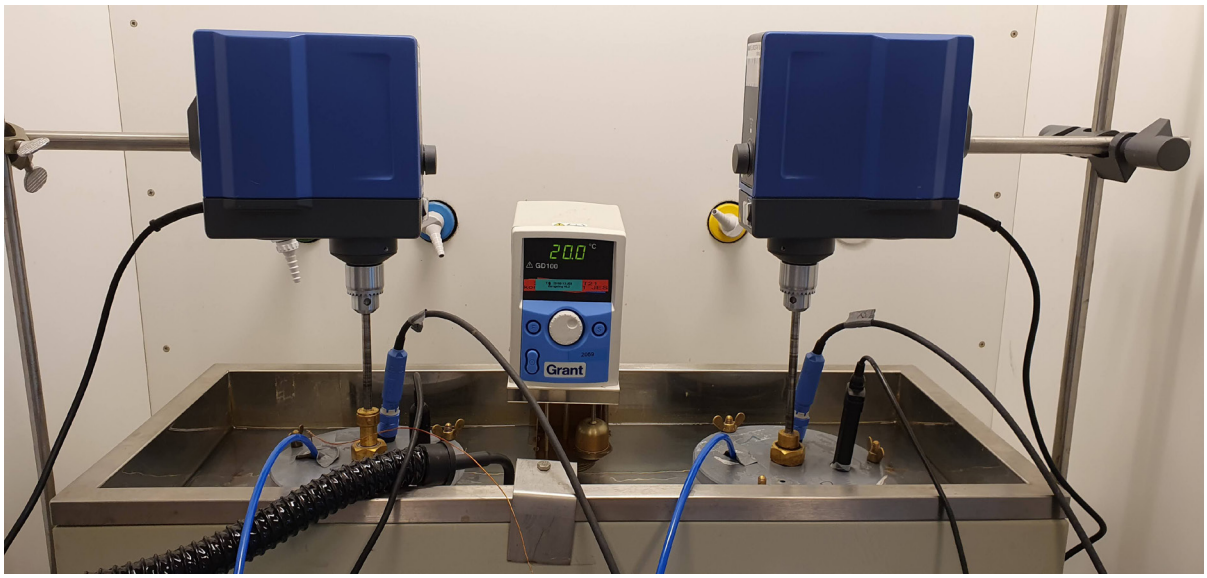
Evaluating the suitability of carbon source from fermentation of primary filter sludge for biological nutrient removal



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by

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Picture on front page: Experimental setup of batch activity test at Källby WWTP, Lund. Photo: Sanna Sahlin

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Preface

This master's thesis has been performed within the framework of the research project Ideal Carbon Utilisation (ICU) at Sweden Water Research, in collaboration with the Department of Chemical Engineering at Lund University. It has been a great pleasure working with this challenging project, and it has given me a thirst to understand even more about the complex world of wastewater treatment processes. I sincerely hope that the final product will contribute to the continued work and development of the ICU-project.

I would like to thank my supervisors at Sweden Water Research, David Gustavsson and Elin Ossiansson, for your immense support and guidance throughout the project. Thank you for always being available for questions and discussion, and for making my time at Sweden Water Research as fun and rewarding as it has been. A special thanks to Elin, for invaluable help during the batch activity tests and for conducting the VFA analysis in the end of the project. Additionally, I would like to thank my supervisor at Lund University, Michael Cimbritz, for your clear feedback and help during the finalisation of the report.

I would also like to thank Victor Ibrahim and Joanna Ekiert Smoter at Källby Wastewater Treatment Plant as well as Per Falås at Lund University, for all your help during laboratory work. Thank you for your expertise and patience in answering all my questions, without your help I would not have been able to conduct all experiments!

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Sanna Sahlin

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Summary

Traditional biological nutrient removal (BNR) processes in wastewater treatment require access to easily biodegradable carbon in order to achieve sufficient removal. However, a lot of the carbon in the influent wastewater is not directly available for BNR bacteria but need to undergo hydrolysis first. Several studies have investigated the possibility to produce an internal carbon source from the generated sludge at wastewater treatment plants (WWTP). The novel process combination of prefiltration and acidogenic fermentation of filtered primary sludge is investigated at a pilot plant at Källby WWTP in Lund, Sweden. The sludge is fermented in two reactors with different retention times, and contains a lot of volatile fatty acids, such as acetate and propionate. The suitability of the produced fermentate for usage in BNR processes was evaluated using batch activity tests.

Nitrate uptake rate (NUR) tests were performed to determine denitrification rates and removal efficiencies with different substrates. The highest specific denitrification rates were obtained with the fermentates, in the range of 7-8.9 mg NO_3^--N eqv/g VSS·h, compared to 5.6 and 4.0 mg NO_3^--N eqv/g VSS·h observed in the tests with acetate and propionate respectively. A mixture of acetate and propionate gave rise to a similar rate as the fermentates (7.8 mg NO_3^--N eqv/g VSS·h), indicating that more complex carbon sources containing many different VFAs stimulates higher rates, as observed in other studies. Similar nitrate removal efficiencies were observed with all carbon sources, in the range of 83-95 % after 3.5 hours.

In the test comparing influent wastewater with filtered wastewater, the highest denitrification rate was obtained with filtered wastewater (4.6 compared with 3.5 NO_3^--N eqv/g VSS·h), which could indicate a slight increase in readily available COD after filtration. However, the overall removal efficiency was higher in the test with influent wastewater (83% compared to 77%), reflecting the higher content of slowly biodegradable COD in the influent.

Additionally, the removal efficiency of the filtered wastewater increased by 13% with the applied addition of fermentate (45 mg COD/L), also taking into account the reduced availability of the added carbon due to a small increase in nutrient loading. The total COD content was significantly lower in this case compared with influent wastewater, indicating a more efficient COD utilisation.

The EBPR activity was tested in anaerobic P release tests. The observed P release rates were low. The tests were inconclusive to determine the suitability of the fermentate as a carbon source for EBPR.

Overall, the fermentates showed good potential to be used as carbon sources for denitrification. More studies are needed to evaluate the applicability of the method in full-scale.

Keywords: Wastewater treatment, batch activity tests, NUR tests, anaerobic P release tests, EBPR, denitrification, removal rates, internal carbon source, fermentate.

Sammanfattning

Traditionell biologisk näringsavskiljning inom avloppsvattenrening, såsom denitrifikation och biologisk fosforavskiljning (EBPR), kräver tillgång på lättnedbrytbart kol för att bra reningsgrad ska uppnås. Dock är en stor andel av kolet i det inkommande avloppsvattnet partikulärt och måste hydrolyseras innan det blir tillgängligt för bakterierna i den biologiska reningen. Flera studier har studerat möjligheten att producera intern kolkälla från slam från avloppsreningsverken. En ny processkombination av förfiltrering och fermentering av filtrerat primärslam undersöks i en pilotanläggning på Källby avloppsreningsverk i Lund, Sverige. Slammet fermenteras i två reaktorer med olika uppehållstid, och innehåller stora mängder lättflyktiga fettsyror, såsom acetat och propionat. För att bedöma det producerade fermentatets lämplighet för användning i den biologiska reningen av näringsämnen har aktivitetstester i labbskala utförts.

Nitrate uptake rate (NUR) tester utfördes för att bedöma denitrifikationshastighet och avskiljningsgrad med olika kolkällor. De högsta specifika denitrifikationshastigheterna uppnåddes med fermentat: 7-8,9 mg NO_3^--N eqv/g VSS·h. I testerna med acetat och propionat som kolkälla observerades hastigheter på 5,6 respektive 4,0 mg NO_3^--N eqv/g VSS·h. En blandning av acetat och propionat uppvisade liknande hastighet (7,8 mg NO_3^--N eqv/g VSS·h) som fermentaten. Detta indikerar att mer komplexa kolkällor som innehåller många olika lättflyktiga fettsyror stimulerar högre hastigheter, vilket också har observerats i andra studier. Liknande avskiljningsgrad av nitrat observerades med alla kolkällor, mellan 83-93% avskiljning efter 3,5 timmar.

I jämförelsetest med inkommande och filtrerat avloppsvatten uppnåddes högre denitrifikationshastighet med filtrerat avloppsvatten (4,6 jämfört med 3,5 mg NO_3^--N eqv/g VSS·h). Detta skulle kunna påvisa att en liten ökning i lättillgängligt COD sker vid filtrering. Dock var den totala avskiljningsgraden högre i testet med inkommande avloppsvatten (83% jämfört med 77%), vilket troligtvis beror på det större innehållet av långsamt nedbrytbart COD i det inkommande vattnet.

Genom att tillsätta fermentat (45 mg COD/L) till det filtrerade avloppsvattnet så ökade avskiljningsgraden av nitrat med 13%. Hänsyn har tagits till att en liten ökning i näringsbelastning sker vid tillsättning av den interna kolkällan. Det totala COD innehållet var betydligt lägre i detta fall jämfört med inkommande avloppsvatten, vilket indikerar ett mer effektivt utnyttjande av COD.

EBPR aktiviteten testades genom anaerobiska P-släpp tester. De observerade hastigheterna av P-släpp var låga. Resultaten från testerna var inte tillräckliga för att kunna bedöma fermentates lämplighet som kolkälla för EBPR.

Överlag visade fermentaten god potential att kunna användas som kolkälla för denitrifikation. Flera tester behövs för att utvärdera metodens tillämpbarhet i fullskala.

Nyckelord: Avloppsvattenrening, aktivitetstester, NUR tester, anaerobiska P-släpp tester, EBPR, denitrifikation, reaktionshastighet, intern kolkälla, fermentat.

Förbättra arbetsförhållandena för mikroberna i avloppsvattnet!

Som i så många andra delar i livet är det våra vänner mikroberna, de encelliga organismerna, som gör grovjobbet i de flesta av våra avloppsreningsverk. Det är de som renar vårt skitfyllda vatten från näringsämnen som kol, kväve och fosfor, och är på så sätt riktiga miljökämpar. Utan deras viktiga arbete skulle vi släppa ut alldeles för mycket näring i våra sjöar och vattendrag, och riskera övergödning och bottendöd. Men för att få en så effektiv rening som möjligt måste mikroberna ha bra arbetsförhållanden. Deras främsta krav är att det finns gott om mat, och precis som vi människor älskar de "snabbmat"!

När avloppsvatten rinner in på avloppsreningsverken är det fullt av kol och andra näringsämnen, som funkar som skitbra mat för mikroberna i den biologiska reningen. Särskilt bakterierna gör ett viktigt arbete i reningen av kväve och fosfor, och de måste ha mat i form av organiska kolfyllda ämnen för att klara av jobbet. Gärna i form av "snabbmat" som går fort att äta, som för bakteriers del består av lättflyktiga fettsyror, som exempelvis ättiksyra. Men många av partiklarna som kommer in till reningsverket är ganska stora, och skulle kräva lång tid och energi att bryta ner, vilket så klart kostar energi och pengar. Därför avskiljs en del av partiklarna tidigt, genom bland annat sedimentering eller filtrering, och på så sätt minskas arbetsbelastningen på mikroberna i nästa steg. De partiklar som tas bort innehåller mycket organiskt material, som vi kan utnyttja genom att producera biogas, men samma material hade bakterierna kunnat använda för mat och energi. Ibland blir det för lite mat kvar i vattnet och då måste mer kol tillföras för att reningen ska bli tillräckligt bra. Oftast används då etanol eller andra alkoholer, vilket som så mycket annat kräver energi och pengar för att produceras. Eftersom vattnet redan innehåller mycket kol från början, vore det självklart bättre att försöka använda det i högre grad istället.

Flera studier har gjorts på att försöka förbättra tillgången på goda kolmolekyler till våra renande bakterier, utan att för den sakens skull ge dem en för hög arbetsbelastning. Bland annat har man gjort försök på att bryta ner kolet i det slam som blir kvar efter sedimentering eller filtrering, och sen använda det som en egenproducerad kolkälla. Ett sådant försök görs i dag i pilotskala på Källby avloppsreningsverk i Lund, där avloppsvattnet filtreras och där det filtrerade slammet bryts ner av andra bakterier. Bakterierna får jobba ostört med att bryta ner det koncentrerade kolet till fettsyror i egna reaktorer, där arbetsförhållandena är extra bra anpassade för det arbetet. Det blir som en extern magsäck för de bakterier som jobbar i reningsbassängerna, som slipper det tidsödande nedbrytningsarbetet och istället får tillgång till redan nedbrutet kol. Det kan de förhoppningsvis käka upp snabbt samtidigt som de renar vattnet bra. Genom en sådan arbetsdelning kan reningen alltså bli mer effektiv!

För att undersöka om de kolpartiklar som produceras i reaktorerna i piloten faktiskt funkar bra som snabbmat så har så kallade aktivitetstester utförts i liten skala. Bakterierna fick tillgång på både kol och kväve, och genom att mäta koncentrationerna av båda ämnena över tid kunde hastigheten på reningsprocessen bestämmas. Den egna kolkällan jämfördes med syntetiskt framställda fettsyror som ättiksyra och propionsyra, och visade sig funka minst lika bra! Liknande experiment utfördes för att undersöka den biologiska fosforreningen. Försök gjordes också med avloppsvatten, både med och utan tillsatts av den egenproducerade kolkällan.

Experimenten visade att bakterierna verkar gilla den nya "snabbmaten" de fick, vilket är lovande för pilotprojektet på Källby. Genom att utnyttja det kol som faktiskt redan finns i avloppsvattnet har reningen möjlighet att bli både smartare och mer effektiv, och våra vänner mikroberna kan få bättre arbetsförhållanden!

List of Abbreviations

AS – Activated sludge

BNR – Biological nitrogen removal

COD – Chemical oxygen demand

DO – Dissolved oxygen

EBPR – Enhanced biological phosphorus removal

ICU – Ideal Carbon Utilisation

NUR – Nitrate uptake rate

MLSS – Mixed liquor suspended solids

MLVSS – Mixed liquor volatile suspended solids

rbCOD – readily biodegradable COD

sbCOD – slowly biodegradable COD

VFA – Volatile fatty acid

WW – Wastewater

WWTP – Wastewater treatment plant

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1 Introduction

Wastewater treatment processes and techniques are continuously being developed in order to reduce the environmental impact and energy demand of wastewater treatment plants (WWTPs). During the last decades, more research has been conducted regarding resource efficiency, aiming towards a more efficient utilisation of the resources present in the wastewater. By increasing the effectivity of the treatment processes, the overall resource and energy requirements of the plant can be reduced.

The main objective of treating wastewater is to remove organic matter and nutrients such as nitrogen and phosphorus. In Sweden, the most common methods of treatment are a combination of biological nutrient removal (BNR) and chemical treatment (Naturvårdsverket, 2020). At several WWTPs, a large parts of the organic matter fraction end up in the sludge and is further utilised by extracting energy in the form of biogas (Energigas Sverige, 2020). This is highly energy efficient but stand in direct conflict with the need for organic carbon in the BNR treatment, which often require access to easily degradable carbon in order to reach satisfactory removal. Especially denitrification and enhanced biological phosphorous removal (EBPR) are vital BNR processes that can be limited by shortage of a suitable carbon source (Liu *et al.*, 2020).

A substantial fraction of the biodegradable organic matter in the wastewater is in particulate form and need to undergo hydrolysis and fermentation before being fully available for BNR microorganisms (Kujawa & Klapwijk, 1999). This further limit the rate of the BNR processes. Thus at some plants, addition of external carbon source, such as ethanol or methanol, might be needed to fulfil the effluent requirements. The usage of externally produced ethanol and methanol, especially if they are of fossil origin, have a large impact on the carbon footprint of the WWTP (Gustavsson & Tumlin, 2013).

These factors have led to an increased interest in utilising the carbon in the wastewater more efficiently by producing an internal carbon source from the sludge produced at the plant. The organic matter in the sludge can hydrolysed and fermented into easily biodegradable carbon, so called volatile fatty acids (VFAs) in a separate treatment step, either in a side-stream or in-line. Several studies worldwide have been conducted on different methods of producing VFAs from sludge, either from primary sludge, biological sludge or a combination (Liu *et al.*, 2020). The ongoing project Ideal Carbon Utilisation (ICU), led by Sweden Water Research, investigates the possibility for internal production of VFAs as a carbon source using a novel process combination of prefiltration and fermentation of resulting primary sludge. This process configuration is studied at pilot scale at Källby WWTP in Lund, Sweden.

Previous studies have shown that VFAs are easily accessible to denitrifying bacteria and phosphate-accumulating organisms (PAOs), resulting in higher removal rates compared to alcohols (Peng *et al.*, 2007; Puig *et al.*, 2008). Hence, a major aim of the project is to be able to use the internally produced VFAs as a carbon source in the BNR at the WWTP. It would ideally lead to increased denitrification and EBPR rates and potentials, which could result in a more compact treatment process overall. Another potential benefit could be lower energy demand by ensuring better utilisation of the influent COD through BNR, resulting in lower need for aeration. Additionally, lower carbon footprint can be achieved due to reduced need for external carbon source addition.

1.1 Aim

The aim of this project was to investigate the suitability of fermentate from filtered primary sludge as a carbon source for usage in BNR processes, by studying denitrification and EBPR rates in ex-situ batch activity tests, as well as carbon utilisation. The removal efficiency of nitrate during denitrification was also studied. The aim was reached through the following research questions:

- How suitable for BNR is the internally produced carbon source compared with externally produced VFAs, such as acetate and propionate?
- What removal rates and efficiencies can be obtained with influent wastewater compared with filtered wastewater?
- What is the effect of adding the internally produced carbon source to the filtered wastewater?

2 Theoretical background

In the following section, the theoretical background of BNR processes is described, as well as the main microbial principles behind denitrification and EBPR. Additionally, fermentation as a method of producing VFAs is explained in the second section. Finally, the theoretical principles behind batch activity tests in wastewater treatment are outlined, with details explaining the background to NUR and EBPR tests for assessing removal rates and potentials. Important design parameters are presented.

2.1 Biological nutrient removal – BNR

BNR is performed in activated sludge (AS) systems or in biofilm systems such as trickling filters, moving bed biofilm reactors (MBBRs) and granular sludge systems. The activated sludge (AS) process is the most widely used BNR treatment technique in Sweden (Falås *et al.*, 2012), and has been used for over hundred years across the globe (Jenkins & Wanner, 2014).

BNR processes utilise the metabolism of naturally occurring microorganisms, mostly bacteria but also other microbes such as protozoa and fungi (Davies, 2006). The basic principle of the AS process is to keep a high concentration of microbes in the basins, thus ensuring a large amount of microorganisms which can do the work, i.e. clean the wastewater (Davies, 2006; Rajasulochana & Preethy, 2016). The sludge is kept in suspension, either by stirring or due to high aeration flow, thereby increasing the contact between the microorganisms and the food (i.e. organic carbon and nutrients) (ibid.).

Several factors affect the performance of BNR processes, ranging from temperature, pH and redox conditions to specific wastewater characteristics and presence of vital nutrients (Davies, 2006). All these environmental conditions affect the composition of the microbial population of the sludge as well as their activity, and thus have a huge impact on the result of the treatment.

The bacterial flora in the AS comprise of up to 300 different species with different metabolism (Davies, 2006). The most dominant bacterial group is the heterotrophs, which feed mainly on organic carbon. There are three important subgroups of heterotrophic organisms in BNR processes: ordinary heterotrophic organisms (OHOs), denitrifying ordinary heterotrophic organisms (dOHOs) and phosphate-accumulating organisms (PAOs) (van Loosdrecht *et al.*, 2016). Another important group of bacteria is the autotrophs, which utilise inorganic forms of carbon (such as CO_2) for synthesis of organic compounds. The most important autotrophic bacteria are the nitrifiers, which can be subdivided into ammonia-oxidizing organisms (AOOs) and nitrite-oxidizing organisms (NOOs) (ibid).

Both denitrification and EBPR are conducted by heterotrophic bacteria that require organic carbon for their metabolism. Thus, these processes are highly dependent on the type of carbon source available in the wastewater. A short description of each process is described in the sections below.

2.1.1 Denitrification

Dentification is a crucial step in biological nitrogen removal in wastewater treatment, removing the produced nitrate from the nitrification step in the form of dinitrogen gas. In wastewater systems, the process is mostly carried out by heterotrophic bacteria, using nitrate as the electron acceptors for the oxidation of organic carbon. During denitrification, nitrate (NO_3^-) is reduced to nitrogen gas (N_2), through the intermediates nitrite (NO_2^-), nitric oxide (NO) and nitrous

oxide (N_2O) following the steps presented in Equation 2.1 (Matějů *et al.*, 1992). Several dOHOs are only facultative denitrifiers, that exhibit denitrification as a facultative trait which is triggered by low oxygen levels and depend on the availability of NO_3^- or NO_2^- (Zumft, 1997).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad \textit{Equation 2.1}$$

The rate of the denitrification process depend on many different factors, such as temperature, pH and the availability of a sufficient carbon source, which is usually expressed as the COD/NO_3^--N ratio. The following reaction (Equation 2.2) describe heterotrophic denitrification with acetate as carbon source under anoxic conditions, showing a stoichiometric ratio of 5 mole acetate per 8 mole NO_3^--N (Xu, 1996). This corresponds to the theoretical relationship of 2.86 g COD/g NO_3^--N. However, this does not take into account the COD used for synthesis of biomass and maintenance, and is also based on the assumption that all COD is exclusively used for denitrification (Kujawa & Klapwijk, 1999; Matějů *et al.*, 1992). Thus, in practice, COD/NO_3^--N ratios required for a satisfactory treatment are in the range of 4-15 g COD/g NO_3^--N removed (Kujawa & Klapwijk, 1999; Zheng *et al.*, 2018).

$$8\,NO_3^- + 5\,CH_3COOH \rightarrow 4\,N_2 + 10\,CO_2 + 6H_2O + 8\,OH^- \qquad \textit{Equation 2.2}$$

The COD/NO_3^--N ratio highly depend on the kind of carbon source used. Several different organic carbon compounds (aside from regular wastewater) have successfully been used for denitrification – from methanol and ethanol (Her & Huang, 1995; Mokhayeri *et al.*, 2008; Peng *et al.*, 2007) to simple pure VFAs (Elefsiniotis *et al.*, 2004; Kujawa & Klapwijk, 1999; Naidoo *et al.*, 1998; Xu, 1996), to more complex mixtures of organic carbon molecules from fermented sludge or other waste products (see section 2.2).

2.1.2 Enhanced biological phosphorus removal (EBPR)

In EBPR, phosphorus removal is caused by bacteria which are able to store phosphate in excess, so called phosphate-accumulating organisms (Smolders *et al.*, 1994). PAOs can store phosphate intracellularly as polyphosphate, in the range of 0.35-0.38 g P/g VSS for PAO, compared to 0.03 g/g VSS for OHOs (van Loosdrecht *et al.*, 2016). The process consists of two steps – P-release and P-uptake. P-release usually occur anaerobically (no electron acceptor present), where PAO take up readily available carbon, mainly VFAs in form of acetate and propionate, and store this as polyhydroxy-alkanoates (PHA). The energy for the storage reaction is believed to come from the hydrolysis of intracellularly stored polyphosphate, leading to a release of orthophosphate (PO_4^{3-}) (Smolders *et al.*, 1994). Later, in the anoxic or aerobic zone, the stored COD (in the form of PHAs) is utilised for energy for growth and the synthetises of polyphosphate, resulting in a net-uptake of phosphorus. The phosphorus is removed from the system as waste activated sludge.

The presence of other organisms competing for the readily available substrate under anaerobic conditions, such as GAOs (glycogen-accumulating organisms), can eventually affect the EBPR efficiency. A study by Oehmen *et al.* (2006) investigating lab-enriched cultures of bio-P sludge showed a slight advantage of PAOs to GAOs when propionate was used as a carbon source compared with acetate, thus leading to a better level of P-removal with propionate. Additionally, temperatures above 20 °C and pH below 7 is believed to favour GAOs, at least in lab-scale systems (van Loosdrecht *et al.*, 2016). However, whether or not GAOs are a problem in full-scale systems is not fully investigated (Nielsen *et al.*, 2019). Though, it is evident that factors

such as pH, temperature and carbon source affect the EBPR process and need to be considered when designing EBPR activity tests (Smolders *et al.*, 1994; van Loosdrecht *et al.*, 2016).

Successful EBPR has been conducted with several different carbon sources, from ethanol (Puig *et al.*, 2008) and volatile fatty acids such as acetate and propionate (Oehmen *et al.*, 2006; Smolders *et al.*, 1994; Zhang *et al.*, 2011), to different fermentation liquids (Cai *et al.*, 2016; Kampas *et al.*, 2009), in both full-scale and lab-scale systems.

2.2 Fermentation of sludge for internal production of carbon source

VFAs such as acetate, propionate, butyrate and valerate, have been shown to be good carbon sources for BNR in wastewater treatment. Through a process of hydrolysation and fermentation, VFA can be produced from organic-rich waste (Lee *et al.*, 2014). In hydrolysis, complex organic polymers such as proteins, lipids and long carbohydrates, are broken down to simpler monomers (amino acids, long chain fatty acids and monosaccharides). These compounds can then be converted to VFAs through a process called acidogenesis, also known as acidogenic fermentation (ibid).

Several different waste products have been used for production of an external carbon source for BNR processes, from food waste and kitchen wastewater (Kim *et al.*, 2016; Pu *et al.*, 2019; Qi *et al.*, 2020; Zhang *et al.*, 2016; Zheng *et al.*, 2018), dairy effluents and industrial wastewater (Sage *et al.*, 2006; Swinarski *et al.*, 2009), to municipal waste (Bolzonella *et al.*, 2001), which have all shown positive results. However, the possibility to produce an internal carbon source by fermentation of wastewater sludge, such as primary sludge and biological sludge, has gained more attention (Barlindhaug & Ødegaard, 1996; Isaacs *et al.*, 1994; Kampas *et al.*, 2009; Lee *et al.*, 2014; Liu *et al.*, 2016). The organic matter in wastewater sludge consist mainly of slowly biodegradable COD, often in particulate form, denoted as XC_B (Barlindhaug & Ødegaard, 1996; Henze *et al.*, 1987). By hydrolysis and fermentation, the organic matter can be converted to soluble biodegradable carbon (S_B) such as VFAs, which can be utilised as a carbon source (Barlindhaug & Ødegaard, 1996). Through this process the soluble biodegradable COD fraction in the wastewater is increased, thus theoretically improving the composition of the wastewater for BNR.

However, a lot of organic waste contain proteins and other compounds that are rich in nutrients (Kampas *et al.*, 2009). Thus, during hydrolysation and fermentation, the soluble nitrogen and phosphorus content is increased along with the S_B and VFA content. The use of fermentation liquid as a carbon source could increase the load of nutrients to the plant, thereby reducing the availability of the produced carbon. One possibility to reduce this effect is by removing nitrogen and phosphorus in advance, through different stripping techniques, adsorption, ion exchange or struvite precipitation (Liu *et al.*, 2020). Another possibility is to extract the VFAs from the fermentation liquid itself, and thereby obtain a more pure carbon source, where extraction methods such as liquid-liquid extraction and membrane technology have been studied (ibid).

2.3 Ex-situ batch activity tests in wastewater treatment

2.3.1 Overview

The aim of conducting batch activity tests in the field of wastewater treatment is to study the performance of a certain biological process. The basic principle is to simulate the process in a small scale and measure and follow the change in concentration of substrates and/or products over time. Tests are performed on biological sludge or biofilms with real or synthetic wastewater. The scope of the study affects which type of sludge and substrate that are used,

which parameters that are measured, under which environmental conditions (redox, pH and temperature) and for how long time the test is run.

