation (2) yield the conversion of anaerobic digestion only. In other words: the difference between both equations is an estimate of the amount of settled material. This assumption is validated by a measurement of the DS of the UFD's content at the end of the experimental period.

The VS destruction is calculated with the data obtained per day; the HRT (i.e. 12/20 days) is not considered in the VS destruction calculation for both CSTR's. Since in the UFD's sedimentation takes place, HRT is not equal to SRT and therefore the (volatile) solids reduction is also calculated on a daily basis.

During stable operation, NH_4 measurements are performed on the feed and digestate. Latter parameter is determined weekly, based on a mixed, proportional sample collected during a week.

3.1.3 Thermal pressure hydrolysis pre-treatment

Thermal hydrolysis pre-treatment is performed in a pressure vessel with a volume of 4 L. The pressure vessel is connected to a second vessel, acting as a buffer for temporary storage of expanding sludge during treatment. The second vessel is connected to a compressor, which keeps the pressure between 6-8 bars. After filling of the first vessel with undiluted sludge, the vessel is put in an oil bath. The oil is heated with a tailor-made heating element to a temperature of 160 ^{0}C. When the oil bath is 2 hours at target temperature, the vessel is released from the oil and cooled to room temperature in cold water (± 12 ^{0}C). Although 2 hour thermal hydrolysis is not applied at full scale (see e.g. STOWA (2012)), this duration is applied to guarantee a minimum heating time for the full sludge mixture due to a lack of mixing.

Initially an HRT of 20 days is applied for all three reactor systems. After a month of operation the HRT is decreased to 12 days.

It was expected that the TS, VS and ALE concentration would increase in time in the UFD's due to sedimentation. After finishing the experimental period, the reactor content will be analysed on (volatile) solids and ALE content.

3.2 Batch tests

3.2.1 Experiments

Batch experiments are performed for investigation of the hydrolysis constant and biological methane potential. The experiments are performed in 400 mL bottles, closed with a aluminium cap and septum. The bottles are filled with 120 mL of substrate-inoculum mixture. A substrate-inoculum ratio of 1:3 based on volatile solids is applied. The bottles are placed in a box filled with water, located on a stirring plateau (75 RPM) in which the water temperature is kept at 35 ^{0}C. A simultaneous batch test is started to check for a pH drop (methanogenesis inhibition) when biogas production is maximal (i.e. at day 1). A pH drop was not observed.

In Table 3.1 the performed batch test series are listed. The **first series** are meant to investigate the effect of heating period during pre-treatment duration on the increase of hydrolysis rate. Also the production of inert soluble COD can be estimated by these tests. Substrate preparation is described in section 3.1.

In the **second series** the biodegradability of ALE in soluble (sodium) and insoluble (calcium) form is investigated. Na-ALE is dosed in acid form to the inoculum. Ca-ALE is prepared by neutralizing acid Na-ALE with 4 M NaOH and dosing it into a 3 M $CaCl_2$ solution with a syringe. The granules are kept in the solution for 5 days at < 4 ^{0}C. This procedure is similar to Lin et al. (2012).

The purpose of the **third series** is to investigate how the biodegradability is affected by the thermal treatment applied in ALE extraction (80 ^{0}C for 30 minutes) and what the combined effect is together with alkali dosing.

Table 3.1. Performed batch tests

	Series 1	Series 2	Series 3
Inoculum	R2	R2	UFD3

Substrates	Raw sludge;
	Heated sludge till target temperature , e.g. t =0 min;
	t=30 min;
	t=60 min;
	t=120 min.
		Na-ALE;
		Ca-ALE.
			Raw sludge;
			ALE extraction heat treatment applied on raw sludge (no chemical addition).

N. B. For every series a blanco test is included.

Breakdown calculation

Based on the gas production from the batch test the anaerobic biodegradability is estimated with the following rules/assumptions:

- The gas production is normalized to standard conditions (0 ^{0}C) by making use of Charles' law;
- The fraction of CH_4 in the biogas is 0.62 (Metcalf and Eddy, 2004), validated by measurements of A Akoz, reporting CH_4 fractions between 0.60-0.64 in biogas from NES (results not published).
- 1 kg COD conversion yields 0.350 m^3 CH_4;
- 1 gr VS equals 1.49 – 1.01 gr COD for resp. sludge – ALE (measured values).

Hydrolysis constant estimation

Both the first-order as the Contois hydrolysis constant is determined. The first one is still frequently used and can also be used in modelling software. Chen and Hashimoto (1980) applied Contois kinetics to biological treatment system. The Chen & Hashimoto model is frequently used by dutch water boards (STOWA, 2011). The main difference between both types of kinetics is that with Contois kinetics substrate is distinguished from anaerobic biomass. A minimum residence time for methanogenic bacteria is thus included in the model. For a more detailed description of both types of kinetics reference is made to Annex A.

Both hydrolysis constants are determined by means of MS Excel. The first order hydrolysis constant (k_h) can be determined from the gas production by the following expression:

$$biogas_t = biogas_{max} \cdot (1 - e^{-k_h \cdot t}) \quad (3)$$

The first order hydrolysis constant is determined by minimizing the coefficient of determination between the measured and modelled values by using the Solver Tool. For Contois hydrolysis constant a numerical scheme is applied with a time step of 0,02 days. The constant is obtained again by making use of the Solver tool.

3.2.2 Continuous test validation

With the determined hydrolysis constants determined of the substrates, the conversion efficiencies obtained in the continuous tests are validated.

Completely mixed systems

For both CSTR's two types of kinetics are used to estimate the performance.

With **first order kinetics** the conversion efficiency of biodegradable substrate can be written as:

$$E\ [\%] = \left[1 - \frac{1}{1 + k_h \cdot \theta}\right] \cdot 100 \cdot f_d \quad (4)$$

θ is the hydraulic residence time and k_h the first order hydrolysis constant (Bruning, 2010). f_d is the fraction of biodegradable substrate.

Chen and Hashimoto (1980) developed a model in which **Contois kinetics** is used. The conversion efficiency equation for completely mixed systems reads:

$$E\ [\%] = \left[\frac{\hat{\theta} - 1}{\hat{\theta} - 1 + K}\right] \cdot 100 \cdot f_d \quad (5)$$

In which K is the Contois hydrolysis constant. Note that, in contrast to the first order constant, an *increase* in hydrolysis rate results in a *decrease* of Contois constant value.

$\hat{\theta}$ is a dimensionless parameter and is defined as:

$$\hat{\theta}[d] = \frac{\theta}{\theta_{min}} = \theta \cdot \mu_{max} \quad (6)$$

θ_{min} is the minimum required SRT at which the involved microbial population can maintain itself [d]. This value is the inverse of the maximum specific growth rate of the microorganisms (μ_{max}). In case of anaerobic digestion the growth limiting micro-organisms are methanogenic bacteria. For 35 ^{0}C a μ_{max} value of 0.467d^{-1} is used (according to Chen and Hashimoto (1980)).

Up-flow digester

The UFD's can mathematically be described by plug-flow equations with biomass retention and biomass recycling. The SRT is not equal to the HRT.

According to Bruning (2010) the residence time is defined in general as: $\theta\ [d] = \frac{hold-up\ in\ the\ system}{flow\ through\ the\ system}$.

For the residence time of solids the following equation applies:

$$\theta_x[d] = \frac{VX_r}{Q\frac{X_0}{X_e}} \quad (7)$$

In which V is the reactor volume, X_r is the solids concentration in the reactor [gr/l], X_0 is the solids concentration in the feed [gr/l] and X_e is the solids concentration in the effluent [gr/l].

The solids concentration in the reactor can be measured at the end of the experimental period and can be estimated by using the difference between VS conversion calculated by van Kleeck and mass balance equations. Note that in case of sludge recycle S_0 is composed of both feed and digested sludge.

With this solids retention time we can use known equations for plug-flow systems to estimate the conversion of biodegradable organics, i.e:

$$E\ [\%] = 1 - \frac{S_e}{S_0} \cdot f_d = 1 - \frac{e^{\frac{-k_h \cdot \theta_x}{1+R}}}{1 + R - R \cdot e^{\frac{-k_h \cdot \theta}{1+R}}} \cdot f_d \quad (8)$$

In which R is the recycle ratio [-]:

$$R\ [-] \equiv \frac{Recycle\ flow}{feed\ flow} \quad (9)$$

3.2.3 ALE extraction optimization

Optimization of the extraction process of alginate like exopolysacharides (ALE) is required for economical reasons. A modified method described by Lin et al. (2008) is used to isolate the ALE from NES. The method is modified with respect to the heating period, which is shortened from 2 hours to 30 minutes in the alkali phase. The method is based on the extraction of alginate from seaweed, described in section 2.3.2. As a concentration step of the ALE solution the alginic acid method is selected.

The reference (non-optimized) method is as follows; the corresponding optimization experiments listed in Table 3.2 are indicated in brackets.

Raw thickened sludge is diluted with a sodium-carbonate (Na_2CO_3) solution to a dry solids content of 3% *(2)*. The sodium-carbonate dosage is set at 7.5 mmol Na^+ /gr VS *(1)*. The sludge mixture is heated to 80^{o}C and kept at that temperature for 30 minutes. After centrifugation at 4.500 RPM (5000 G) for 20 minutes the pellet is discarded. The supernatant pH is adjusted to 2.0 by addition of 4 M HCl *(3)*. After centrifugation at 4.500 RPM (5000 G) for 40 minutes *(4)* the precipitate is analysed on volatile solids content, which is assumed to be ALE.

Parameters to be optimized are:

- Required Na^+ dosing to achieve successful ion exchange with bound Ca^{2+};
- Relation pH in alkali phase and yield of ALE;
- Maximum TS concentration during alkali & acid phase;
- pH at which ALE precipitates and can be separated from liquid;
- Required centrifugation time to precipitate ALE.

In Table 3.2 the ALE-extraction optimization experiments are presented. The variable values used in the method of Lin et al. (2008) are indicated in bold.

Table 3.2. ALE extraction optimization experiments.

Exp. No.	Parameter optimized	Variable
1a	Na^+ dosing	Na_2CO_3 dosage: 1.5, 3.0, 4.5, 6.0, **7.5**, 9.0 mmol Na^+/gr VS sludge
2a	Relation pH – ALE yield	Na_2CO_3 dosage: 1.5, 3.0, 4.5, 6.0, **7.5**, 9.0 mmol Na^+/gr VS + pH at 10 by addition of NaOH
2b		pH at 8.5, 9.0, 9.5, 10.0, 10.5, 11.0 by addition of NaOH
3a	Maximum TS concentration without limiting ALE yield.	DS concentration sludge 1%, **2**%, 3%, 4%, 5%, 6%, 7%
3b		Initial DS: 7%, dilution till 3% in acid phase
4a	Required pH to reach precipitation of ALE	HCl dosage to pH 1.0, 1.5, 2.0, 2.5, **3.0**, 3.5, 4.0, 4.5, 5.0, 5.5
4b		HCl dosage to pH 2.0, afterwards NaOH dosage to pH 2.5, 3.0, 3.5, 4.0
5a	Required centrifugation time for ALE separation -	second centrifugation step: 1, 2, 3, 4, 5, 10, 20, 30, **40**, 50 minutes
5b	Different separation equipment for acid ALE separation	Replacement of second centrifugation step by GFC filter, sieve and belt filter

* In experiment no. 2b-5 the Na_2CO_3 dosage applied is adapted according to the results of experiment 1a.

3.2.4 Interactions between digestion/TPH and ALE extraction

To investigate the relation between digestion/thermal pressure hydrolysis and degradation of ALE several experiments are performed. All ALE extraction experiments are performed according to the standard procedure described in the previous section. The experiments performed are listed in Table 3.3.

Table 3.3. Substrates of which ALE yield is determined.

Sludge substrate
TPH heating time raw sludge * • 0 minutes (only brought at temperature); • 30 minutes; • 60 minutes; • 120 minutes.
Digestate CSTR fed with untreated sludge (HRT: 20 days); Digestate UFD fed with untreated sludge (HRT: 20 days); Digestate CSTR fed with TPH treated sludge (HRT: 20 days).
Thickened digestate from CSTR fed with untreated sludge (HRT 20 days) at a dry solids concentration of 5%.

** The same sludge substrates are used for the first batch test series described before.*

3.2.5 Protein content of ALE

To get a better understanding of the possible protein contamination of ALE and the influence of heating and pH on this process, the following experimental series are carried out.

ALE is extracted from raw sludge with 3 different alkalis: NaOH, Na_2CO_3 and $NaHCO_3$. Besides extraction with reference heating time and period (80 ^{0}C, 30 minutes), the extraction is also performed at room temperature (20 ^{0}C) for 1 and 24 hours. From all samples ALE yield is determined, followed by protein content characterization by three different methods. The modified Kjeldahl, modified Lowry and Bradford assay is used (for more details, see following section). Since protein content is expected to be correlated with the hydrophobic properties of ALE, water absorption properties of the samples are also evaluated.

Besides NES, waste activated sludge (WAS) from WWTP Amersfoort is analysed on the same parameters above. For this sludge only the standard extraction procedure is applied.

3.3 Physical and chemical parameter characterization

3.3.1 Total and organic solids

The total and organic solids content of samples were performed according to APHA (1998). DS and VS measurements for the continuously fed digester systems are performed once per day, for the batch tests duplicate samples are taken.

3.3.2 Biogas production

The biogas produced in the continuous reactor experiments is collected in gas bags. The gas bags are emptied on working days and the volume of gas produced is measured with a domestic gas meter. The volume of gas produced in the batch test is measured with syringes (30-60 mL).

3.3.3 Particle size distribution

Particle size distribution of raw, untreated sludge is measured with a SALD-2300 Laser Diffraction Particle Size Analyzer, including a SALD-MS23 sample unit for wet samples. The measurement is performed only once at the onset of the experimental period, to check blockage risk of the up-flow digesters. The sample is 4 times analyzed.

3.3.4 COD and nutrients

COD (total and soluble) and nutrients (total nitrogen, ammonium) are measured using Hach Lange photometric cell tests (Knechtel, 1978, Koroleff, 1972, Koroleff, 1969). The photo spectrometer used is a Hach Lange DR2800. If heating of the sample is required, a Hach Lange LT100 is used. Prior to sampling, the samples are homogenized for 5 minutes using an IKA Ultra-Turrax homogenizer. In case of soluble COD and ammonium measurements the samples are centrifuged at 4500 RPM for 30 minutes (MSE GF8) and filtered over a 0.45 µm membrane of Hach Lange. Dilutions are based on weight.

3.3.5 Protein

For the quantification of protein three methods are used. Lin et al. (2010) used the Bradford method for the detection of proteins in ALE, which is also used in this research. Bradford Coomassie Bleu is obtained from Thermo Scientific. Bovine serum albumin (BSA) is used as internal standard. The Modified Lowry method is the second method used for protein quantification. Modified Lowry reagent and Folin-Ciocalteau is obtained from Thermo Scientific. BSA is used as internal standard. The third method applied for protein detection is a modified version of the Kjeldahl-N method. From a diluted ALE sample total nitrogen, soluble ammonium and nitrate is measured. Latter two compounds are subtracted from the total nitrogen value. For the conversion of insoluble N to protein, it is assumed that all the insoluble N originates from protein and the protein consists for 16% of nitrogen (Raunkjær et al., 1994). Total-N, NO_3 and NH_4 are measured with Hach Lange cuvette tests. All parameters are measured with a Hach Lange DR 2800 photospectrometer.

3.3.6 Water absorption capacity

The water absorption capacity is determined according to a modified version of Pilosof et al. (1985). ALE material is neutralized with NaOH and dried at 60 ^{0}C for 4 days. Dried ALE is added to demineralised water at a concentration of 30 gr TS/L and Turrax homogenization is applied for 5 minutes. The mixture is put in measuring cylinders and centrifuged at 250 G (1000 RPM). As soon as the target speed is obtained, the centrifuge is switched off and the volume of ALE material is measured (0,1 mL accuracy). The water absorption capacity is calculated by the total volume of the sample – the amount of separated water, expressed as mL of water absorbed per gr VS of ALE sample. As the accuracy of this method is low, the results obtained are indicative.

4 Results

In this chapter the results from the experiments are presented. After characterization of the sludge (4.1), ALE extraction optimization experiments are discussed (4.2), followed by the behaviour of ALE during digestion (4.3) and NES digestibility behaviour during the ALE extraction procedure (4.4). Subsequently the results dealing with protein content of ALE are presented (4.5). This chapter is finalized with the results of the digestion experiments of both the batch and continuous systems (4.6).

4.1 Sludge characterization

Several batches of sludge were characterized upon delivery on DS, VS, pH, conductivity, $COD_{(soll)}$ and NH_4 concentration. The results of the measured parameters are listed in Table 4.1. Although the value of some values seem overestimated (COD_{sol}, NH_4), also in literature similar values are found; see e.g. Kim et al. (2003), Eskicioglu et al. (2006) and Val del Río et al. (2011). Most likely pre-hydrolysis of the sludge occurs in the storage and thickening process. Although on average the residence time of sludge in the sludge buffer tanks is limited to a few hours (pers. comm. L. M. M. de Bruin, RHDHV), new sludge is mixed with older sludge both in the excess sludge buffer tank and extraction tank between belt press and sludge storage. Likely most of the soluble compounds originate from the older fraction of the sludge mixture.

The high organic content of the sludge is surprising: 80.7%. This is significant higher than 60-70% reported in literature about AGS with nitrogen and phosphorus removal (de Kreuk et al., 2005a, de Kreuk et al., 2005b). Also incidental measurements of the VS fraction of sludge in the SBR's at WWTP Epe yield organic fractions of 69-71%.

Table 4.1. Overview raw sludge characterization measurements.

	DS	**VS**	**pH**	**Conductivity**	**COD_{tot}**	**COD_{SOL}**	**NH_4-N_{sol}**	**ortho PO_4-P**
	[%]	*[% of DS]*	*[-]*	*[mS/cm]*	*[g O_2/L]*	*[mg O_2/L]*	*[mg NH_4-N/L]*	*[mg PO_4-P/L]*
Average	4.78	80.73	5.98	3.16	60.8	1145.4	438.3	131.1
Std. dev.	0.63	1.67	0.22	1.69	9.6	14.5	135.4	79.64

In Figure 4.1 a photo is displayed of untreated NES, made with a microscope. Granules are not visible; the structure seems more like flocculent sludge. The granular structure could have been (partly) disturbed by the filter belt press and sludge pumps.

A particle size distribution measurement is performed once on the raw sludge. The particle size distribution curves are shown in Annex B. The average particle size was 109-127 µm whereas no particles where detected larger than 900 µm. This result indicate that the risk of blockage of the pores in the UFD's is negligible (pore diameter 5 mm).

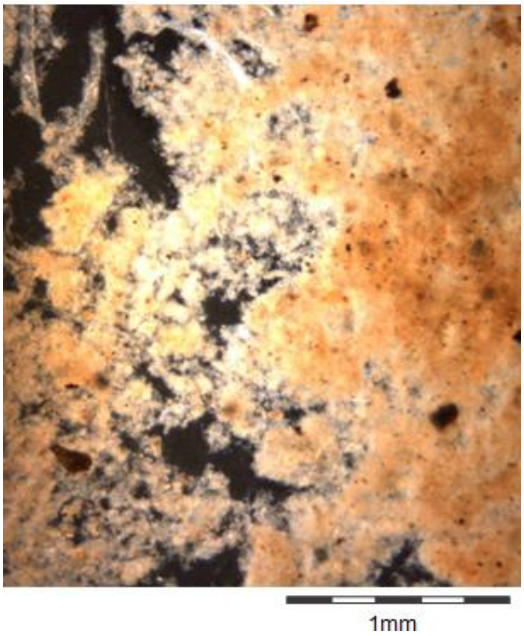

Figure 4.1. Microscopic picture of untreated Nereda excess sludge

4.2 ALE extraction optimization

Several experimental series are performed to minimize cost of chemicals and equipment required for the ALE extraction procedure. In the next sections the relation between several parameters and ALE-yield is discussed. The parameters are: Na^+ dosing, pH in alkali phase, minimum pH required for ALE precipitation in acid phase, maximum DS concentration and centrifugation time required for effective ALE seperation. Note that the enhancement of ALE-yield can be caused by intercellular material contamination. The extent of this contamination is however not of importance for this thesis; the objective is to find parameters in the extraction procedure which influences the yield of ALE.

4.2.1 Na^+ dosing and relation with pH in alkali phase

To reach effective ion-exchange with the Ca^{2+}-ions in the alginate gel, a certain dose of Na^+ is required. The dose is varied between 1.5 – 9 mmol Na^+/gr VS in sludge by means of Na_2CO_3 dosing. The result is depicted in Figure 4.2. In this figure the result of a similar experiment is shown, with as only difference that the pH of the sludge mixture is brought to 10 after Na_2CO_3 dosing by means of NaOH.

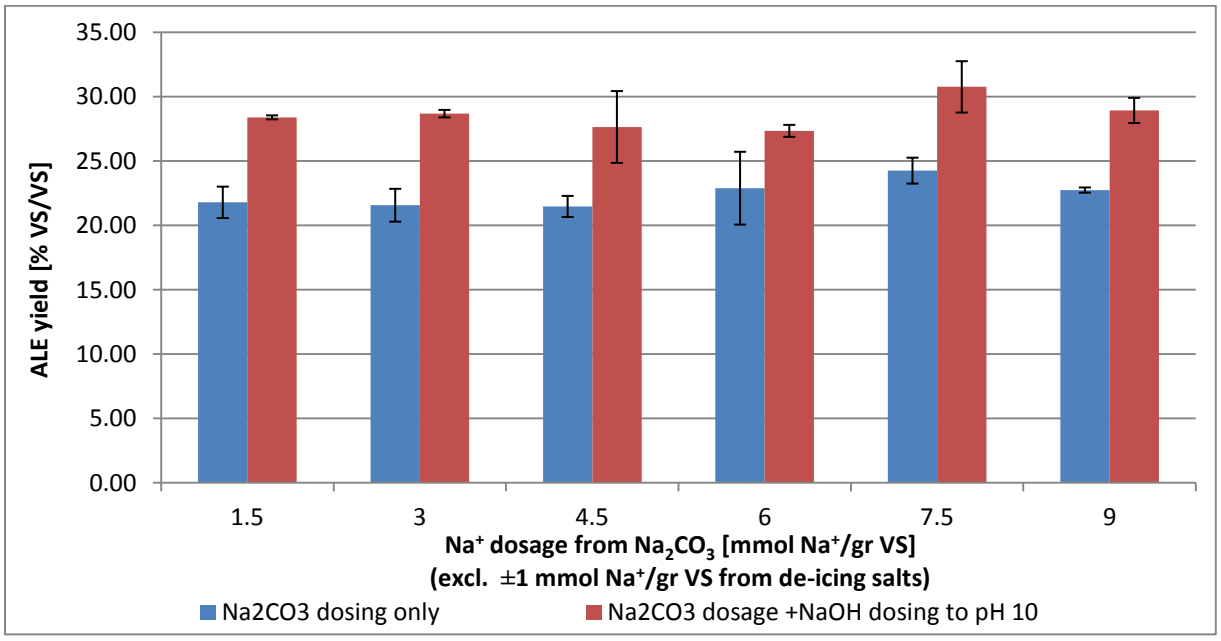

Figure 4.2. ALE-yield with different sodium carbonate dosages for both with and without additional NaOH dosing to pH 10.

From the figure it is clear that the ALE yield is around 22% VS/VS and not lowered by a lower Na^+ dosing within the range tested. By addition of NaOH the yield is enhanced to 28% VS/VS. Note that these experiments were performed when the conductivity of the sludge was relatively high: 1 mmol Na^+/gr VS extra compared to sludge obtained for later experiments. The cause is expected to be NaCl contamination of the wastewater and subsequently the sludge due to de-icing salts from streets (it was freezing outside). In later experiments (where conductivity was lower) Na_2CO_3 dosing of 1.5 mmol Na^+/gr VS reduced yield (see experiment with varying DS concentration).

During the experiments it was observed that the pH decreased by approx. one unit during the heating time to 80 ^{0}C; during the subsequent 30 minutes the pH did not change significantly anymore. NaOH is dosed when target temperature was reached; not prior to the heating. This reduced the extraction time of the NaOH compared to Na_2CO_3 which was dosed prior to heating. If NaOH was also dosed prior to heating, ALE-yield would have been higher. The relation between extraction time and ALE-yield is shown by Lin (2013) and Hernández-Carmona et al. (1999).

In the following experiments the pH of the sludge mixture was increased by addition of NaOH (when target temperature was reached). The Na_2CO_3 dosing was kept constant at 3 mmol Na^+/gr VS. The result is presented in Figure 4.3. The pH reached with3 mmol Na^+/gr VS by Na_2CO_3 dosage only is around 7.5.

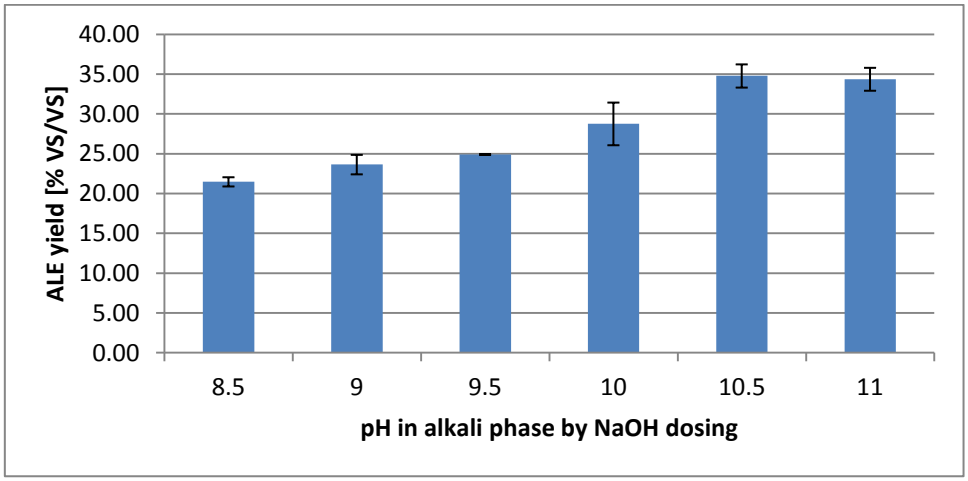

Figure 4.3. ALE yield as a function of pH in alkali phase. pH is increased by NaOH dosing.

Also in the figure above the relation between pH and ALE-yield is observed, ranging from 22 to 34% VS/VS for pH values of 8.5 - 11 resp.

4.2.2 Relation DS concentration and yield

Extraction of ALE with a higher DS concentration of the sludge results in a lower chemical demand and more efficient use of the equipment. ALE-yield is measured at increasing DS concentrations; the results are presented in Figure 4.4. Note that the Na_2CO_3 dose was set at 1.5 mmol Na^+/gr VS. This lower dose was applied due to a temporal shortage of Na_2CO_3. The yield at standard DS concentration (3%) is therefore lower (16% vs. 22%) compared to 3.0 mmol Na^+/gr VS. Sludge used for this experiment did not contain elevated concentrations of de-icing salts anymore, contrary to the Na^+ dosing experiment. The yield seems to be limited at DS concentration from 6-7%.

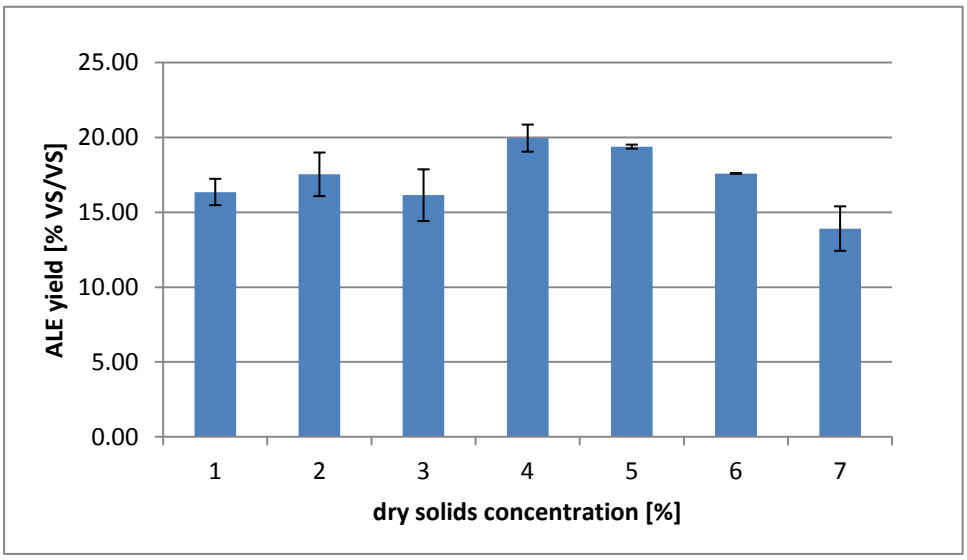

Figure 4.4. ALE yield at increasing DS concentrations of sludge-alkali solution.

This experiment repeated with CSTR digestate used instead of raw sludge yielded similar results: from 5% DS and higher the yield was lowered.

4.2.3 ALE precipitation & separation

After alkali extraction, the ALE solution is separated from the sludge by centrifugation. The supernatant with ALE is too dilute, requiring concentration. ALE is concentrated by means of acid precipitation.

Figure 4.5 shows the result of two experimental series. In the first one the pH is varied by HCl dosing (after alkali phase). In the second series the pH is initially brought to 2, and increased to a higher value by NaOH dosing.

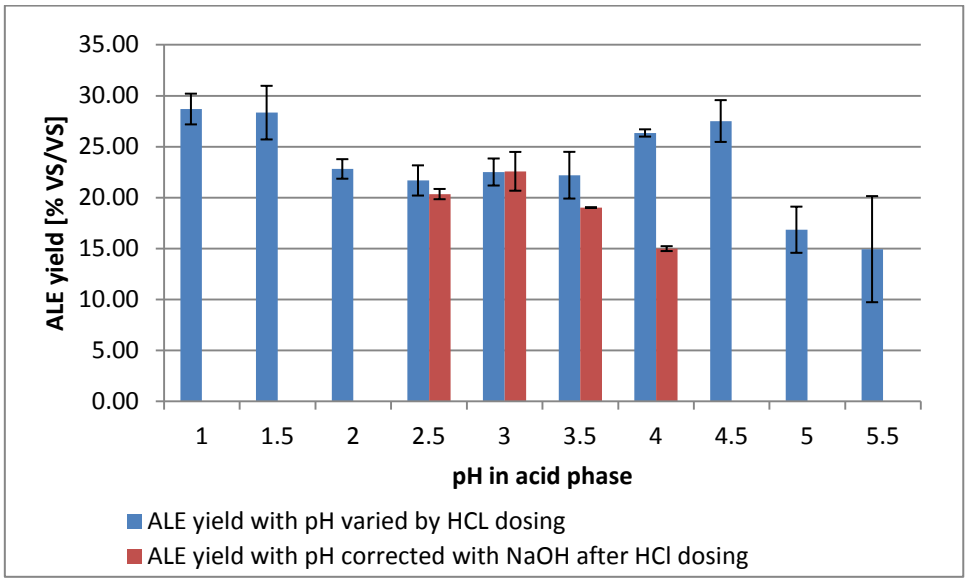

Figure 4.5. ALE yield as a function of pH, controlled by solely HCl dosing and NaOH correction after pH being brought to 2.

The data presented in the figure suggest that a pH value of 4-4.5 yields most ALE. It should be noted however that the alkali solution was prepared at once for all experiments. The experimental series started with the lowest pH value (i.e. pH 1). The experiments with higher pH values (pH 4-5) were thus performed later, resulting in a longer alkali extraction time (at room temperature). This might have influenced the result.

pH correction with NaOH after HCl dosing did not result higher ALE-yield values compared to a lower HCl dosing: at pH 4 by solely HCl dosing an ALE yield of 26% is obtained, whereas by NaOH correction the yield is 15%. pH values higher than 4.5 result in a loss of ALE.

Although the ALE yield is not affected at a pH of 4-4.5, the harvested material shows to be more fluffy compared to ALE precipitated at lower pH values. This indicates that the transition between soluble-insoluble phase of ALE is reached at a pH value of 4-4.5.

In Figure 4.6 the relation between centrifugation time and ALE-yield is depicted, indicating that the yield is rather independent of centrifugation time within the range tested. The sample harvested after 5 minutes centrifugation was less sticky/better separable from centrifuge container bottom compared to the sample centrifuged for 40-50 minutes.

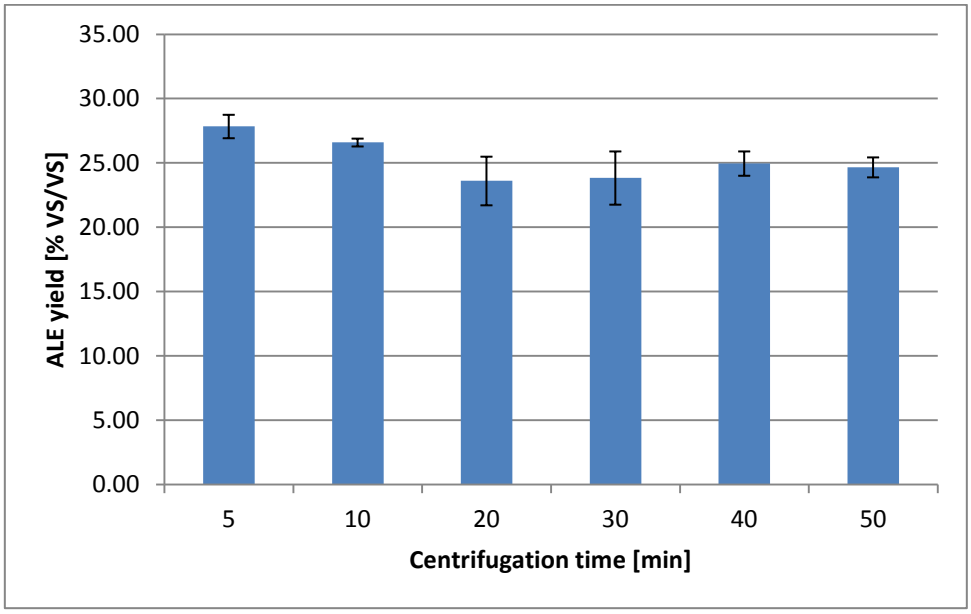

Figure 4.6. ALE yield as a function of centrifugation time in acidic phase (second separation step).

Instead of centrifugation GFC filters and a sieve was used to evaluate the applicability of other separation methods. The filters were clogged directly after acid ALE dosing. The principle of filtration is not usable for acid ALE separation.

4.3 ALE stability during digestion

Digestate of the different digester systems were analyzed on ALE content in order to asses to what extent ALE is degraded anaerobically. In Table 4.2 the yields of ALE from R1, R2and UFD digesters are presented. The digestion performance results are presented in section 4.6. Also corrected values are presented to normalize results to undigested VS values. In Figure 4.8 the ALE yield from TPH pre-treated (undigested) sludge with increasing treatment periods is depicted

The corrected yield of the reference digester is 25%, which is comparable to the yield of raw sludge. During TPH treatment ALE yield is reduced already (from 20 % to 13%) and seems to be modified to an easier biodegradable form: after digestion only 7.3 % is left over. Note that the extracted acid ALE had a darker colour and was more mucous, which possibly indicates a composition change. ALE-yield of the UFD reactor is reduced to 17.7%, which is probably caused by ALE retention within the digester (more than the other solids). The content of UFD reactor content is also analysed on ALE-content; these results are presented in section 4.6.

Table 4.2. ALE recovery from digestate from reference, TPH and UFD digesters.

Substrate from with ALE is extracted	**ALE yield** *[% VS/VS]*	**Corrected ALE yield *** *[% VS/VS]*
Digested sludge from R1 (HRT: 20 days)	43.9 ± 4.4	25.3
Digested sludge from R2 (HRT: 20 days)	14.4 ± 0.6	7.26
Digested sludge from UFD3 (HRT: 20 days)	35.1 ± 2.0	17.7

* Correction is applied to normalize results to undigested conditions. This is done to enable comparison with undigested sludge (ALE yield of ± 23% VS/VS).