When determining the process kinetics on a specific carbon source, a synthetic substrate is used. In order to ensure that the process is not limited by lack of important nutrients, a nutrient solution can be prepared for the synthetic media. van Loosdrecht *et al.* (2016) also suggest using the effluent of the plant for the creation of a synthetic wastewater, providing that the effluent is of good quality and does not contain any toxic compounds.

Since the pH and temperature affect the rate of the biological process (affecting the microbial metabolism), it is recommended to conduct the test at constant temperature and pH, thereby ensuring consistent environmental conditions throughout the test. Both denitrification and P-release affect the pH, which will be discussed in the coming sections. Any variation in the rate of the process due to temperature or pH can thus be omitted.

Important parameters for NUR tests and anaerobic P-release tests are described in the two sections below.

2.3.2 Nitrate uptake rate (NUR)

NUR test is a common method for determining denitrification kinetics, where the concentrations of NO_3^- and NO_2^- are followed during the course of the test. By following the change in soluble COD (SCOD), the carbon source utilisation can be estimated. The NUR tests have also been used as a means of determining the COD characteristics of a wastewater or complex carbon substrates (Ekama *et al.*, 1986; Kujawa & Klapwijk, 1999; Naidoo *et al.*, 1998).

In this study, SCOD was determined by filtration through filters with 0.45 µm in pore size, and includes both biodegradable soluble carbon (S_B) and inert (non-degradable) soluble compounds (S_U). S_B can in turn be divided in to S_{VFA} (fermentation products) and S_F (fermentable organic matter), as defined by the notation system put forward by Corominas *et al.* (2010). Several important parameters for design of NUR tests such as COD/NO_3^--N ratio or F/M (Food-to-microorganism ratio) are often expressed in terms of S_B and rbCOD (readily biodegradable carbon) (Ekama *et al.*, 1986; van Loosdrecht *et al.*, 2016). However, the exact S_B content of a wastewater or a complex carbon source (such as a fermentation liquid) might not be known beforehand. Thus, for practical reasons, NUR tests (and other activity tests) are often designed in relation to SCOD (Kujawa & Klapwijk, 1999).

Denitrification rates

Depending on the substrate used, two or three linear phases of nitrate reduction can be observed during a NUR test (Figure 2.1), with denitrification rates r_{D1}, r_{D2} and r_{D3} respectively (mg NO_3^--N/L·h), determined by linear regression. When a mono-component carbon source is used, such as acetate or propionate, only two phases are observed – a fast phase which is assumed to primarily depend on the denitrification on S_B, containing readily biodegradable COD (eg. acetate and other VFAs), and a slow phase due to endogenous denitrification (Figure 2.1A). The endogenous denitrification rate depend on decay and lysis of internal cell material in the biomass. Complex carbon sources containing a mixture of carbon compounds (such as fermentation liquids) or real wastewater can give rise to a three phased denitrification curve, with a second (middle) phase assumed to relate to the denitrification on XC_B, i.e. slowly biodegradable COD, which has a slower rate than denitrification on S_B but higher rate than endogenous denitrification (Figure 2.1B). It is often assumed that all three phases occur simultaneously during the denitrification process when assessing NUR kinetics (Kujawa & Klapwijk, 1999).

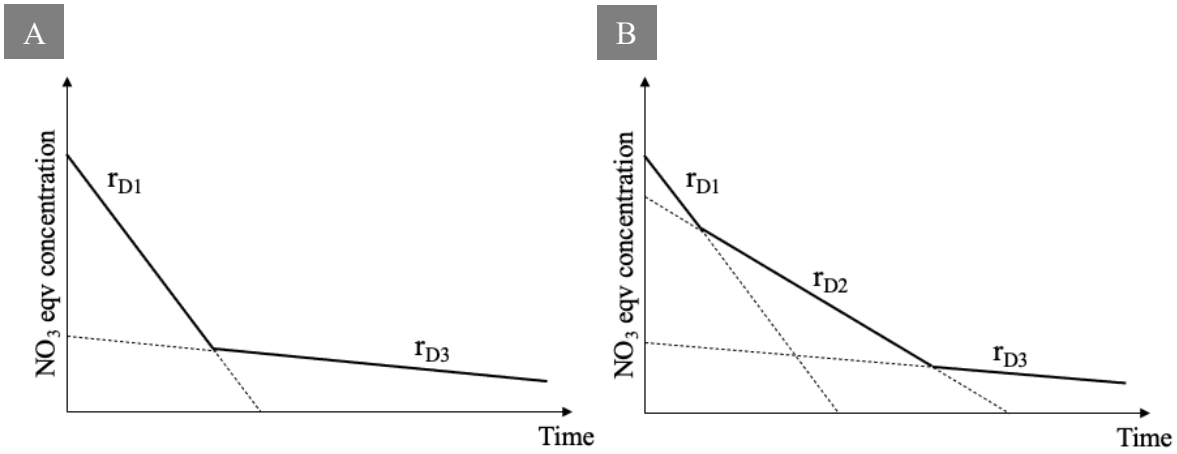

Figure 2.1. Schematic figure of two NUR curves (thick lines), showing the different phases of nitrate reduction with linear reduction rates r_{D1}, r_{D2} and r_{D3} (extrapolated dotted lines). Inspired by Kujawa and Klapwijk (1999).

A: Mono-component carbon source (S_B). B: Complex carbon sources/wastewater (S_B+XC_B).

In the activated sludge model ASM1 (IAWQ no. 1) put forward by Henze *et al.* (1987), the sbCOD is treated as if it is particulate (for modelling purposes), and is thus often denoted as XC_B, even though some slowly biodegradable compounds might be soluble. Furthermore, the model assume that denitrification only occurs on S_B, i.e. XC_B must first be converted through hydrolysis and fermentation to S_B before being available as an electron donor for denitrification. It is further assumed that no utilisation of electron acceptors (nitrate or oxygen) occur during the hydrolysis on XC_B. The denitrification rate on XC_B is therefore limited by the kinetics of hydrolysis and fermentation.

Kujawa and Klapwijk (1999) put forward a simplified model of additive denitrification rates, where the highest slope (r_{D1}) is assumed to reflect the simultaneous denitrification on S_B (r_{DSB}), hydrolysed XC_B (r_{DXCB}) and endogenous material (r_{Dendo}), each rate adding to the total rate (Equation 2.3). The second rate (r_{D2}) reflects the combination of r_{DXCB} and r_{Dendo} (Equation 2.4), whereas the third and last rate relates to the basic endogenous denitrification (r_{Dendo}) (Equation 2.5). Thus, according to Kujawa and Klapwijk (1999), the denitrification on the S_B fraction of a carbon source/wastewater (r_{DSB}) can be determined by subtracting r_{D2} from r_{D1} (Equation 2.6). The denitrification on X_{CB} can be determined in a similar manner (Equation 2.7).

$$r_{D1} = r_{DS_B} + r_{D_{XC_B}} + r_{D_{endo}} \qquad \textit{Equation 2.3}$$

$$r_{D2} = r_{D_{XC_B}} + r_{D_{endo}} \qquad \textit{Equation 2.4}$$

$$r_{D3} = r_{D_{endo}} \qquad \textit{Equation 2.5}$$

$$r_{DS_B} = r_{D1} - r_{D2} \qquad \textit{Equation 2.6}$$

$$r_{D_{XC_B}} = r_{D2} - r_{D3} \qquad \textit{Equation 2.7}$$

However, Sage *et al.* (2006) argue that simultaneous denitrification on all different COD fractions does not imply additive rates per se. The denitrification rate on S_B can be said to be

proportional to the consumption rate of S_B, which according to Monod's theory is proportional to the maximum specific growth of heterotrophic denitrifies (μ_{DN}) when the S_B concentration is not rate limiting, as illustrated by the following Equation 2.8:

$$r_{DN} = \mu_{HD} \cdot \frac{S_B}{S_B + K_{S_B}} \cdot X_{HD} \qquad \textit{Equation 2.8}$$

where K_{SB} is the half saturation coefficient on S_B (g COD/L), X_{HD} is the heterotrophic denitrifying concentration (g COD/L).

In this case, when the S_B concentration is not rate limiting, Sage *et al.* (2006) explain that theoretically, the maximum denitrification rate will not increase due to the hydrolysis of XC_B or endogenous material into S_B; i.e. the rates would not be additive. The rate is only governed by the total specific biomass growth rate μ_{DN}.

Sage *et al.* (2006) mean that additive rates are in theory based on the assumption that some species of denitrifying bacteria use exogenous S_B whereas other utilise the S_B produced from XC_B or endogenous sources. This would mean that the rate is governed by the sum of specific growth rates of different species in the biomass ($\sum\mu_{DN,i}$, where *i* denotes a different species), and the highest denitrification rate (r_{D1}) could thus be described as suggested by Kujawa and Klapwijk (1999) (Equation 2.3). This assumption is not unreasonable, since it has been shown that different rates occur due to different carbon sources, and the resulting S_B from hydrolysis on XC_B and endogenous carbon could have a different composition compared with the exogenous S_B. As Sage *et al.* (2006) argue, the reality is probably between these two opposing hypotheses.

Under the assumption of simultaneous denitrification on all different COD fractions, a continuous hydrolysis and production of S_B will of course contribute to the overall denitrification through a constant supply of available carbon (assuming denitrification only occur on S_B as stated previously). But rather than leading to an increased rate (i.e. additive effect) it could be argued that the effect is instead a longer period of high denitrification rate (r_{D1}). However, the contribution of XC_B and endogenous activity to the highest denitrification rate could be estimated by assuming that additive rates apply.

Another definition of the different denitrification rates is put forward by van Loosdrecht *et al.* (2016), where only the endogenous denitrification rate (r_{D3}) is subtracted from both r_{D1} and r_{D2} (as defined by Kujawa and Klapwijk (1999)) for the rate on S_B and XC_B respectively (see Equations 2.9 and 2.10).

$$r_{D_{S_B}} = r_{D1} - r_{D3} \qquad \textit{Equation 2.9}$$

$$r_{D_{XC_B}} = r_{D2} - r_{D3} \qquad \textit{Equation 2.10}$$

As the denitrification rates depend on the MLVSS (mixed liquor volatile suspended solids) concentration, the rates will be expressed as specific denitrification rates (mg NO_3^--N/g VSS·h), which is given by Equation 2.11 below.

$$k_D = \frac{r_D}{MLVSS} \qquad \textit{Equation 2.11}$$

Due to the uncertainty of the actual additive effects of denitrification rates, the rates in this study will primarily be expressed and discussed as total rates (i.e. k_{D1}, k_{D2} and k_{D3}).

Denitrification potential

The denitrification potential (p_{DN}) is a measure of the amount of NO_3^- which can be reduced on a given quantity of a carbon source (g NO_3^--N/g COD) or a specific COD fraction of a wastewater (g NO_3^--N/L wastewater). It is related to the total COD needed for both the reduction of NO_3^- to N_2 and the anoxic growth of heterotrophic denitrifying bacteria (as well as other COD "lost" due to degradation and growth by other microbes). The anoxic growth can be described by the yield coefficient Y_{HD} (g COD/g COD), which is related to p_{DN} according to Equation 2.12 presented below. The anoxic growth yield is specific for each activated sludge and also depend on the substrate used (Kujawa & Klapwijk, 1999).

$$1 - Y_{HD} = 2.86\, p_{DN} \qquad \textit{Equation 2.12}$$

The denitrification potential on a carbon source *x* can be experimentally determined by estimating the amount of COD utilised (ΔCOD_x) for the denitrification of a certain amount of NO_3^- (ΔNO_3^--N eqv_x) (Equation 2.13). Several studies use the assumption of separable and additive denitrification rates in order to determine the amount of NO_3^- removed by specific COD fractions (S_B, XC_B or endogenous), which imply that the denitrification potentials also are additive (Bolzonella *et al.*, 2001; Kujawa & Klapwijk, 1999; Naidoo *et al.*, 1998). This is illustrated in the schematic NUR curve presented in Figure 2.2, where $\Delta(NO_3\text{-N eqv})_1$ represents the amount removed due to S_B, and $\Delta(NO_3\text{-N eqv})_2$ is the amount removed due to XC_B. It can also be argued that since the rate reflecting denitrification on XC_B (denoted as r_{D2}) is assumed to reflect the continuous production of S_B, the contribution of XC_B to the nitrate reduction in the first phase can be estimated through the definitions illustrated in Figure 2.2 below.

$$p_{DN} = \frac{\Delta NO_3\text{-}N\ eqv_x}{\Delta COD_x} \qquad \textit{Equation 2.13}$$

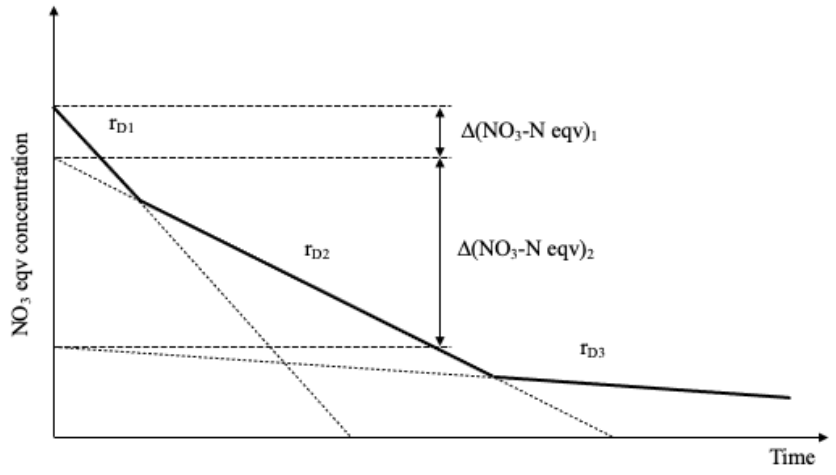

Figure 2.2. Schematic figure of a NUR curve, illustrating the amount of NO_3^--N eqv removed due to different carbon fractions, as defined by Kujawa and Klapwijk (1999), Naidoo et al. (1998) and others.

Furthermore, these assumptions of additivity are the basis for the determination of the different COD fractions using the NUR test, as put forward by Ekama *et al.* (1986) (Equation 2.14). Ekama *et al.* (1986) mean that estimates of rbCOD determined using NUR test give accurate results compared with other determination methods, based on OUR (oxygen utilisation rate). However, Sage *et al.* (2006) argue that this assumption of additive denitrification potentials is used by several authors but is not supported by experimental evidence or literature references. Their own study showed that the relationship between the p_{DN} of a complex substrate and its individual components could not validate this assumption, thus leading to the conclusion that additive effects of potentials cannot be regarded as a universal rule (Sage *et al.*, 2006).

$$\Delta COD_x = \frac{2.86 \cdot \Delta NO_3\text{-}N\ eqv_x}{1-Y_{HD}} \qquad \textit{Equation 2.14}$$

Depending on how the amount of NO_3^- denitrified on a specific carbon source is defined, the resulting p_{DN} may differ, which in turn affect the estimation of anoxic growth yield (Equation 2.12). Zhang *et al.* (2016) uses the Equation 2.15 for calculation of p_{DN}, where $N_{initial}$ and $N_{end,XCB}$ are the NO_3^- eqv concentrations measured at the initial sampling point and the final sampling point at full XC_B consumption respectively, whereas COD_{in} is the total exogenous carbon added and COD_e is the measured COD in the effluent. Thus, as it seems, Zhang *et al.* (2016) assign a larger amount of NO_3^- removal to the added carbon compared with Kujawa and Klapwijk (1999) and others.

$$p_{DN} = \frac{N_{initial} - N_{end_{XC_B}}}{COD_{in} - COD_e} \qquad \textit{Equation 2.15}$$

Due to the varying definitions of denitrification potential and difficulties in assessing the endogenous denitrification rate, no exact calculations of p_{DN} will be made in this study. Instead the amount of utilised substrate in relation to amount of NO_3^- removed will be discussed in relation to the measured total difference in SCOD during different phases of the NUR curve (primarily the first reduction phase), divided by the measured change in NO_3^- equivalents during corresponding phase ($\Delta SCOD_x/\Delta NO_3^-$-N eqv_x). This parameter can be seen as the inverse of a simplified p_{DN} definition, similar but not equal to that used by Zhang *et al.* (2016).

F/M ratio

The F/M ratio, expressing the amount of carbon in relation to bacteria, governs the shape of the response curve. Ekama *et al.* (1986) explains that a too low ratio will result in a rapid depletion of S_B, whereas a too high ratio could make it difficult to establish when S_B is fully consumed or, in the worst case, lead to biomass inhibition. A S_B to MLVSS ratio of 0.05-0.1 g COD/g VSS is recommended by van Loosdrecht *et al.* (2016).

Due to variations in SCOD concentration in wastewater and other complex carbon sources, as well as variations in the MLVSS concentration, it can be difficult to obtain the same F/M ratio in all tests. It will also depend on the fraction of wastewater/substrate to the total volume in the reactor (f_{ww}), as addition of substrate will dilute the activated sludge mixed liquor. For both Naidoo *et al.* (1998) and Kujawa and Klapwijk (1999), the ratio varied between 0.02 – 0.07 mg COD/MLVSS.

COD/NO_3^--N ratio
The COD/NO_3^--N ratio will determine whether maximum denitrification rate on a specific carbon source is reached during the test and if the denitrification will be complete before the substrate is fully utilised. The ratio is specific for each carbon source. Tests can be conducted beforehand in order to determine suitable COD/NO_3^--N ratios. van Loosdrecht *et al.* (2016) recommends a ratio twice the stoichiometric relationship, and by assuming an anoxic growth yield of 0.5 g COD/g COD, a ratio of 10 is obtained. However, often ratios around 4-6 are sufficient for complete denitrification (Elefsiniotis *et al.*, 2004; Zhang *et al.*, 2016).

Redox, pH and temperature
Denitrification occurs under anoxic conditions, and it is recommended to keep the redox conditions of the sludge constant prior to starting the activity test (van Loosdrecht *et al.*, 2016). It is advisable to wash the sludge before the test to remove soluble compounds (residual nitrates and SCOD), which will affect the redox conditions. The sludge should be sparged with N_2 gas before the start of the activity test to remove any dissolved oxygen (DO).

During denitrification, one mole of OH^- is produced per mole NO_3^- reduced (Equation 2.2) leading to an increase in pH. In order to keep the pH constant, addition of acid is needed. NUR tests are often conducted at pH 7-7.5. To avoid any fluctuations in pH, the sludge, the substrate and eventual nutrient solutions can be adjusted to the pH set point before the test starts.

A temperature of 20-25 °C is a common temperature for conducting NUR tests. However, in real WWTP the temperature can be lower and higher (3-30°C), thus when assessing real operational kinetics, it is recommended to conduct the test at current temperatures.

dPAO and anoxic P-release
In activated sludge systems with EBPR, anoxic P-uptake and P-release can occur, which can deteriorate the measurement of the activity of heterotrophic denitrifies (dOHOs).

Several studies have investigated the activity of dPAOs, i.e. denitrifying phosphate-accumulating organisms, which is believed to utilise stored COD (PHB) for reduction of nitrate and uptake of phosphate (Barker & Dold, 1996; Hu *et al.*, 2002; Lanham *et al.*, 2018). Anoxic P-uptake is not as efficient as aerobic uptake, and slower growth of PAOs has also been observed during anoxic conditions (ibid). However, it could affect the evaluation of carbon utilisation for heterotrophic denitrifies, if a substantial part of the available NO_3^- is reduced by dPAO using internally stored COD. Although, the specific denitrification rates of dOHOs are significantly higher than those obtained with dPAOs (Hu *et al.*, 2002). Additionally, when nitrate is limiting, dOHOs tend to outcompete dPAOs for nitrate (ibid). If needed, an aeration period prior to the start of the anoxic batch test can be introduced in order to remove stored COD present in the biomass, thus reducing the unwanted effect of dPAO activity (van Loosdrecht *et al.*, 2016). This would also reduce other residual SCOD present in the sludge. A washing step of the sludge can also be preferable, as this also might decrease intracellular stored COD (ibid).

Anoxic P-release is also of concern when assessing the denitrification rates, where available carbon is utilised by PAO or GAO bacteria instead of heterotrophic denitrification. Kujawa Kujawa and Klapwijk (1999) observed a release as high as 7-10 mg PO_4^{3-} mg/L under the first 30 minutes of an anoxic batch test using acetate as substrate (C_{NO3} = 25-30 mg/L). Other studies have seen effective phosphate release using acetate, propionate and formate, even under anoxic conditions, provided that the sludge studied had some degree of EBPR activity (Gerber, 1986; Iwema & Meunier, 1985). However, anoxic P-release was not observed with other organic

substrates tested (Gerber, 1986). Gerber (1986) argue that this observation indicates that phosphate release is dependent on carbon source rather than the anaerobic redox state. Furthermore, it is hypothesised that release and uptake of phosphate occur simultaneously, indicating that it is the net effect that is observed during measurements (Barker & Dold, 1996; Gerber, 1986).

As anoxic P-release would reduce the available carbon for denitrification, it is important to follow the PO_4^{3-} concentration during NUR tests.

2.3.3 Anaerobic P-release test

In order to assess the EBPR activity based on a carbon source, the most important step to study is the anaerobic P-release and simultaneous uptake of carbon. A high P-release suggest a high storage of COD and thus good conditions for P-uptake in the following step. However, it should be noted that a high P-release does not automatically result in a good overall removal (Gerber, 1986). For a more complete analysis of the EBPR activity, resulting in measures of the total P-removal, both anaerobic and following anoxic/aerobic steps should be studied.

By following the concentration of both PO_4^{3-} and SCOD, the amount of P released per C utilised ratio can be determined, called the P/C-ratio. Common values reported in the literature are in the range of 0.25-0.75 P-mole/C-mole, with acetate as a carbon source (Smolders *et al.*, 1994). The P-release rate can also be estimated with linear regression (Zhang *et al.*, 2011).

F/M-ratio

In contrast to NUR tests, van Loosdrecht *et al.* (2016) recommends a lower F/M ratio when conducting P-release tests, in the range of 0.025-0.050 mg COD/mg VSS (expressed as rbCOD/MLVSS). A lower F/M ratio is preferable in order to ensure that full consumption of rbCOD occur for the duration of the test, especially when including subsequent anoxic/aerobic P uptake.

Redox, pH and temperature

Activated sludge used in anaerobic P-release is usually collected from the preceding step, i.e. the aerobic zone. Before the tests, it is advisable to keep the redox conditions of the sludge constant, i.e. under aeration. The actual tests should be conducted under anaerobic conditions, in the absence of any electron acceptors, thus residual DO and other electron acceptors should be removed directly prior to the test by sparging with N_2 gas. N_2 sparging can continue throughout the test in order to limit oxygen intrusion. Since nitrification can occur during aeration step, ATU (allylthiourea) should be added in order to inhibit aerobic ammonium oxidation (nitritation), thus hinder NO_2^- and NO_3^- production.

The pH is affected by the P-release process, but the exact mechanism is complex. Both the release of phosphate, usually assumed to be in the form of $H_2PO_4^-$, and the uptake of VFAs affect the pH. A study by Marcelino *et al.* (2009) showed that the pH changed significantly in the beginning of the anaerobic phase. But depending on the initial pH in the reactor, the change in pH varied. At an initial pH at 6.5-7, an increase in pH could be observed, whereas at initial pH 7.2-7.5 the pH profile decreased. Anaerobic P-release tests are usually conducted in the pH range of 7-7.5, thus depending on the pH set point, the pH should be regulated with either acid or base.

As with NUR tests, 20-25°C are common temperatures for conducting P-release tests.

3 Methodology

In this chapter, a detailed description of the methodology used is presented. In the first section, the pilot plant at Källby WWTP is described. Secondly, the procedure used during the batch activity tests is outlined. In the following sections, the preparation of substrates, nutrient stock solutions and activated sludge are presented, after which the handling of important parameters such as pH, DO and temperature is described. Thereafter, the analytical methods used for determining different concentrations are presented. Finally, the data analysis is explained.

3.1 Pilot plant description

The ICU pilot plant is situated at Källby WWTP in Lund, Sweden. A schematic overview of the pilot plant is presented in Figure 3.2, and a picture showing tank T1-T5 as well as the filter is presented in Figure 3.2.

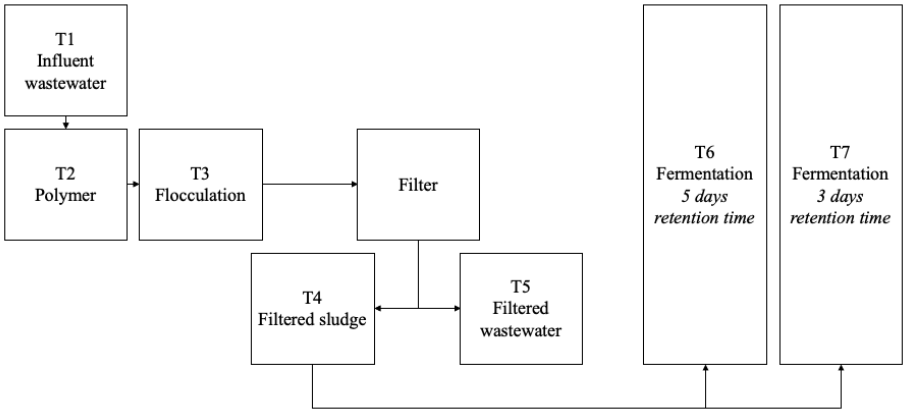

Figure 3.1. Schematic overview of the pilot plant at Källby WWTP.

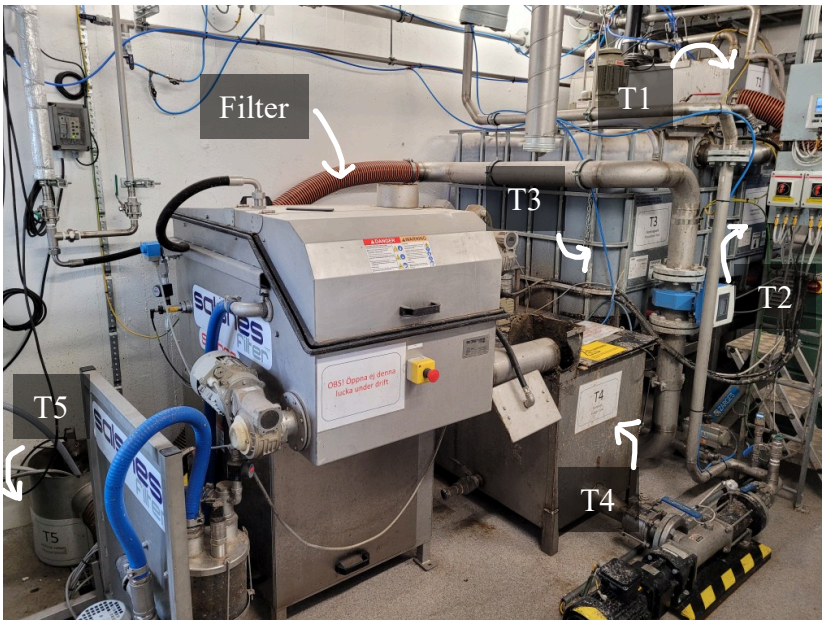

Figure 3.2. Photo of the pilot plant at Källby WWTP: influent wastewater tank (T1), polymer and flocculation step (T2 and T3), collected sludge tank T4 and filtered wastewater tank (T5). (Photo: Elin Ossiansson).

A side-stream (6-18 m^3/h) of the influent wastewater (WW) to the WWTP, is pumped flow proportional to the pilot plant into tank T1 (Figure 3.2). In the proceeding tanks (T2 and T3), polymer is added to the wastewater and flocculation occurs. The water is then filtered using rotating belt filtration (Salsnes SF1000), where the water phase and the resulting primary sludge are separated.

The pore size of the filter is 350 µm. However, a filter mat has developed, especially due to polymer addition, thus decreasing the actual working pore size. Mainly particles smaller than 10-40 µm end up in the filtered water (Tebini, 2020).