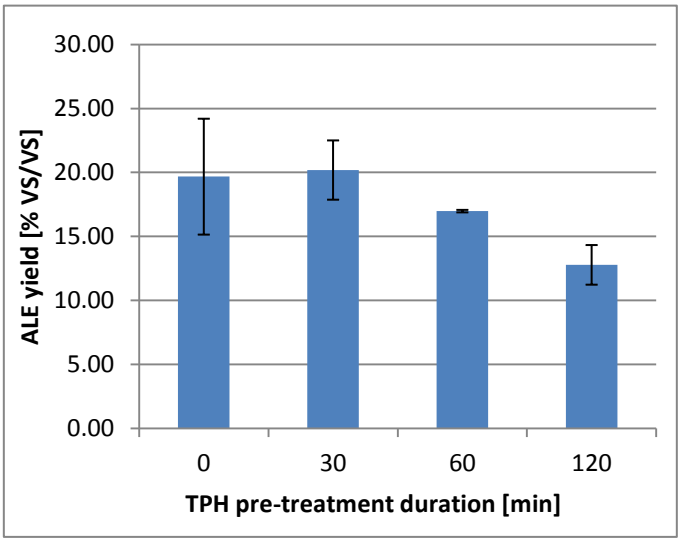

Figure 4.8. ALE-yield as function of thermal pressure hydrolysis pre-treatment duration applied on raw sludge.

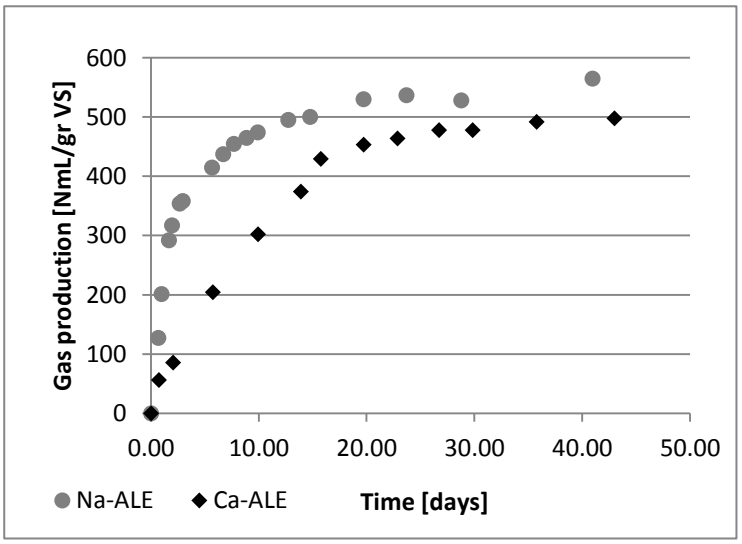

Figure 4.7. Normalized cumulative gas production obtained from Na-ALE and Ca-ALE degraded anaerobically in batch tests.

Figure 4.7 presents the cumulative gas production obtained in batch tests in which both Ca- and Na-ALE were investigated separately. The biodegradability and Contois and first order kinetics hydrolysis constants are listed in Table 4.3. Ca-ALE is degraded at a lower rate; the extent of degradation is almost similar: 99 and 87% for resp. Na- and Ca-ALE. Note that a higher hydrolysis rate results in a lower Contois K and a higher first order kintetics hydrolysis constant k_h.

Table 4.3. Anaerobic biodegradability and Contois and first order kinetics hydrolysis constants of both Na- and Ca-ALE.

substrate	Biodegradability – 41 days *[%]*	Contois hydrolysis constant (K) *[-]*	R^2 *[-]*	First order hydrolysis constant (k_h) *[-]*	R^2 *[-]*
Na-ALE	99 ± 1.4	1.09	0.90	0.32	0.90
Ca-ALE	87 ± 0.2	3.44	0.99	0.10	0.99

4.4 NES digestibility reduction during ALE-extraction

To assess the extent to what the biodegradability of NES is affected during ALE extraction, a batch test is performed in which both raw sludge and sludge heated to 80 ^{0}C for 30 minutes is digested (applied in ALE extraction procedure). In contrast to the ALE-extraction procedure, no chemicals are added. The result is depicted in Figure 4.9. A decrease in biodegradability can be observed due to thermal treatment applied for 30 minutes at 80 ^{0}C. After 35 days the gas production is around 13% (relative) lower than the raw sludge.

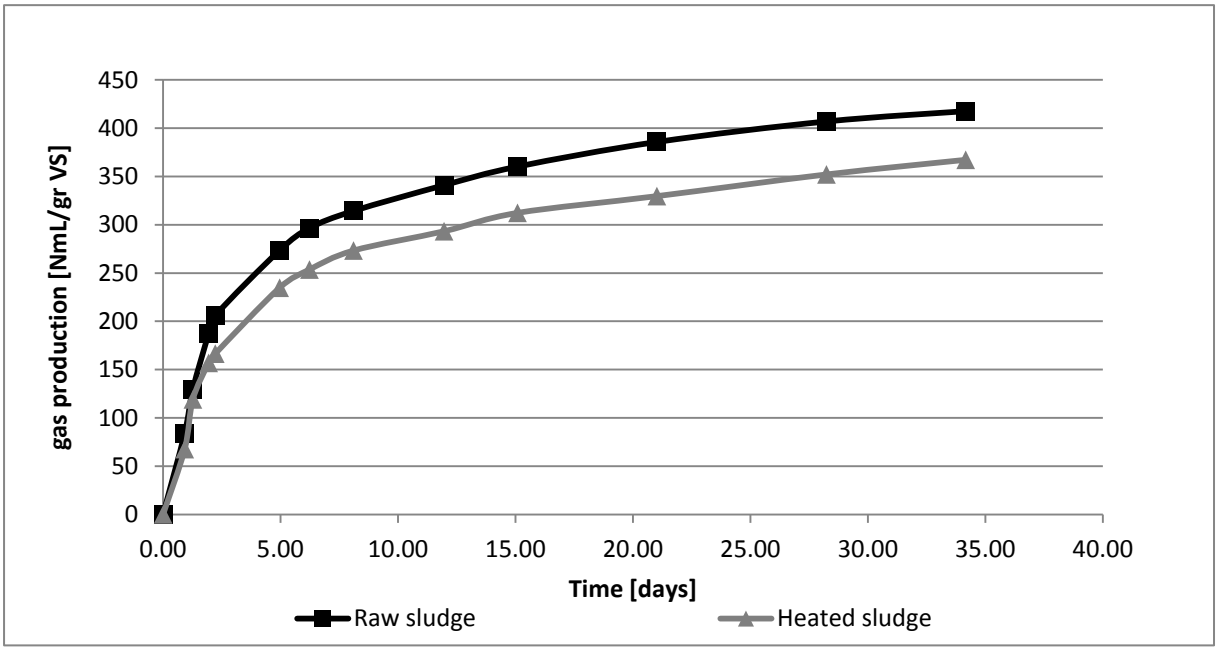

Figure 4.9. Normalized cumulative gas production in time for untreated AGS and sludge prepared according to the ALE-extraction procedure.

4.5 Protein content of ALE

The results of ALE-yield, protein content and water absorption capacity of the extracted ALE is presented in Table 4.4. Besides the ALE from raw NES, ALE is extracted and characterized from digestate of two digester systems (CSTR and UFD). Furthermore raw NES is analyzed on soluble protein content. As a reference ALE is extracted and analyzed on protein content from secondary sludge of WWTP Amersfoort. Latter WWTP is a conventional activated sludge system with primary clarification and partly biological/partly chemical P-removal. The alkali dosage used is 3 mmol Na^+/gr VS.

In first instance brown colour interference developed in the total-N measurement (Koroleff-method). Stronger dilution (30-40 times) resulted finally in a clear sample and appropriate readings.

Table 4.4. ALE yield, soluble protein content measured with 3 methods and water absorption capacity detected in ALE extracted from NES with 3 alkali ion-exchangers at 2 temperatures and 2 durations. Digested NES from CSTR and UFD digesters is also analyzed on earlier mentioned parameters. Raw sludge of Epe is analyzed on soluble protein content and ALE extracted from sludge from WWTP Amersfoort is characterized on protein content and presented as reference.

Material analyzed	Ion exchange alkali + pH	Temperature	Duration extraction period	ALE yield	Protein modified Kj-N	Protein Lowry	Protein Bradford	Water absorption capacity
	[-]	*[^{0}C]*	*[hour]*	*[% VS/VS]*	*[mg/g VS]*	*[mg/g VS]*	*[mg/g VS]*	*[ml/gr VS]*
ALE extracted from untreated sludge	NaOH 13.0	80	1	47.70±2.2	536±19.3	496±1.7	230±0.5	5.8±0.7
		20	1	30.20±0.4	595±11.2	555±30.9	230±3.4	6.0±0.7
		20	24	32.60±1.6	520±13.5	480±21.7	203±0.5	5.6±2.3
	Na_2CO_3 9.2	80	1	25.14±1.1	434±10.2	217±20.6	30±1.3	1.2±0.4
		20	1	6.95±0.01	652±49.2	225±25.7	29±1.3	3.4±0.7
		20	24	11.22±1.0	352±28.4	288±27.8	36±6.0	1.3±0.8
	$NaHCO_3$ 7.0	80	1	16.71±0.3	513±16.0	726±95.0	20±2.2	2.4±1.1
		20	1	3.99±2.1	599±78.9	228±11.6	44±3.2	3.1±0.9
		20	24	8.35±0.1	342±2.1	226±7.5	25±5.0	3.9±1.1
ALE from CSTR digestate (HRT 20 days)	Na_2CO_3	80	1	25.3*	472±44.7	235±4.7	22±3.6	2.8±1.3
ALE from UFD digestate (HRT 20 days)	Na_2CO_3	80	1	17.7*	331±20.1	150±2.9	12±3.4	5.9±2.1
Raw sludge Epe	-	-	-	-	398±7.8	190±8.9	53±1.3	n. d. **
ALE extracted from WAS from WWTP Amersfoort	Na_2CO_3	80	1	21.33±1.5	268±12.9	121±8.4	4±2.6	n. d.

* ALE-yield is corrected for VS destruction during digestion.

** n.d. = not determined

Several trends are visible in the data presented in Table 4.4. ALE yield varies from 4% to 48% VS/VS at resp. 20 ^{0}C with $NaHCO_3$ and 80 ^{0}C with NaOH. The ALE yield is enhanced with a higher pH applied in the alkali phase, both at 80 ^{0}C and room temperature. A longer extraction time results in a higher yield at 20 ^{0}C. Also the temperature has the same effect.
Protein content shows a less clear relation. Modified Kj-N method results in a protein content of around 50% of ALE, rather independent of the type of extraction temperature, duration or base used. The Modified Lowry method yields a protein content of 50% in case of extraction with NaOH; using Na_2CO_3 or $NaHCO_3$ the content ranges from 22-29% (except one outlier in case of extraction with $NaHCO_3$ at 80 ^{0}C).
Bradford's assay detects only a small fraction of what is detected by the other two methods. In case of Na_2CO_3 and $NaHCO_3$ applied for extraction the protein content ranges from 1-4%. Independent of temperature, the protein content is measured at 20-23% when NaOH is used as extraction agent. Linked to this, the water absorption capacity is doubled to a value of 5.6-6.0 mL/gr VS.

ALE extracted from sludge digested with a CSTR system (HRT = 20 days) show similar characteristics compared to raw sludge with the same extraction method. ALE extracted from sludge digested in an UFD digester shows a lower ALE-yield and a lower protein content: 330, 150 and 12 mg/gr VS for resp. mod. Kj-N, Lowry and Bradford method.
Raw sludge shows somewhat lower results for protein content compared to ALE, indicating that the majority of soluble protein of NES is present in ALE/EPS.

4.6 Digestibility of aerobic granular sludge

4.6.1 Effect of temperature pressure hydrolysis on biodegradation

Batch tests

Biogas production of raw sludge and pre-treated sludge was measured. Pre-treatment periods varied: substrates treated for 0, 30, 60 and 120 minutes were used. A treatment period of 0 minutes means that the sample is only heated till target temperature (160 ^{0}C). The results are shown in Figure 4.10. To make the figure readable curves are displayed rather than points. The points are indicated in Figure 4.11. Publishing standard deviation bars would make the figure unreadable. The average standard deviation of this batch test series was calculated at 1.6% of the measured gas production. In Figure 4.10 a clear increase in degradation rate can be observed, as more biogas is produced in the first few days due to thermal treatment of the sludge. Also the duration of treatment show a positive correlation with the biogas production. The gas production from the batches fed with the substrate of 120 minutes thermal treatment seems to be flattened from 30 days onwards, indicating that all the biodegradable substrate is depleted. As other batches fed with substrate treated for a shorter time show a higher gas production, this result tend to indicate that inert compounds are formed during long pre-treatment. Note that the gas production of raw sludge is very similar to that of raw sludge in the experiment discussed in section 4.4.

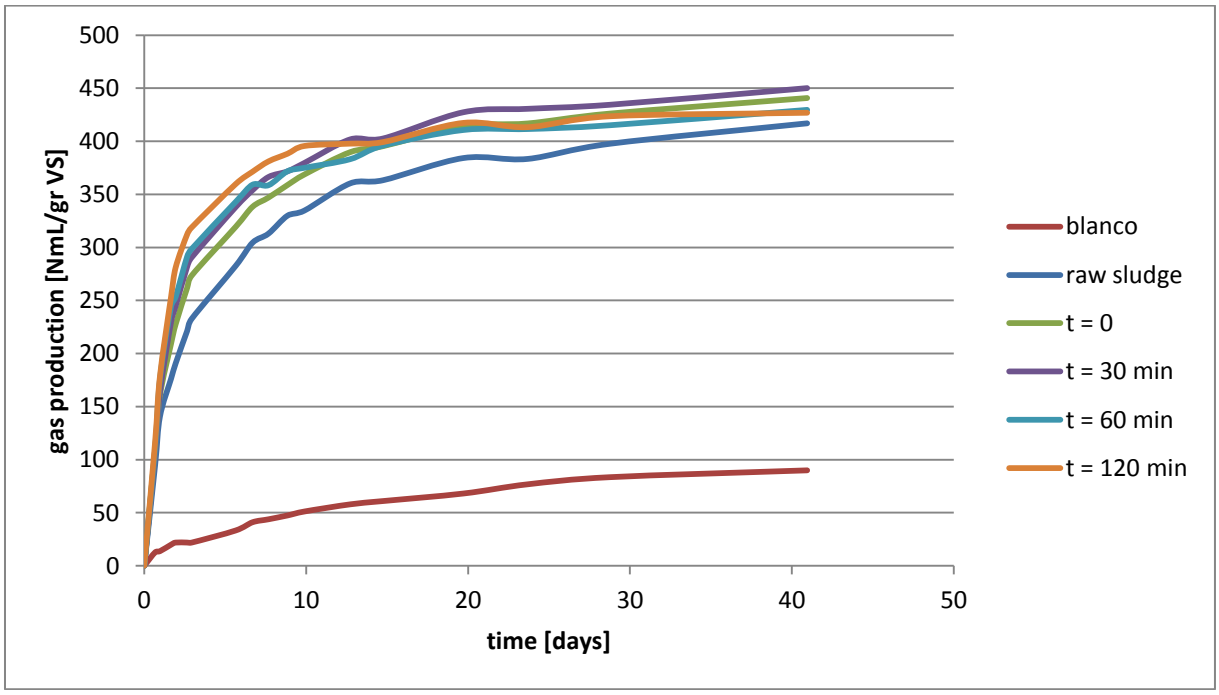

Figure 4.10. Normalized cumulative gas production from raw sludge and sludge treated with thermal pressure hydrolysis for 0, 30, 60 and 120 minutes. 0 minutes treatment means that sludge is only brought at target temperature (T=160 ^{0}C).

Figure 4.11 shows the gas production translated into VS breakdown. The calculated VS breakdown yield results which are very close to the results obtained by a mass balance applied on the initial and final DS-VS content. Figure 4.11 also displays the modelled breakdown curve. The curve represent both first order as Contois kinetics, since their behaviour is very similar. Because the majority of data points is located at start of experiment (gas production is initially the highest), the difference between measured and modelled values is higher from ±10 days onwards. In other words: the accuracy of the hydrolysis constant correlates with the gas production rate. The lower the gas production (i.e. breakdown), the less accurate the modelled value will be.

In Table 4.5 first order and Contois kinetics hydrolysis constants are indicated, including the coefficient of variation. For both types of kinetics it is observed that the hydrolysis rate is doubled when 2 hour during thermal pre-treatment is applied compared to raw sludge. The hydrolysis rate can only be determined if hydrolysis is the rate limiting step in the process, i.e. methanogenic activity is not limited. A pH drop was not observed in an additional batch test fed with the same substrate-inoculum, indicating that methanogenesis inhibition did not occur or to a limited extent.

In Figure 4.12 the solubilization of COD is depicted. Note that the COD_{sol} of raw sludge is measured at 1.1 gr/L and COD_{tot} at 60.9 gr/L. During heating of the sludge till 160 ^{0}C around 13 gr/L COD is solubilized. After reaching 160 ^{0}C the increment in sol COD shows a linear relation with heating duration. After 2 hours of heating the COD_{sol} concentration is 26 gr/L, equaling 43% of COD_{tot}.

COD_{sol} concentrations of the batch tests at the end of the experiment were also determined to investigate the effect of refractionary soluble COD formation. Unfortunately it was not possible to distinguish the COD_{sol} from inoculum and substrate in the batches. After correction for inoculum negative COD_{sol} values were found; the data is therefore not published.

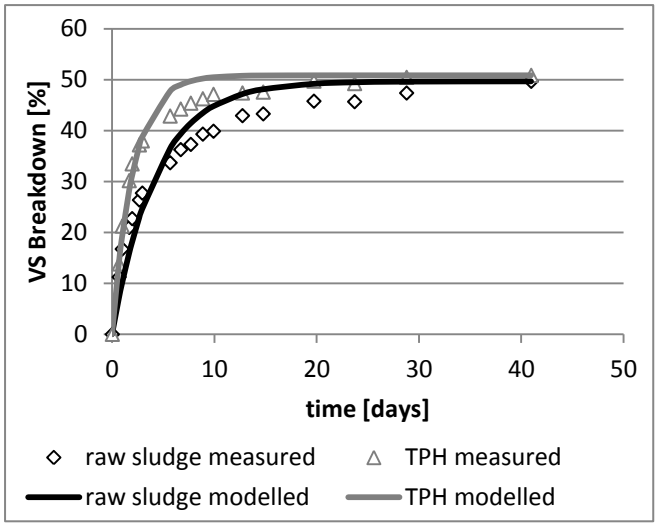

Figure 4.11. Measured and modeled breakdown points/ curves for raw sludge and thermal treated sludge (2 hours). The modeled curves are both first order and Contois kinetics; these curves are very similar.

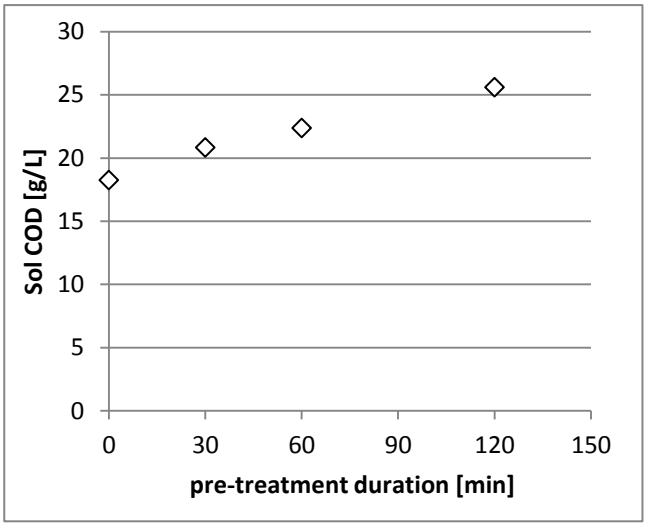

Figure 4.12. COD-solubilisation as a function of pre-treatment duration. The standard deviation was too small to be visible.

Table 4.5. Hydrolysis constants and coefficient of determination for raw sludge and thermal pre-treated sludge treated for 0, 60 and 120 minutes.

	First order kinetics		Contois kinetics	
substrate	k_h	R^2	K	R^2
Raw sludge	0.232	0.916	1.48	0.914
t = 0 min	0.290	0.914	1.19	0.911
t = 60 min	0.402	0.925	0.86	0.923
t = 120 min	0.485	0.962	0.71	0.961

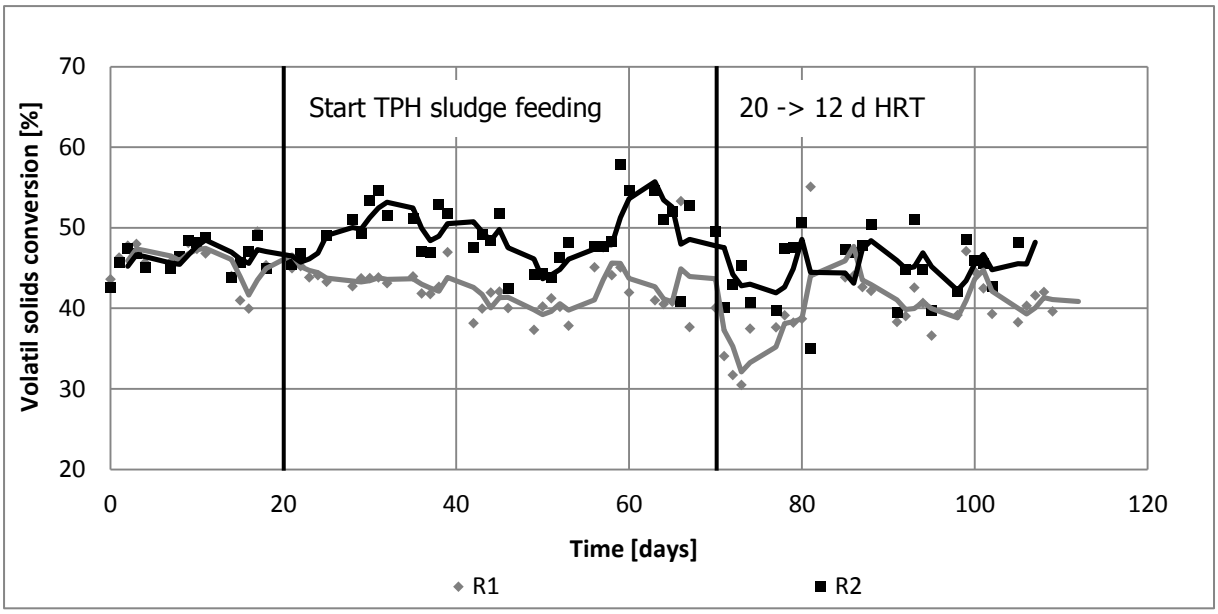

Figure 4.13. VS conversion in time for R1 (digester fed with raw sludge) and R2 (digester fed with thermally treated sludge). Calculated data points and a moving average curve is presented, latter curve with a period of 4 measuring days.

4.6.2 Continuous tests

In Figure 4.13 the calculated VS conversion is shown in time for both digesters fed with raw and thermal pressure hydrolysis treated sludge. Conversion is calculated with the mass balance; the van Kleeck conversion values were very similar to mass balance values and therefore not presented. In Table 4.6 the average values of DS & VS conversion and specific gas production are listed, both for the HRT of 12 and 20 days. The VS conversion is calculated as the average of the van Kleeck and VS balance equation results. In this table also the periods in which the applied HRT was 12 and 20 days is indicated, corresponding to the dates shown in Figure 4.13.

From approximately day 80 onwards a volume increase in R2 was observed. Later on it became clear that the heating spiral leaked water into the digester, resulting in dilution. Based on the van Kleeck equation, the VS – DS removal is corrected for this effect. Note that the HRT is also lowered to 9.2 days due to the dilution.

Figure 4.13 and Table 4.6 show an increase of solids destruction during digestion for both retention times applied. VS conversion with an applied HRT of 12 days resulted in 42.5% and 45.7% conversion for resp. R1 and R2; a relative increase of 8%. In case of an HRT of 20 days, the relative difference is higher: 13%; the corresponding VS conversion values are 42.4% and 47.9% for resp. R1 and R2.

No clear relation can be found between HRT/ pre-treatment and specific gas production. The experimental set-up is vulnerable for gas leakages; the obtained gas production is therefore mainly indicative.

Table 4.6. VS, DS and specific gas production for R1 & R2 fed with resp. raw and thermally treated sludge. Values are indicated for both 12 and 20 days HRT; VS destruction is calculated as the average of the van Kleeck and VS-balance equations.

	HRT = 12 days Day 70 - 112			**HRT = 20 days Day 24 - 70**		
	VS conversion	**DS conversion**	**Spec. gas production**	**VS conversion**	**DS conversion**	**Spec. gas production**
Digester	*%*	*%*	*N L/kg VS removed*	*%*	*%*	*N L/kg VS removed*
R1	42.5	34.6	1020	42.4	34.1	950
R2	45.7	36.5	1080	47.9	40.5	860

4.6.3 Modified schwarting-Uhde reactor performance

Separate from the 2 CSTR's, 3 up-flow digesters (UFD) according to the Schwarting-Uhde concept were operated in series to digest untreated aerobic granular sludge. Sedimentation of particles is expected, enabled by up-flow operation. Due to this solids retention, a higher solids conversion is expected. To distinguish between measured DS-VS conversion caused by settling and further breakdown, two equations are used. With the mass balance the combined effect of sedimentation and conversion is calculated; the van Kleeck equation determines only the biological degradation of solids.

Figure 4.14 shows the result of both calculations. VS conversion (+ storage) is indicated by points and its moving average trend line with a period of 4 measuring days. The green curve represents the difference between the mass balance and van Kleeck equations. Surprisingly the van Kleeck equation shows the same initial decreasing pattern as the mass balance. Most likely the additional breakdown in the first part of the experimental period (day 1-40) is caused by additional breakdown of the inoculant rather than storage. Still, the difference between both curves remains more or less constant till day 70, indicating that till that day the sedimentation rate is more or less in equilibrium with the additional breakdown. Note that from day 70 onwards the HRT was decreased to 12 days, resulting in higher up-flow velocity. In addition to that the recycle pumps of the second and third UFD were switched off as the first reactor was able to provide neutralized sludge. These two parameter changes will result in less sedimentation and possibly preferential flow path development. As a result: the conversion is lower and the retention of solids seems to be stopped (difference between mass balance and van Kleeck equation is close to 0).

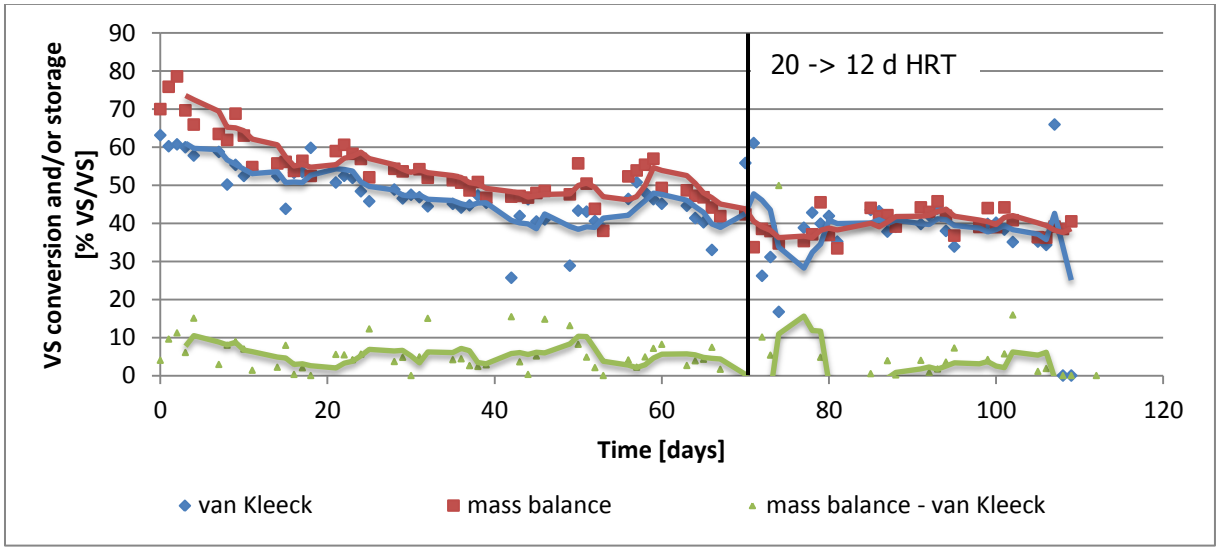

Figure 4.14. VS conversion in time for UFD reactors, calculated by mass balance and van Kleeck equation.

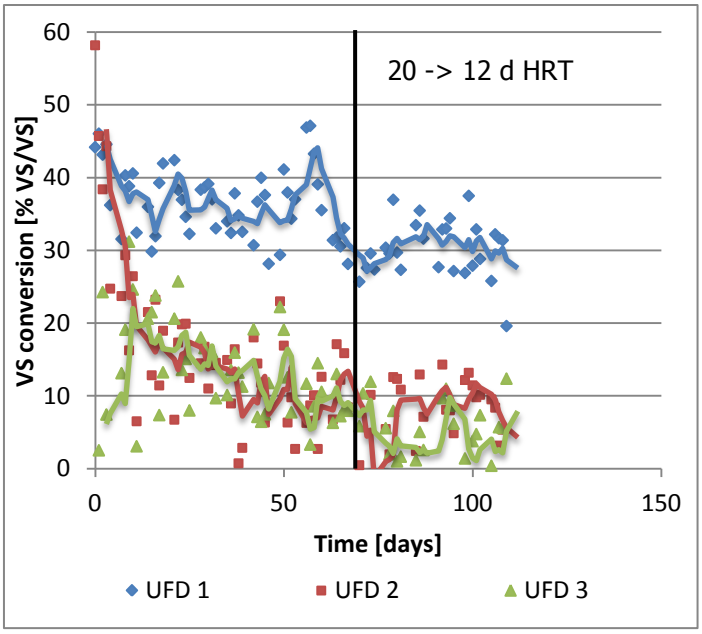

Figure 4.15 VS conversion calculated with mass balance equation for every up-flow UFD separately. Trend line is moving average with period of 4 measuring days.

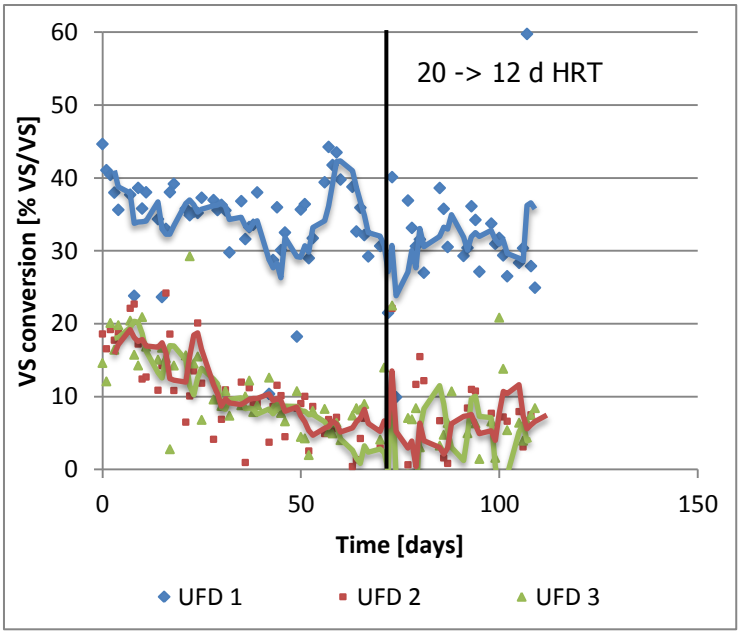

Figure 4.16. VS conversion calculated with van Kleeck equation for every UFD separately. Trend line is moving average with period of 4 measuring days.

In Figure 4.16 and Figure 4.15 the VS conversion is presented, calculated with resp. van Kleeck and mass balance equation. The conversion is calculated separately for the three UFD's. These figures enable us to investigate if sedimentation occurs in an earlier (first reactor) or later stage (second or third reactor).

The difference in VS conversion calculated with both equations in the first UFD digester is low. This indicates that sedimentation doesn't play a dominant role in the first digester. Most likely this is caused by the feed sludge which is still quite viscous (4.5 % DS).

For the second UFD digester the differences are more apparent. Based on the mass balance the VS conversion is high, whereas the calculation with the van Kleeck equation shows moderate results. The initial high conversion efficiency seems to be mainly caused by settling of solids. VS conversion calculated with van Kleeck shows for both UFD 2 and 3 a similar pattern. For these reactors partly sedimentation but also further degradation of sludge plays a role in the calculated conversion. Note that digestate samples from UFD 1 & 2 are taken directly from the reactor and don't represent the full amount of sludge produced by the reactor concerned. This makes the sampling more vulnerable for temporary fluctuations.

Average DS-VS conversion values for the separate and combined digesters are listed in Table 4.7 for both HRT periods applied. Also the specific gas production is indicated. The VS breakdown in the first UFD digester is rather independent of the HRT applied (33 – 34%). Expected was that due to shorter HRT, the conversion process would take place more in the last part of the system (e.g. UFD 2 & 3). The opposite seems however to be the case.

Table 4.7. DS, VS conversion for separate and combined UFD digesters, calculated for both 12 and 20 day HRT periods. Conversion is calculated based on the van Kleeck equation. Specific gas production is indicated as well.

	HRT = 12 days			HRT = 20 days		
	DS conversion	**VS conversion**	**Spec. gas production**	**DS conversion**	**VS conversion**	**Spec. gas production**
digester	*[% DS/DS]*	*[% VS/VS]*	*[N L/kg VS removed]*	*[% DS/DS]*	*[% VS/VS]*	*[N L/kg VS removed]*
UFD 1	26.7	32.7		27.1	33.9	
UFD 2	3.5	4.6		4.9	6.7	
UFD 3	5.1	6.9		5.71	8.0	
UFD 1 + 2	29.2	35.9		30.7	38.4	
UFD 1 + 2 + 3	32.8	40.3	824	34.7	43.3	773

Sedimentation effects

The difference between van Kleeck and mass balance conversion is used to estimate the amount of stored solids. After finishing the experiments, the content of the UFD digesters is analyzed on solids and ALE-content. The results are shown in Table 4.8. After the digesters were opened, a very viscous sludge mixture was found, which seemed to have good gelling properties.

Several trends can be observed from the data in the table:

- Solids accumulate as expected: in UFD 1 the DS content inside the digester is 15% higher than the digestate. For UFD 2 & 3 the solids concentration is 50% higher. This difference is caused by the higher settling potential as the sludge fed to the digester contains fewer solids and is less viscous. Besides that latter 2 digester are not recycled, whereas the first one was recycled. The resulting upflow velocity and mixing is reduced, enabling more solids to settle.
- ALE-yield is low compared to earlier analysis performed on digested sludge, see section 4.3. Since the digestate of the UFD reactors contained less ALE compared to the CSTR digester fed with raw sludge, it was expected that part of the ALE is retained inside the system. This is not confirmed with the data in Table 4.8. It seems that part of the ALE is retained and degraded within the digesters.
- The VS fraction of the solids within the digester is significantly lower than the digestate: 65% within digesters vs. 72-74% of digestate. This result indicates that the retained solids are further biodegraded than the non-retained sludge/digestate. As this effect does not occur in the first UFD digester indicates again that solids retention is not a major process in the first digester.
- The difference in VS fraction between digestate and reactor content infirms the assumption used earlier that the settled solids have the same VS fraction as the sludge in the digester concerned. This assumption was made for using the van Kleeck equation for quantification of VS conversion solely, whereas the mass balance equation was used for both sedimentation and conversion processes. Using both equations the settled amount of solids can be estimated. Note however that the digester content is analysed at the end of the experimental period, whereas the difference between van Kleeck and mass balance conversion values were the largest during the first 70 days of the experiment. Solids within the digesters will not have been degraded that far at that moment as they are during analysis after 112 days.