The filtrated wastewater (WW) ends up in tank T5 before it is returned back to the WWTP. The sludge is collected in tank T4 and is subsequently pumped to the hydrolysis-fermentation reactors T6 (five days hydraulic retention time) and T7 (three days hydraulic retention time), where acidogenic hydrolysis and acidogenic fermentation of the primary filter sludge take place.

To date, the fermented filtered primary sludge at the pilot plant is returned back to the WWTP directly, but the plan is to return it through the filter, thus separating the larger particles from the fermentation liquid.

The influent WW (T1), the filtered WW (T5) and the filtered fermented primary sludge (T6 and T7) where used in the batch activity tests experiments in this study. The water samples T1 and T5 were collected from 24-hour flow proportional samplers, by pouring water from the sample cans into 2-L plastic cans. The water in the sampling cans was mixed before sample collection to ensure a representative sample. The sludge samples were collected from the fermentation reactors into small plastic jars by using a sampling tube connected to each the reactor.

3.2 Ex-situ batch activity tests

3.2.1 Overview of conducted tests

A total of 12 parallel batch activity tests were conducted during the testing period, March 3rd to April 29th 2021. An overview of the conducted NUR tests is shown in Table 3.1 below, presenting the test number, dates of the conducted tests and the carbon source/substrate in each reactor.

The following carbon sources and substrates have been tested:

- Acetate
- Propionate
- Acetate + propionate
- Filtered fermented primary sludge (T6)
- Filtered fermented primary sludge (T7)
- Influent WW (T1)
- Filtered WW (T5)
- Filtered WW (T5) + filtered fermented primary sludge (T6)
- Blank (no carbon source)

In the tests using filtered fermented primary sludge (T6 or T7), the supernatant (called the fermentate) of the sludge have been used. For more details on the preparation of the fermentate and the other substrates, see section 3.3.

Table 3.1. Overview of conducted NUR tests, with test number, date and carbon source/substrates presented.

NUR test #	Date	Reactor A	Reactor B
N1	03-03-2021	Acetate*	Acetate**
N2	03-16-2021	Acetate**	Fermentate (T6**)
N3	03-18-2021	Acetate	Fermentate (T6)
N4	03-24-2021	Influent WW (T1)	Filtered WW (T5)
N5	03-29-2021	Blank	
N6	04-06-2021	Acetate + propionate	Fermentate (T6)
N7	04-08-2021	Filtered WW (T5)	Filtered WW (T5) + Fermentate (T6)
N8	04-19-2021	Fermentate (T6)	Fermentate (T7)
N9	04-21-2021	Filtered WW (T5)	Filtered WW (T5) + Fermentate (T6)
N10	04-29-2021	Propionate	Fermentate (T6)

* With nutrient solution and ATU, without pH regulation.

** With nutrient solution and ATU.

The conducted P-release tests are shown in Table 3.2, presenting the test number, dates and carbon source/substrate in each reactor.

Table 3.2. Overview of conducted P-release tests, with test number, date and carbon source/substrates presented.

P-release test #	Date	Reactor A	Reactor B
P1	03-10-2021	Acetate*	Acetate**
P2	03-22-2021	Acetate	Fermentate (T6)
P3	03-29-2021		Blank

* With nutrient solution and ATU, without pH regulation.

** With nutrient solution and ATU.

3.2.2 Important design parameters

All tests were conducted at 20 °C and pH 7.0 (regulated manually). The activated sludge used was collected from Källby WWTP, from line 3, tank B3.

In order to create similar conditions in all conducted tests, the initial soluble COD concentration was determined based on the concentration in the influent WW (T1) and filtered WW (T5). The median value of the SCOD concentration in the wastewater (both influent WW and filtered

WW) was around 215 mg/L during the period May 2020 to February 2021. However, it was later realised that the SCOD value determined by the lab was based on a filtration with 18.5 µm filter and not on 0.45 µm filtration, thus leading to an overestimation of SCOD. This was not known at the time when the experiments were designed, and therefore the value of 215 mg SCOD/L was used as the basis for all tests.

The aim was to obtain as similar SCOD/NO_3^--N and SCOD/VSS ratios as possible in all NUR tests. A SCOD/NO_3^--N ratio over 5 was deemed sufficient, and with a NO_3^- start concentration of 20 mg/L, a SCOD start concentration of around 100 mg/L was needed in the reactors. Thus, a dilution factor (total volume to wastewater volume, V_{TOT}:V_{WW}) of 2 was necessary in order achieve a start SCOD concentration of 105-110 mg/L in the test conducted with wastewater. As this dilution affect the MLVSS concentration in the reactors, it was decided to use the same dilution in all tests, thus reaching similar SCOD/VSS ratios as well. Therefore the volume of the activated sludge and the substrate was 1 L respectively in all tests. For simplicity, the same ratios of substrate to sludge were used in the EBPR tests. Due to time constraints, only anaerobic P-release tests were conducted when assessing EBPR activity.

Test N1 and P1 was conducted during 2 and 2.5 hours respectively. Test N2-N3, N6 and P2 were 3.5 hours long. All other tests, N4, N5/P3 and N7-N10, were 4 hours long.

3.2.3 Experimental set up

In the beginning of the test period, the laboratory work was conducted at the laboratory at Källby WWTP. At the end of the period, the equipment was moved to Lund University and the apparatus hall at the Department of Chemical Engineering, due to renovation work at the laboratory at Källby WWTP. A schematic picture of the experimental set up is presented in Figure 3.3, and a picture of the experimental set up at the laboratory at Källby WWTP is shown in Figure 3.4.

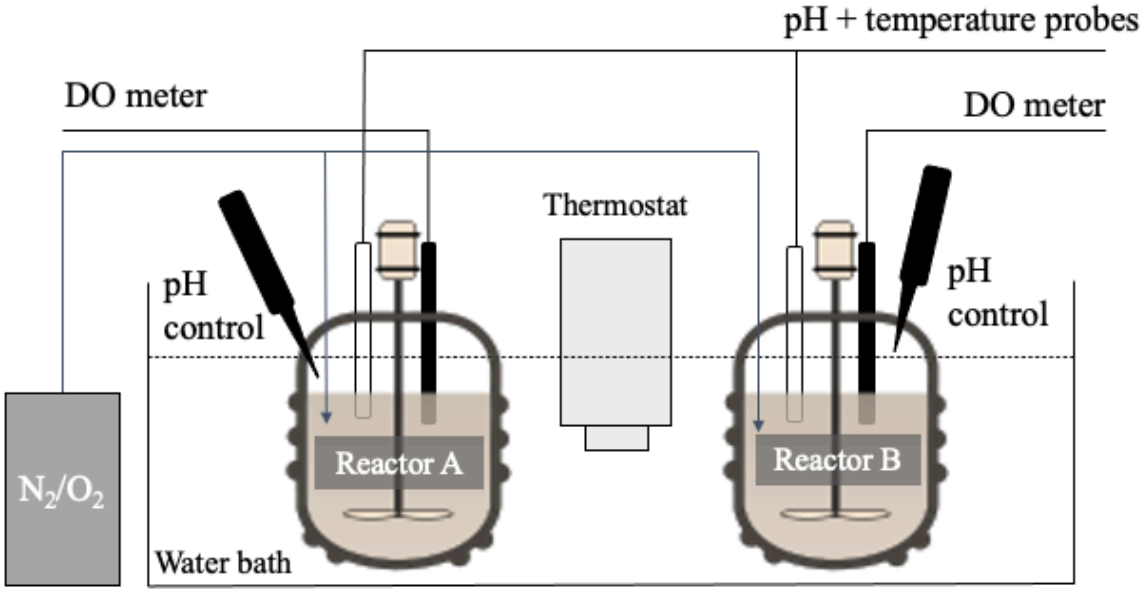

Figure 3.3. Schematic picture of experimental setup.

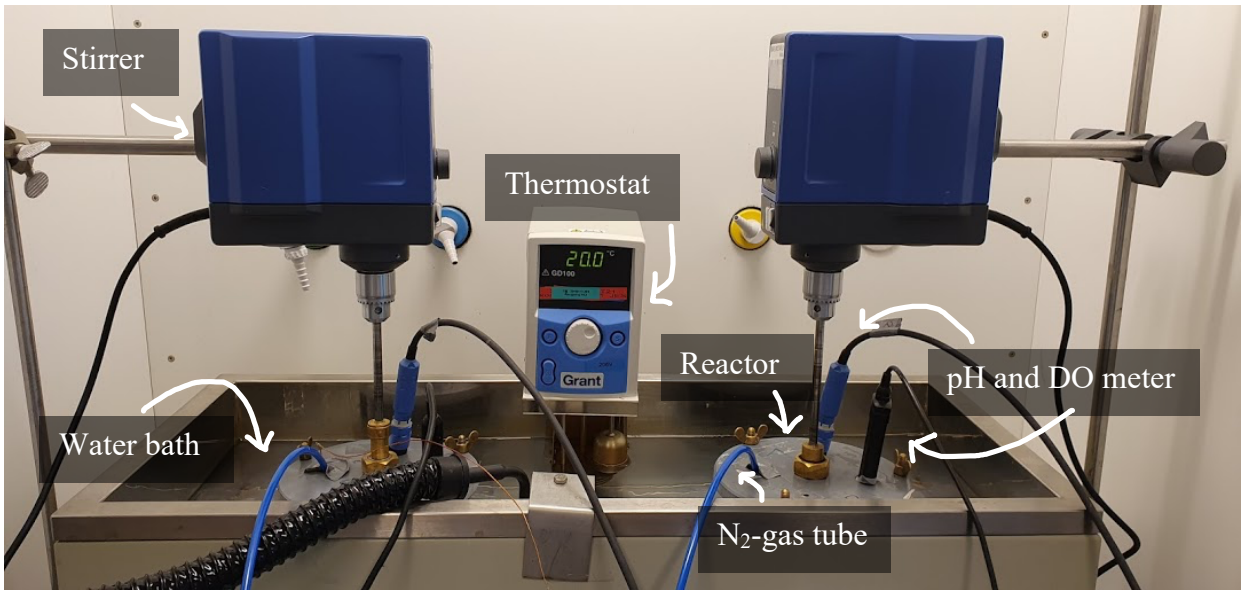

Figure 3.4. Experimental set up at Källby WWTP. (Photo: Sanna Sahlin)

Two batch activity tests were conducted simultaneously using two glass reactors (A and B) with a 2-L working volume each, placed in a water bath. The temperature in the water bath was controlled at 20°C using a Grant Instrument GD100 immersion thermostat and water bath circulator. The reactors were equipped with stirrers (IKA EUROSTAR 20 Digital) and four ports used for sampling, addition of different solutions and placement of different probes and tubes. A mixing rate of 70 rpm was used in the majority of the tests[1].

The pH probes (Endress+Hauser) were calibrated before being used the first time and checked before each test, and the DO meters (Hach Company) were checked periodically, see section 3.6 for a more detailed description. In the beginning of the testing period, a digital thermometer was used when measuring the temperature, alternating between the reactors. At a later stage, the internal thermometers in the pH probes were used for temperature measurement.

The pH was regulated manually using pipettes, through the addition of HCl (1 M/0.5 M) or NaOH (1 M/0.5 M). Pipettes were also used for addition of ATU and NO_3^- stock solutions. See Appendix A for detailed description on the preparation of these stock solutions.

Aeration was supplied via a Stellar dual outlet air pump W60. Each outlet was connected to a plastic tube to glass dispersion tube (PYREX) to ensure good dispersion of air in the reactors. A N_2 gas tank (2 bar) connected with a plastic hose was used in order to supply N_2 gas to the reactors. A flow meter enabled regulation of N_2 gas flow during the test. The total working flow ranged from 10 L/min down to 1 L/min. However, exact flow regulation was not available at Lund University.

3.2.4 Sample collection

Sample syringes á 10 mL connected with small plastic tubes were used for sampling. In order to achieve homogenous samples, the syringes were filled and emptied several times, around 2-3 times, before the samples were collected.

[1] 60 rpm was used in test N1, and 80 rpm for a short while in reactor P1:A.

The sludge samples for analysis of total COD (TCOD), MLSS (mixed liquor suspended solids), and MLVSS (mixed liquor volatile suspended solids), were collected in test tubes á 50 mL. Samples for analysis of soluble compounds (SCOD, NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}, VFA) were immediately filtered through folded filter papers with pore size 18.5 µm in order to remove large sludge particles. The resulting liquid was then quickly filtrated through a 0.45 µm filter (ABLUO GVS/Sartorius) and collected in test tubes á 15 mL. 1.5 mL of the filtered samples were frozen in Eppendorf tubes and saved for VFA analysis.

The collected samples were stored in a fridge overnight before analysis with Hach cuvette tests and other methods (for more detailed description, see section 3.73.7). During the tests conducted at Lund University, several parameters were instead analysed using ion chromatography (Metrohm), which in some cases could be started directly after the test was concluded.

3.2.5 Experimental procedure

The experimental procedures for conducting NUR and P-release test were rather similar, and the used methodology was based on the procedures outlined in chapter 2.2 and 2.4 in Experimental Methods in Wastewater Treatment (van Loosdrecht *et al.*, 2016). An overview of the procedure is presented in Table A.1 in Appendix A.

Nitrate uptake rate (NUR) tests

For the NUR tests, activated sludge was collected from the end of the pre-denitrification tank, line 3[2] at Källby WWTP, using a 2-L sampling tool and pouring the sludge into 2-L plastic cans.

The activated sludge was washed before test started, for which a detailed methodology is described in section 3.5. A sample of the sludge was taken in order to measure MLSS, MLVSS, TCOD and SCOD. Ammonium (NH_4^+) and orthophosphate (PO_4^{3-}) concentrations in the activated sludge was also measured in some tests. The soluble compounds were measured after the same filtration procedure as described in section 3.2.4, i.e. after filtration with both folded filter (18.5 µm) and 0.45 µm filter.

The prepared anoxic sludge was poured into the sealed reactors (1 L in each reactor), after which the mixing started. In the first tests two tests (N1 and N2) 2 mL of ATU stock solution (10 g/L) were added before aeration started, in order to inhibit nitrification and limit accumulation of NO_3^- and NO_2^- before the test. The ATU concentration in the reactor of was 20 mg/L in the reactor before addition of substrate, with a final concentration of 10 mg/L during the test. The sludge was aerated for at least 30 minutes, striving to reach endogenous conditions and removal of residual SCOD, and letting the sludge adjust to the temperature before the test.

At the end of the aeration period, around 10-20 minutes before the test started, a sample of the washed and aerated sludge was collected, after which aeration was stopped and dispersion of N_2 gas started from the bottom of the reactor. Sparging of N_2 gas continued for at least 10 minutes, until the DO concentration was 0.0 mg/L in both reactors. The gas flow varied between the tests, between 1-10 L/min[3].

Before the test started, DO and temperature were checked, and pH value noted. Small samples of the prepared substrates were collected for analysis of total COD, SCOD, NH_4^+ and PO_4^{3-}.

[2] In the majority of the test, the AS-samples were collected from site B3:7, but during test N3 the sludge was collected from B3:4 when the aerated volume in the AS-tank was larger.

[3] In the last three tests (N8-N10) conducted at Lund University, no data of the exact flow used are available.

When DO levels were below 0.0 mg/L, the substrates were added to the reactors. In the first tests, N1 and N2, nutrient solutions were added directly afterwards (2.5 mL macronutrient solution and 5 mL micronutrient solution NUR). Nitrate was also added immediately, by pipetting 4 mL of nitrate stock solution (10 g/L) into the reactors. The test started (time 0) when nitrate was added. Manual pH regulation was started directly after the start of the test, with some time lag due to sampling. In the majority of the tests, the DO concentration increased slightly directly after addition of the substrates. The N_2 sparging continued from the bottom of the reactor until the DO concentration reached 0.0 mg/L again, after which N_2-sparging continued in the headspace of the reactor.

The first sample was collected at minute 1, allowing for some mixing before sampling. During the first 30 minutes, samples were collected every 10 minutes (minute 1, 10, 20, 30). Then every 15 minutes for an hour (minute 45, 60, 75, 90) and then every 30 minutes until the end of the test (minute 120, 150, 180, 210, 240)[4]. All samples were immediately stored in fridge or freezer after collection.

The concentrations of TCOD, MLSS and MLVSS was checked in the first and final samples. The concentrations of all soluble compounds (SCOD, NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}, VFA) were followed during the course of the test, at different time steps. A schematic overview of the different collected samples during the course of the test is presented in Table A.2 in Appendix A.

P-release rate tests

The procedure for P-release test follow the similar steps as described in Table A.1 and in the above section. The activated sludge was collected at the end of the aerobic zone of the AS-tank, from line 3 at Källby WWTP[5]. The sludge was prepared as described in section 3.5 and added to the reactors. ATU was added before aeration of the sludge in test P1 but omitted in test P2 and P3. Aeration was conducted, striving to reach endogenous conditions.

A sludge sample was collected after aeration in order to check initial concentrations, as described previously. Before the test started, the rectors were sparged with N_2 gas from the bottom of the rector for at least 10 minutes, to ensure anaerobic conditions. The test started with addition of substrate. Nutrient solutions were added directly afterwards in test P1, by pipetting 2.5 mL of macro solution and 1.5 mL[6] of micro solution EBPR into the reactors. Nutrient solutions were omitted in the other tests.

Sample collection started at minute 1, then planned to continue at minute 10, 20 and 30, i.e. around every 10 minutes during the first half hour. Then collection occurred every 15 minutes during the proceeding hour, and then every 30 minutes until the end of the test.

The concentrations of MLSS, MLVSS and TCOD as well as all soluble compounds were checked in the washed and aerated sludge, and in the first and final samples. During the rest of the test, only SCOD and PO_4^{3-} concentration were followed. Samples for VFA analyses were saved in test P2 and P3. A schematic overview of the collected samples for different compounds is presented in Table A.3 in Appendix A.

[4] Some discrepancies in test time occurred during the different tests, and the actual sampling time usually offset by 1 minute, i.e. minute 1, 11, 21 etc.
[5] Site B3:10.
[6] Too high addition of micronutrient solution, see section 3.4.3 for more details

3.3 Preparation of substrates

3.3.1 Preparation of external carbon sources

The preparation of acetate and propionate stock solutions (20 g COD/L) is presented in Appendix A. In the tests with synthetic substrates, substrates solutions with concentration of 220 mg COD/L were prepared using the stock solutions. 11 mL stock solution was diluted with tap water to a total volume of 1 L, mixed in volumetric flasks. The mixture of acetate and propionate (ratio 50/50 in terms of COD) used in test N6 were prepared by addition of 5.5 mL of each stock solution.

3.3.2 Preparation of fermentate

Filtered fermented primary sludge was collected from the pilot plant, either from tank T6 or T7, as described in section 3.

Two different preparation methods were tested March 15th:

- Filtration through 10 µm filter
- Centrifugation and filtration of supernatant through folded filter (18.5 µm)

Filtration through 10 µm filter

To be able to filtrate the sludge through 10 µm filter, the sludge was diluted beforehand. The diluted sludge was also pre-filtered through 100 µm filter, thus enabling a potentially quicker filtration through 10 µm filter. However, the filtration step through 10 µm was still extremely slow, and only a very small volume passed through the filter after 1 hour. Since the sludge was already diluted, a much larger volume of the resulting fermentate would be necessary, compared with undiluted fermentate, in order to create a substrate with up to 220 mg/L SCOD concentration. The resulting volume from the filtration through of 10 µm after 1 hour was not sufficient. Thus, this preparation method of the fermentate was deemed unpractical, as it would require a too long preparation time and the result was unsatisfactory. Therefore, the other tested method using centrifugation described below was developed and used during the batch activity tests.

Centrifugation and filtration through 18.5 µm filter

A weighted amount fermented sludge was added to at least one centrifugation tube and centrifuged at 5 000 rpm[7] for 5 minutes. The resulting supernatant, called the fermentate, was filtrated into a beaker using folded filter of pore size 18.5 µm. The beaker was sealed with parafilm and stored in a fridge before usage.

The substrate was prepared by adding between 24.5-25 mL of fermentate to a volumetric flask filled with tap water, resulting in a total volume of substrate of 1 L. A sample for analysis of TCOD, SCOD, PO_4^{3-} and NH_4^+ concentrations was collected, as previously mentioned in section 3.2.5.

The concentration of COD and SCOD in the filtered fermented primary sludge varied during the testing period, between 8000-10 000 mg COD/L. Hence, the amount of fermentate was adjusted between the tests, to maintain a concentration of around 220 mg COD/L in the preprepared substrate and a resulting concentration of 110 mg COD/L in the reactors. In tests N2, N3 and P2, the fermentate was assumed to have a SCOD concentration of 8500 mg/L, which led to an addition of 25 mL fermentate. In tests N7 and N8, the concentration was assumed to be around 9000 mg COD/L, giving rise to an addition of 24.5 mL fermentate. In the test with

[7] At Lund University, the maximum centrifugation speed was 4 500 rpm.

fermentate mixed with wastewater (tests N6 and N9), 4.5 mL was added for an assumed addition of 45 mg COD, i.e. based on an assumption of 10 000 mg COD/L in the fermentate (see section 3.3.3 below for more details).

3.3.3 Preparation of wastewater

Influent WW (T1) and filtered WW (T5) were collected from the pilot plant as stated in section 3. One litre of wastewater per reactor was prepared in volumetric flasks.

During tests N4 and N9, characterisation of the wastewaters, both influent WW (T1) and filtered WW (T5), into different COD fractions was conducted simultaneously, according to the methodology described in Tebini (2020). The lab personnel at Källby conducted test on Tot-P and NH_4^+ during test N4. In the later tests, N7 and N9, small samples of filtered WW (T5) were collected for analysis of PO_4^{3-}, NH_4^+ and SCOD. A sample of the mixture of filtered WW (T5) + fermentate was also taken for analysis of soluble compounds.

A large change in temperature occurred during test N4 when the substrates were added to the reactors, as the wastewater samples had been stored in a fridge around 5-10 °C. The temperature quickly adjusted to the test temperature of 20 °C, but the rapid change in temperature in the start of the test was not ideal. Thus, in test N7 and N9, the volumetric flasks were added to the water bath before the test, so that the wastewater could adjust to the test temperature beforehand.

In test N6 and N9, 4.5 mL fermentate was added to one of the volumetric flasks in order to obtain a mixture of fermentate and filtered WW. The addition of 4.5 mL fermentate per L wastewater is based on the following assumptions and calculated according to Equation 3.1.

- Fermentate with a $COD_{filt,0.45\ \mu m}$ concentration of 10 000 mg COD/L
- 1 m^3 fermentate produced per day
- 220 m^3 wastewater per day passing the filter in the pilot plant

$$\frac{10\,000\ mgCOD_{filt,0.45\ \mu m}/L \cdot 1\ m^3 fermentate/d}{220\ m^3 \frac{wastewater}{d}} = 45\ mg\ COD_{filt,0.45\ \mu m}/L\ wastewater$$

Equation 3.1

3.3.4 Preparation of blank test

For the blank tests (N5 and P3), 1 L of tap water was prepared as substrate for each reactor.

3.4 Preparation of nutrient stock solutions

All nutrient solutions were prepared at Lund University in the beginning of the project. The compounds were dissolved in distilled water using a magnetic stirrer, and the flasks were transported to Källby WWTP and stored in a fridge during the testing period.

Addition of nutrient solutions may be needed to ensure that no shortages of important nutrients occur, as this can affect the rate of the biological process and thus lead to an underestimation of the removal rates. Nutrient additions are primarily important when testing pure substrates such as acetate. The aim with using nutrient solution is to mimic the wastewater composition, i.e. to create a synthetic wastewater, which can be comparable to real wastewater.

Several studies have performed experiments with synthetic wastewater, as it gives the ability to control the experimental conditions to a large extent compared with real wastewater. However, the exact composition of nutrient solutions varies, both between studies conducting similar experiments but also between NUR-test and test evaluating EBPR-activity.

A thorough review of the prepared nutrient solutions is presented in the section below, as this is often missing in other studies.

3.4.1 Macronutrients

The composition of macronutrients in the stock solution used during test N1, P1 and N2 is presented in Table 3.3 below. The solution was prepared according to the recipe for synthetic media used by Smolders *et al.* (1994), which is presented in the second column, with minor additions. The stock solution was concentrated 200 times to the concentration presented in the third column, thus allowing for a small addition between 1-10 mL depending on wanted final concentration in the reactor. The concentration presented in the fourth column corresponds to an addition of 2.5 mL macronutrient stock solution to the reactor.

The media presented by Smolders *et al.* (1994) is the basis for the synthetic wastewater composition suggested by van Loosdrecht *et al.* (2016) in Experimental Methods in Wastewater Treatment, with some minor details changed. Some of these differences were corrected in this study. For example, the media used by Smolders *et al.* (1994) contained 15 mg P/L (added in the form of $NaH_2PO_4 \cdot 2H_2O$), thus a corresponding addition of 65.9 mg/L KH_2PO_4 was included to the macronutrient solution, to ensure that no shortages of PO_4^{3-} occur for both anoxic and anaerobic growth (even if the effect may be small).

Furthermore, the synthetic media used by Smolders *et al.* (1994) included a concentration of 400 mg COD/L, which is around four times higher than the COD concentrations used in this study. Thus, it was decided to regulate the addition of macro- and microsolutions to the reactors in relation to the COD concentration, and thereby keeping the same ratio between COD, PO_4^3 and NH_4^+ and other nutrients. As can be seen in Table 3.3, the final concentration in the reactor is a fourth of the original suggested concentration. 26.8 mg/L NH_4Cl corresponds to a NH_4^+-N concentration of 7 mg/L, and 16.5 mg/L KH_2PO_4 corresponds to 3.75 mg/L PO_4^{3-}-P. This gives a ratio of COD:PO_4^{3-}-P of 26.7 and COD:NH_4^+-N of 14.3 with an assumed COD concentration of 100 mg/L. Mokhayeri *et al.* (2008) presented a similar solution of macronutrients, with ratios of COD:PO_4^{3-}-P and COD:NH_4^+-N of 30, and other studies show similar or higher ratios (De Vleeschauwer *et al.*, 2019; Lu *et al.*, 2006).

Table 3.3. Macronutrient solution based on Smolders et al. (1994).

Macronutrients	Original concentrations [mg/L]	Concentrated stock solution [g/L]	Final concentration in reactor [mg/L]
NH_4Cl	107	21.4	26.8
$MgSO_4 \cdot 7H_2O$	90	18	22.5
$CaCl_2 \cdot 2H_2O$	14	2.8	3.5
KCl	36	7.2	9
KH_2PO_4	65.9	13.8	16.5
Yeast extract	1	0.2	0.25

van Loosdrecht *et al.* (2016) recommended sterilization of the stock solutions before usage. Unfortunately this was overlooked, resulting in a change in turbidity and transparency of the solution after a few weeks. Before test P1, it was noted that the solution was cloudy, probably due to the yeast extract as well as the omitted sterilization. As there was not possible to remake the solution directly, the solution was used during the test P1 but was remade March 15th, without addition of yeast extract. However, the new solution was only used once, during test N2, as it was decided to perform the following tests without addition of nutrient solutions.

3.4.2 Micronutrient solution NUR

The composition of the micronutrient solution used during the NUR test is based on the trace metal solution presented by Vishniac and Santer (1957), as recommended by van Loosdrecht *et al.* (2016). The micronutrients and their corresponding concentration in the stock solution are presented in
Table 3.4 below, as well as the final concentration in the reactor after addition of 5 mL (assuming total volume of 2 L in the reactor). $CaCl_2 \cdot 2H_2O$ was used instead of $CaCl_2$ and the concentration was changed accordingly.

Table 3.4. Micronutrient solution NUR based on Vishniac and Santer (1957).