Table 4.8. DS/VS analysis results of UFD reactor contents, including ALE yield and average DS/VS values of digestate of UFD reactors during last 40 days of operation.

	DS reactor content	VS	Average DS digestate	Average VS digestate	ALE-yield
Digester	*[%]*	*[% DS]*	*[%]*	*[% of DS]*	*[% VS/VS]*
UFD 1	3.85	74	3.34	75	23 ± 0.8
UFD 2	4.50	65	3.06	74	20 ± 1.0
UFD 3	4.49	66	2.97	72	20 ± 0.2

The increase in SRT compared to HRT is estimated by two methods:

- The quantity of settled material can be estimated by the difference between calculated VS conversion of the Van Kleeck (VK) and mass balance (MB) equations.
- The quantity of settled material is measured at the end of the experimental period (values of Table 4.8).

The results are listed in Table 4.9. The difference between both methods is significant. This is caused by several reasons:

- The solids concentration in the reactor is only measured at the end of the experimental period and does not account for a gradual increasing DS concentration in time;
- DS samples of digested sludge of UFD 1 and 2 are instantaneous measurements and are thus subject to temporal variations. This influences the accuracy of the measurement.

Nonetheless, the data in Table 4.9 seems to confirm the hypothesis that sedimentation is not a major process in the first UFD as the increase in SRT is limited (or even negative). In the second and third UFD this effect is more abundant.

Table 4.9. HRT and SRT values of three up-flow digesters determined by two different methods.

digester	HRT 12 days			HRT 20 days		
	HRT	SRT 'measured'	SRT VK – MB	HRT	SRT 'measured'	SRT VK – MB
	[days]	[days]	[days]	[days]	[days]	[days]
UFD 1	4	3.4	4.3	6.7	7.2	7.9
UFD 2	4	5.1	5.0	6.7	9.7	11.1
UFD 3	4	6.0	4.84	6.7	10.7	7.8

4.6.4 NH_4 measurement results

From raw and pre-treated sludge and digestate from the digester systems samples were analyzed on the NH_4-N content. The average result and standard deviations of these measurements are shown in Table 4.10.

Table 4.10. NH_4-N measurement results.

Sludge analyzed	HRT 12 days	HRT 20 days
	[mg NH_4-N/L]	[mg NH_4-N/L]
Raw sludge	438 ± 135	
Digestate raw sludge digester	1416 ± 108	1444 ± 67
TPH treated sludge	520 ± 37	
Digestate TPH digester	1468 ± 65	1730 ± 149
Digestate UFD digester	1396 ± 67	1565 ± 98

4.6.5 Comparison batch and continuous test results

Having determined the hydrolysis constants for both first order and Contois kinetics, the VS conversion performance can be estimated and compared with the lab results. The major difference between both type of kinetics can be observed in Figure 4.17. A minimum SRT is incorporated in Contois kinetics, whereas it is not taken into account for first order kinetics. The measured and estimated values are listed in Table 4.11. The values estimated with Contois kinetics/Chen and Hashimoto model are slightly closer to the measured VS destruction. Surprising is the fact that in all cases the predicted conversion is lower than the measured breakdown. In Figure 4.11 it was observed that the modelled hydrolysis constant is overestimated from day 6-30, since the gas production pattern did not represent exact exponential shape behaviour. Based on that it, is expected that the breakdown in CSTR systems with a long retention time (e. g. 20 days) is also overestimated. This is however not the case. Nonetheless, the difference between estimation and measured conversion for 20 days HRT is smaller compared to the difference in case of 12 days HRT. The results in Table 4.11 suggest that the hydrolysis constants are underestimated. A reason for this could be that the normalization of biogas volume is too strict. For this correction calculation it is assumed that the temperature of the biogas is equal to the sludge temperature in the batches (e.g. 35 ^{0}C). Temperature of the measuring syringes is however lower, resulting in a reduced biogas volume.

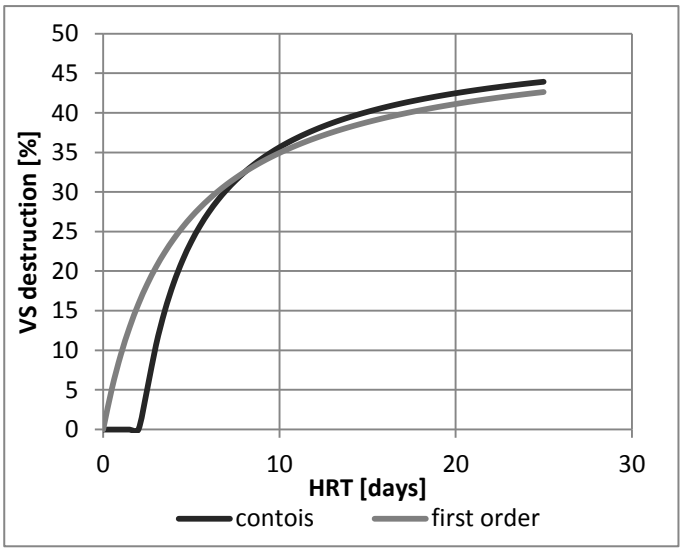

Figure 4.17. VS destruction as function of HRT calculated with Contois and first order kinetics. Kinetic constants are taken for the raw sludge.

Table 4.11. Measured and modelled VS conversion in CSTR's digesters for both untreated and thermally pre-treated sludge. Estimated conversion is based on the determined hydrolysis constants for both first order and Contois kinetics. Latter parameter is applied in the Chen and Hashimoto model for translation to a full scale CSTR system.

	HRT = 12 days			HRT = 20 days		
	measured	first order	Contois	measured	first order	Contois
Sludge type	*[% VS/VS]*	*[% VS/VS]*	*[% VS/VS]*	*[% VS/VS]*	*[% VS/VS]*	*[% VS/VS]*
Raw sludge	42.5	36.8	37.8	42.4	41.1	42.5
TPH treated sludge	45.7	43.5	44.2	47.9	46.2	47.0

Estimating the solids concentration in the digestate for the up-flow system has shown to be a bit more difficult. It was already noted in the section dealing with the hydrolysis constant determination that from day 10 onwards the modelled hydrolysis rate was overestimated. This effect resulted in a greatly overestimated breakdown for the second and third UFD. For the first digester the prediction was close to reality. The results are presented in Table 4.12.

Table 4.12. Measured and modelled VS conversion in up-flow digesters.

	HRT = 12 days			HRT = 20 days		
	Measured VK	Measured MB	Modelled	Measured VK	Measured MB	Modelled
digester	*[% VS/VS]*	*[% VS/VS]*	*[% VS/VS]*	*[% VS/VS]*	*[% VS/VS]*	*[% VS/VS]*
UFD 1	32.7	31.6	25.5	33.9	35.9	34.7
UFD 2	4.6	6.4	20.2	6.7	7.0	17.5
UFD 3	6.9	3.0	10.3	8.0	6.5	5.5

5 Discussion

In this chapter the results presented in chapter 4 are discussed and interpreted. First attention is paid to digestion of raw, pre-treated sludge and the up-flow digester, followed by a discussion on methanogenesis inhibition by free ammonia. Subsequently the optimized ALE extraction procedure and the interactions with digestion is elaborated on. Finally the protein content of ALE is discussed.

5.1 Digestibility of raw aerobic granular sludge

NES from WWTP Epe shows good biodegradability values during anaerobic digestion compared to other WWTP's with CAS. In a CSTR at 20 days HRT a VS conversion of 42,4% was obtained, whereas the maximal breakdown reached after 40 days is 50% VS/VS. A similar breakdown was found when a HRT of 12 days was applied. This is likely an overestimation. The modelled value is around 38% VS conversion.

The relatively high organic content and low metal dosing are expected to be the main reasons of the high biodegradability of NES. WWTP Epe is a full scale SBR system in which Nereda sludge is used to treat domestic and slaughterhouse wastewater. No primary clarification takes place, resulting in a sludge mixture of both primary and secondary sludge. During the whole experimental period the VS fraction of the sludge was stable around 80%. This is high compared to other WWTP's in the Netherlands. Also literature reporting about the organic content of AGS report a lower content, often around 60- 70% (see e.g. de Kreuk et al. (2005b), de Kreuk et al. (2005a). Sludge taken from SBR reactor (4 m depth from top) also showed lower organic values (69-71%). Large SBR-systems thus allow keeping the well settle able solids/granules in the reactor, whereas light solids/fluffy granules are discharged. In other words: SBR operation allows segregating between relatively young sludge from the reactor whereas older granules are kept in the reactor. This explains why the excess sludge from WWTP Epe is rich in organics. It is already known that a shorter SRT results in a higher organic content and anaerobic biodegradability of the sludge, see e.g. Bolzonella et al. (2005), Smith and Carliell-Marquet (2008) and Yu et al. (2012). A higher SRT in the wastewater system induces further mineralization of sludge, leaving less organic material for anaerobic digestion (Canales et al., 1994). The final sludge quantity though, after anaerobic digestion, is rather independent of the SRT in the wastewater treatment system (Gossett and Belser, 1982).
Additional to the high organic content of the sludge, a very limited amount of aluminium is dosed for additional phosphorus removal. The impact of metal dosing on anaerobic biodegradability is well known (see e.g. Johnson et al., 2003, Kindzierski and Hrudey, 1986, Dentel and Gossett, 1982)), but will play a minor role (if any) in the digestion process of NES as the dosed amount is limited.

5.2 Digestibility enhancement by thermal pressure hydrolysis

A clear increase in VS conversion was observed as a result of temperature pressure hydrolysis, both in the continuous reactors as in the batch tests. A VS conversion of 48 and 46% was found for digestion in a CSTR with a HRT of 20 and 12 days resp., a relative increase of resp. 13 and 7% compared to untreated sludge digestion. This is relatively low compared to claims made by manufacturers of TPH systems (STOWA, 2005). In this research sludge was pre-treated for 2 hours at 160 ^{0}C. As mixing was abundant in the heating vessel, a longer heating period was applied to ensure proper heating of the full sludge volume. Depending on the manufacturer, a heating period of 0,5-1 hr is applied. This shorter residence time will possibly result in a smaller increase of VS conversion compared to system tested in this research.
The enhancement of VS reduction is not enough to afford the investment of a full scale TPH installation. Although the final economic evaluation depends on a lot of variables (sludge deposit contract, external sludge treatment etc.), it can be said that a minimum absolute increase in DS removal of 15% is required to make the investment of a TSO unit beneficial (pers. comm. W. Wiegant, STOWA 2012). As the additional DS destruction is around 6% - 2% for resp. HRT values of 20 and 12 days, it is not beneficial to construct a TPH unit (Val del Río et al., 2011).

5.3 Digestibility enhancement by application of up-flow digester

It is difficult to draw strong conclusions about the digestion performance of the up-flow digesters, modified according to the Schwarting-Uhde concept. Several issues are playing a role, which are discussed in this section. Afterwards the process performance for NES is evaluated.

5.3.1 Uncoupling SRT – HRT

The up-flow operation mode allows solid material to settle to the bottom, resulting in an extended SRT compared to the HRT. Analysis of the DS content of UFD 1 showed that this did not significantly differ from the average of feed and digestate. As a consequence, the calculated SRT value did not differ significant from the HRT value. In the second and third UFD digester solids did clearly accumulate. Also the difference between calculated VS conversion with van Kleeck and mass balance equations did not show a significant difference for UFD1. This finding could have been expected, as the DS concentration and subsequently viscosity of the sludge is too high for good settling properties (Metcalf and Eddy, 2004). The measured DS concentration in the second and third UFD digester differed significantly compared to the calculated average concentration. Although the SRT was enhanced, the obtained additional removal was low. The settled biomass showed a very high viscosity. This suggest that a gel-like substance was retarded, which is poorly biodegradable. It is thus questionable to what extent NES retention in the digester results in enhanced degradation. Besides that it is economically unattractive to place three UFD in series at full-scale. A CSTR system used for post-digestion yielded better results and seems to be a more attractive alternative (Boersma, 2013).

5.3.2 Benefits of plug-flow conditions

From process engineering it is known that the largest fraction of substrate is extracted directly after dosing to a CSTR system. This is visualized in Figure 5.1. In this figure the total/fractional discharge in time is depicted of substrate dosed at day 0. The breakdown of the compound is modelled with first order kinetics (k_h=0,237, raw sludge value). The HRT used is 20 days. Due to the high initial extraction of not yet degraded substrate, in total 20 % of this substrate is extracted without being converted. CSTR digesters operate at low substrate concentrations as a result of a generally applied HRT of 20 days (STOWA, 2011). This makes conventional systems robust to temporal variations and overloading; the drawback is though that the system is low-loaded and not efficient. Plug-flow systems are known to be more efficient, enabling high-loadings and omitting the risk of direct extraction of substrate (Bruning, 2010, Liu, 1998). The HRT is thus more homogeneously distributed over the sludge in a plug-flow system, resulting in a net better conversion of substrate. The drawback of using PF reactors for anaerobic digestion is the requirement of inoculum addition to the substrate for acidification prevention. Inoculum is added to the feed by recycling of digested sludge. When very high recycle ratios are applied, PF reactors are going to behave as CSTR-systems, which is undesirable. On the other hand: not enough inoculants added will result in (partly) acidified conditions, which can worsen the net conversion of the system. In this research a recycle ratio of 2 was applied. This ratio is quite low for PF systems with sludge recycle (van Balkom, 2012). The benefit of partial plug-flow conditions is expected to be the major benefit of the UFD-system.

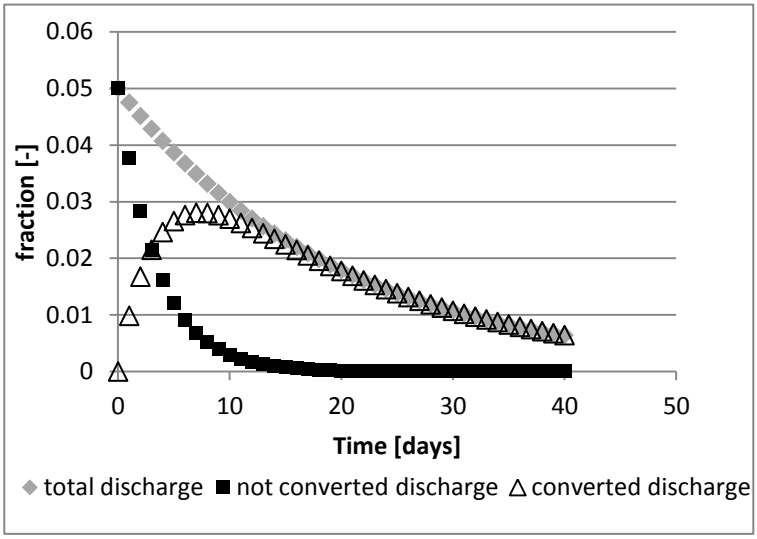

Figure 5.1. Fractions (not)converted discharge of a instantaneous loading in a CSTR system in time. Hydrolysis constant: 0,237 (raw sludge). HRT: 20 days.

Mixing requirements

Mixing is essential in anaerobic digestion, as several groups of bacteria are involved for substrate degradation. Mixing ensures exchange of compounds and bacteria. As mixing in the UFD digesters is only supplied by means of recycling and rising gas bubbles, it is questionable if this enough to reach optimal conditions. This remark is especially important for UFD 2 and 3. Since the breakdown is low in those digesters, mixing by rising gas bubbles also low, possibly worsening the conversion efficiency. Mixing is also needed for prevention of the development of preferential flow paths. Preferential flow paths result in short-cuts in the digester, reducing the HRT and thus VS conversion. Switching off of the recycle pump of the second and third up-flow digesters enhanced settling, but will probably have induced preferential flow path development. This resulted during the 12 days HRT period in a decrease of VS conversion efficiency, although the substrate load was higher. Further research is required to investigate to what extent sludge recycling and rising gas bubbles can replace mechanical mixing or how mechanical mixing can be incorporated in the up-flow digester.

5.3.3 Applicability of UFD for NES digestion

The obtained VS conversion results obtained for this thesis work are rather limited compared to CSTR systems. At an HRT of 12 and 20 days the conversion is 40.3 and 43.3 resp., whereas for digestion in a CSTR 42.5 -42.2 % VS resp. is converted. This indicates that the additional benefit of the UFD system seems negligible. Several possible reasons can have induced this effect:

- Biodegradability of raw sludge is high, resulting in a high conversion in the CSTR system. This results into the situation that only a small fraction of biodegradable substrate can be digested additionally;
- No mixing occurred anymore in UFD 2 and 3 in the period of 12 days HRT as the recycle pumps were switched off. This could have resulted in the development of preferential flow paths, reducing the VS breakdown.
- Sedimentation of inorganic solids/gelling components could have reduced the effective digester volume, reducing the conversion efficiency;
- Mixing is only supplied by recycling, which could limit the exchange of substrates to the different bacterial groups involved in the digestion process;
- Calculation of VS conversion is difficult because solids are retained.

Compared to removal efficiencies mentioned in literature, the results obtained in this thesis are low. Gessler and Keller (1995) and STOWA (2006) reported VS conversion values of 60-70% for a primary/secondary sludge mixture at a HRT of 20 days. The origin of the sludge (bio-phosphorus removal, extended aeration etc.) is not known. It is not likely that similar values can be obtained for NES digestion, as the maximum biodegradability is estimated at 50% VS.

Modelling was possible, although significant differences were observed in the modelled and measured VS breakdown. The main problem of UFD modelling with first order hydrolysis kinetics is the overestimation of degradation rate of the last 20% of biodegradable substrate. This part is poorer biodegradable; more time is required for full breakdown. This results in an overestimation of the VS conversion of the second and third UFD. Further optimization of the model can be done by using multiple hydrolysis constants for the easily and poorer biodegradable substrates. This method has already been applied to two-stage digestion systems, see e.g. Blumensaat and Keller (2005) and Vavilin et al. (2001).