Micronutrients	**Concentration in stock solution [g/L]**	**Final concentration in reactor [mg/L]**
EDTA	50	125
$ZnSO_4 \cdot 7H_2O$	22	55
$MnCl_2 \cdot 4H_2O$	5.06	12.65
$CuSO_4 \cdot 5H_2O$	1.57	3.93
$CoCl_2 \cdot 6H_2O$	1.61	4.03
$FeSO_4 \cdot 7H_2O$	4.99	12.48
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.10	2.75
$CaCl_2 \cdot 2H_2O$ [8]	7.4	13.85

Vishniac and Santer (1957) suggested an addition of 10 mL per litre synthetic wastewater, based on achieving a successful cultivation of *Thiobacillus denitrificans* with mentioned concentration. The addition of 5 mL of micronutrient solution to the 2 L working reactor volume was selected in relation to the addition of macronutrients, in order to obtain the same ratios of macro- and micronutrients as recommended by van Loosdrecht *et al.* (2016).

The trace element solution suggested by Vishniac and Santer (1957) has been used in other studies, not limited to usage during NUR tests but also when performing EBPR tests or other unrelated experiments, and with different amounts used. For example, De Vleeschauwer *et al.* (2019) added 1 mL of the micronutrient solution per litre synthetic wastewater, in their study about EBPR performance.

Vishniac and Santer (1957) recommended adjustment of pH to 6 using KOH, but as only small additions of stock solutions were used it was not deemed necessary to change the pH. As

[8] Original recipe included $CaCl_2$ with a concentration of 5.54 g/L. The concentration of $CaCl_2 \cdot 2H_2O$ was changed accordingly to give the same molar concentration of $CaCl_2$.

previously explained, the solution was unfortunately not sterilized, although this was recommended by both Vishniac and Santer (1957) and van Loosdrecht *et al.* (2016).

3.4.3 Micronutrient solution EBPR

The composition of the micronutrient solution used during P-release test P1 is presented in

Table 3.5 below, and is based on the solution used by Smolders *et al.* (1994), as suggested by van Loosdrecht *et al.* (2016). An addition of 0.3 mL per L synthetic wastewater/media is recommended, for a media with a COD concentration of 400 mg/L. As the media used in this study contained around 200 mg COD/L, an addition of 0.15 mL was planned. However an error occurred (due to something as simple as a typo) and 1.5 mL was added instead. The final concentration achieved in the reactor after addition of 1.5 mL is presented in

Table 3.5, i.e. a 10 times larger concentration than intended (and also larger than the originally recommended addition of 0.3 mL/L).

Potassium iodide (KI) was not available at the lab at Lund University, and was thus omitted from the prepared stock solution. However, the loss of potassium ions (which is one of the more important ions concerning EBPR test, especially for P uptake as potassium and magnesium is required for uptake of orthophosphate) could be more than sufficiently counteracted by the addition of KH_2PO_4 in the macronutrient solution. The loss of iodide on the other hand was not compensated.

Furthermore, the original recipe included $FeCl_3 \cdot 6H_2O$ which was not available either, but was replaced with $FeCl_2 \cdot 4H_2O$, with the same molar concentration of Fe.

Table 3.5. Micronutrient solution EBPR based on Smolders et al. (1994)

Micronutrients	**Concentration in stock solution [g/L]**	**Final concentration in reactor**[9] **[mg/L]**
EDTA	10	7.5
$ZnSO_4 \cdot 7H_2O$	0.12	0.09
$MnCl_2 \cdot 4H_2O$	0.12	0.09
$CuSO_4 \cdot 5H_2O$	0.03	0.0225
$CoCl_2 \cdot 6H_2O$	0.15	0.1125
$FeCl_2 \cdot 4H_2O$ [10]	1.1	0.825
$Na_2MoO_4 \cdot 2H_2O$	0.06	0.045
H_3BO_3	0.15	0.1125
KI [11]	0.18	-

Several other studies have used the same nutrient solution recommended by Smolders *et al.* (1994), but with different additions to the synthetic wastewater/media. Lu *et al.* (2006) added around 0.6 mL per L to their synthetic media, whereas Zheng *et al.* (2018) added 0.5 mL per L

[9] After addition of 1.5 mL stock solution, i.e. 10 times higher concentration than intended.
[10] Original recipe contained on $FeCl_3 \cdot 6H_2O$ with a concentration of 1.5 g/L. The concentration of was $FeCl_2 \cdot 4H_2O$ was changed accordingly to give the same molar concentration of Fe.
[11] Not available at the lab at Lund University

synthetic wastewater. Coats *et al.* (2017) based their addition of micronutrients on Lu *et al.* (2006), but exact volume is unknown.

As only a small volume of the nutrient stock solution was added, no adjustment in pH of the solution was deemed necessary. The solution was not sterilized. Neither Lu *et al.* (2006) nor Zheng *et al.* (2018) mention sterilization, and Coats *et al.* (2017) actually state that the substrate was unsterilized. As previously explained, the solution was unfortunately not sterilized, although this was recommended by both Vishniac and Santer (1957) and van Loosdrecht *et al.* (2016).

3.4.4 Solubility and turbidity of micronutrient solutions

Both micronutrient solutions contained large concentrations of ethylenediamine tetra-acetic acid (EDTA, $C_{10}H_{16}N_2O_8$), 50 g/L and 10 g/L for micronutrient solution NUR and EBPR respectively. EDTA is added for its binding properties, and can form soluble complexes with calcium and heavy metal ions, thus enabling the dissolution of these compounds (National Center for Biotechnology Information, 2021). However, there were some issues with reaching a fully dissolved state of the nutrient solutions, with settling of particles at the bottom of the glass flask and an evident turbidity when shaken. This is believed to be due to the high amount of EDTA. Similar problems have occurred in other projects (Homlin W., 2020), where even less EDTA was used (5 and 15 g/L in two different solutions). Nonetheless, the solution was used in its imperfect state, but was stirred thoroughly with a magnetic stirrer to ensure the addition of a homogenous solution.

3.4.5 Omitting nutrient solution in test N3-N10 and P2-P3

Due to a large increase in SCOD after addition of nutrient solution in test N1, N2 and P1, which disturbed the SCOD result, it was decided to omit the nutrient solution in the following tests. No apparent effect on achieved process rates were observed.

3.5 Preparation of activated sludge

The collection of activated sludge is described in detail in section 3.2.5. After collection, the activated sludge was washed in order to remove soluble compounds such as NH_4^+ and PO_4^{3+} as well as residual SCOD. Eventual toxic compounds can also be removed. Furthermore, van Loosdrecht *et al.* (2016) mentions that the washing procedure also can decrease the levels of intercellular stored COD among PAOs, and thus it is not recommended to use the sludge for EBPR tests that start with an anoxic or aerobic phase. However, this effect might be beneficial in the NUR test, by decreasing the ability of PAOs to denitrify with internally stored COD.

The washing procedure can be conducted with tap water, effluent wastewater (if it is of good quality) or with a synthetic washing medium (van Loosdrecht *et al.*, 2016). For example, the macro- and micronutrients solutions described under section 3.4 can be used for this purpose.

Two different methods of washing the sludge were tested: centrifugation and settling. The aim with both these methods was to separate the biomass from the liquid phase, removing the supernatant and then resuspending the sludge in the washing media. Tap water was chosen as the washing media. The methodology is based on the procedure described by van Loosdrecht *et al.* (2016).

Centrifugation

Centrifugation of a small portion of the sludge was tested, in order to check which speed and centrifugation time would be required to achieve a sufficient separation of the biomass and the

supernatant. van Loosdrecht *et al.* (2016) recommend a mild centrifugation of 2000-3000 rpm, as higher speeds can harm the flocs. When lower centrifugations speeds were tested, for between 1-5 minutes, no good results were achieved. A sufficient separation could be achieved with higher speeds closer to 5 000 rpm for at least 5 minutes. However, the centrifuge available could only take very small volumes of sludge, around 200 mL maximum, and 1 L total sludge is required per reactor. Thus, the method based on centrifugation was deemed unpractical.

Settling

Different procedures of washing the sludge through settling was tested in the beginning of the experimental period (March 9th and March 12th). The basic principal is to let the sludge settle, remove the supernatant slowly and then resuspend the sludge to the original volume in the washing media, and then repeat one or two times. In order to speed up the settling process, the sludge was diluted 3 times. The SCOD concentration after 1, 2 and 3 consecutive washing steps was analysed, and washing the sludge two times was deemed sufficient. The choice was a compromise between achieved concentration of soluble compounds and time requirements.

One litre sludge was prepared for each reactor, i.e. a total volume of 2 L sludge was washed. The methodology for washing 1 L sludge is described below, and illustrated in Figure 3.5.

The sludge was poured into a measuring glass á 1 L, then further separated into a total of 3 measuring glasses á 1 L, to a volume of around 333 mL in each. The measuring glasses were filled with tap water to final volume of 1 L (Figure 3.5:A). The sludge in each glass was gently mixed with a magnetic stirring rod for a few seconds, before settling began. The sludge settled for 15 minutes, which was sufficient for the biomass to settle below the original volume of 333 mL sludge (Figure 3.5:B). After settling, the water phase was slowly separated into a large beaker. Care was taken to avoid losing biomass in the process. However, sometimes small parts of the biomass floated at the top of the measuring glass, and it was difficult to avoid losing this part of the biomass

.

The washing cycle was repeated one more time, by filling the measuring glass with tap water up to a total volume of 1 L, gentle mixing, and then settling for 15 minutes and removing the water phase. The remaining sludge in the three measuring glasses was combined in one measuring glass, and filled up to the original volume of 1 L using tap water, which replaced the volume of the removed supernatant. The tap water was poured using the two other used measuring glasses, thus rinsing the measuring glasses and saving biomass simultaneously. The MLVSS concentration was kept as close to the original concentration as possible.

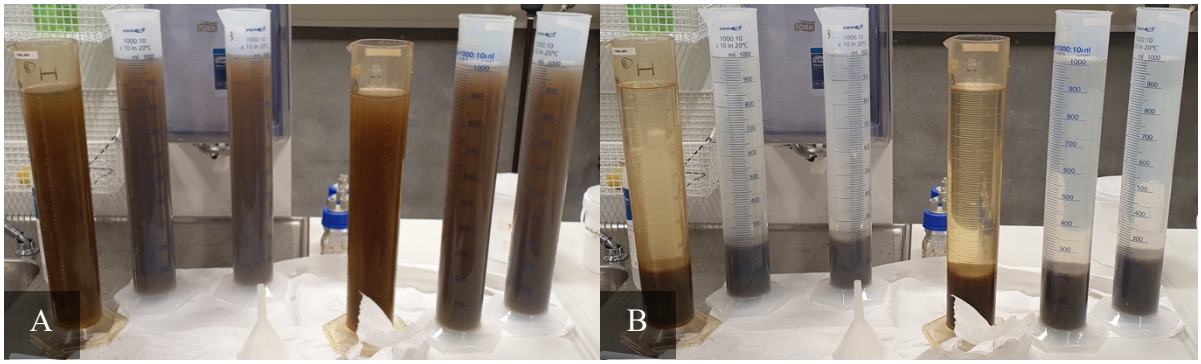

Figure 3.5. Washing sludge procedure. Picture A: activated sludge diluted with tap water. Picture B: sludge with supernatant after 15 minutes settling time. (Photo: Sanna Sahlin)

3.6 Important parameters: pH, dissolved oxygen and temperature

3.6.1 pH

The pH in the reactors was regulated manually at pH set point 7.0 with addition of HCl and NaOH (1/0.5 M), as described in section 3.2.3. van Loosdrecht *et al.* (2016) recommends keeping the pH constant with no larger fluctuations than ± 0.1. However, as the pH was controlled manually, it was difficult to achieve such desirable results. The average pH during regulation were between 7-7.09 in the different reactors, with an average standard deviation of 0.12. However, the pH fluctuations were a bit higher than illustrated by the standard deviation; pH values +0.1-0.4 above and –0.1-0.2 below the desired set point of pH 7.0 were reached in the majority of the tests. More exact data on the initial pH in the reactor, the average pH during regulation, and the minimum and maximum pH values reached are presented in Table A.18 in Appendix C.

During the first two tests (N1 and P1), one pH probe was used for both reactors, and the pH was only regulated in one of the reactors (reactor B), and measured periodically in the other (reactor A). Two new pH probes (Digital pH sensors Orbisint CPS11D from Endress+Hauser) were used in the remaining tests. The probes were connected to a Liquiline transmitter from Endress+Hauser, used for following the measurement of both pH and temperature. Before being used, the probes were calibrated according to two step calibration, calibrating at pH 4.00 and 7.00 using buffer solutions at respective pH. The result of the calibration for probe 1 and 2 respectively is presented in Table A.6 in Appendix A.

The pH probes were checked at pH 8.00 before test N2, N3 and P2, and at pH 7.00 before tests N4-N10 (thus also test P3). The pH probes were left in the pH buffer solution for two minutes for stabilization before measurement. The result of the pH check is presented in Table A.7 in Appendix A. The pH probe used during test N1 and P1 was checked by the lab personnel at Källby WWTP.

3.6.2 DO

Two DO meters were used for following the DO concentration before (during aeration period and N_2 sparging) and during the tests, one in each reactor. Hach HQ10 portable LDO DO meter and Hach HQ40d portable multi meter were used in reactor A and B respectively, connected with luminescent/optical DO sensors. The DO meters were checked periodically by measuring the DO concentration in the air. Values of 8-10 mg/L were deemed acceptable.

The aim of measuring the DO concentration during the aeration period was to ensure a sufficient supply of DO. During N_2 sparging in the beginning and during the test, the DO measurements was used to ensure anoxic and anaerobic conditions, i.e. at concentrations of 0.0 mg/L or lower, ideally the DO concentration should be under range. Hach HQ10 measured the DO concentration with one decimal digit, whereas Hach HQ40d measured with two decimal digits.

Uncertainties

During test P2 (March 22th) it was noted that the DO sensors were not submerged in the sludge in the beginning of the tests (i.e. during the aeration and N_2 sparging period). The sensors were close to the surface but did not reach fully into the sludge. Thus, the measurements of the DO concentration in the before the tests start are uncertain. This is of major concern the first measurements are the most important as the aim is to reach DO of 0 before the tests start. Therefore, it was tested to add 0.5 L pure tap water earlier during test P2, in order to submerge the DO

probes and get more accurate measurements. The substrate was changed accordingly. After start of N_2 sparging, the DO concentration changed from around 8 to 0 under 20 minutes.

However, it was not possible to add tap water earlier in all tests, especially with tests conducted with real wastewater as this would destroy the experiment. The amount of sludge could not be increased either as this would change the ratio between carbon and VSS. Therefore, it was accepted that the DO measurements were uncertain in the beginning in the following tests.

This can have contributed to the slightly higher DO values than desired in the beginning of some of the tests. In tests N3, N4, N5 and N8, the DO concentration directly after addition of substrate was measured at around to 3-4 mg/L, but quickly declined to below 0.0 mg/L in less than 10 minutes in almost all cases. This could have had an effect on the readily available carbon in the reactors in the beginning of the test. Another possibility is that oxygen intrusion occurred when pouring the substrate into reactors, and therefore it is advisable to pour the substrate as slowly as possible. Some residual DO could also be present in the substrates, which should be checked in future tests.

3.6.3 Temperature

The temperature in the reactors was controlled at 20 °C in a water bath as described in section 3.2.3. van Loosdrecht *et al.* (2016) recommend to avoid fluctuations of the temperature ±1-2 °C above desired value. The Grant thermostat used to regulate the water bath temperature was set to 20 °C during the majority of the tests, which was sufficient in order to reach average temperatures of 19.7-20.1 °C in the reactors. However, in the last tests conducted at Lund University (N8-N10), some discrepancies between the temperature in the water bath and the reactors were noted, and the set point for the thermostat was varied between 20-21.5 °C in order to regulate the temperature to the desired values. The available cooling machine was not needed for sufficient temperature regulation, as explained previously. The temperatures did not fluctuate more than ±0.5 °C in any test. More detailed data of the temperature variation is presented in Table A.19 in Appendix C.

As mentioned in section 3.3.3, a change in temperature after addition of substrate was noted during test N4, due to the low temperatures of the substrates (T1 and T5). Thus, during following tests with wastewater (tests N7 and N9), the substrates including T5 were adjusted to temperature before the start of the tests by placing the volumetric flasks with the substrates in the water bath.

3.7 Analytical methods

3.7.1 Hach LCK cuvette tests

During the first part of the experimental period, when the tests were conducted at the lab at Källby WWTP (N1-N7 and P1-P3), all compounds of interest (COD, SCOD, NO_3^-, NO_2^-, PO_4^{3+} and NH_4^+) were analysed using Hach LCK cuvette tests. In the end of the testing period at LTH, mainly COD/SCOD was analysed using Hach cuvettes. Only a few other samples, where the sample volume was too small for ion chromatography analysis, were analysed with cuvette tests for other compounds.

The concentration of the compounds was analysed using spectrophotometry. At Källby WWTP, the tests were analysed in a DR 3900 Laboratory Spectrophotometer from Hach, whereas at Lund University, the instrument DR 2800 Portable Spectrophotometer from Hach was used.

Cuvettes with different concentration ranges were used depending on expected concentration in the sample. The exact cuvette types used are described in more detail in Appendix A. Each tests were conducted according to the procedure specified for each cuvette. NH_4^+ cuvettes were stored in fridge (+2-8 °C) in-between the tests as recommended, whereas all other cuvettes were stored in room temperature (+15-25 °C).

3.7.2 Ion chromatography

When the tests were conducted at Lund University (tests N8-N10) all soluble compounds except COD/SCOD were analysed with ion chromatography. The ion chromatography system ECO IC combined with 863 Compact Autosampler from Metrohm was used. A total volume of 8 mL was needed for each sample. Detailed description of sampling procedure is presented in Appendix A.

3.7.3 MLSS/MLVSS

Duplicate measurements of MLSS/MLVSS concentration were conducted for each sample, to increase the reliability of the result.

Whatman Glass Microfiber Filters (GF/A; GE Healthcare) with pore size 1.6 µm were used for filtration of the sludge samples. In order to remove residuals on the filters, a washing step was conducted. The filter papers were washed with distilled water, excess water was removed with a vacuum pump and the filters were put in an oven at 105-106 °C degrees to be dried completely. After 30 minutes in the oven, the filters were marked with numbers, and then dried for another 30 minutes. All washed and dried filters were weighted and stored before usage.

MLSS and MLVSS were determined according to following procedure. Around 5 mL sludge was measured with a pipette and poured into a small plastic disc and weighted. The small sludge sample was filtrated through a 1.6 µm filter (washed and labelled) using a glass vacuum filter set and a vacuum pump. The plastic disc was rinsed with distilled water, to ensure that the whole weighted sample was poured onto the filter. The process was repeated for each sample, including duplicates. The same pipette tips was used, rinsed with distilled water between different samples.

The resulting filters with solid sludge were dried in an oven at 105-106 °C for 1 hour, after which each filter was weighted for MLSS result. Then the filters were incinerated at 550 °C for 1 hour, in order to remove all volatile solids. The filters were placed in aluminium trays, with additional trays as lids for protection. When the oven started at low degrees, around 22-30 minutes were needed as a warmup period. The filters were put in a desiccator in order to cool down before being weighted and MLVSS concentration could be determined. The dried filters could be stored in the desiccator for a longer period if necessary, before being weighted or before incineration.

3.7.4 VFA analysis

The plan was to analyse the VFA samples using gas chromatography. Unfortunately, this was not possible for all tests due to problems with the gas chromatography at Lund University and time constraints in the last phase of the project. A few samples in test N10:B with fermentate were analysed using the gas chromatography (Hewlett Packard HP 6890 Series GC System with DB-WAX UI column) at the laboratory of Bulltofta Water Treatment Plant operated by VA SYD. During the analysis, the column was heated from 80 to 230°C. The results were evaluated using GC Chem station Rev B.04.03 (16) software from Agilent, and compared to standard solution concentration curves for the VFAs.

3.8 Calculations and data analysis

3.8.1 MLSS/MLVSS calculations

MLSS is determined by measuring the dry weight of the suspended solids (SS) in a specific volume of an activated sludge sample. The density of the activated sludge samples was assumed to equal that of pure water, i.e. $\rho_{AS} = 1000 \frac{g}{L}$. Thus, the volume of the activated sludge sample was calculated according to the simple formula $\frac{w_{AS}}{\rho_{AS}}$, where w_{AS} denotes the weight of the sludge sample.

The MLSS concentration was determined according to Equation 3.2 below, where $w_{(SS+filter)}$ denotes the total weight of suspended solids and the filter combined and where w_{filter} denotes the weight of the filter. The difference is equal to the resulting weight of the dried suspended solids, i.e. w_{SS}, weight of SS.

$$MLSS \left[\frac{mg}{L}\right] = \frac{w_{(SS+filter)} - w_{filter}}{volume} \cdot 1000 \qquad \textit{Equation 3.2}$$

MLVSS is the mixed liquor volatile suspended solids, and is determined as the solids lost after incineration. Thus, the MLVSS concentration was determined according to the following Equation 3.3 where $w_{(\text{ignition residuals + filter after incineration})}$ is the weight after incineration. The original filter weight was corrected by removing 0.0008 g, which according to the laboratory at Källby is lost during incineration.

$$MLVSS \left[\frac{mg}{L}\right] = \frac{w_{(SS+filter)} - w_{(ignition\ residuals+filter\ after\ ignition)} - 0.0008\ g}{volume} \cdot 1000 \qquad \textit{Equation 3.3}$$

The average MLSS and MLVSS for each sludge samples was calculated, as well as the percentage of VSS to SS.

3.8.2 Oxidized nitrogen equivalent

During denitrification, NO_2^- is formed, and an accumulation of NO_2^- during a NUR test is not always negligible. Thus, a weighted sum of NO_3^- and NO_2^-, called oxidized nitrogen equivalent (NO_3^--N_{eqv}), was determined, to better represent the overall reduction of NO_3^- and NO_2^- to nitrogen gas. NO_3^--N_{eqv} is determined according to Equation 3.4, as suggested by Kujawa and Klapwijk (1999). The weighted sum is based on the relative electron accepting capacity of the two compounds, where the same amount of electrons (around 0.214 mol e^-) is needed to reduced 1 g of NO_2^--N_{eqv} to 1 g N_2 as is needed for the reduction of 0.6 g NO_3^--N_{eqv} to 0.6 N_2 (Kujawa & Klapwijk, 1999; van Loosdrecht *et al.*, 2016). Furthermore, it is assumed that no accumulation of other intermediates NO and N_2O occur (Naidoo *et al.*, 1998).

$$NO_3^- - N_{eqv} = NO_3^- - N + 0.6 \cdot\ NO_2^- - N \qquad \textit{Equation 3.4}$$

The NO_2^- concentration in all samples in tests N1-N7 was not analysed (Table A.2). Therefore, in order to determine the change in NO_3^--N_{eqv} for all tests, the NO_2^- concentration at all sample points were estimated by simple regression analysis, assuming a linear relationship between the sample points. During test N8-N10, the NO_2^- concentration in all samples was measured using ion chromatography and no estimation was needed.

3.8.3 Regression analysis and calculations of rates

The volumetric denitrification rates (r_D), P-release rates (r_P) and substrate utilisation rates (t_C) were determined through linear regression analysis. The specific denitrification rates (k_D), P-release rates (denoted as k_P) and specific substrate utilisation rates (k_C) were determined according to Equation 3.5, where $MLVSS_{initial}$ denotes the MLVSS concentration in the first sample of the test.

$$k_i = \frac{r_i}{MLVSS_{initial}} \qquad \textit{Equation 3.5}$$

3.8.4 Nitrate removal efficiency

The nitrate removal efficiency is defined as the accumulated percentage of removed NO_3^--N eqv at each time step *x*, and calculated according to Equation 3.6 below.

$$Nitrate\ removal\ efficiency_x = \frac{NO_3^- - N\ eqv_{initial} - NO_3^- - N\ eqv_x}{NO_3^- - N\ eqv_{initial}} \qquad \textit{Equation 3.6}$$

3.8.5 Carbon utilisation

The total amount of externally added SCOD utilised during the whole test was estimated according to Equation 3.7 below. This is based on an assumption that the SCOD present in the sludge (biomass) did not contribute as a carbon source for denitrification, and that all measured SCOD utilised came from the external carbon source added.

$$\Delta SCOD_{tot} = SCOD_{first\ sample} - SCOD_{final\ sample} \qquad \textit{Equation 3.7}$$

SCOD utilised during the different phases of nitrate reduction was calculated in the same manner, as illustrated by Equation 3.8 below. Subscript *i* denotes the phase.

$$\Delta SCOD_i = SCOD_{start,phase\ i} - SCOD_{end,phase\ i} \qquad \textit{Equation 3.8}$$

4 Results and discussion

The results are presented and discussed in four major sections.

In sections 4.1 and 4.2, the results obtained in the conducted NUR tests are discussed. In section 4.1, the different denitrification rates and carbon utilisation patterns observed in the NUR tests with fermentates are discussed and compared with the corresponding parameters obtained in NUR tests with synthetic substrates. Additionally, the anoxic P release is analysed. In section 4.2, the results obtained in the NUR tests with wastewater as substrates are discussed and analysed.

In section 4.3, the anaerobic P release tests are presented and discussed. Finally, some important design parameters and additional uncertainties are presented and analysed in section 4.4.

4.1 Comparison of NUR tests with synthetic substrates and fermentate

4.1.1 Overall nitrate removal efficiency

Over the whole course of the test (3.5-4 hr), all carbon sources showed a good overall removal efficiency of NO_3^-, as illustrated by Figure 4.1 and Figure 4.2 presented below.

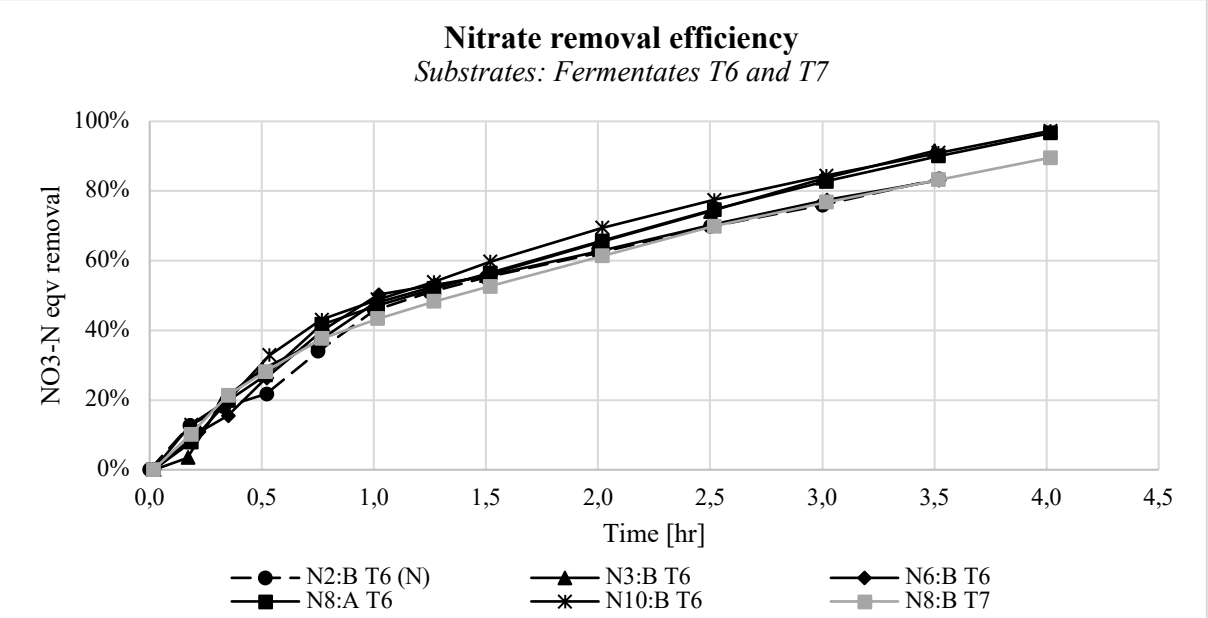

Figure 4.1. Nitrate removal efficiency for fermentates (T6 and T7). Tests conducted with nutrient solution are illustrated with a dotted line and denoted with (N) in the description.