Further research is required to investigate the extent of non-biodegradable solids accumulation, the risk preferential flow path development and sludge recycle requirements. A two stage process with a thermophilic and mesophilic stage, a combination of a CSTR and a UFD in series (or v.v.) can also be considered.

5.4 Methanogenesis inhibition by ammonia

As a result of enhanced digestion, the ammonia concentration could cause inhibition of methanogenesis. Methanogenesis is inhibited by (among others) free ammonia (Angelidaki and Ahring, 1994). The concentration of free ammonia is dependent on the concentration of ammonium, pH and temperature (Vasconcelos Fernandes, 2010). When a digester is operated at mesophilic conditions and is stable, a concentration of 3 gr NH_4-N/L is indicated as a safe limit (STOWA, 2012). The degree of ammonia inhibition depends greatly on the degree of adaptation of the inoculum to the ammonia; sludge with concentrations up to 5 g NH_4-N/L showed a limited reduction of methanogenic activity (Van Velsen, 1979, Koster and Lettinga, 1984). Within this research an ammonium concentration higher than 2 gr NH_4-N/L was not obtained. It can thus be concluded that ammonia inhibition was of negligible impact.

5.5 ALE extraction

Extraction of ALE from NES can be optimized in several ways. The process steps involved are discussed in this section. First attention is paid to the limitations of using NES for ALE extraction

5.5.1 Different properties AGS and NES

It is questionable to what extent the NES resemble similar properties as Nereda slude in the SBR tanks. In the previous section the high organic content was already indicated as a benefit for anaerobic biodegradability. 80% of NES is organic, whereas AGS contain around 70% of organic components. The opposite is that ALE extracted from NES will likely posses characteristics which are not completely similar compared to ALE extracted from AGS. A consequence of lighter, fluffier, flocculent sludge like behaviour of sludge directly influences the characteristics of extracted ALE (Seviour et al., 2009a, Lin et al., 2012). Further research is required to characterise the ALE from NES and quantify the difference in properties.

5.5.2 Extraction of ALE from NES in alkali phase

A sodium dose of 3 mmol Na^+/gr VS has shown to be enough to reach effective ion-exchange with Ca^{2+} ions bound to the ALE. An ALE yield of 23% VS/VS was reached. Although effective extraction was obtained at a Na^+ dose of 1.5 mmol Na^+/gr VS, this was also partly caused by the elevated concentration of NaCl in the sludge, originating from de-icing salts from streets. Earlier research has shown that a clear relation exists between heating temperature, duration and ALE-yield (Lin, 2013). It is however questionable if no contamination of intercellular material takes place at higher temperatures and extraction duration. Besides that, McHugh (1987) and Hernández-Carmona et al. (1999) noted that higher temperatures and prolonged extraction times lead to breakdown of uronic acids and thus lower viscosities of the final product.

For extraction at elevated pH values the risk of intercellular material contamination is even more abundant. Also alkaline hydrolysis of ALE has to be considered (Wedlock and Fasihuddin, 1990, Hernández-Carmona et al., 1999).

A DS concentration of 5% and more has shown to limit the extraction efficiency of ALE from WAS in the alkali phase. Further research is required what the limiting mechanism is in this process. Possible explanations could be the maximum solubility of ALE in the alkali solution, limited diffusion of alkali into the sludge, presence of divalent cations etc.

Further research is required to investigate the exact relation between parameter changes in alkali phase and ALE quality. Also pre-treatment methods as acid dosing prior to alkali dosage or extrusive extraction requires further investigation (Hernández-Carmona et al., 1998, Vauchel et al., 2008). Treatment with acid prior to the alkali phase is interesting as an acid waste stream is produced in the ALE precipitation process. Also homogenization of sludge has shown to be an effective way to increase the amount of harvested EPS from AGS (McSwain et al., 2005). If ALE extraction is applied prior to digestion, homogenization will also enlarge the available surface area for enzymes, enhancing the hydrolysis rate. Seperation of sludge from ALE solution can be done by centrifugation; other methods as flotation or filtration can also be considered (Hernández-Carmona et al., 1999).

5.5.3 Acid precipitation of ALE

Separation of acid ALE precipitates has shown to be a possible with less HCl dosed and shorter centrifugation time. A pH value of 4 is enough to reach precipitate formation of acid-ALE and a few minutes of centrifugation is enough to separate acid ALE and the residual solution.

Haug and Larsen (1963) found that alginates with higher guluronic acid content compared to mannuronic acid is precipitating at higher pH values (3-4). This is in line with the high GG-block content of ALE from AGS (Lin et al., 2010). Note that the combined effect of a higher pH and shorter centrifugation period will probably lead to a less effective separation, as the structure of harvested ALE at higher pH's was more fluffy and less easily separated.

5.6 Interactions between ALE extraction and digestion

In this section the interactions between ALE extraction and digestion is discussed. First the anaerobic biodegradability of ALE is discussed, followed by an elaboration of the digestibility of sludge treated with ALE extraction conditions. Finally the results are used to formulate recommendations for a full scale system in which both ALE extraction and digestion is combined.

5.6.1 Biodegradability of ALE

The biodegradability of ALE seems to be mainly dependent on the bound cation to the GG-blocks. Na-ALE is fully biodegradable, whereas Ca-crosslinks hinder biodegradation. Ba^{2+} forms a stronger gel complex compared to Ca^{2+} gels, which explains why Leenen (1996) did not notice any form of biological breakdown of Ba-Ca-alginate. Ba^{2+} and Sr^{2+} are not expected to be present in drinking water and subsequently not in waste-water. The main chelating ion will thus be Ca^{2+} in ALE. It is not known to what extent the extracted ALE has the same characteristics as ALE produced in AGS. Due to extraction changes in molecular conformation can occur, making ALE poorer or better biodegradable. Also the storage of acid ALE could have resulted in partial acid hydrolysis, releasing the biodegradable MM and MG blocks from the cross linked GG-blocks.

Other researchers found similar results regarding breakdown of the EPS and cellular core of AGS. Nielsen et al. (1996) reported a decrease in both carbohydrate and protein content of EPS during anaerobic storage and a subsequent deterioration of dewaterability. Adav et al. (2009b) researched protein hydrolysis during storage of aerobic granules and found that after 40 days of storage the protein content decreased from 460 mg g VSS^{-1} to 290 mg g VSS^{-1}. Adav et al. (2007b) and Pijuan et al. (2009) also noted the anaerobic degradation of the (protein) core during storage, which resulted in destabilization of the granule. Also release of Ca^{2+} was observed, indicating degradation of ALE (Adav et al., 2009a).
Degradation of ALE during thermal pressure hydrolysis was expected, as alginate is very sensitive to temperatures above boiling point (Smidsrod et al., 1963, McDowell, 1961, Rhim, 2004)

5.6.2 Behaviour of NES during ALE extraction

In section 4.4 a decrease (relative difference of 13%) in digestibility of NES was pointed out as a consequence of heat treatment applied in ALE extraction procedure. This seems contradictory to what is expected; but other researchers detected the same phenomenon. Ge et al. (2011b) reported a decrease in methane potential of WAS with thermal pre-treatment from 65 ^{0}C onwards. Around 60-65 ^{0}C there seems to be an optimum temperature. Note that this pre-treatment was applied for 2 days. Digestibility is enhanced again at temperatures of around 90 ^{0}C (Val del Río et al., 2011). Lin (2013) showed a higher specific gas production from NES after ALE extraction compared to raw sludge. However, the initial increase was only 6% (absolute), whereas the ALE yield was around 22% VS/VS. Ca-ALE has shown to be not easily biodegradable. If ALE is poorly degraded in raw NES, sludge without ALE should have a ± 25% higher initial specific gas production compared to raw NES. The relatively small increase in specific gas production indicates that some components are turned into non-biodegradable organic material as a result of 80 ^{0}C heating. From a digestibility perspective ALE extraction applied at 80 ^{0}C is not beneficial. Latter limitation is obviously only valid if ALE is extracted from rough sludge prior to digestion. Further research is required to what extent ALE-yield is affected at lower temperatures of extraction, with a shorter heating period and how this relates to the digestibility.

5.6.3 Implications for full scale systems

It is expected that in the future ALE extraction and digestion will be combined as NES treatment to reduce sludge disposal costs. Biogas is considered as a low value product, whereas ALE has a higher value. It is thus the objective to prevent ALE from converted to biogas, whereas the digestibility should not be reduced of the non-ALE components. Roughly distinguished 3 process schemes are possible for the combination of both processes, which are depicted in Figure 5.2. The benefits and drawbacks of the processes are discussed.

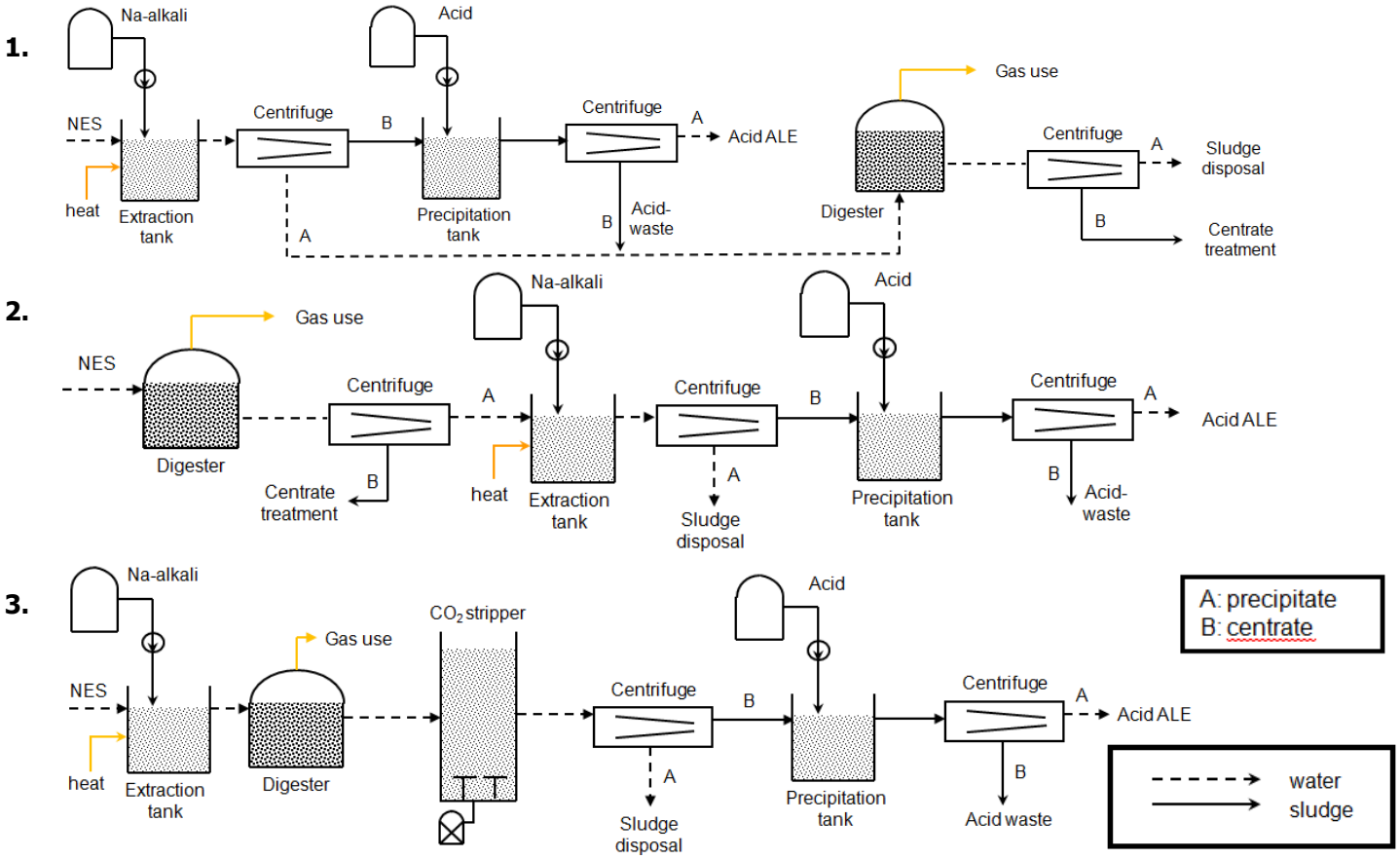

Figure 5.2. Process schemes of sludge treatment lines in which both digestion and ALE extraction is applied on NES. 1. ALE extraction applied prior to anaerobic digestion. 2. ALE is extracted from digested NES. 3. Alkali extraction is applied before digestion, ALE is separated and precipitated after digestion.

1. **ALE extraction prior to digestion.**
   - ***Benefits:***
     - ◊ Risk of ALE breakdown during digestion is omitted;
     - ◊ ALE is extracted from NES, making the sludge better dewaterable, allowing high DS loaded systems and thus a compact digester (Li and Yang, 2007). On the other hand a higher Na^+ concentration is present in the sludge, which is known to worsen the biodegradability (Jin et al., 2004). It is not clear how a high sodium concentration influences the dewaterability of sludge from which ALE/EPS is extracted. Possibly dry digestion can be considered if extensive dewatering is possible.
     - ◊ If appropriate extraction temperature is applied, sludge underwent a thermal pre-treatment step. A more efficient digestion process is the result (Skiadas et al., 2005).

- ***Drawbacks:***
  - ALE extraction unit is possible larger compared to option 2, as the dewaterability of NES is lower. First a solution has to be found to extract ALE effectively at high DS concentrations. If latter is not feasible, this drawback is not applicable.
  - It is not clear what the effect of the sodium concentration is on the behaviour of anaerobic bacteria. Values from 3 g Na^+/L are reported to inhibit the digestion process, although the extent of inhibition is very sludge specific (Feijoo et al., 1995, McCarty and McKinney, 1961). On top of that: a fraction of the added sodium can be removed by dewatering.
  - The sludge needs to be neutralized, as the pH is probably too high for methanogenic activity (Yuan et al., 2006). Latter parameter is dependent on the type and dosage of alkali used. Neutralization can be done by dosing of the acid waste originating from the ALE precipitation process. Drawback is however that the sludge is diluted.

2. ALE extraction after digestion

- ***Benefits:***
  - Possibility of a compacter ALE extraction unit;
  - As the total volume of treated sludge is smaller, the chemical dosage can be lowered. This point requires further investigation;
- ***Drawbacks:***
  - Larger digester is required as the extent of dewatering is limited;
  - The extent to which ALE is broken down is not fully clear;
  - An up-flow digester is not applicable, as some of the ALE will be retained and broken down;
  - Need for CO_2 stripper to reduce acid dosing in ALE extraction procedure

3. Digestion between alkali and acid phase

- This combination is not feasible, as the ALE enters the digester in the soluble sodium form, which is easily biodegradable. The major fraction of ALE will be broken down, making acid precipitation afterwards otiose. Thermal treatment combined with alkali dosing could however be combined as pre-treatment step for NES digestion. Thermal treatment enhances the biodegradability rate, whereas also sodium alginate is released. The mixture is expected to be better biodegradable. Further research is required to investigate the influence of heat, alkali and combinations, including the economic evaluation of such a process.

The benefits and drawbacks are summarized in an multi criteria analysis in Table 5.1. Based on the current knowledge it is advised to apply ALE extraction before digestion. The major reason is that the risk of ALE breakdown is ruled out, whereas the digestibility of sludge can be enhanced and applied at a compacter scale. Further research is required on the influence of anaerobic digestion on the composition of alginate. It could be that due to anaerobic digestion a fraction of the MG and MM building blocks is broken down, whereas the GG blocks are preserved. If so, anaerobic digestion prior to ALE extraction acts as an ALE enrichment method.

Table 5.1. Multi criteria analysis scenario's for combined ALE-extraction and digestion.

Scenario	Compact digester	Compact extraction installation	ALE breakdown omitted	Sludge pre-treatment	Reuse acid waste
1	+	-	+	+	+
2	-	+	+-	-	-
3	-	++	- -	-	-

5.7 Protein content ALE

No consensus is reached about the role of functional groups in EPS for granulation processes. Issues as differing wastewater composition, varying WWTP configuration and operation parameters, different EPS extraction and different characterization methods make it difficult to obtain comparable results. The results obtained in this thesis exemplify this statement: the protein content varied from 2-65% depending on the ALE extraction and protein detection method. In the next part of the discussion attention will be paid to protein quantification methods and ALE-extraction methods, both focusing on how these issues could have influenced the (measured) protein content of ALE. These sections are continued with a literature overview containing most of literature dealing with polysaccharide and protein quantification of EPS from AGS. Afterwards the implications for aerobic granulation mechanisms will be discussed.

5.7.1 Protein detection methods

The exact binding mechanism of the protein detection methods need to be clear for a proper understanding of strengths and weaknesses of the methods used. In Annex C a review is presented of the protein detection methods used in this research. In this section the major findings are summarized.

Lowry method

This method is based on the Biuret reaction of copper with peptide bonds. Glycosylated proteins and humic acids can results in overestimation and a lot of interfering components are investigated (salts, sugars etc.).

Bradford Assay

Coomassie Brilliant Bleu reacts with basic amino acids (Arg, Lys, His) present in the protein. Latter components are involved in the Maillard reaction. Maillard reaction products are thus not detected by the Bradford assay. Bovine serum albumin is often used as internal standard. This standard contains a higher fraction of basic amino acids compard to several wastewater samples.A major limitation of the assay is the molecular weigth (MW) requirement of > 3 KDa. MW distributions of wastewater, sludge, EPS and Granulan show that the majority of protein is below this limit, although there are exceptions. Also soluble proteins often do not meet latter requirement. The quantity of interfering components is limited.

Total nitrogen – Koroleff method

In this measurement all nitrogen is converted to nitrate with peroxo-disulphate. The accuracy of the method is similar or even better than Kjeldahl-N method.

5.7.2 ALE extraction methods

Extraction methods of EPS determine to a large extent the EPS composition and quantity harvested. When a higher pH is applied normally the yield increases, but the risk of intercellular contamination is also higher (Adav and Lee, 2008). For the extraction of ALE ion-exchange of Na^+ with Ca^{2+} is required. In this research three sodium-alkalis were used: NaOH, Na_2CO_3 and $NaHCO_3$. Furthermore the extraction took place at 80 ^{0}C and room temperature (20 ^{0}C).