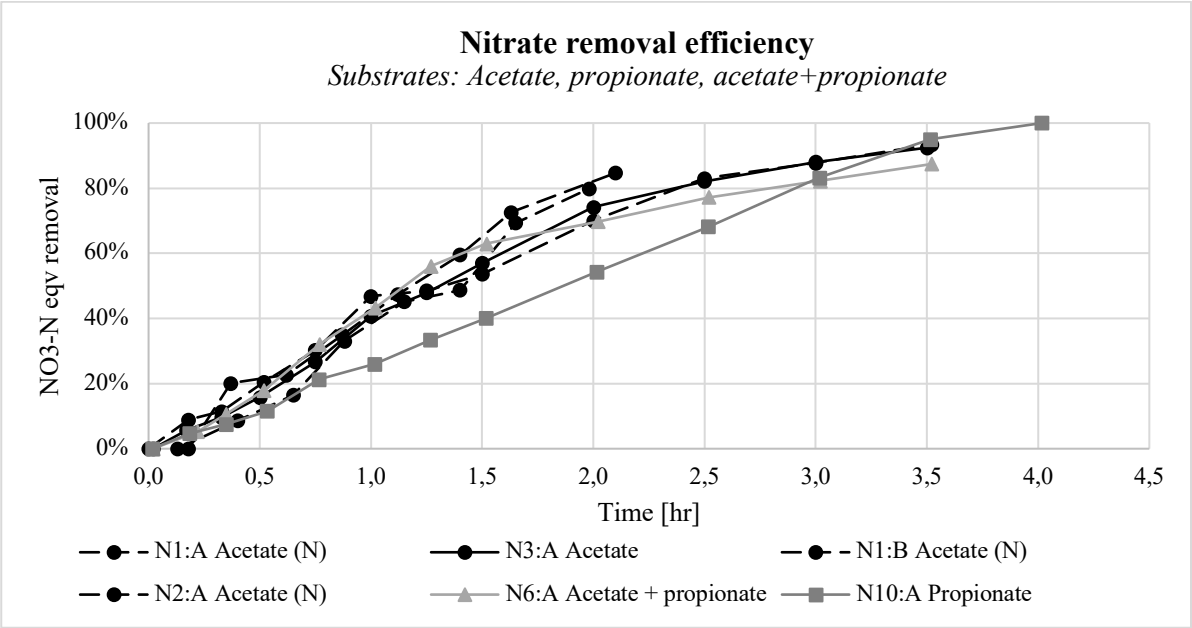

Figure 4.2. Nitrate removal efficiency for acetate (with and without addition of nutrient solution), propionate and mixture of acetate + propionate (50/50). Tests conducted with nutrient solution are illustrated with a dotted line and denoted with (N) in description.

Tests conducted with fermentate showed a removal efficiency between 83-91% after 3.5 hours (all tests), and 90-97% removal after 4 hours (test N8 and N10). After 3.5 hours, acetate showed a removal of 92% in both tests, whereas propionate showed a removal of 95%. The combination of acetate and propionate gave rise to a removal of 87% after 3.5 hours, similar to what was obtained with fermentate. Furthermore, no significant accumulation of NO_2^- occurred at the end of the tests for any carbon source (see Figure A.1 and Figure A.2).

The difference in nitrate removal between synthetic substrates and fermentate is illustrated in Figure 4.3, showing the removal efficiency for fermentate and acetate in test N3, and the removal efficiency for propionate (N10:A) and the mixture (N6:A).

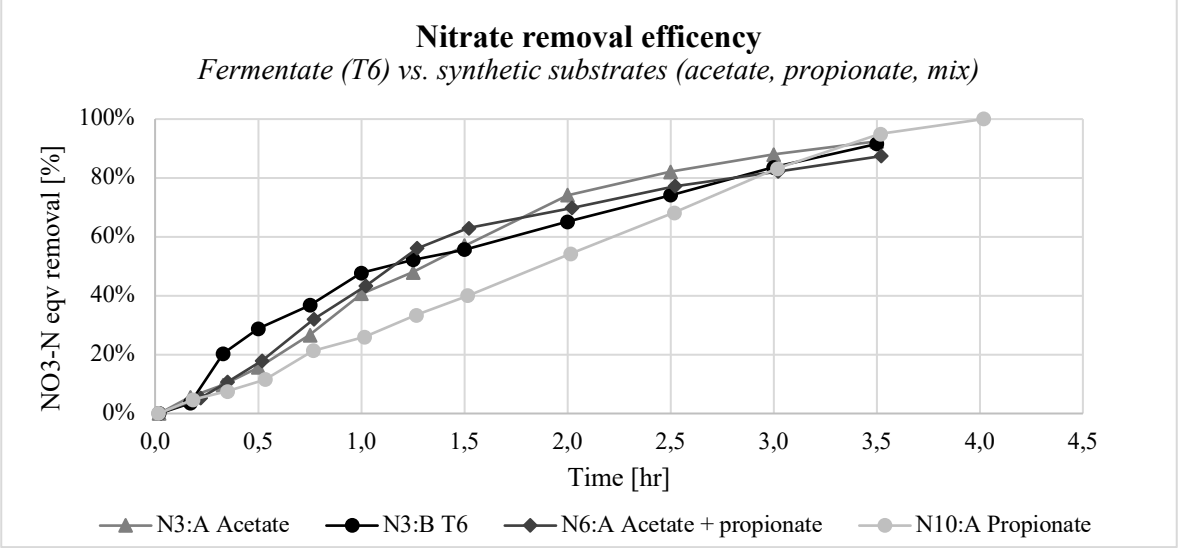

Figure 4.3. Nitrate removal efficiency for acetate and fermentate T6 (test N3), acetate + propionate (N6:A) and propionate (N10:B).

A higher removal efficiency by fermentate can be seen in the beginning, up to around 48% removal after 1 hour, compared with 26% and 41% removal for propionate and acetate respectively, and 43% for the mixture. However, after around 1 hour, the rate of nitrate removal changes for fermentate, whereas the removal rate for the synthetic substrates is maintained for a longer period, before decreasing. As an example, this leads to a higher removal of 74% for acetate compared with 65% for the fermentate after 2 hours. But the slightly higher second removal rate maintained by the fermentate compared with the synthetic substrates results in a very similar overall removal efficiency. Regardless of the most preferable removal rates, the similar removal efficiencies over the whole course of the tests indicate that the fermentate is comparable to synthetic VFAs as a carbon source for denitrification.

However, it should be noted that there was a small discrepancy between the SCOD in the substrates with fermentate (varying between 215-249 mg COD/L, see Table A.12), and the synthetic substrates, which had a constant SCOD concentrations of around 220 mg/L when measured. The difference was around 12% at the most. This discrepancy was due to the fact that the fermentate substrates were prepared with assumed SCOD concentrations in the fermentation liquid of 8500 mg/L and 9000 mg/L during different tests (see section 3.3 for more details). Ideally, the substrates should have been prepared according to the actual SCOD concentration in the fermentation liquid, or at least based on the latest measurement before the test, but this did not happen due to time constraints. However, in the reactors, the initial SCOD concentrations were very similar between the different substrates, and thus the C/N-ratios in the beginning of the tests were also in the same range (average value 5.4±0.3) (Table A.9). Thus, the small difference in SCOD concentration in the substrates might not have had that large effect.

On the other hand, it is likely that there was a difference in readily available COD in the reactors, since the substrates where matched in SCOD content and not VFA content. The SCOD measured in the synthetic substrates consist of VFAs only, whereas some part of the SCOD in fermentate might be inert (non-degradable) or more slowly biodegradable, i.e. not all SCOD in the fermentate substrates might be directly available for denitrification. This could explain the difference in over how long period a high removal rate was maintained between the substrates (see Figure 4.3). However, data on the fermentates T6 and T7 from November and December 2020 show that 91% of the SCOD in T6 consist of VFA on average, whereas 78% of the SCOD in T7 were VFAs (Table A.10). Thus, the difference in VFA content in the reactors might not be that large, but still have an effect. Unfortunately, since VFA analysis could not be conducted for all tests (only for some samples in test N10:B) due to time constraints and problems with the gas chromatography, the exact composition of VFA in the different substrates and reactors are unknown.

4.1.2 Highest specific denitrification rates (kD1)

The specific denitrification rates (k_{D1} and k_{D3}) obtained from NUR curves for acetate, propionate, mixture of acetate + propionate (50/50) and fermentates (T6 and T7) are presented in Table 4.1 below. When only two phases of nitrate reduction were observed, the rates were divided into k_{D1} and k_{D3} as per Figure 2.1 in section 2.3.2. Whether or not the slowest rate, k_{D3}, reflect the endogenous denitrification rate will be discussed.

Table 4.1. Specific denitrification rates for all different test with fermentates and synthetic substrates.

Specific denitrification rates mg NO_3^--N/g VSS·h	**Carbon source**	k_{D1}	k_{D3}
N1:A	Acetate	5.4	-
N1:B	Acetate	5.2	-
N2:A	Acetate	5.9	1.8
N2:B	Fermentate (T6)	7.0	2.4
N3:A	Acetate	5.9	1.9
N3:B	Fermentate (T6)	7.1	2.5
N6:A	Acetate + propionate	7.8	2.13
N6:B	Fermentate (T6)	8.7	2.3
N8:A	Fermentate (T6)	8.9	2.7
N8:B	Fermentate (T7)	8.0	2.5
N10:A	Propionate	4.2	1.5*
N10:B	Fermentate (T6)	8.7	2.6
N5:A	Blank	-	1.0

* Regression between two points only

A selection of NUR curves from the tests with fermentate and synthetic substrates are presented in Figure 4.4 below. As can be seen, only two phases of nitrate reduction could be observed, even for the fermentates T6 and T7. NUR graphs for all tests are presented in Figure A.4 in Appendix B. In all cases, the first volumetric nitrate reduction rate (denitrification rate r_{D1}) could be determined with linear regression, confirmed by the high values of the coefficient of determination (r^2), with an average value of 0.987 (varying between 0.965-0.999). A similar good fit was found for r_{D3}, with values of r^2 varying between 0.984-1, with an average of 0.995 (note: the value of 1 was obtained for test N10:A with propionate, were only two values were used for determining r_{D3}).

The similar high specific denitrification rates (k_{D1}) obtained with and without nutrient solution for acetate and fermentate (see test N1-N3 in Figure A.4), indicate that omitting the nutrient solution did not have any significant effect. Although, it should be noted that no comparable tests exist for the other synthetic substrates used (acetate + propionate and propionate), as these tests were only conducted without nutrient solution. See section 4.4.2 for a more detailed discussion about the effect of omitting nutrient solutions.

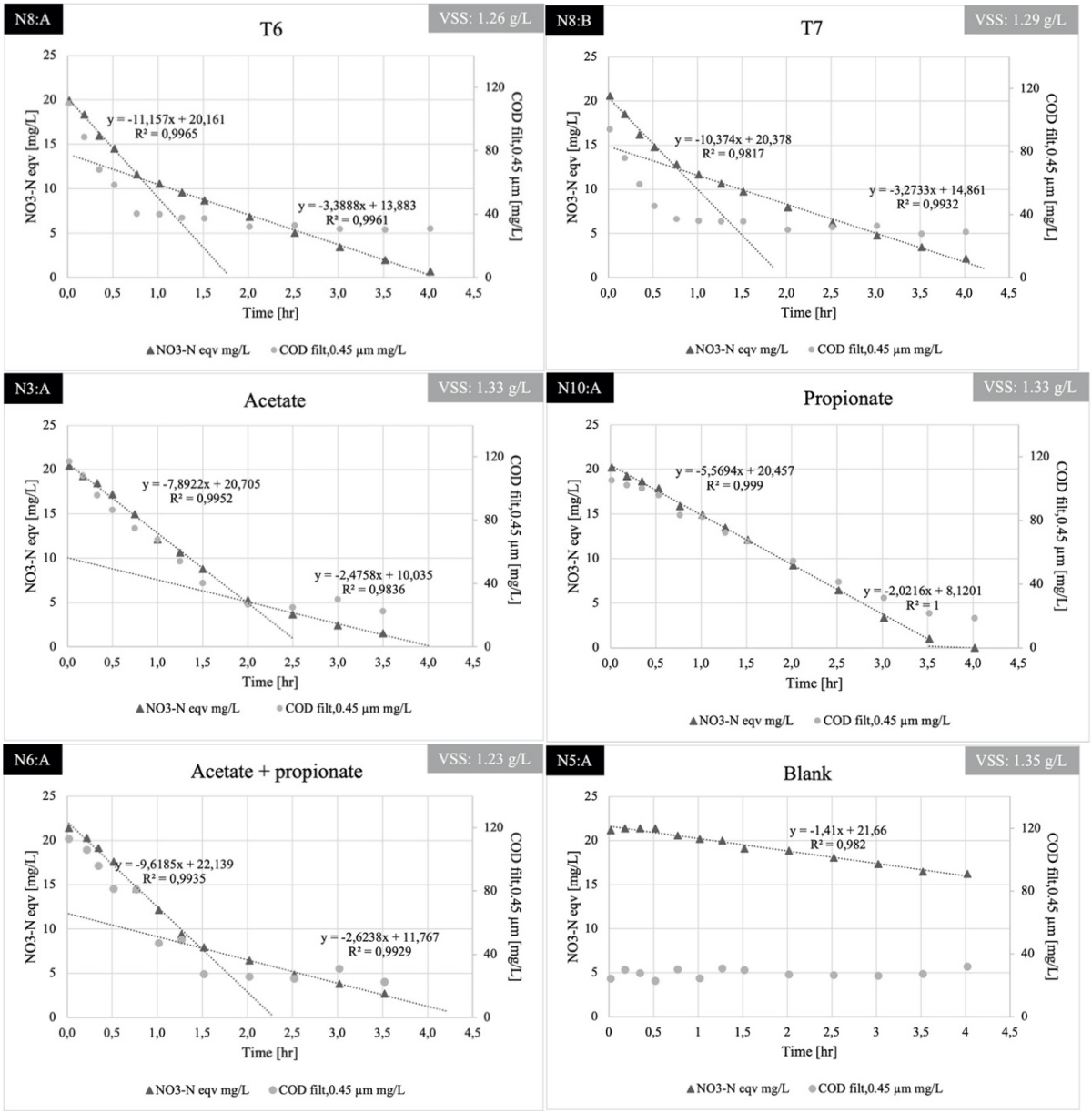

Figure 4.4. Selected NUR graphs for all different carbon sources (fermentates T6 and T7, acetate, propionate, mixture of acetate + propionate) as well as blank test. The test number is noted in the upper left corner, and the initial VSS concentration in each reactor is noted in the upper right corner.

As can be seen in Table 4.1, the two different fermentate sources give rise to the highest specific denitrification rates in the first phase of the nitrate reduction (k_{D1}) of all tested carbon sources, with the highest rate obtained with fermentate T6 at 8.9 mg NO_3^--N/g VSS·h (N8:A), and 8.0

mg NO_3-N/g VSS·h for fermentate T7 (N8:B). However, during the testing period, the k_{D1} for T6 varied between the tests, between 7.0-8.9 mg NO_3^--N/g VSS·h, with an average of 8.1±0.95 (CoV 12%). But in every test, the reactor with addition of fermentate T6 still showed the highest k_{D1} value. This is in line with the observed high removal efficiency seen with fermentate in the beginning of the tests, compared to the synthetic substrates (Figure 4.3).

In comparison, the mixture of acetate and propionate (50/50) gives a k_{D1} of 7.82 mg NO_3^--N/g VSS·h, which is similar to the average value obtained for the fermentation liquids. This is reasonable as older data from November and December 2020 of the VFA content in the fermentate show that around 60% of the VFA-C consists of acetate and propionate (Table A.11). The slightly higher rate obtained with fermentate could indicate the presence of other VFAs, such as butyrate and valerate.

On the other hand, the highest denitrification rates on acetate and propionate separately were much lower, with an average of 5.6±0.35 mg NO_3^--N/g VSS·h for acetate and a value of 4.2 for propionate. This is in line with other studies where fermentation liquids from waste products and mixtures of VFAs have shown higher rates compared to sole carbon sources such as acetate and propionate (Barlindhaug & Ødegaard, 1996; Bolzonella *et al.*, 2001; Kampas *et al.*, 2009; Liu *et al.*, 2016) or similar rates (Zhang *et al.*, 2016). A mixture of carbon compounds is believed to stimulate a larger variety of bacteria, a synergistic effect, and thereby has the potential to increase the denitrifying activity of the biomass (Lee *et al.*, 2014; Liu *et al.*, 2020; Zhang *et al.*, 2016). Combining the k_{D1} rates obtained with acetate and propionate results in rates in the range of 9-10 mg NO_3^--N/g VSS·h, which is close to the rates obtained with the mixed carbon sources. This could indicate that different microbial species utilise acetate and propionate respectively, leading to more or less additive rates. Additionally, the higher rate obtained with acetate compared to propionate indicate that the denitrifies utilise acetate faster than propionate, which is reasonable since acetate has a lower molar mass and a more simple metabolic pathway for utilisation (Elefsiniotis & Wareham, 2007).

A small difference can be observed between fermentates T6 and T7 (test N6), where T6 gave rise to a 10% larger k_{D1} rate. This could be explained by the larger ratio of VFA to SCOD in T6 (around 90%) compared to T7 (around 80%), as mentioned in section 4.1.1. This indicate that T6 contain more readily available carbon, which in turn reflect the different retention times in the two fermentation reactors (five and three days in reactor T6 and T7 respectively). But as only one test with T7 was conducted, and since it was observed that the k_{D1} values for T6 varied rather much between the test, more test comparing T6 and T7 should be conducted before drawing any conclusions. Furthermore, a slightly lower start C/N-ratio was observed in reactor N8:B with T7 as substrate (4.57 compared to C/N ratio of 5.52 in N8:A with T6, see Table A.9), probably due to a small spillage when pouring substrate T7 into the reactor. This could have affected the maximum dentification rate obtained with T7, making the comparison uncertain. For example, in their studies with fermentation liquids from food waste, Zhang *et al.* (2016) observed that C/N ratios between 2-4 resulted in slightly lower denitrification rates in the beginning compared with C/N ratios between 6-10. In the future, it is advisable to investigate optimal C/N ratio for the fermentates for more accurate comparisons.

The variation in k_{D1} observed for fermentate T6 can be an indication of a slight variation in composition of VFAs and other biodegradable material in the fermentate between the tests. The rates in test N2 and N3 are similar (around 7), whereas the rates in N6, N8 and N10 are as high as 8.7-8.9 mg NO_3^--N/g VSS·h. One factor affecting the composition of the fermentate could be the temperature in the reactor. In the first tests (N2 and N3) conducted in the beginning of

March, the temperature was around 17 °C, whereas the temperature was 19-20 °C in the tests conducted in April (N6, N8 and N10) (Table A.14). This could suggest seasonal variations in the fermentate composition, resulting in different denitrification rates. More tests should be conducted over the whole year in order to gain a more representative value of k_{D1} for fermentate T6, as well as investigating any eventual seasonal variations. However, it is also possible that the variation reflect a difference in biomass activity overall, and that similar variations in rates would have been observed for the other substrates as well if more tests had been conducted.

Important to note is the fact that the denitrification rates will depend on the acclimation of the biomass to the different carbon sources. The tests in this study was conducted with activated sludge from Källby WWTP which was not acclimated to the different substrates before the tests. This means that the biomass could be more acclimated to some of the substrates compared to others, depending on the composition of the influent WW to the WWTP. Higher adaption to different substrates is believed to result in higher rates and removal efficiencies (Barlindhaug & Ødegaard, 1996; Elefsiniotis *et al.*, 2004). Thus, in order to compare the highest denitrification rate possible for each substrate, a period of acclimatising the biomass to the different substrates would have been needed (Sage *et al.*, 2006).

4.1.3 Carbon utilisation in the first phase of nitrate reduction

In almost all cases, the change in SCOD concentration match the highest reduction in NO_3^- rather well. This can be clearly seen in Figure 4.4, where a change in substrate utilisation rate is observed at the same time point as the nitrate reduction rate changes. Thus, it is reasonable to assume that the highest rate reflects the utilisation of readily available carbon to large extent.

Four different parameters illustrating the carbon utilisation are presented in Table 4.2: the measured change in SCOD concentration during the first phase ($\Delta SCOD_1$), the specific substrate utilisation rate during the first phase (k_{C1}), the measured SCOD utilised per removed NO_3^--N equivalents during the first phase (expressed as $\Delta SCOD_1/NO_3^-$-N eqv_1, where subscript *1* denotes the first phase), and lastly the percentage of $\Delta SCOD_1$ to the total SCOD utilised during the test ($\Delta SCOD_1/\Delta SCOD_{tot}$). Since the SCOD measurements in tests N1 and N2 were disturbed by the addition of nutrient solution, these test are omitted.

It should be noted that the change in SCOD concentration ($\Delta SCOD$) is used as a simplified estimation of the amount of externally added SCOD utilised during the tests. Some of the removed SCOD can originate from the activated sludge itself, but it is assumed that this amount is negligible. The amount of SCOD utilised in the first phase of the tests is ($\Delta SCOD_1$) assumed to reflect the amount of readily available COD.

A discrepancy between the amount of added external SCOD and the measured utilised amount in the first phase ($\Delta SCOD_1$) can be observed in the tests with synthetic substrates. A measured difference of 84-90 mg SCOD/L can be seen, but a known addition of 110 mg SCOD/L was made, showing a discrepancy of 20-30%. It can to some extent be explained by measurements errors (20% in COD measurements), but it can also reflect that some SCOD is lost during the addition of the substrates to the reactors. The first samples were taken after around 1-2 minutes after substrate addition, meaning that some of the added SCOD can have been utilised before the measurements started. Thus, it should be noted that the measured difference in SCOD is not an exact measure of the utilised substrate overall, but can function as a rough estimation for comparison.

Table 4.2. Parameters illustrating the carbon utilisation for all different test with fermentates and synthetic substrates.

	Carbon source	$\Delta SCOD_1$	k_{C1}	$\Delta SCOD_1$/ ΔNO_3^--N eqv_1*	$\Delta SCOD_1$ /$\Delta SCOD_{tot}$
		SCOD mg/L	mg SCOD/g VSS·h	mg SCOD utilised /mg NO_3^--N re-moved*	mg SCOD/mg SCOD
N3:A	Acetate	90 mg/L	34.5	6.0	96 %
N3:B	Fermentate (T6)	68 mg/L	44.4	7.1	96 %
N6:A	Acetate + propionate	86 mg/L	46.4	6.3	95 %
N6:B	Fermentate (T6)	66 mg/L	49.9	6.2	83 %
N8:A	Fermentate (T6)	70 mg/L	71.7	8.4	88 %
N8:B	Fermentate (T7)	57 mg/L	59.7	7.3	87 %
N10:A	Propionate	84 mg/L	18.7	4.4	96 %
N10:B	Fermentate (T6)	73 mg/L	69.5	8.2	88 %

* During the first phase of nitrate reduction.

As can be seen, a larger amount of SCOD is utilised in the test with synthetic substrates (84-90 mg/L, avg. 86 mg/L) compared with fermentate (57-73 mg/L, avg. 66 mg/L). This indicate that the synthetic substrates contain a larger amount of readily available substrate. This is not un-reasonable, as the substrates are matched in SCOD content and not VFA content, as discussed in section 4.1.1. This is also supported by the higher percentage of SCOD utilised in the first phase for the synthetic substrates (95-96%), in relation to 83-88%[12] for the fermentates. This could explain why the higher denitrification rate (k_{D1}) is maintained during a longer period with synthetic substrates compared with the fermentates (Figure 4.3), i.e. due to larger amount of available substrate.

On the other hand, the specific substrate utilisation rates (k_{C1}) show that the complex carbon sources (fermentate and the mixture of acetate + propionate) is utilised at a higher rate (44-72 mg/g VSS·h) compared to the sole carbon sources acetate (34.5 mg/g VSS·h) and propionate

[12] It should be noted that the high percentage of SCOD utilised in the first phase for test N3:B with fermentate (96%) is not deemed to be representative value. The SCOD concentration in the last measurement was rather high, leading to a unusually low $\Delta SCOD_{utilised}$ value.

(18.7 mg/g VSS·h). This confirms the observation in section 4.1.2 that a complex carbon source stimulates the activity of a larger variety of different microbial species, resulting in a faster total utilisation. Interestingly, the sum of the utilisation rate for acetate and propionate gives a rate in the similar range as the complex carbon source, at 53 mg/g VSS·h. This could mean that different organisms prefer to use acetate whereas other prefer propionate, leading to an increased total utilisation rate when both carbon sources are present (additive effect). Important to note is that not all SCOD is necessarily used for denitrification in the first phase, some is used for anoxic growth and some parts can be used and stored by both denitrifying and non-denitrifying bacteria. Furthermore, the slower uptake rate for propionate could reflect the fact that it has a higher molecular weight compared to acetate, requiring a more complex metabolic pathway (Elefsiniotis & Li, 2006).

The parameter $\Delta SCOD_1/\Delta NO_3^-$-N eqv_1 is an expression of the amount of SCOD utilised for the removal of a certain amount of NO_3^- in the first phase, also taking into account the COD used for anoxic growth, and other COD "lost" through uptake by non-denitrifying bacteria. Some COD might also be stored by denitrifying bacteria, and used at a later stage (Abufayed & Schroeder, 1986). However, it should be noted that eventual contribution to nitrate removal due to endogenous respiration is not included, as it was difficult to assess the endogenous denitrification rates (see section 4.1.4). Thus, ΔNO_3^--N eqv_1 is calculated as the total change in NO_3^--N eqv concentration in the first phase. This parameter may be an underestimation of the amount of carbon needed for removal of NO_3^- in the different tests.

The parameter vary a lot for the tested fermentate substrates, with values in the range from 6.2-8.4 mg SCOD/mg NO_3^--N eqv. These values are common in wastewater treatment, as reported by Zheng *et al.* (2018). The variation could be an indication of a variation in composition in the fermentate during the testing period, as discussed in section 0. This in turn could result in different amounts of COD "lost", either by varying anoxic growth yields or different uptake by other bacteria or microbes. It could also be a reflection in different biomass activity between the tests.

The lower $\Delta SCOD_1/\Delta NO_3^-$-N eqv_1 value obtained with propionate (4.4 mg SCOD/mg NO_3^--N eqv) compared with acetate (6.0 mg/mg) as a sole carbon source, can be explained by the slightly lower theoretical C/N ratio required for propionate. Elefsiniotis and Li (2006) reports a stoichiometric value of 1.53 mg VFA-C/NO_3^--N for propionate, including requirements for growth of biomass, with a corresponding value of 1.82 mg/mg for acetate. This indicate a lower anoxic growth yield for propionate. The higher value for acetate could also indicate that more acetate is stored or taken up by different bacteria compared with propionate, resulting in a higher ratio due to larger SCOD "loss".

It should be noted that the anoxic growth yield, the substrate utilisation rates and other parameters are highly dependent on the biomass, and will reflect the relative acclimation of the biomass to the specific substrates (Kujawa & Klapwijk, 1999; Rahman *et al.*, 2014).

4.1.4 Lowest specific denitrification rates (k_{D3})

As can be seen in Table 4.1, there is a large variation in the low specific denitrification rates (k_{D3}) between the different carbon sources.

The k_{D3} value obtained during the blank test (N5) was around 1 mg NO_3^--N eqv/g VSS·h, and is assumed to reflect an endogenous rate since no exogenous carbon was added. However, other studies have shown lower values for endogenous rates, in the range of 0.2-0.7 mg NO_3^--N eqv/g

VSS·h (Bolzonella *et al.*, 2001; Isaacs *et al.*, 1994; Kujawa & Klapwijk, 1999). Naidoo *et al.* (1998) reported assumed endogenous rates in the range of 0.9-2, but argue that it is possible that slowly biodegradable COD absorbed to the sludge could have influenced these rates. It can be seen that the SCOD curve in the blank test is rather constant (see Figure 4.4), varying up and down between 24 and 32 mg COD/L (average 27.5±2.8 mg/L), indicating no substantial uptake, as the variation is within the error margins of COD measurements. But the small variation in SCOD could indicate the production of S_B due to hydrolysis of XC_B present in the biomass, and thus not reflect fully endogenous conditions. It is possible that a more extensive washing procedure and a longer aeration period before the test would have resulted in a lower denitrification rate. Thus, it is recommended to conduct additional blank tests with longer aeration time in order to assess the endogenous rate with more reliability.

It should be noted that it was difficult to assess how much of the measured SCOD in the reactors originated from the washed and aerated sludge. The SCOD in the sludge (sample 0) varied between 19-33 mg SCOD between the test, with an average value of 24±4 mg/L. Theoretically, the sludge would then contribute with half this amount in the reactors (i.e. around 10-15 mg COD/L). However, in the blank test, the SCOD concentration in the sludge (sample 0) was measured to 28 mg/L, whereas the start concentration was as high as 24 mg/L after addition of 1 L tap water. This discrepancy could be due to measurements errors, especially at so low concentrations, even though low range COD cuvettes were used (LCK 314, 15-150 mg/L). For simplicity it will be assumed that the sludge contributed with around 20-30 mg SCOD/L as a base line in all reactors, as measured in the blank test.

The tests with synthetic substrates gave rise to k_{D3} values in the range of 1.5-2.1 NO_3^--N eqv/g VSS·h, which differ a bit from the rate measured in the blank test, but are in the same range as the assumed endogenous rates reported by Naidoo *et al.* (1998). The slightly higher rate could reflect the usage of stored COD, meaning that the denitrifiers might take up excess carbon in the first phase for storage, and use it for denitrification in a later stage when all readily available substrate has been consumed (Abufayed & Schroeder, 1986; Çokgör *et al.*, 1998). This could explain the high C/N uptake rates observed in the first phase (4-6 mg SCOD/mg NO_3^--N eqv). Utilisation of residual biodegradable COD in the sludge could also affect the rate as discussed by Naidoo *et al.* (1998). This stresses the importance of ensuring endogenous conditions and full removal of residual biodegradable COD before conducting NUR tests, as explained by Kujawa and Klapwijk (1999). This is a lesson for future NUR tests.

It should also be noted that Naidoo *et al.* (1998) observed second rates in the range of 1-3 mg NO_3^--N/g VSS·h, which were assumed to reflect denitrification on XC_B, showing that it is difficult to completely distinguish between the rate on slowly hydrolysable material and endogenous rates, which other studies have reported as well (Sage *et al.*, 2006).

Based on this, it is difficult to regard the slower rates in the tests with synthetic substrates as fully endogenous. Nevertheless, the SCOD curves for the synthetic substrates, presented in Figure 4.5 below, show that the SCOD concentration was reduced to around 20-30 mg/L after the first reduction phase in all tests. This is in line with the assumed SCOD base line of the activated sludge in the reactors, indicating that all exogenous S_B had been utilised before the slow rate begin. Thus, full consumption of the added substrates can still be assumed.

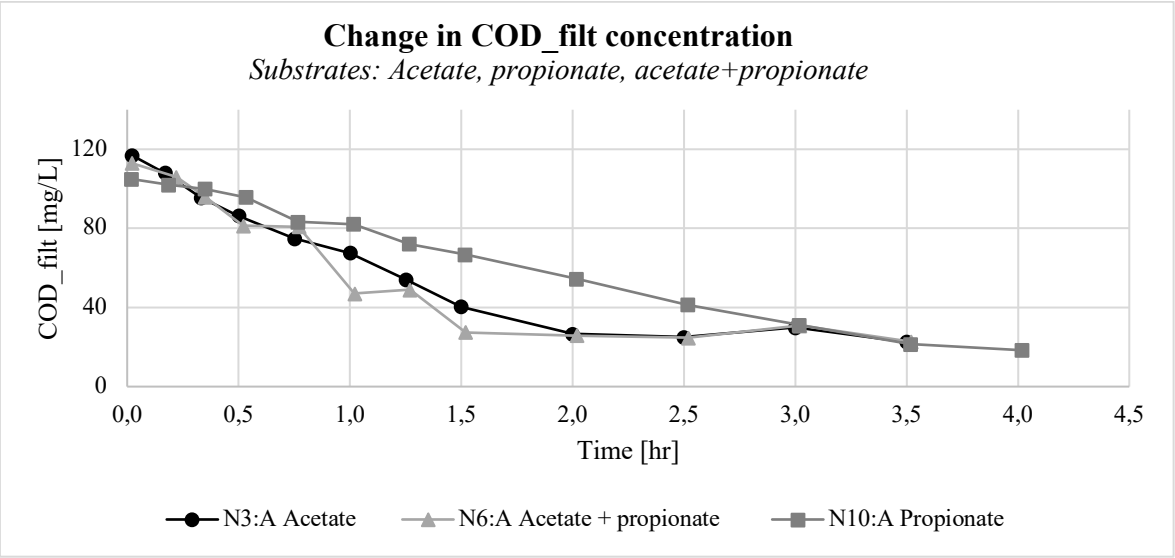

Figure 4.5. Change in SCOD concentration during NUR tests with acetate, propionate and acetate + propionate.

In comparison, the k_{D3} values obtained with fermentate show a much larger difference from the blank k_{D3} value, with rates in the range of 2.3-2.7 NO_3^--N eqv/g VSS·h (average 2.5±0.16, CoV 6.5 %). These rates are in the same range as the second rates observed by Naidoo *et al.* (1998), 1-3 mg N/VSS·h, which were believed to reflect denitrification on XC_B as mentioned previously. A slight decline in SCOD concentration can also be seen in the test with fermentate during this phase, as illustrated in Figure 4.6. Furthermore, the SCOD concentration in the reactors after the first reduction phase is in the range of 37-53 mg/L, which is above the assumed base line of the sludge. The base line is not reached until later, although for test N6 it is not reached at all (lowest value 39.5 mg/L). This indicates that some of the SCOD present in the fermentate is not readily available for denitrification, but could be inert (non-degradable) or slowly biodegradable. Overall, full consumption of the exogenous substrate cannot be assumed after the first reduction phase, in difference with the synthetic substrates.

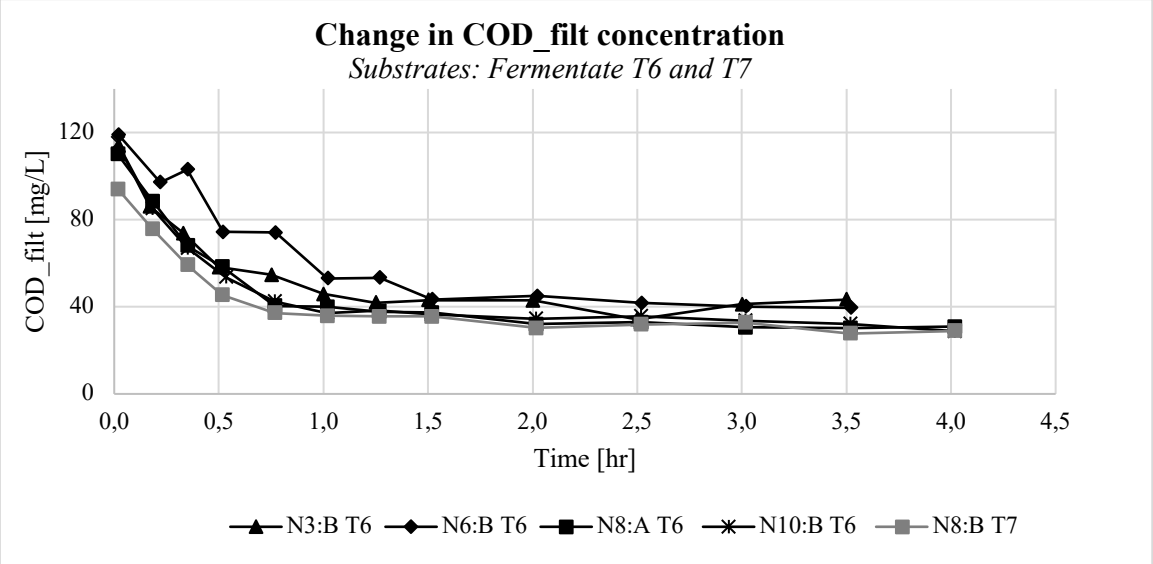

Figure 4.6. Change in SCOD concentration during NUR tests with fermentate T6 and T7

Additionally, the total COD in the fermentate substrates were actually larger than the synthetic substrates. The filtered fermentate contain some other COD fractions as well (< 18.5 µm), which might need to be hydrolysed before being utilised (particulate biodegradable COD, XC_B). The ratio of COD_{filt} to unfiltered COD in the fermentate substrates varied between 65-79% (Table A.12) indicating that there was a 21-35% additional COD in the fermentate reactors, not shown in the SCOD measurements. All this indicate that it is reasonable to assume that the slowest rate k_{D3} reflect a rate based on hydrolysis of XC_B or utilisation of soluble slowly biodegradable carbon present in the fermentate substrates, as well as eventual residual biodegradable COD in the sludge and endogenous denitrification based on cell decay.

In all slower rates, the measured SCOD utilisation per NO_3^--N eqv is lower than the theoretical requirement of 2.86 mg COD/mg NO_3^--N. Therefore, the carbon necessary for NO_3^- reduction must have been supplied by either stored COD taken up during the first phase, by hydrolysis of slowly biodegradable COD (either in the substrate or present in the biomass) or from endogenous material through cell decay (Abufayed & Schroeder, 1986). This supply of carbon must have occurred at a similar rate to that of carbon utilisation for denitrification, and thus not registered in the SCOD curve (ibid).

4.1.5 Example of VFA utilisation in NUR test with fermentate as substrate

The VFA composition of some samples from test N10:B, with fermentate T6 as substrate, were analysed. The gas chromatography analysis showed reasonable results for acetate and propionate. Other VFAs, for example butyrate, which been present in previous analyses of the fermentate were not registered. This might be due to some measurement errors, but this has not been evaluated. Other VFAs showed unreasonable peaks and were thus omitted from the results (a curve with all analysed VFAs are presented in Figure A.5 in Appendix B).

To illustrate the VFA utilisation in a test with fermentate, the change in total SCOD, acetate and propionate during test N10:B is presented in Figure 4.7. The sum of acetate and propionate (total VFA-C) is also shown, as well as the difference between total SCOD and the sum of VFA-C.

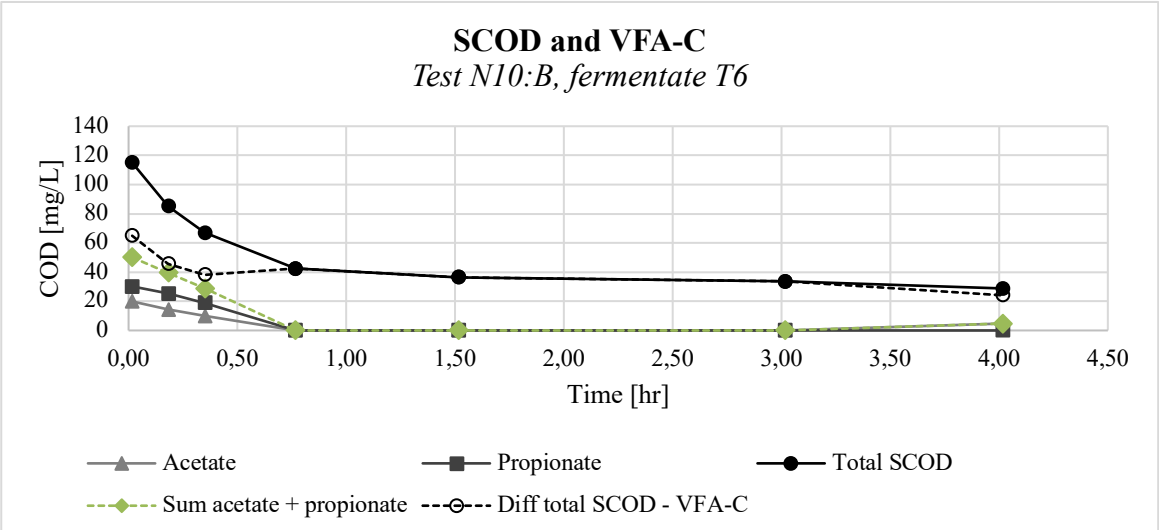

Figure 4.7. Change in total SCOD, acetate and propionate in test N10:B fermentate T6.

As can be seen, acetate and propionate make up 50 mg COD/L in the first sample, constituting 44% of the initial SCOD in reactor, also including the SCOD present in the biomass. Both VFAs are fully consumed at time 0.77 hr, and at the same time a total SCOD of 73 mg/L has been consumed (Table 4.2). Thus, 68% of the SCOD utilised during the first phase consists of acetate and propionate. This ratio is in line with older data, which showed that acetate and propionate made up 60% of the VFA-C in the fermentate, as mentioned previously (Table A.11).

The difference between total SCOD and VFA-C in the beginning indicate the presence of additional readily available carbon, which could reflect the utilisation of other VFAs which were not registered in the VFA analysis, for example butyrate. But interestingly, at time 0.35 h, almost all available SCOD is made up of acetate and propionate, resulting in a constant base line, which could indicate that VFAs are utilised first before any other SCOD compounds. Thus, the assumption that the first denitrification phase reflects the utilisation of readily available carbon seem reasonable.

Furthermore, both VFAs are utilised at rather constant rates, estimated to around 19 mg COD/g VSS·h for acetate and 30 mg COD/g VSS·h for propionate. This indicates that both are consumed simultaneously. This is in contrast to other research, which have seen a clear preference of acetate before propionate, where the consumption of propionate only has occurred after acetate has been fully consumed (Elefsiniotis & Wareham, 2007). The rather high utilisation rate of propionate also suggests a slight preference, which could be due to high acclimatisation of the biomass to propionate. It could also be due to a concentration effect, where the higher rate only reflect the slightly higher availability of propionate. Such an effect was observed by Elefsiniotis and Wareham (2007), although the VFA-C concentrations in their tests were significantly larger overall. The difference in VFA-C between acetate and propionate in the reactor were rather small (19.9 mg/L compared with 30.2 mg/L), indicating that a concentration effect may not have had that large significance for the utilisation of VFAs.

4.1.6 P-release during anoxic conditions in tests with sole carbon sources

A slight P-release occurred in all tests under anoxic conditions. This can be seen in Figure 4.8 and Figure 4.9 below, showing the change in PO_4^{3-}-P concentration for fermentates (T6/T7) and synthetic substrates respectively, in relation to the initial PO_4^{3-}-P concentration. A negative value indicates a net-uptake, whereas a positive value shows a net-release.

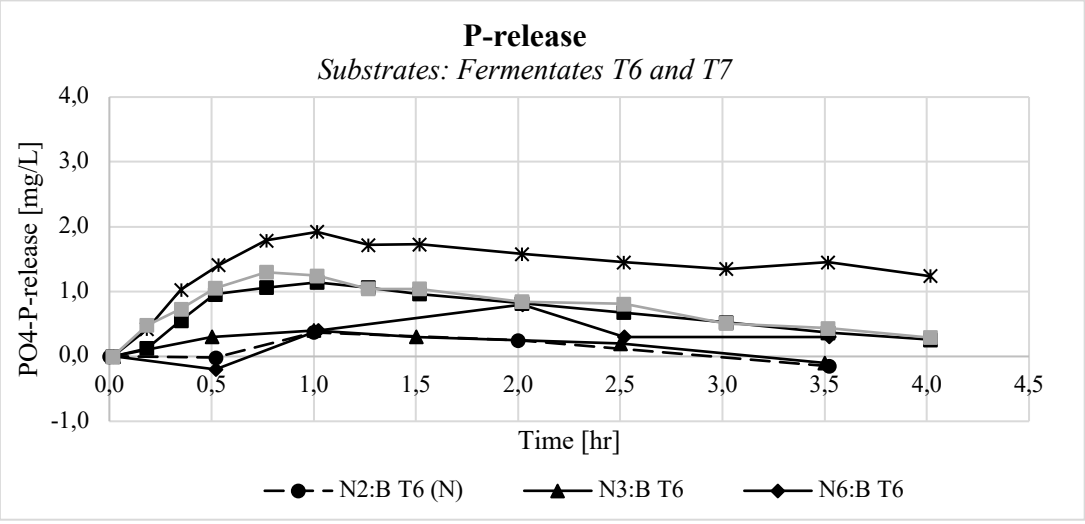

Figure 4.8. Anoxic P-release curves for tests with fermentate (T6 and T7).

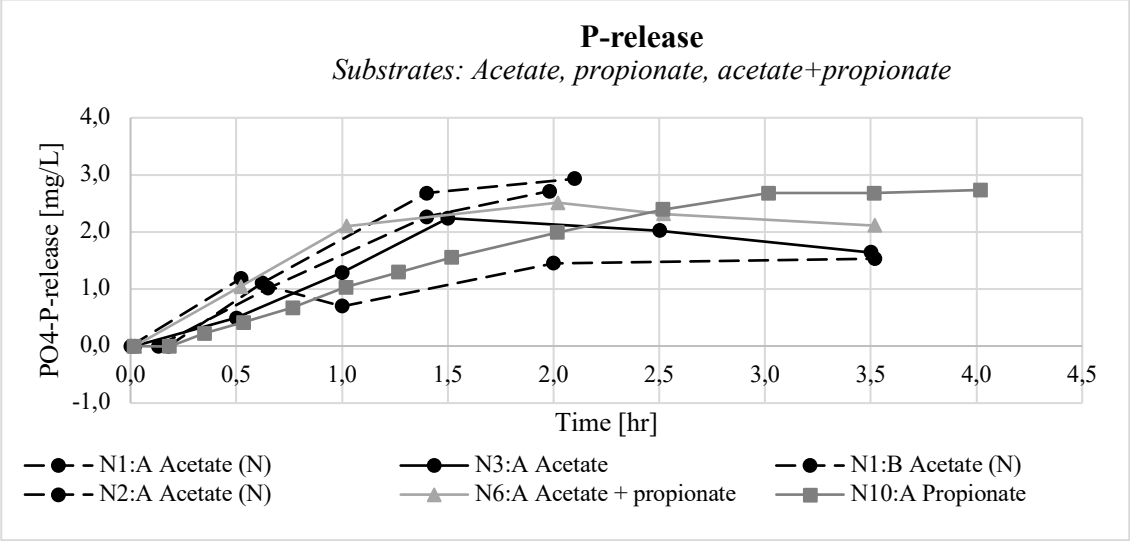

Figure 4.9. Anoxic P-release curves for tests with synthetic substrates.

It is evident from both figures that the P-release follow the availability of readily available carbon to a large extent. In the tests with fermentate (Figure 4.8), the P-release is the highest in the beginning, reaching a plateau as the substrate is less available, and then declining in the second phase when the readily available substrate has been consumed. Similar trends can be seen in the tests with synthetic substrates (Figure 4.9). This is in line with other studies, showing that net anoxic P-release occur when readily degradable substrate is available (less competition between different organisms), reducing as the substrates become limiting, after which uptake of phosphorus can occur if NO_3^- is still present (Gerber, 1986; Iwema & Meunier, 1985).

A bit higher net-release can be seen in the tests with synthetic substrates; max total release of around 3 mg/L, compared to max 2 mg/L with fermentate. This can be explained by the higher VFA content in the synthetic substrates, i.e. more available COD for the PAOs to use as well. This indicate that some of the available substrate is utilised for storage by non-denitrifying organisms during the first phase, suggesting a slight overestimation of the amount SCOD used per NO_3^- removed. However, it should be noted that the amount P release is low compared to other tests. For example, Kujawa and Klapwijk (1999) observed a total release of 10 mg/L, and other studies have shown P-release up to 20-30 mg/L under anoxic conditions (Barker & Dold, 1996; Gerber, 1986). This suggests that the activity of PAOs in the sludge at Källby WWTP are rather small, which is also confirmed by the low EBPR activity observed in the anaerobic P-release tests (see section 4.3 for more details). Overall, the effect of anoxic P-release on measured denitrifications rates and other parameters can be regarded as small.

4.2 Comparison of NUR tests with wastewater

4.2.1 Denitrification rates for wastewater

The experiments with wastewater were designed with a low dilution factor of 2, in order to have a high SCOD concentration in the reactor, and thus a high C/N-ratio and good F/M ratio for determination of denitrification kinetics. As explained, there was a discrepancy between the SCOD measurements by the lab and the definition for SCOD measurements used in this study, resulting in lower SCOD concentrations in the experiments with wastewater than expected; initial concentrations in the reactor were at 60-70 mg/L instead of 100-110 mg/L. This means that the start SCOD/N ratio was significantly lower in the test conducted with wastewater,

around 3-3.5 mg SCOD/mg NO_3^--N eqv (Table A.9). This in turn could mean that maximum denitrification rate is not reached in the tests, due to carbon limitation. Furthermore, the F/M ratio in the tests with wastewater were also in the low range, around 0.04-0.05 mg COD/mg VSS. As explained, a low F/M ratio could mean a rapid depletion of VFA and other readily available carbon, which might make it difficult to assess an eventual rate on this carbon fraction.

However, van Loosdrecht *et al.* (2016) put forward that a final total biodegradable carbon concentration (S_B+XC_B) of 30-70 mg/L in the reactors is both common and ideal when conducting tests with real wastewater, since it is difficult to obtain higher levels due to the dilution effect. One possibility is to increase the MLVSS concentration, by concentrating the sludge, and thereby be able to add more wastewater in relation to sludge and still keep a good F/M ratio (Naidoo *et al.*, 1998; van Loosdrecht *et al.*, 2016). This could be advisable to do in future tests. As the discrepancy in SCOD concentration was realised after the first test was conducted, it was decided to use the same procedure in the following tests for comparison reasons.

Figure 4.10 show the NUR curves for test N4, with influent (raw) WW (T1, N4:A) and filtered WW (T5, N4:B), illustrating that mainly two phases can be observed clearly, which was the case for the rest of the tests as well (Figure 4.13). The specific denitrification rates obtained for all tests with wastewater are presented in Table 4.3 below. As only two phases could be observed, the rates are denoted as k_{D1} for the fast rate, and k_{D3} for the slow rate, in the same manner as for the tests with sole carbon sources. However, whether k_{D1} reflect denitrification on S_B in the wastewater or on XC_B will be discussed, and same goes for k_{D3}. The measure change in SCOD in the first phase of the NUR curves ($\Delta SCOD_1$) is also presented, as an estimation of the SCOD utilised.

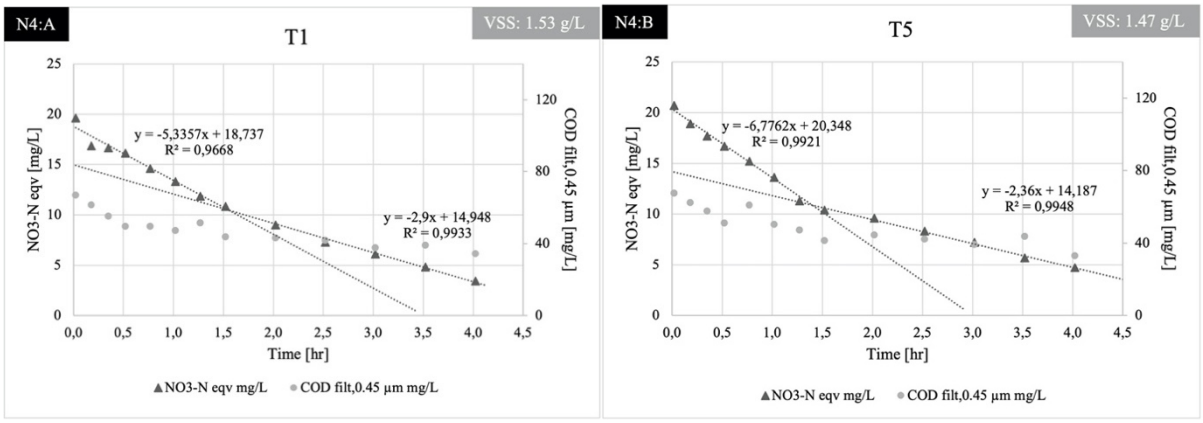

Figure 4.10. NUR curves for test N4:A influent WW (T1) and N4:B filtered WW (T5).

Table 4.3. Denitrification rates and $\Delta SCOD_1$ for tests with wastewater.

	Carbon source	k_{D1}	k_{D3}	$\Delta SCOD_1$
		mg NO_3^--N/ g VSS·h		mg/L
N4:A	Influent WW (T1)	3.5	1.9	23
N4:B	Filtered WW (T5)	4.6	1.6	26
N7:A	Filtered WW (T5)	3.6	2.2	18
N7:B	Filtered WW (T5) + Fermentate (T6)	4.6	2.4	27
N7:A	Filtered WW (T5)	3.7	1.9	20
N7:B	Filtered WW (T5) + Fermentate (T6)	4.5	2.1	45

The faster rates obtained with filtered WW (T5) were in the range of 3.5-4.6 mg NO_3^--N/g VSS·h, and the test with influent WW (T1) showed a rate of 3.5. These rates are slightly lower compared with the ones achieved with sole carbon sources, which could indicate that the fraction rbCOD is very low and quickly depleted, resulting in that denitrification on this fraction is not observed. This would mean that the first rate reflect denitrification on slowly biodegradable carbon. However, Naidoo *et al.* (1998) obtained similar initial rates with wastewater (in the range of 3-5 mg N/g VSS·h in the most of the tests), using similar C/N and F/M-ratios, which they argued reflected the use of readily available carbon in the wastewaters. The lower rates compared to the sole carbon sources could be due to the lower C/N ratios, as explained above.

Several studies have estimated that the rbCOD constitutes around 10-20% of the total COD, and around 20-50% of SCOD (Isaacs *et al.*, 1994; Naidoo *et al.*, 1998). The influent WW had a total COD concentration of 560 mg/L, indicating a rbCOD concentration of 50-110 mg/L (Table A.13) corresponding to around 25-55 mg/L in the reactors. The COD in the filtered WW on the other hand varied between 260-330 mg/L, showing a significant reduction by the filter in the pilot plant. But as the SCOD concentration is rather constant after filtration (Table A.13) it is likely that a similar rbCOD concentration can be assumed for the filtered WW as for the influent WW. As can be seen in Table 4.3, the estimated substrate utilisation in the first phase ($\Delta SCOD_1$) varies between 18-26 mg/L, which matches the assumed rbCOD concentration in the different wastewaters rather well. Based on this, it is reasonable to assume that the first reaction rate reflect the usage of readily available carbon to a large extent.