Both a higher pH and a higher temperature applied during ALE extraction have five possible effects, which are discussed below.

1. Maillard reactions are enhanced;
2. Cell lysis occurs, releasing intercellular material;
3. Protein denaturation is enhanced;
4. At a higher pH hydroxyl groups are dissociated;
5. Hydrolysis of proteins occurs at elevated temperatures.

Maillard chemistry

Maillard reactions compromise the binding of unprotonated basic amino groups to carbonyl groups of sugars and 'hide' therefore proteins from the Bradford assay (see Chiu et al. (2009)). These reactions are enhanced at higher temperatures and higher pH values. It is not clear to what extent Maillard reactions play a role in the different extraction procedures of ALE. The difference between protein concentration measured by Lowry and Bradford's assay can be caused by either Maillard chemistry, protein MW reduction, the presence of mainly small proteins or combinations of these mechanisms. The difference between Lowry – Bradford is not increased at higher temperatures, which seems to be an indicator that Maillard reactions are of minor importance during extraction. Maillard reactions are known to increase the heat stability, solubilising capacity and gelling properties of proteins (Oliver et al., 2006). Heating prior to a gelling property measurement of a sugar-protein mixture could therefore disturb the result.

Protein denaturation

It is expected that, as a result of heat treatment, protein denaturizes, is made insoluble and is precipitated during centrifugation (Levy and Benaglia, 1950). Renkema et al. (2002) showed however that over 70% of soy protein remains soluble after heat treatment at 95 ^{0}C for 1 hour. In other words: a major part of protein can remain in solution after heat treatment. In this research also no correlation is found between the temperature of extraction and protein content of ALE, indicating that most of the protein seems to remain in solution.

ALE contamination with intercellular material/protein

Contamination of EPS with intercellular material is a known risk in EPS extraction from sludges (Frølund et al., 1996). The pH reached in this research by using NaOH was measured at 12,9, which will likely have resulted in cell lysis. The high ALE-yield (48% VS/VS) seems to be an indicator of this mechanism. As a result: more protein is released and measured by both the Bradford and Lowry assay. The increase measured by latter method is from around 250 gr/gr VS to 550 gr/gr VS. As the protein content did not differ significantly between Na_2CO_3 and $NaHCO_3$ extraction, no significant cell lysis is expected in case of Na_2CO_3 extraction. Furthermore: Seviour et al. (2009a) reported low levels of cell lysis till a pH of 10-11 applied in the alkali phase using a 2-keto-3-deoxyoctonate (KDO) as a marker. The pH in alkali phase with Na_2CO_3 used did not exceed 9.5. This result seems to confirm the expectation that no significant cell lysis occurs during extraction in case when Na_2CO_3 is used as alkali.

Dissociation of hydroxyl groups

Alkaline treatment causes charged groups, such as carboxylic groups in proteins and polysaccharides, to be ionized since their isoelectric points are typically below pH 4 to 6. This also causes a repulsion between EPS components and increases their water solubility (Nielsen and Jahn, 1999). This repulsion is believed to hinder Maillard reactions. Based on this the high Bradford protein content of ALE extracted with NaOH can be explained. Due to the high pH intercellular proteins are released into the solution. The high pH also causes repulsion between proteins and sugars, (partly) prohibiting Maillard reactions to occur. This could be a reason for the detected elevated concentrations of proteins by the Bradford assay.

Hydrolysis of proteins at elevated temperatures

Heat treatment seems to result in hydrolysis of proteins. Comte et al. (2007) determined the molecular weight of proteins in EPS from two different activated sludge waste water treatment plants, by using both physical methods and ion exchange. By comparing the results, it became clear that the molecular weight of the EPS extracted by heating (80 ^{0}C, 10 minutes) was significantly lower compared to the ion exchange methods. 89% of the EPS molecules had a molecular weight (MW) of 0.7 to 2.7 KDa. Hydrolysis during heating was attributed as responsible mechanism. In line with these

results, Karapanagiotis et al. (1989) showed that the MW of proteins in EPS after heating showed a more heterogeneous pattern, suggesting deterioration of molecular integrity.

5.7.3 Literature

The available literature about EPS characterization of AGS was investigated. The result is summarized in Table 5.2 & Table 5.3. The substrate on which AGS was grown, the used EPS extraction methods and protein & carbohydrate detection methods are indicated. The PN/PS ratio is obviously the most important result.

Table 5.2. Protein/polysaccharides ratio reported in literature, including EPS extraction methods, protein and carbohydrate detection methods and substrate on which granules are grown.

Wastewater type/carbon source	*EPS extraction method(s)*	*Protein detection method*	*Carbohydrate detection method*	*PN/PS ratio*	*Reference*
Glucose + peptone	1, 2	1	1	7.9-10.9 6.7-7.6	McSwain et al. (2005)
Glucose + peptone	1	3	2	6.0	McSwain et al. (2004)
Phenol	3, 4, 5, 6, 7, 8, 9, 10	2	2	2.4 – 6.2	Adav and Lee (2008)
Phenol	3, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20	1	2	0.8 – 3.5	Adav and Lee (2011)
Acetate	20	1	2	1 – 110	Adav et al. (2010b)
Sucrose + peptone	21	3	3	8.7	Zheng et al. (2005)
Glucose + acetate	22	1	3	4.9	Zhang et al. (2007)
Phenol	3, 4, 5, 6, 7, 8, 9, 10	3	2	4.0	Adav et al. (2009b)
Phenol	3, 5, 6, 7, 8, 9, 10	1	2	0.9 – 1.3	Adav et al. (2007a)
Sodium acetate	6	2	2	2.7	Adav et al. (2009a)
Domestic and industrial wastewater	23	2	1	± 3	Li et al. (2008)
Sodium acetate + peptone	1	1	1	1.5 – 2.0	Li et al. (2006)
Sodium acetate	1	4	3	1.3	Wang et al. (2007)
Glucose	24	1	3	1.1	Wang et al. (2006)
Municipal sewage	25	5	3	No protein detected	Lin et al. (2010)
Ethanol + sodium acetate	1	5	3	0.64 - 1.6	Jiang et al. (2003)
Liquid fraction of pig slurry	Sludge analysis, total and soluble fraction	2	2	4.6 – 2.0 (tot. – sol. resp.)	Val del Río et al. (2011)
Synthetic wastewater, simulating urban wastewater	Sludge analysis, total and soluble fraction	2	2	3.9 – 2.6 (tot. – sol. resp.)	Val del Río et al. (2011)
Sodium acetate	Cell	4	3	0.07– 0.33	Tay et al. (2001b)
Sodium acetate	Idem	4	3	0.07 – 0.2	Liu and Tay (2002) (Tay et al., 2001a)

[1] *In all cases of synthetic wastewater also the required nutrients were dosed.*

Table 5.3. List of used methods for EPS extraction, protein and carbohydrate detection in EPS analysis methods listed in Table 5.2.

EPS extraction method	
1	Cation-exchange resin (DOWEX)
2	NaOH + heat (80 ^{0}C)
3	Centrifugation
4	Heat (80 ^{0}C) + centrifugation
5	Formaldehyde + NaoH + centrifugation
6	Ultrasound + 5
7	5 + ultrasound prior to centrifugation
8	Formamide + NaOH + centrifugation
9	Ultrasound + 8
10	8 + ultrasound prior to centrifugation
11	Centrifugation + NaCl + centrifugation
12	11 + ultrasound prior to last centrifugation step
13	11 + EDTA prior to last centrifugation step
14	11 + (formaldehyde + NaOH) prior to last centrifugation step
15	11 + (ultrasound + formaldehyde + NaOH) prior to last centrifugation step
16	11+ (formaldehyde + NaOH + ultrasound) prior to last centrifugation step
17	11 + (formamide + NaOH) prior to last centrifugation step
18	11 + (ultrasound + formamide + NaOH) prior to last centrifugation step
19	11 + (formamide + NaOH + ultrasound) prior to last centrifugation step
20	Centrifugation + heat (50 ^{0}C) + centrifugation
21	Centrifugation + EDTA
22	Sonication + centrifugation
23	Centrifugation + NaCl + heating (80 ^{0}C) + centrifugation
24	PBS + centrifugation + PBS + heating (60 ^{0}C) + centrifugation
25	Na_2CO_3 + heat (80 ^{0}C) + centrifugation
Protein detection methods	
1	Modified Lowry method according to Frølund et al. (1996)
2	Modified Lowry method
3	Lowry method
4	Bicinchoninic acid method (BCA)
5	Bradford assay
Carbohydrate detection methods	
1	Anthrone method according to Frølund et al. (1996)
2	Anthrone method
3	Dubois method

The results in Table 5.2 show clearly that in almost all cases a significant amount of protein was detected in the EPS, in most cases even more compared to polysaccharides. For anaerobic granular sludge similar results are reported, see e.g. Batstone and Keller (2001) & D'Abzac et al. (2010).

5.7.4 Synthesis: protein in ALE

The Bradford assay seems to be not useful for protein quantification in ALE. In this research soluble proteins were measured. A big discrepancy was already found between soluble proteins detected by Lowry and Bradford's assay in raw sludge. This indicates that the majority of the soluble proteins present doesn't meet the criteria of MW > 3kDa. ALE extraction with NaOH could release intercellular protein, which were possibly detected afterwards by the Bradford assay. As presumably with ALE extraction with Na_2CO_3 and $NaHCO_3$ negligible cell lysis occurs, the amount of large soluble proteins remained low for detection with Bradfords assay. Taking into account the incompatibility of Bradford's assay with low MW proteins and the heating effect, it seems that Lin et al. (2010) used a method which is not suitable for the purpose of protein detection in ALE. Results obtained in this thesis confirm this. The results of Jiang et al. (2003) shows that with Bradford's assay protein can be detected in EPS extracted by cation-ion-exchange. The results obtained by Liu and Tay (2002), Tay et al. (2001b), Tay et al. (2001a) are dealing with sludge cell analysis rather than EPS and are therefore not directly comparable with the other results dealing with EPS.

The Koroleff method has shown to be a robust method for which no interfering components were detected. Only if samples contain too much colour the measurement will be disturbed (encountered by Lin (2013)). A dilution factor of 30 resulted in a very clear sample, making the risk of colour-interference negligible. The reason for the big discrepancy between the Modified Kjeldahl-N method and Lowry detection method is not clear. Probably the assumption of nitrogen composition of 16% in protein deviated from reality.

Seviour et al. (2012) noted some unpublished research results where the yield of Granulan harvested from AGS decreased from 320 to 30 mg EPS/gr DS sludge after protein precipitation and fractional precipitation. It was not noted to what extent the yield was lowered after protein precipitation only. Also H-NMR-spectra of Granulan indicated the presence of protein groups (Seviour et al., 2010b).

Taking into account the amount of experimental data and literature, there seems to be a lot of data present stating that a significant of protein is present in the ALE/EPS of AGS. In the research of Lin et al. (2010) the carbohydrate content of ALE was estimated at 47%, with sodium alginate used as standard. The findings presented in this thesis suggest that the other 50% contains a significant portion of protein.

ALE extracted from WAS originating from WWTP Amersfoort showed a lower content of protein. This is in line with research of McSwain et al. (2005) and Adav and Lee (2008). The gelling properties of the ALE extracted are likely much lower compared to ALE extracted from AGS, as shown by Lin et al. (2010) and Seviour et al. (2009a).
A relation between protein content and the application of digestion prior to extraction was not observed. This finding indicates that the protein in the ALE is not broken down to a larger extent than the ALE.

5.7.5 Granulation mechanisms

Based on the data discussed in the previous sections, in this section the components in EPS responsible for aerobic granulation are discussed.

Several authors found a relation between flocculation properties and protein content of EPS. Dignac et al. (1998) extracted EPS from activated sludge and characterized the samples on polysaccharide and protein content. The protein content was higher compared to polysaccharides; around 25% of the amino acids were negatively charged, highlighting the specific role of proteins in floc formation. Ras et al. (2008) made the same suggestion.
Adav et al. (2010b) found a strong correlation between excreted protein content and aggregation index with a high organic loading rate, whereas this was not observed for polysaccharides.
Also Li et al. (2008) noted that proteins were responsible for the hydrophobic interactions, whereas polysaccharides were hydrophilic components. Orive et al. (2006) reported an increase in hydrophilicity of seaweed-alginate as a result of a purification process in which protein was removed.

Although proteins can be involved in the flocculation/granulation mechanism, polysaccharides are mainly responsible for the integrity of the floc itself. According to Lee et al. (2010), both Adav et al. (2008) & Adav et al. (2010a) hydrolyzed proteins, α- and β-polysaccharides and lipids using enzymes. Hereby the stability change of the granules were analyzed. Although proteins were abundant in the core region, removal of proteins had a minimal impact on granule stability. Hydrolysis of β-polysaccharides caused disintegration however. The conclusion was drawn that the granule structure is principally composed of a β-polysaccharides network as backbone for embedded proteins, lipids, α-polysaccharides and cells. The crucial role of polysaccharides rather than proteins in gel formation was also noted by Seviour et al. (2009a).

Thus far it seems that polysaccharides are responsible for gel flexibility and strength. The binding with divalent cations seems not to be restricted to polysaccharides only. This is confirmed by results from Li et al. (2009), showing that Mg^{2+} augmentation resulted in more dense and compact granules and an increase in polysaccharide content of the EPS, whereas the protein content remained equal. As discussed in chapter 2, Mg^{2+} cannot serve as a chelating ion for alginate. The finding that Mg^{2+} resulted in enhanced granulation makes that the granulation process cannot be understood as a property of the ALE solely. As other sugars can also form gels, most probably more sugars and/or proteins are involved in aerobic granulation (Morris, 1998). As Mg^{2+} can serve as an activating agent for enzymes, it may be the case that enzymes are (indirectly) involved in the granulation process (Ryszard Jan and Waldemar, 2002).

The presence of protein and its influence on the gelling properties is also shown in a study of thermal pre-treatment on two types of AGS Val del Río et al. (2011). One SBR was fed with synthetic wastewater, the other with the liquid fraction of pig slurry waste. AGS of latter SBR was rich in proteins. During thermal pre-treatment at temperatures of 60 ^{0}C, 90 ^{0}C and 115 ^{0}C (20 minutes) an increase in viscosity was observed (contrary to what is often claimed, see Bougrier et al. (2008)). The sludge sample treated at 115 ^{0}C was present as a gel-block. Treatment at 140 ^{0}C did not result in this gelling behaviour. The concentration soluble proteins did not increase during treatment at 60 ^{0}C, 90 ^{0}C and 115 ^{0}C (remained at 15% solubilisation), whereas after treatment at 140 ^{0}C over 40% of the proteins were in soluble phase. Alginate cannot be held responsible for this gelling behaviour, as alginates depolymerizes as a result of thermal treatment (McDowell, 1961). Leo et al. (1990) reported a decrease in viscosity of alginate at sterilization temperatures. Especially for temperatures above 100 ^{0}C alginates showed to be very sensitive. Gel strength of alginate is among others dependent on molecular size/chain length; depolimerization thus results in viscosity loss (Martinsen et al., 1989). Yamagiwa et al. (1995) found a rapid decrease in viscosity of alginate from temperatures of 40 ^{0}C. Most likely the responsible mechanism is Maillard reaction products formation linked to protein

denaturation. Maillard reactions enhance emulsifying and gelling properties of proteins (Hattori et al., 1997). Also the heat stability and solubility is enhanced (Oliver et al., 2006, Aoki et al., 2001). At 140 ^{0}C protein degradation occurs, resulting in a loss of gelling capacity (Kinsella, 1979).

5.7.6 Consequences for ALE characteristics

In the research of Lin et al. (2010) ALE building blocks were fractionated according to Leal et al. (2008). The principle behind this method is that the various building blocks of alginate precipitate at different pH values (Haug and Larsen, 1963). For alginate obtained from brown seaweed this is an appropriate method, as protein content is rather low: 3-15% (Fleurence, 1999, Mabeau and Fleurence, 1993). Orive et al. (2002) showed that protein contamination in alginate was correlated to the different building blocks. G-rich alginate contained 37% less protein compared to M-rich alginate. If ALE contains a significant portion of protein; it is likely that this will have influence the block fractionation measurement.

6 Conclusion and recommendations

Two aspects of Nereda excess sludge (NES) treatment were the subject of this thesis. First anaerobic digestion enhancement were studied, achieved by means of thermal pre-treatment and an up-flow digester concept instead of a CSTR. Secondly the optimization of ALE-extraction and characterization was investigated. Finally the interaction between ALE extraction and digestion was discussed. In this section the questions posed in the introduction are answered, followed by recommendations for further research.

6.1 Conclusions

6.1.1 Digestibility of Nereda excess sludge

NES showed to be well biodegradable during anaerobic digestion. 42% of the VS fraction is converted in a CSTR with an applied HRT of 20 days. The increase in VS conversion due to thermal pressure hydrolysis is limited to 3-6% (absolute) for an HRT of 12 and 20 days resp. As untreated sludge is already good biodegradable, the enhancement caused by pre-treatment is relatively low. For the same reason the up-flow digester concept yielded marginal additional breakdown values. It is not clear which processes are dominating the degradation process in the up-flow digesters. A lack of mixing, preferential flow path development and accumulation of inert solids are believed to influence the conversion efficiency; the extent of each factor is not clear.

Modelling of the digestion in the CSTR's showed to yield breakdown values close to values obtained in the lab-scale digestion systems. The applicability of modelling for up-flow digester seemed limited.

6.1.2 ALE extraction

A significantly lower alkali dosage was required to reach effective ion-exchange of Na^+ with Ca^{2+}. A dosage of 3 mmol Na^+/gr VS was sufficient. Besides that, the minimum pH to reach crystallization was measured at 4-4.5. Also the required centrifugation time for effective separation could be reduced to a few minutes.

A clear relation was detected between pH/temperature applied in alkali phase and the amount of ALE extracted. It is questionable if no intercellular components where extracted together with ALE at higher pH/temperature values.