The slower rates (k_{D3}) on the other hand varies between 1.6-2.2 mg NO_3^--N eqv/g VSS·h. Since the higher rates are believed to reflect the rates on readily available carbon, it is likely that the slower rates reflect denitrification on slowly hydrolysable material to a large degree. As already mentioned, Naidoo *et al.* (1998) observed rates in the range of 1-3 mg NO_3^--N/g VSS·h, which was assumed to reflect denitrification rates on slowly biodegradable carbon. This is further supported by the observed reduction of SCOD in the first phase in the NUR curves, where SCOD is reduced to between 37-44 mg/L, which is higher than the assumed base line of 20-30 mg/L. Thus, it is reasonable to assume that the slower rates reflect denitrification on XC_B and

other slowly degradable carbon, and that a fully endogenous rate is not reached. This indicates that full consumption of the COD in the wastewater is not reached either during the NUR tests. The slightly lower k_{D3} rates compared with the k_{D3} obtained with fermentate could be due to lower COD concentration overall.

4.2.2 Influent wastewater (T1) and filtered wastewater (T5)

The nitrate removal efficiency curve obtained during test N4 conducted with influent WW (T1) and filtered WW (T5) is presented in Figure 4.11 below.

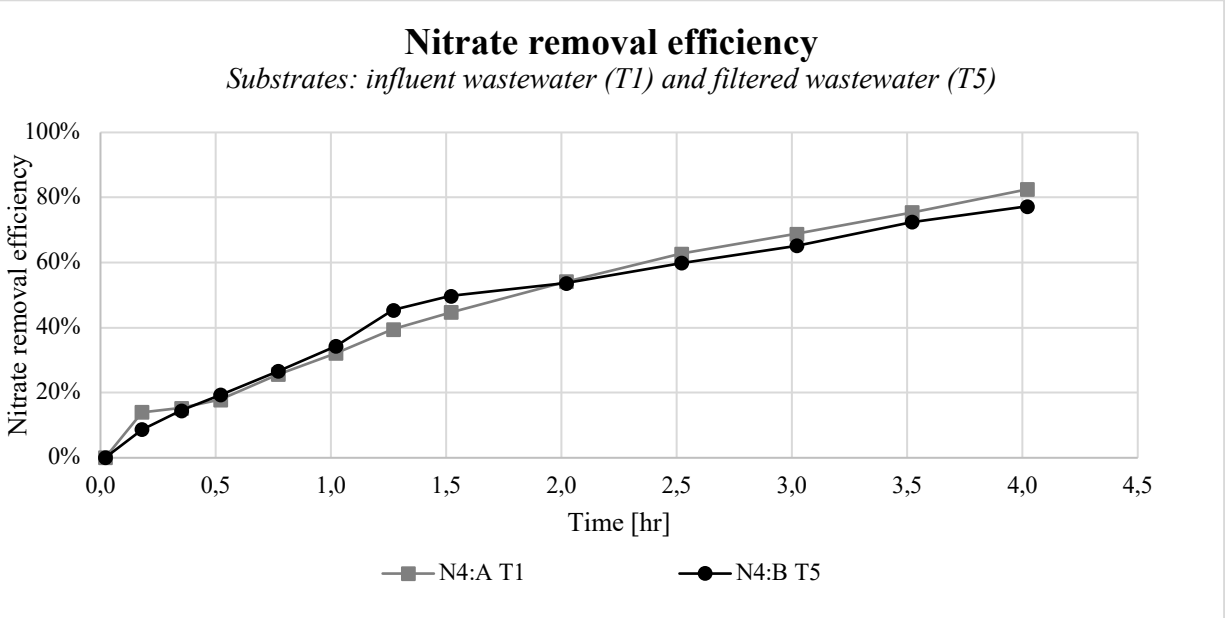

Figure 4.11. Nitrate removal efficiency curves for test N4 with influent WW (T1) and filtered WW (T5).

Test N4 showed a significantly larger fast denitrification rate for filtered WW (T5) compared with influent WW (T1) (4.6 compared with 3.5 mg NO_3^--N/g VSS·h), even though the SCOD and concentration in the two substrates were similar (124 and 112 mg/L in filtered WW and influent WW respectively). However, the concentration of COD particles < 0.1 µm constituted 97% of the SCOD in the filtered WW, compared to 92% in influent WW, according to the COD characterisation of the wastewater conducted (Table A.16 and Table A.17). This could indicate that the filtration affect the amount of readily available COD to a certain degree, possibly due to shear stress or release of attached SCOD from larger particles, but more tests comparing influent WW (T1) and filtered WW (T5) should be conducted before any far-reaching conclusions are drawn. The overall nitrate removal over the whole course of the test (4 hours) was slightly higher for influent WW compared with filtered WW (83% removal compared with 77%, see Figure 4.11), which reflect the higher amount of XC_B present in the influent wastewater, resulting in a higher k_{D3} rate.

4.2.3 Filtered wastewater with and without addition of fermentate

The nitrate removal efficiency curves obtained during test N7 and N9 conducted with filtered WW T5 and filtered WW with the addition of fermentate T6 (T5+T6) are presented in Figure 4.12.

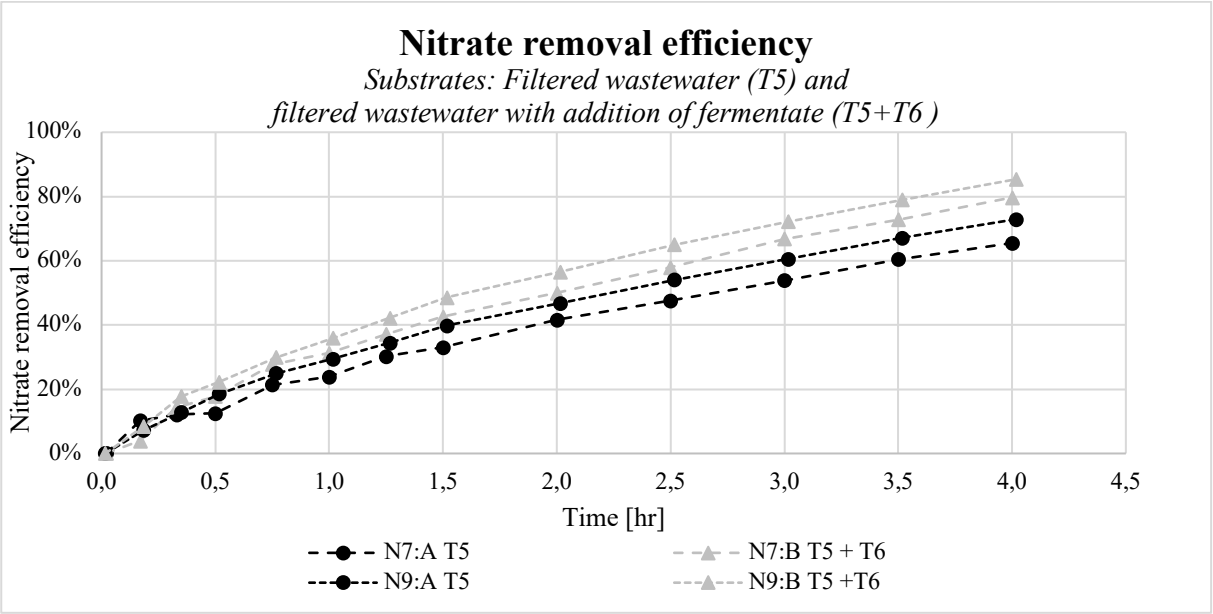

Figure 4.12. Nitrate removal efficiency curves for tests N7 and N9 with filtered WW (T5) and filtered WW with addition of fermentate (T5+T6).

The NUR curves for test N7 and N9, showing the curves with filtered WW (T5) with and without addition of fermentate (T6), are presented in Figure 4.13. In test N7, the reactor with T5 obtained a k_{D1} rate of 3.6 mg NO_3^--N/g VSS·h, whereas the reactor with filtered WW with fermentate (T5+T6) showed a k_{D1} rate of 4.6, a 28% increase. Similarly, in test N9, the k_{D1} in the reactor with T5+T6 was 22% higher compared to the reactor with only filtered WW (T5). The higher rates could to some extent reflect the higher C/N-ratio in the tests with fermentate addition (Table A.9), but it is more likely that it reflect the increase in readily available substrate. The SCOD concentration increased with 48-50% with the addition of the fermentate. During the whole course of the tests, nitrate removal of 80% and 85% were achieved in the reactors N7:B and N9:B with filtered WW (T5) with fermentate (T6) respectively, compared with 66% and 73% achieved with only filtered WW (Figure 4.12). This corresponds to an 17-22% increased removal efficiency overall.

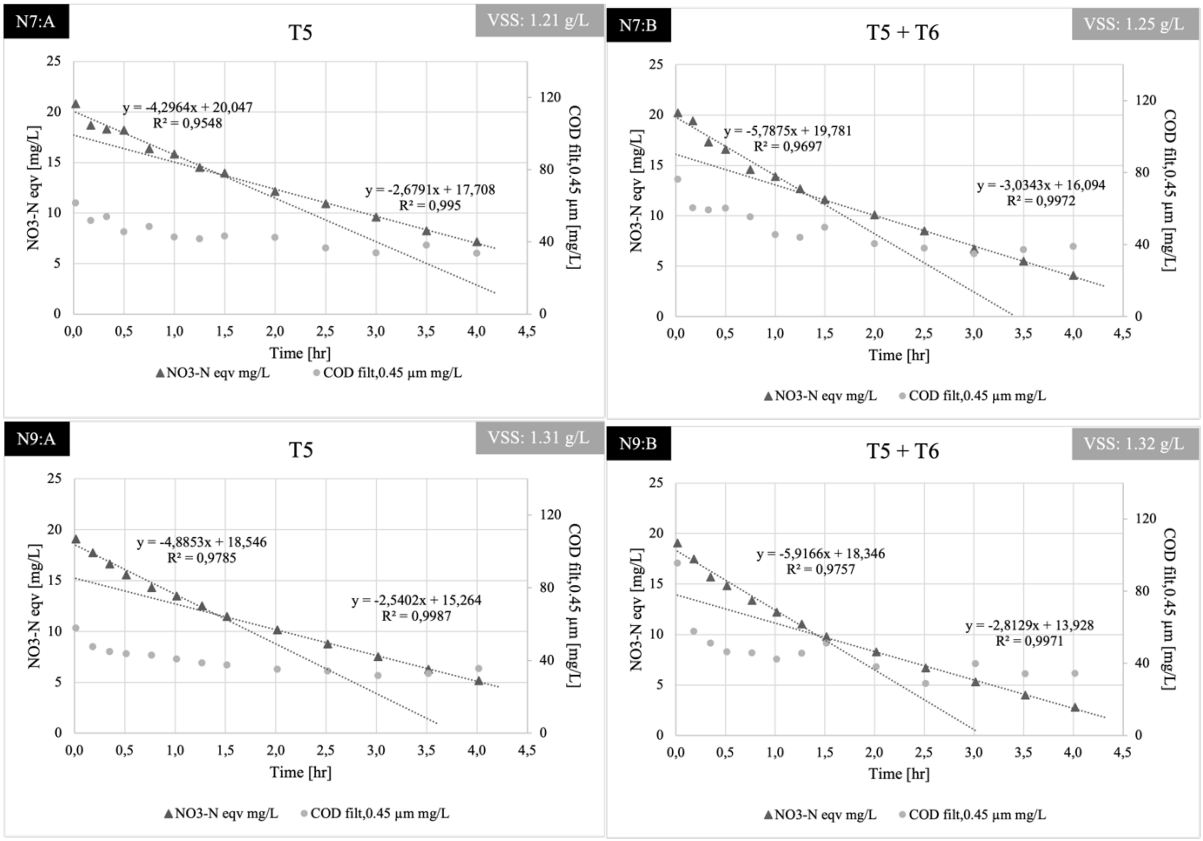

Figure 4.13. NUR curves for test N7 and N9, with filtered WW (T5) and filtered WW with addition of fermentate (T5+T6).

However, a complicating factor is the increase in soluble PO_4^{3-} and NH_4^+ also occurring due to the addition of fermentate. When comparing the content of filtered WW (T5) with filtered WW with fermentate (T5+T6), the PO_4^{3-} content increased with 17-18%, and NH_4^+ concentration increased with 3-9% in test N7 and N9 (see Table A.12 in Appendix C for more details). This increased load of nutrients will reduce the overall availability of the added substrate, as explained by Liu *et al.* (2020). This means that the estimated increase in removal efficiency is lower in reality, as some of the added substrate will be needed to compensate for the small increase in nutrient loading. In order to estimate the "real" removal efficiency, the small increase in NH_4^+-N concentration measured between the substrates is assumed to result in a similar potential increase in NO_3^--N eqv concentration. It is further assumed that this extra amount would not be removed during the batch tests, assuming same removal rates. Based on these assumptions, the "real" removal efficiency in test N7:B and N9:B can be grossly estimated to around 74% and 83% respectively, corresponding to a 13% increase in removal efficiency compared to the tests with only filtered WW (T5). However, it should be noted that higher removal efficiencies could be obtained if the biomass is acclimated to the added fermentate.

Compared with the nitrate removal efficiency obtained with influent WW in test N4 of 83% (Figure 4.11), the removal efficiencies achieved with the combination of filtered wastewater with fermentate addition (T5+T6) in the latter tests do not show a large difference. Although, it should be noted that the tests were conducted at different days, thus not reflecting the same wastewater. Since the filtered WW showed higher removal rates in general in test N4 (removal efficiency of 77%) compared to test N7 and N9, it can be assumed that the addition of fermentate would have resulted in even higher removal efficiencies, maybe even slightly higher than 83% if assuming a 13% increase (estimated to 87%).

Regardless, it is interesting that removal efficiencies in the same range (around 80-85%) are achieved with both influent WW and filtered WW with fermentate addition, even though the substrates differ in COD composition. For example, the difference in total COD is substantial: 560 mg COD/L in influent WW (T1) compared with around 310-390 mg COD/L in filtered WW with added fermentate (T5+T6) (Table A.13). Additionally, the ratio of SCOD/COD is around 20% for influent WW and 50-60% for T5+T6, with a significantly larger SCOD concentration in the latter. Thus, it is evident that a large part of the influent COD has been removed through filtration, as observed by Tebini (2020) as well. But a small part of this organic material is returned in a more soluble form to the filtered WW through the addition of fermentate. To conclude, the filtered WW with addition fermentate show both a high removal efficiency, higher than without addition and to some extent comparable to that of influent WW. At the same time the amount of total COD is significantly lower compared with the influent WW, thus showing a potentially better utilisation of the COD, achieving removal efficiencies in the same range even though a large part of the COD is removed.

However, it should be remembered that in the actual process at Källby WWTP, the influent wastewater has undergone primary sedimentation before reaching the activated sludge process, meaning that the composition of the wastewater is slightly different compared with the influent (raw) wastewater tested in this study. For a better comparison with the process used at Källby WWTP today, tests comparing the rates and removal obtained with sedimented wastewater and filtered WW with fermentate addition (T5+T6) should be conducted.

4.3 Anaerobic P-release tests

The calculated specific P-release rates, k_P, are presented in Table 4.4 below. All k_P values were determined for 2.5 hour duration for comparison reasons.

Table 4.4. Specific P-release rates.

Specific P-release rate	**Carbon source**	k_P
		mg PO_4^{3-}-P/ g VSS·h
P1:A	Acetate[13]	1.8
P1:B	Acetate[14]	1.6
P2:A	Acetate	1.5
P2:B	Fermentate (T6)	1.6
P3	Blank	0.4

The P-release curves for all tests are presented in Figure 4.14 below, and the corresponding SCOD curves are presented in Figure 4.15. The P-release curves are adjusted for the initial concentration of PO_4^{3-}-P for comparison reasons. The SCOD values for test P1:A are omitted due to an error with the heating block.

[13] Including nutrient solution and ATU, no pH regulation
[14] Including nutrient solution and ATU

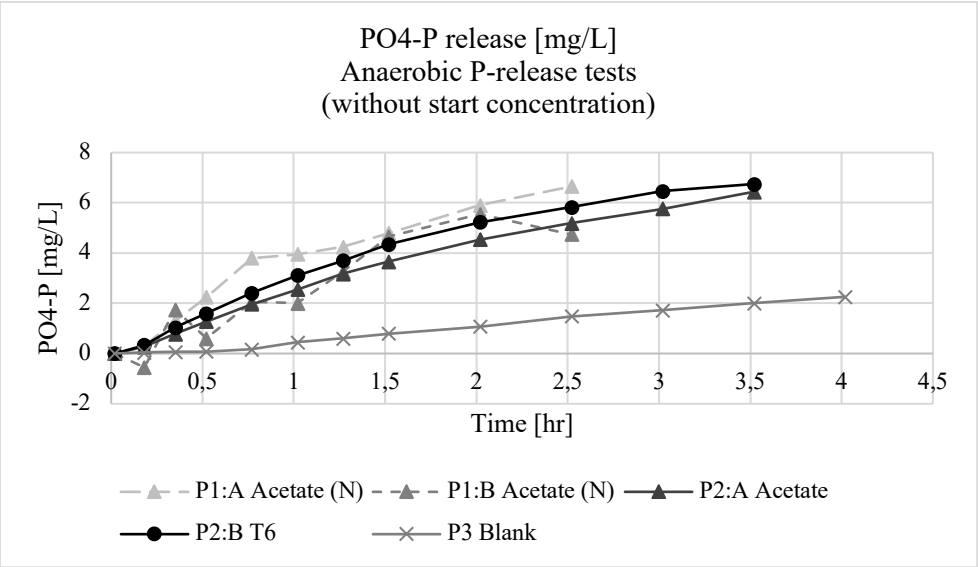

Figure 4.14. P-release curves for all anaerobic P-release tests.

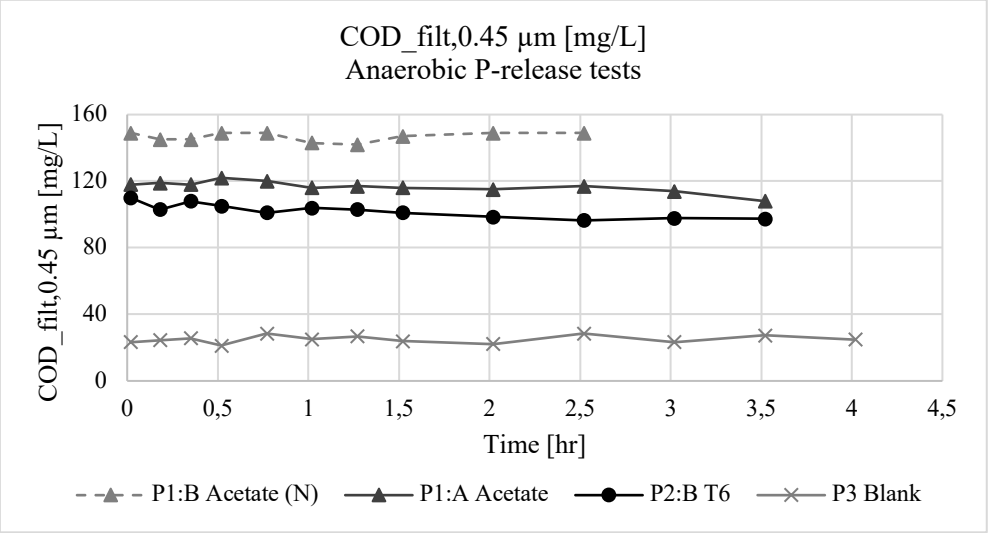

Figure 4.15. SCOD curves for all anaerobic P-release tests.

As can be seen, only a rather small P-release can be observed for both acetate and fermentate, reaching a concentration of 6-7 mg/L after 3.5 hours in test P2. This is in large contrasts to the 20-80 mg/L phosphorus released in tests with acetate and propionate with EBPR enriched sludge (Oehmen *et al.*, 2006). However, this is not unreasonable since fully enriched EBPR sludge are not usually obtained in WWTP.

The P-release rates for acetate varied between 1.5-1.8 mg P/g VSS·h, and 1.6 mg P/g VSS·h was obtained for fermentate. These rates are in the same range as those obtained in the study conducted by Zhang *et al.* (2011), investigating EBPR activity in different WWTPs in batch tests with acetate, were the majority of the tested sludges showed rates in the range of 0.2-3.6 mg P/g VSS·h. These rates were regarded as low in intensity, resulting in subsequently low P-uptake in the following steps. Other studies have shown P release rates in the range of 5-20 mg P/g VSS·h (Zhang *et al.*, 2011). Based on this, the EBPR activity in the sludge at Källby WWTP can be regarded as low.

Furthermore, the SCOD curves presented in Figure 4.15 show an extremely small uptake. The substrate utilisation rate in test P2:A and P2:B was 1.0 and 2.8 mg COD/g VSS·h respectively (calculated for 2.5 hr duration). It corresponds to COD/P ratios of 0.7 mg COD/mg P released for test P2:A with acetate, and 1.75 mg COD/mg P released for P2:B with fermentate. This is very low compared with the acetate uptake rate measured by Zhang *et al.* (2011), in the range of 13-44 mg acetate/g VSS·h[15], with the lowest amount of acetate utilised per P released around 3 mg/mg P. Smolders *et al.* (1994) reported common values for acetate in the range of 0.25-0.75 P-mol/C-mol. This can be grossly converted to around 1.4-4 mg COD/mg P. From this it can be seen that the utilisation is low, but maybe not unreasonable in relation to the low P release.

It should be noted that the F/M ratio was a higher than suggested by van Loosdrecht *et al.* (2016), in the range of 0.07-0.08 mg SCOD/mg VSS, which could indicate too much rbCOD for the duration of the test. However, as a very low substrate utilisation was seen regardless, this is not believed to have had a large effect.

The EBPR activity could have been disturbed by too high NO_3^- values in the beginning of the tests, especially in the tests without ATU addition. However, the concentrations were low: the initial concentration of NO_3^- in test P2 was 0.6-0.7 mg/L, compared with 0.2-0.3 in test P1 with ATU, and it is likely that the NO_3^- concentration reduced quickly, either due to the continued N_2 sparging or due to eventual denitrification. Some SCOD could theoretically have been used for denitrification of NO_3^-, but since no large drop can be seen in the beginning, this effect does not seem to be significant. However, longer period of N_2 sparging prior to the tests could have removed all NO_3^- before the tests which would have been preferred. In future tests, it is recommended to analyse the NO_3^- concentration using Hach cuvettes in order to make sure that no electron acceptors are present before starting the anaerobic test.

Even though the EBPR activity seem to be low, the similar rates obtained with fermentate and acetate indicate that the fermentate has a good potential to be used as a carbon source for P-removal. It is recommended to conduct more tests with a sludge with known higher EBPR-activity in order to estimate the suitability of the fermentate with more reliability.

4.4 Experimental design parameters and uncertainties

4.4.1 Effect of nutrient solutions and ATU on COD

Other problems apart from dissolution problems described in section 3.4 also arose with the use of EDTA. Since EDTA is a compound which contains carbon, it can be oxidized to carbon dioxide and water in the presence of an oxidizing agent. Thus, the concentration of EDTA will theoretically show in the COD analysis result. This occurred during the batch activity tests. In the first three tests (N1, N2 and P1), when nutrient solutions were added, a large increase in the SCOD concentrations were noted, with concentrations around 170-200 mg/L in the beginning of the tests. This was much higher than expected, as the concentration in the substrates were around 220-250 mg/L and in the sludge around 50 mg/L (including ATU), which would correspond to a theoretical start SCOD concentration of 135-150 mg/L in the reactors.

This discrepancy is illustrated in Figure 4.16 and Figure 4.17, which show the COD concentration in the test with nutrient solutions and without (with corresponding carbon sources). As can

[15] COD conversion factor for acetate is 1.07 g/g, hence the utilisation rates are comparable.

be seen, there is a major disturbance occurring due to the nutrient solution. The difference between SCOD concentration with and without nutrient solution in the NUR tests were between 50-80 mg/L, whereas a difference of around 30 mg/L is seen for EBPR test. However, it should be noted that a too large amount of micro-solution EBPR was added to the reactors in test P1 (1.5 mL instead of 0.15 mL). Thus, it is possible that the effect on COD would have been much lower for the EBPR test if the correct amount had been used.

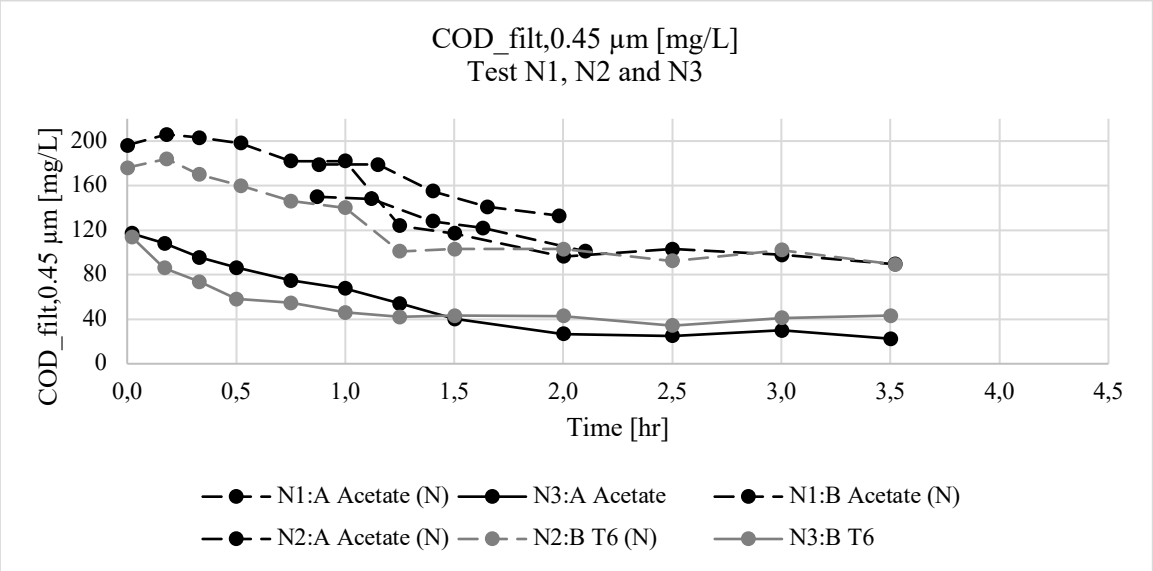

Figure 4.16. Difference in SCOD concentration with and without nutrient solution in test N1, N2 and N3.

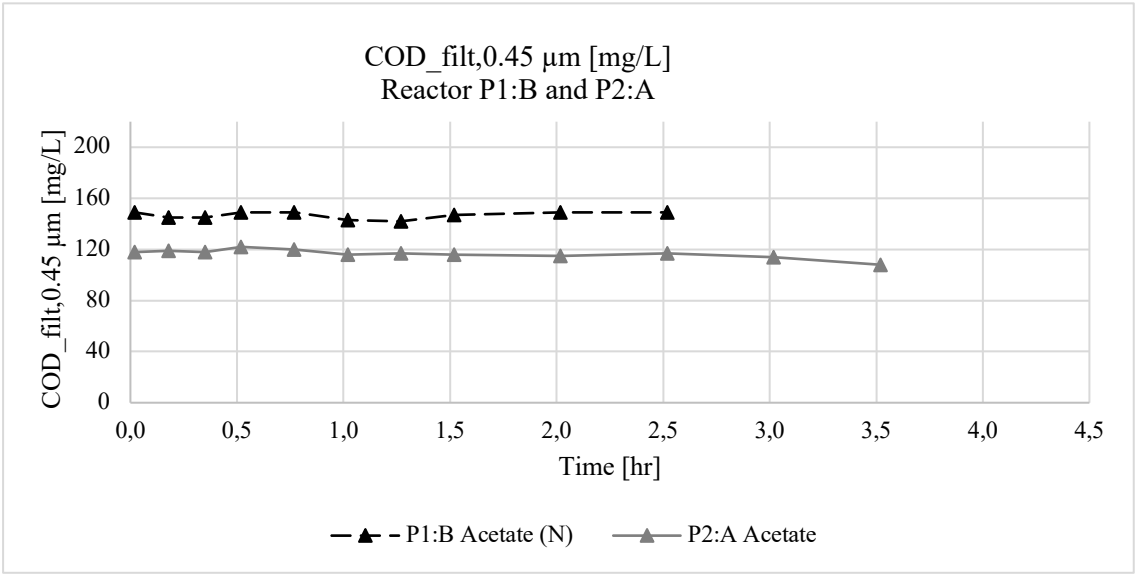

Figure 4.17. Difference in SCOD concentration with and without nutrient solution in test P1:B and P2:A.