6.1.3 Protein content ALE

The amount of protein detected in the ALE extracted varied form 3-60%. Depending on the alkali used for extraction and protein detection method applied. The Bradford detected generally very low values. The other two methods used (modified Kjeldahl nitrogen and modified Lowry method) yielded protein values of 25-60%. Although the exact protein content could not be indicated; in further research it should be taken into account that a significant part of the ALE extracted from NES consists of protein.

6.1.4 Interactions digestion & ALE extraction

ALE seemed to be slowly biodegradable in the Ca-ALE form. The standard ALE-extraction method (80 ^{0}C, 30 minutes) negatively resulted in a biodegradability reduction of 13% (relative).

6.2 Recommendations for further research

The topics dealt with in this thesis are all not very extensively studied. Several aspects are still unclear and a lot of questions remain unanswered, which are posed here for further research.

6.2.1 Digestion of NES

Temperature pressure hydrolysis showed very moderate results. An investigation of low temperature pre-treatment is necessary (60 – 80 ^{0}C).

Digestion in up-flow systems is surrounded by a lot of questions:

- Is the accumulation of solids beneficial to the conversion efficiency of the system and how can this process be controlled?
- To what extent is preferential flow path development present in the digester system and what are the available methods to counteract this development?
- Is lack of mixing a conversion limiting issue and how can this be resolved?
- In which combination can multiple reactor concepts be combined to enhance the overall conversion efficiency of the system?

6.2.2 ALE extraction

It is recommended to perform a detailed characterization study to how the ALE extraction conditions influence the composition of ALE. Temperature, extraction duration and pH have shown to be important parameters, both for ALE-yield and quality, see Hernández-Carmona et al. (1999). Also the extent of intercellular material/DNA contamination requires further investigation.
As a further optimization step the use of extrusive extraction and/or pre-treatment of sludge with acid to reach more effective ion-exchange can be used. Also here the influence of type of extraction and ALE quality has to be considered.

6.2.3 Interaction digestion and ALE-extraction

ALE extraction applied prior to anaerobic digestion seemed to have several benefits. During extraction sludge is heated, which serves as low temperature pre-treatment step. The optimal temperature has still to be found. As the EPS is extracted, higher dry solids concentrations can be reached, which allows for a compact digestion system. In further research the influence of lower temperatures and/or heating periods on the anaerobic degradation rate and methane potential has to be evaluated, including the relation with the amount and quality of ALE extracted.

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Annexes

Annex A - Background information kinetics

Anaerobic digestion involves several groups of bacteria/enzymes. Each group can be modelled separately with different types of kinetics(Tomei et al., 2009). As hydrolysis is generally the rate limiting step (Eastman and Ferguson, 1981), modelling is often limited to the hydrolysis phase only; the other processes are dominated by the hydrolysis rate .

For sludge digestion mainly two types of kinetics are used to describe the conversion of organic material: first order kinetics and Contois kinetics. Contois kinetics are frequently used by water boards, whereas in most scientific literature first order kinetics are applied. In this section both types of kinetics are characterized and the differences are explained. Note that for sludge digestion modelling one overall hydrolysis constant is calculated, whereas actually every component in the sludge has its own specific hydrolysis rate (Miron et al., 2000).

First order kinetics

First order kinetics refer to a first order chemical reaction. The reaction rate is dependent on the substrate concentration and a substrate specific constant. The equation reads:

$$\frac{-dS}{dt} = kS$$

Where k is the maximum specific substrate utilization rate [d^{-1}] and S is the substrate concentration [gr/L] (Vavilin et al., 2008).

Although the amount of anaerobic biomass is important for the digestion process, it is not considered in the kinetic formulation. The application of first order kinetics is thus only valid if enough anaerobic biomass is present; i.e. at low loading rates. Latter prerequisite is normally obtained by applying a high SRT value.

Contois kinetics

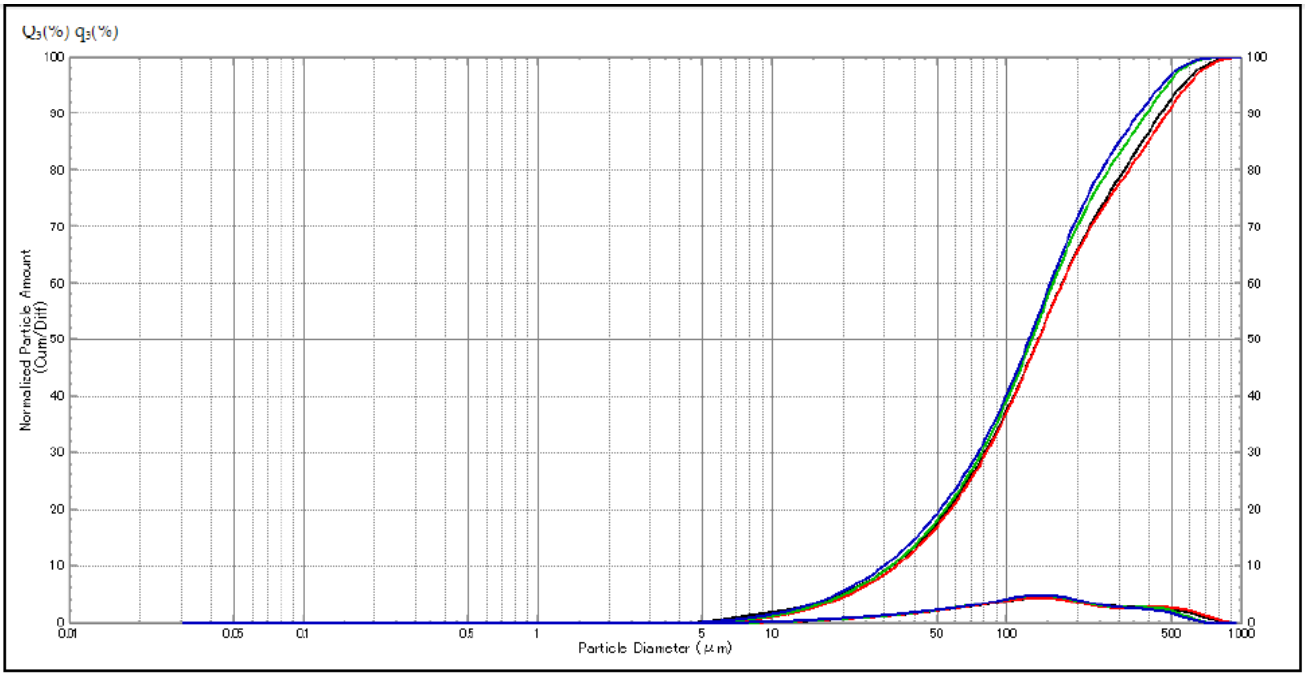
Contois kinetis is, just as Monod kinetics, an example of biological growth kinetics. Biological growth kinetics are based on two fundamental relationships: growth rate and substrate utilization rate (Kruger, 2002). With regard to anaerobic digestion, the main difference between Monod and Contois kinetics is the independency of effluent concentration on substrate concentration in case of Monod. Contois kinetics also takes into account the influent concentration. Essentially: the Contois kinetics account for organic loading (Trösch and Niemann, 1999).
By using Contois kinetics a distinction is made between substrate concentration and the concentration of predominant micro-organisms (micro-organisms degrading the substrate). By applying latter distinction, Contois kinetics can successfully be applied at high loading rates and low SRT values. It is known that methanogenic bacteria are susceptible to wash-out of a reactor system at low SRT's (1-5 days) caused by a low specific growth rate of latter organisms. Contrary to first order kinetics, Contois kinetics will account for latter aspect. On the other hand: Contois and first order kinetics yield similar results when low loading rates (i. e. high SRT values) are applied.

The equation for the substrate conversion rate reads:

$$\frac{-dS}{dt} = \frac{\mu_m X S}{Y(B X + S)}$$

In which X is the micro-organism concentration [g/L], μ_m = maximum specific growth rate of the micro-organism [d], Y the growth yield coefficient [gr/gr] and B a kinetic parameter (Chen and Hashimoto, 1980).

Annex B – Particle size distribution measurement results



	File Name	Sample ID	Sample No.	Median D(μm)	Modal D(μm)	Mean V(μm)	Std Dev	Ref Index
1	Slib voeding – 004			134.009	138.246	123.498	0.443	3.00–0.20i
2	Slib voeding – 003			134.325	138.246	127.652	0.436	3.00–0.20i
3	Slib voeding – 002			125.941	138.246	115.212	0.414	3.00–0.20i
4	Slib voeding – 001			122.735	138.246	109.930	0.415	3.00–0.20i

Annex C - Mechanisms of protein quantification

The exact determination of protein in a sample requires the Kjeldahl procedure. As this procedure is time and sample consuming, several colorimetric methods are developed for the detection of protein, like the Lowry method, Biuret method and Coomassie Blue G-250 dye binding (Bradford assay). As distinctly different reaction principles are used, the measured protein content differs as well (Sapan et al., 1999). In this section some experiences with the Lowry and Bradford assay in wastewater treatment are discussed. Subsequently the molecular binding mechanisms involved in both methods are examined, including the Koroleff method used for total nitrogen measurements.

Experiences of Lowry and Bradford assays in wastewater treatment

In the field of wastewater analysis the Bradford assay underestimates the protein content, both for total and soluble proteins. This is frequently attributed to molecular structure (Raunkjær et al., 1994). The Bradford assay requires proteins with a macromolecular structure: 8-9 peptide bonds and a molecular mass of at least 3 kDa (Walker, 1996, Sedmak and Grossberg, 1977). Especially soluble protein doesn't meet latter criteria and are therefore not detected by the Bradford assay (Lazarova and Manem, 1995). Confer et al. (1995) and Pehlivanoglu-Mantas and Sedlak (2008) investigated among others the molecular size distribution of dissolved nitrogenous components, concluding that the major fraction (67%) of the total dissolved organic nitrogen in WWTP effluent has a molecular weight of < 1 kDa. However, Higgins and Novak (1997a) determined the molecular size of protein in EPS originating from activated sludge samples, resulting in an average value of 15 kDa. Seviour et al. (2010a) reported that protein in EPS of AGS was present in the medium and low molecular weight fractions. The majority of the proteins were present with a molecular weigth of around 1 kDa.

On the contrary: overestimation with the Lowry method occurs in case of glycosylated proteins and colour interference from amino acids (Fountoulakis et al., 1994, Legler et al., 1985, Wu et al., 1978). Ras et al. (2008) compared the Lowry method and Bicinchoninic acid (BCA) assay for protein quantification in wastewater and concluded that both methods were accurate within a range of 0-0.8 g L^{-1}.

Lowry method

The Lowry method is based on the amplification of the Biuret reaction by subsequent reaction with Folin phenol reagent (Folin-Ciocalteu). In the modified Lowry assay two unstable reagents described by Lowry et al. (1951) are replaced by one stable reagent.

In the Biuret reaction, copper sulphate is added to the sample in a strong alkaline solution in the presence of tartrate, where copper is reduced. A purplish-violet colour is produced, resulting from a tertradentate copper complex between a cupric ion and four peptide bonds (Sapan et al., 1999).

After the Biuret reaction, Folin-Ciocalteu reagent is added and gets reduced, producing a characteristic blue color. The Folin-Ciocaltue reagent consists of phosphomolybdic-tungstic mixed acid, which are the chromagen:

$$3H_2O \cdot P_2O_5 \cdot 13WO_3 \cdot 5MoO_3 \cdot 10H_2O$$
$$3H_2O \cdot P_2O_5 \cdot 14WO_3 \cdot 4MoO_3 \cdot 10H_2O$$

The copper-peptide complex facilitates electron transport to the acid mixture, especially in the presence of amino acids (Peterson, 1979). In this way proteins induce a reduction of the mixed acids by losing 1, 2 or 3 oxygen atoms from tungstate and/or molybdate. The reduced species have a characteristic blue colour (λ=750nm).
Besides humic acids, also various sugars (fructose, galactose, sucrose), lipids and salts are known for their interference. Interference of alginic acid/alginate with the Lowry method is not studied. For an extensive overview of interfering components in the Lowry protein assay, reference is made to Peterson (1979), Gregory and Sajdera (1970), Eichberg and Mokrasch (1969) and Box (1983) .

Bradford assay

In the Bradford assay the dye Coomassie Brilliant Blue (CBB) is used. The dye binds with proteins, resulting in a dye-protein complex with increased molar absorbance (Sapan et al., 1999). This method is however very specific and lacks response to a wide range of chemical classes, including a diversity of nitrogenous components (see Compton and Jones (1985)). The binding is limited to Arginine (Arg) and, to a lesser extent, Histidine (His) and Lysine (Lys) (de Moreno et al., 1986, Congdon et al., 1993). Based on these findings, Compton and Jones (1985) concluded that a protein should have a macromolecular structure and an active basic amino acid (i.e. Arg, Lys, His) in order to bind with the dye. Several other authors mention the requirement of a molecular weight of at least 3 kDa (Walker, 1996, Sedmak and Grossberg, 1977, Noble and Bailey, 2009). Lys, His and Arg (latter two to a lesser extent) are involved in the Maillard reactions and will form Maillard reaction products after heating. These products are not detected by Bradford Assay (Ames, 1992).

CBB exists in three ionic species: cationic (red, pH < 0.39), neutral (green, pH ≈ 1.3) and anionic (blue, pH > 1.3) species (Compton and Jones, 1985). The neutral and anionic species can form complexes with proteins. Both species bind to proteins by electrostatic attraction between the dissociated sulfonic group, the neutral species binds also by hydrophobic interactions, see figure 1. Arg, Lys and His can react with the sulfonic groups, because their positively charged guanidino group in the pH range (0-1.3) of their formation. The cationic species does not form a complex with proteins as both sulfonic groups are unavailable for reaction with Arg, Lys or His becaused they are proton neutralized. (Georgiou et al., 2008)

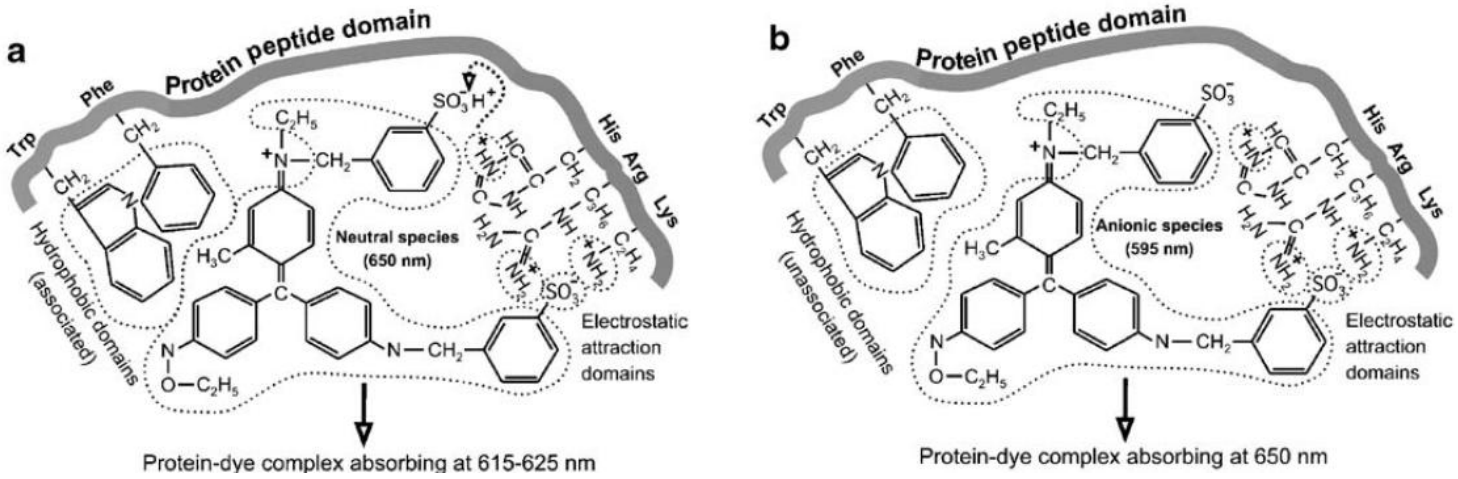


Figure 1 28 Mechanism of CBB binding to proteins of anionic species. a. The neutral species binds to protein both by hydrophobic interactions (via Phe and Trp) and by electrostatic attraction between the dissociated sulfonic group and Arg (or Lys and His). b.The anionic species binds to proteins the same as the neutral species, except for the hydrophobic interactions (figure from Georgiou et al. (2008)).

In table 1 the fraction of basic amino acids for proteins in wastewater, sludge, EPS and Bovine Serum Albumin (BSA) is indicated. Bovine Serum Albumin is a protein which is often used as an internal standard. The data in the table indicates that the basic amino acid composition varies depending on the substrate; on average the fraction basic components is however significantly lower than in case of BSA. This data suggests that a correction is required, as otherwise protein content is underestimated.

Table 0.1. Fraction of basic amino acids in proteins from wastewater, sludge, EPS and BSA.

Substrate analysed	**Fraction of basic amino acids in protein (Arg, Lys and His)** *[%]*	**Reference**
Activated sludge	16.2	Hackler et al. (1957)
Raw wastewater	13.2	Burleson et al. (1980)
EPS of three activated sludges	9.8 – 15.5 – 13.8	Hejzlar and Chudoba (1986)
Activated sludge from industry and lab reactor	Resp. 6.1 – 6.6	Higgins and Novak (1997a)
Raw wastewater and WWTP effluent	Resp. 9.5 – 12.0	Dignac et al. (2000)
Bovine serum albumin	20.9	Lewis et al. (1950)

Total Nitrogen - Koroleff method

The method of Koroleff (1972) was proposed as a much less dangerous and time-intensive method of Kjeldahl-nitrogen. In this method nitrogen is oxidized to nitrate at a high temperature by peroxo-disulphate in an alkaline medium (Nydahl, 1978). Nitrate can be measured photometrically with 2,6-dimethyl-phenol (ISO 7890-1). The method of Koroleff yield results similar or even more accurate than the Kjeldahl-N methods for waste-water samples, shown by Smart et al. (1981), Smart et al. (1983), Langner and Hendrix (1982) and Hosomi and Sudo (1986).

CV of the author

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