The COD concentration in the micronutrient solutions with EDTA were tested (diluted 200 times) on March 12th. The resulting COD concentrations are presented in Table 4.5 below. As can be seen, the nutrient solutions show very high COD values.

A study conducted by Anderson *et al.* (2007) showed that the oxidation of EDTA in COD analysis often is incomplete, depending on the dichromate concentration in the vials. Anderson suggested a theoretical conversion factor of 0.944 g COD/g EDTA. By calculating a theoretical COD value in the micro-nutrient solutions (see Table 4.5), it could be seen that the obtained value during measurement was 40-60 percent lower, i.e. indicating that an incomplete oxidation occur.

Table 4.5. COD results for the micro-nutrient solutions for EBPR and NUR.

	EBPR	**NUR**
Measured COD [mg/L]	6100	20200
Theoretical COD [mg/L]	9440	47200
Measured/theoretical %	65%	43%

Using the conversion factor of 0.944 g COD/g EDTA, an estimated contribution from EDTA in the reactors can be calculated. In the NUR test, EDTA was added in a concentration of 125 mg/L, which result in 118 mg COD/L fully oxidized. This show a very large effect. On the other hand, in the EBPR test, addition of 1.5 mL resulted in a theoretical concentration of 7.5 mg EDTA/L in the reactors, corresponding to 7.08 mg COD/L fully oxidized, i.e. a not so large effect. This effect would also be insignificant if the correct amount of nutrient solution had been added.

However, due to the large disturbance caused by the nutrient solutions on the COD measurements and other uncertainties regarding the amount of nutrient solution added to the reactors, it was decided to omit the nutrient solutions in the rest of the tests. Incorrect COD measurement affected the possibility to determine C/N or C/VSS ratios for example. It was especially important to stop the usage of nutrient solution (even though potential negative effects), as VFA data became unavailable, and SCOD results were the only measure of carbon utilisation during the activity tests.

In the beginning of testing period, it was also noted that the SCOD concentration of the washed and aerated sludge increased with addition of ATU (35-40% higher SCOD concentration than original sludge, see Table A.20). Additionally, only small effects were seen in inhibiting nitrification during aeration, due to low soluble NH_4^+ present after washing. Thus, it was decided to not use ATU in the following tests in order to avoid disturbances in the COD analyses, and get a more correct estimation of the amount of SCOD in the washed and aerated sludge.

4.4.2 Effect of omitting nutrient solutions

No large effect in the highest denitrification and P-release rates obtained with acetate were seen when omitting the nutrient solutions, as is evident when comparing test N2:A and N3:A, and P1:B and P2:A respectively. The same conclusion could be drawn with fermentate (comparing N2:B and N3:B), though it was expected that the effect would be small with fermentate since it is likely to contain some important micro- and macronutrients naturally.

It should be noted that small amounts of NH_4^+ and PO_4^{3-} were present in the initial samples in test N3:A (acetate) and N6:A (acetate + propionate) (0.15-0.2 mg NH_4^+ mg/L and 0.3-0.5 mg PO_4^{3-}-P/L). A small decline in NH_4^+ concentration over the course of the tests is indicated by the last measurements. But in test N10:A with propionate, the amount of ammonium was very low in the first sample (0.01 mg/L) and was almost completely removed in the beginning of the test. This could mean that the test with propionate was limited by insufficient amount of

ammonium. van Loosdrecht *et al.* (2016) recommended a concentration of at least 0.05 g NH_4^+-N/g SCOD for denitrification tests, which is in line with the general ratio of COD:N:P of 100:5:1 (Davies, 2006), and this suggests that there were too low amounts NH_4^+ and PO_4^{3-} in all tests with synthetic substrates. In the test with fermentate, average concentration of NH_4^+ and PO_4^3 concentration in the initial samples were 4.2±0.4 mg/L and 1.7±0.7 mg/L respectively, resulting in a better ratio but not ideal.

Overall, the effect of omitting the nutrient solutions seem to be negligible, but cannot be disregarded completely. More tests comparing the activity with and without nutrient solutions with different carbon sources should be conducted.

4.4.3 pH regulation

No large differences in the kinetics rates obtained in the tests with unregulated pH (N1:A and P1:A) compared to the tests with pH regulation were observed. Thus, the imperfect pH regulation explained in section 3.6.1 does not seem to have had that large effect on the obtained denitrification rates and P-release rates.

For a more exact pH regulation, it is recommended to use automatic pH control. It is also advisable to adjust the pH of the substrate and the sludge in the reactor to the desired set point before starting the tests, in order to avoid extremely high or low initial pH values.

4.4.4 Mixing conditions and discrepancy in MLVSS concentration

The reactors were slowly mixed at a low rpm of 70 in the majority of the tests. Higher speeds of around 90-100 rpm resulted in unstable equipment in the reactors (such as the pH and DO probes). A low speed can have the beneficial effect of reducing potential oxygen intrusion during the test, but there is also a risk of insufficient mixing and eventual anaerobic pockets occurring (van Loosdrecht *et al.*, 2016). Similar low speeds in the range of 60-100 rpm have been used in other tests (Liu *et al.*, 2016; Zhang *et al.*, 2011).

In a few test (N1, N2, N3 and N6), a substantial difference between the MLVSS concentration in the first and final sample in some of the reactors was noted, especially in the reactors with acetate as substrate (Table A.21). The highest difference occurred in sample N3:A, where the final MLVSS concentration was only 68% of the initial sample. In tests N1, N2 and N6, the percentage were between 76-85%. This could indicate insufficient mixing and non-homogenous conditions. Unfortunately, it was difficult to assess the mixing conditions in large detail during the tests, as the reactor lid and the water bath obscured the view. In-homogenous conditions is of major concern as it could have led to unrepresentative NUR curves and errors in the calculated denitrification rates.

Especially the tests with acetate can have been affected, leading to a possible overestimation of the actual working MLVSS concentration and thus underestimating the specific denitrification rate with acetate. Although, no other indications of inhomogeneous-conditions, such as unstable removal rates, was observed in the tests. The specific denitrification rate observed with acetate in this study was on average 5.6 mg NO_3^--N/g VSS·h. Other studies have shown k_{D1} rates in the range of 12-16 mg/g VSS·h (Peng *et al.*, 2007; Zhang *et al.*, 2016), which are significantly larger. Although, others have observed similar rates in the range of 3-7 mg/g VSS·h (Kujawa & Klapwijk, 1999; Naidoo *et al.*, 1998), showing that the rates obtained are not unreasonable. The rates from other studies are not directly comparable due to different process configurations and temperatures (between 20-25 °C), but can give a general indication of reasonable ranges of

k_{D1} for acetate. Nevertheless, it is advisable to conduct more tests with acetate in order to reduce the uncertainties and gain a better comparison.

In all other tests the difference between $MLVSS_{final}$ and $MLVSS_{initial}$ were less than 10%, indicating that the mixing itself might not be the sole problem in the first tests. On average, the percentage $MLVSS_{final}/MLVSS_{inital}$ was 89% in all NUR tests. No substantial difference was noted in the EBPR tests. Another explanation for the discrepancy could be different sludge properties, with more floating sludge or difference in settling properties in the beginning of the testing period in March, requiring higher mixing rates for more homogenous sludge samples.

Overall, the discrepancy in the MLVSS is a major uncertainty, and more tests should be conducted in order to gain more reliable results. The effect of different mixing speeds should be investigated if possible.

4.4.5 Washing and aeration

The washing procedure seem to have worked adequately, with only a 3-4% decrease in MLVSS concentration on average in all NUR tests, and negligible difference in the EBPR-tests. All soluble compounds tested (SCOD, NH_4^+ and PO_4^{3-}) decreased. In the test without ATU addition, the SCOD concentration in the aerated and washed sludge in the NUR test was 62% of the original concentration on average, and 79% in the EBPR tests. NH_4^+ and PO_4^{3-} decreased to less than 1 mg/L. The aeration period is likely to have affected the ammonium concentration, especially in the test without ATU, and a slight nitrification can be seen. However, as already explained, the effect of ATU was small, as the NO_3^- concentration in the tests with ATU in the washed and aerated sludge was between 0.2-0.4, and only around 0.5-0.9 mg/L in the tests without ATU. For more details, see Table A.20 in Appendix C.

It should be noted that the aeration period before the tests varied between 40-60 minutes. This can have led to differences in the amount of biodegradable COD present in the activated sludge before the tests started, as different amounts could have been removed during aeration, and thus contributed to different rate in the latter phases of the nitrate reduction curves. However, it is difficult to assess how large this difference is, as both the washing and the aeration affected the COD concentrations. Differences in biodegradable COD in the sludge could have occurred due to the washing procedure as well. It can also have varied due to differences in the original samples of activated sludge from the WWTP. In future tests, it is advisable to keep more similar lengths of the aeration period in all tests.

5 Conclusions

Through a series of batch activity tests, the suitability of using fermented filtered primary sludge as a carbon source for BNR process has been evaluated.

- Complex carbon sources containing a mixture of VFAs showed higher denitrification rates in the first phase of the NUR tests compared with sole carbon sources. The highest specific denitrification rates in the first phase of the NUR tests were obtained with the fermentates, varying between 7.0-8.9 mg NO_3^--N eqv/g VSS·h. Similar rates were obtained with the mixture of synthetic acetate and propionate (7.8 mg NO_3^--N eqv/g VSS·h). Significantly slower rates were obtained with sole carbon sources, with rates for acetate of 5.6 mg NO_3^--N eqv/g VSS·h on average, and 4.0 mg NO_3^--N eqv/g VSS·h for propionate.

- Similar nitrate removal efficiencies were obtained with fermentates and synthetic substrates over the whole course of the NUR tests, in the range of 83-95% after 3.5 hours.

- Full substrate consumption can be assumed to have occurred with synthetic substrates after the first reduction phase, whereas the fermentates seem to require a longer period before being fully consumed.

- Based on the conducted tests, the fermentates are comparable to the synthetic substrates as a carbon source for denitrification, and can be regarded as a suitable substrate.

- The highest specific denitrification rate observed in the test with filtered wastewater (4.6 NO_3^--N eqv/g VSS·h) was higher compared with the rate obtained with influent wastewater (3.5 NO_3^--N eqv/g VSS·h). This could be due to a slight increase in readily available COD during filtration. However, the overall removal efficiency was higher in the test with influent wastewater (83% compared to 77%), reflecting the higher content of XC_B in the latter substrate.

- The removal efficiency of the filtered wastewater increased by 13% with the applied addition of fermentate (45 mg COD/L), also taking into account the reduced availability of the added carbon due to a small increase in nutrient loading. The total COD content was significantly lower in this case compared with influent wastewater, indicating a more efficient COD utilisation.

- Since the general EBPR activity observed in the batch activity tests was low, it was difficult to evaluate the suitability of the fermentate as a carbon source for EBPR.

6 Future work

Batch activity tests are a viable method for studying process kinetics in small scale. Below, several suggestions for future works are presented.

- Study seasonal variations and effect of different retention times in the fermentates and how this affect the denitrification kinetics.

- More tests are required for a more reliable comparison between denitrification rates obtained with influent wastewater and filtered wastewater, as well as with filtered wastewater with added fermentate.

- It is recommended to ensure fully endogenous conditions in the activated sludge before the tests, through a more extensive washing and aeration procedure. This could enable a more clear difference between the denitrification rates on slowly biodegradable COD verses endogenous material, which would allow a better evaluation of substrate utilisation.

- Conduct more EBPR tests with a sludge with more prominent EBPR activity, to better evaluate the suitability of the fermentates as carbon sources for P release.

- Since a lot of samples for VFA analysis were collected, it would be interesting to study the VFA utilisation pattern in the different NUR tests in detail.

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Appendices

Appendix A

Methodology

An overview of the experimental procedure used in the different batch activity tests is presented in Table A.1 below.

Table A.1. Overview of experimental procedure.

Step	Experimental procedure
1	Preparation: Check calibration of probes and meters (pH, DO and thermometers), check pipettes, check stock solutions (macro/micro-nutrients, HCl, NaOH, NO_3^--N). Prepare water bath, reactors and containers for samples (correctly marked).
2	NUR: Fresh sludge sample collected at end of pre-denitrifying anoxic zone (2-4 L). P-release: Fresh sludge sample collected at end of aerobic zone (2-4 L).
3	Prepare carbon sources and substrates. 1 L substrate prepared for each reactor. Take sample of substrate media, check initial concentration: TCOD, SCOD, NH_4^+-N, PO_4^{3-}-P.[16] If the substrate is cold, let it stand in the water bath to adjust to temperature.
4	Take sample of sludge for MLSS/MLVSS determination and COD concentrations. Wash the sludge two times, removing residual SCOD, NOx and other nutrients. Pour 1 L sludge into 3 measuring glass á 1 L (around 333 mL sludge). Add tap water, stir gently, let settle for 15 minutes, separate water phase. Repeate.
5	Pour 1 L washed sludge in each sealed 2-L reactor in the water bath. Make sure pH- and DO probes are situated in the reactor properly. Start gentle mixing (70 rpm).
6	Aerate the sludge in order to remove additional residual COD and achieving endogenous conditions. Let sludge adjust to the water bath temperature 20 °C. Wait for at least 30 min to ensure stable conditions. Add ATU (10 mg/L) before aeration in order to inhibit nitrification.[17]
7	Take sample of the aerated sludge, check initial concentration: TCOD, SCOD, NO_3^--N, NO_2^--N, NH_4^+-N, PO_4^{3-}-P, MLSS, MLVSS. Stop aeration.

[16] NH4-N and PO4-P were not analyzed in substrates with acetate and propionate.
[17] ATU was not added in the majority of the tests as it increased COD concentration in the reactor and only inhibited minor nitrification.

8	Activation of N2-sparging from bottom of the reactor, for at least 10 min (5-10 L/min). Continue sparging throughout the test (from bottom during the beginning until no DO is detected, then through headspace 1 L/min).[18]
9	Verify temp, pH and DO of sludge before starting the test.
10	Add 1 L substrate media and 4 mL nitrate stock solution (10 g/L) to each reactor. Test started after addition of nitrate. Start concentration in reactor: Substrate media SCOD ≈ 110 mg/L.[19] NO3-N = 20 mg/L Add nutrient solutions.[20] NUR: 2.5 mL macro + 5 mL micro NUR. P-release: 2.5 mL macro + 1.5 mL micro EBPR.
11	Sampling at minute: 1, 10, 20, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240.[21] All samples immediately filtered through 0.45 µm syringe filter, stored in fridge. 1.5 mL saved for VFA analysis, stored in freezer. Sludge sample for analysis of MLSS, MLVSS and TCOD taken at minute 1 and at the end of the test, stored in fridge. TCOD analyses are conducted at the same day as the test.
12	Stop test after 2-4 hours. Clean probes, reactors and other equipment.

[18] Flowrate of N2-gas varied between the tests.
[19] Start concentration SCOD varied between the tests.
[20] Nutrient solutions were not added in the majority of the tests as it increased COD concentration in the reactor significantly.
[21] Time between test 1 and 2 was often 10 minutes instead of 9 minutes, leading to actual sample times offset with 1 minute, i.e. sampling time 1, 11, 21 etc.

Schematic overviews of the sampling of different compounds are presented in Table A.2 (NUR-tests) and Table A.3 (EBPR-tests).

Table A.2. Schematic overview of sampling of different compounds (NUR tests).

Time [min]		**1**	**10**	**20**	**30**	**45**	**60**	**75**	**90**	**120**	**150**	**180**	**210**	**240**		
Sample	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Substrate**	**Anox**
TCOD	x	x												x	x	x
SCOD	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
NO3-N	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
NO2-N	x	x			x		x			x		x		x		
PO4-P	x	x			x		x			x		x		x	x	x
NH4-N	x	x												x	x	x
MLSS	x	x												x		x
MLVSS	x	x												x		x
VFA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Table A.3. Schematic overview of sampling of different compounds (P-release tests).

Time [min]		**1**	**10**	**20**	**30**	**45**	**60**	**75**	**90**	**120**	**150**	**180**	**210**	**240**		
Sample	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Substrate**	**Anox**
TCOD	x	x												x	x	x
SCOD	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
NO3-N	x	x												x		
NO2-N	x	x												x		
PO4-P	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
NH4-N	x	x												x	x	x
MLSS	x	x												x		x
MLVSS	x	x												x		x
VFA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Preparation of stock solutions

1. **Preparation of NO_3^- stock solution**

NO_3^- stock solution with concentration 10 g NO_3^--N/L, used during the NUR tests, was prepared at Lund University before the testing period, and stored in a fridge during the testing period.

The solution was prepared according to the following description: 18.04 g of potassium nitrate (KNO_3) was measured and added to 250 mL distilled water, corresponding to a concentration of 72.16 g KNO_3/L, which in turn is equal to 10 g NO_3^--N/L.

The concentration of the stock solution checked at Källby WWTP (February 19th). 1 mL of solution was mixed in a 1-L volumetric flask with distilled water, in order to achieve 1/1000 dilution. The result from the concentration check is presented in Table A.4 below.

Table A.4. Check of NO_3^- concentration in nitrate stock solution

Sample	Dilution	Raw data	Result	Comparison
1	1000	9.85 mg NO_3^--N/L	9.85 g NO_3^--N/L	10 g NO_3^--N/L
2	1000	9.78 mg NO_3^--N/L	9.78 g NO_3^--N/L	10 g NO_3^--N/L

2. **Preparation of ATU stock solution**

Stock solution of allylthiourea (ATU, $C_4H_8N_2S$), with a concentration of 10 g/L was prepared at Lund University in the before the testing period started. 1 g of ATU was measured and mixed with 100 mL distilled water, and the solution was stored in fridge.

3. **Preparation of HCl and NaOH for pH regulation**

Solutions of hydrochloric acid (HCl) and sodium hydroxide (NaOH) were prepared before the first test N1 at Källby WWTP, in concentrations of 1 M. The solutions was stored in volumetric flasks á 1 L. 83.3 mL of a 12 M HCl-solution was added mixed with distilled water to a total volume of 1 L, and 39.9 g NaOH was weighted and mixed with 1 L distilled water.

In tests N5/P3, N6 and N7, a diluted HCl-solution of 0.5 M was used in order to achieve a more fine-tuned pH regulation.

4. **Acetate**

A 250 mL stock solution of acetate with concentration 20 g COD/L was prepared at Lund University in the beginning of the project. The following recipe was used:

- 25.634 g sodium acetate per L equal a concentration of 20 g COD/L.
- 250 mL stock solution → 6.4085 g added to 250 mL distilled water.

The stock solution was stored in fridge for usage in the different batch activity tests. The COD concentration of the stock solution was tested February 22nd by mixing 2 mL stock solution with distilled water in a 100 mL volumetric flask to achieve a 1/50 dilution. The result from the COD check is presented in Table A.5 below.

5. Propionate

Propionic acid ($C_3H_6O_2$) was used to prepare a 250 mL stock solution of propionate with the concentration of 20 g COD/L. The following recipe was used:

- 13.362 mL propionic acid (99%) per L equal a concentration of 20 g COD/L.
- 250 mL stock solution → 3.3405 mL mixed with distilled water, total volume 250 mL.

The stock solution was stored in fridge. The COD concentration was also checked February 22nd, by preparing a 1/50 dilution (see above description). The result is presented in

Table A.5.

Table A.5. COD concentration check of acetate and propionate stock solutions

Sample	Dilution	Raw data	Result	Comparison
Acetate	50	392 mg COD/L	19.6 g COD/L	20 g COD/L
Propionate	50	405 mg COD/L	20.25 g COD/L	20 g COD/L

pH calibration

Calibration results of pH probes are presented in Table A.6. The results from the pH probe check before every tests are presented in Table A.7.

Table A.6. Calibration result of pH probes.

pH probe	Slope [mv/pH]	Zero point [pH]
1	59.14	6.98
2	58.76	7.00

Table A.7. pH probe check.

pH check	**8.00**	**8.00**	**8.00**	**7.00**	**7.00**	**7.00**	**7.00**	**7.00**	**7.00**	**7.00**
pH probe	**N2**	**N3**	**P2**	**N4**	**N5/P3**	**N6**	**N7**	**N8**	**N9**	**N10**
1	7.97	7.97	7.97	6.97	6.97	6.97	6.97	6.97	6.97	6.97
2	8.01	8.00	8.00	7.00	7.00	7.01	7.00	7.01	6.99	7.01

Hach LCK cuvette tests

1. Chemical oxygen demand – COD

Two different COD cuvettes were used for analysis of COD and SCOD concentration in the samples: LCK 114, in the range 150-1 000 mg/L, and LCK 314, in the range 15-150 mg/L. Both cuvette tests use potassium dichromate ($K_2Cr_2O_7$) as an oxidant. For LCK 114, the green coloration of Cr^{3+} is evaluated photometrically (wavelength ~620 nm), whereas for LCK 314, the reduction in yellow coloration of Cr^{6+} is evaluated (wavelength ~420 nm) (Hach Company, 2021).

Chloride ions can be oxidized during the COD-analysis. In order to reduce chloride interference, chloride ions are masked with mercery sulphate (Balmér, 2015). However, both cuvettes have a chloride concentration limit of 1500 mg/L chloride.

Heating blocks of from Hach, heater LT200, was used in order to heat the COD-samples 148±2 °C during 2 hours according to the specified procedure.

When the COD concentration was believed to be under 150 mg/L, LCK 314 cuvettes were used, whereas LCK 114 cuvettes were used for samples over 150 mg/L. During tests when an overlap occurred (test N2), both cuvettes were used for different samples.

2. Nitrate and nitrite

For analysis of NO_3^-, LCK 339 was used, with the range 0.23-13.50 mg/L NO_3^--N/L. As the range was lower than 20 mg/L which was the start concentration in the reactor, the samples were diluted 2 times until the concentration was below 10 mg/L.

The cuvette test LCK 342 was used for analysis of NO_2^-, in the range of 0.6-6.0 mg NO_2^--N /L. No dilution needed.

3. Phosphate

During the experimental period, three different cuvettes for analysis of PO_4^{3-} were used:

- LCK 349 in the range 0.05-1.50 mg PO_4^{3-}-P /L
- LCK 348 in the range 0.5-5.0 mg PO_4^{3-}-P /L
- LCK 350 in the range 2-20 mg PO_4^{3-}-P /L

In the start of the experimental period (during tests N1-N5 and P1-P3), LCK 349 and LCK 350 were used in combination depending on expected concentration. Sometimes, the samples were diluted up to 2 times if the concentration was expected to be in-between the two ranges (between 1-2 mg/L). In the following tests, N6-N10, LCK 348 was mostly used, which often did not require any dilution of the samples. For samples with high expected PO_4^{3-}-P concentration, higher than 2 mg/L and possibly higher than 5 mg/L, LCK 350 was used.

4. Ammonium

Two different cuvettes for analysis of ammonium (NH_4^+-N) were available: LCK304 in the range of 0.015-2.000 mg NH_4^+-N/L and LCK 303 in the range 2.0-47.0 mg NH_4^+-N/L.

In the beginning of the testing period (during tests N1-N2 and P1), only LCK 303 was available, thus some samples appeared under range (< 2 mg/L). When only LCK 304 cuvettes where available, samples which were expected to have large NH_4^+ concentration was diluted up to 10

times (eg. during test N5). When both cuvette tests were available, they were used depending on expected concentration.

LCK 304 required a large sample volume of 5 mL, thus sometimes dilution was necessary even though the concentration was expected to be low, which was the case during test N3. The sampled volume was increased during the following test to ensure enough sample for ammonium analysis without dilution.

Ion chromatography

Detialed description of analysis of soluble compounds using the ion chromatography system at Lund University is presented below. The lower limit and the OK upper region for each substance of interest are presented in Table A.8.

Table A.8. Concentration ranges for different substances for ion chromatography system

Substance	Unit	Lower limit	OK upper region
NO_3^-	mg N/L	0.05	15
NO_2^-	mg N/L	0.02	3
PO_4^{3-}	mg P/L	0.1	15
NH_4^+	mg N/L	0.1	15

As the OK upper region for nitrate was around 15 mg/L, and the start concentration in the reactors were 20 mg/L, samples 1-13 were diluted 2 times (4 mL sample + 4 mL distilled water). The possibility to only dilute the first samples, before the concentration dropped below 15 mg/L, was discussed. However, it was difficult to filtrate enough sample through 0.45 syringe filters and it was decided to dilute all samples. Sample 0 and other additional samples such as anoxic sludge sample were analysed without dilution (total 8 mL). At least one blank sample with distilled water was analysed during each session.

The samples were prepared and shaken before placement in the autosampler. Each sample was registered in the computer. An equilibration period of around 30 minutes was conducted before the real analysis began. Each sample was analysed for 30 minutes.

Appendix B

Results: Figures

Nitrite curves for all tests are presented in Figure A.1 (synthetic substrates), Figure A.2 (fermentate) and Figure A.3 (wastewater).

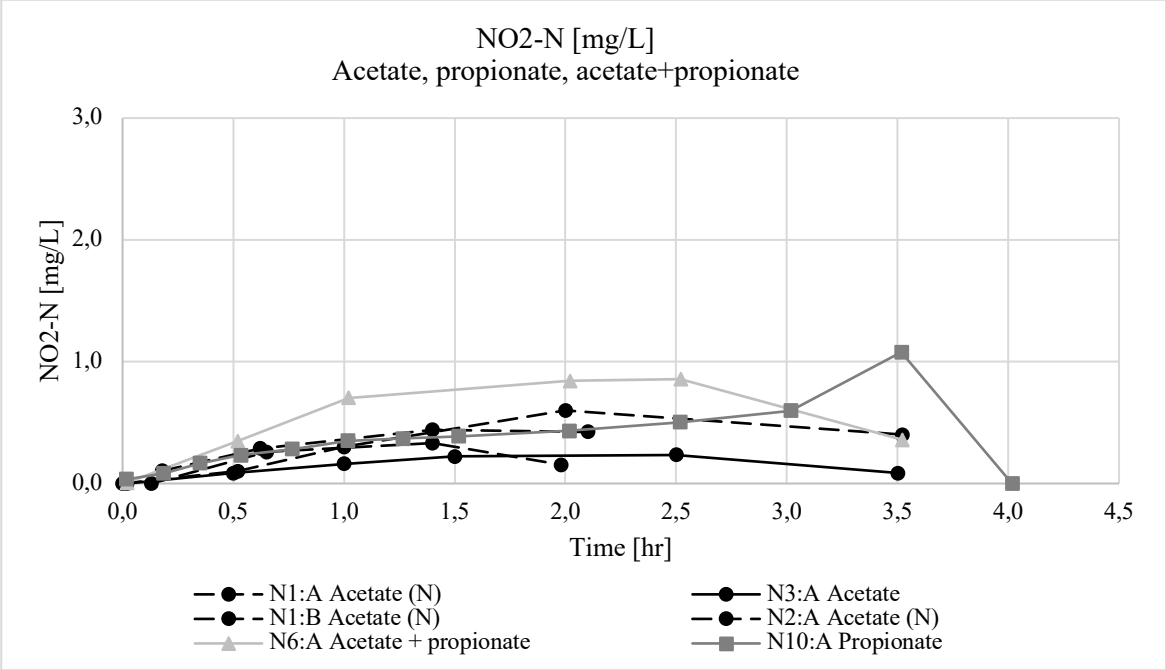


Figure A.1. Nitrite curves for synthetic substrates.

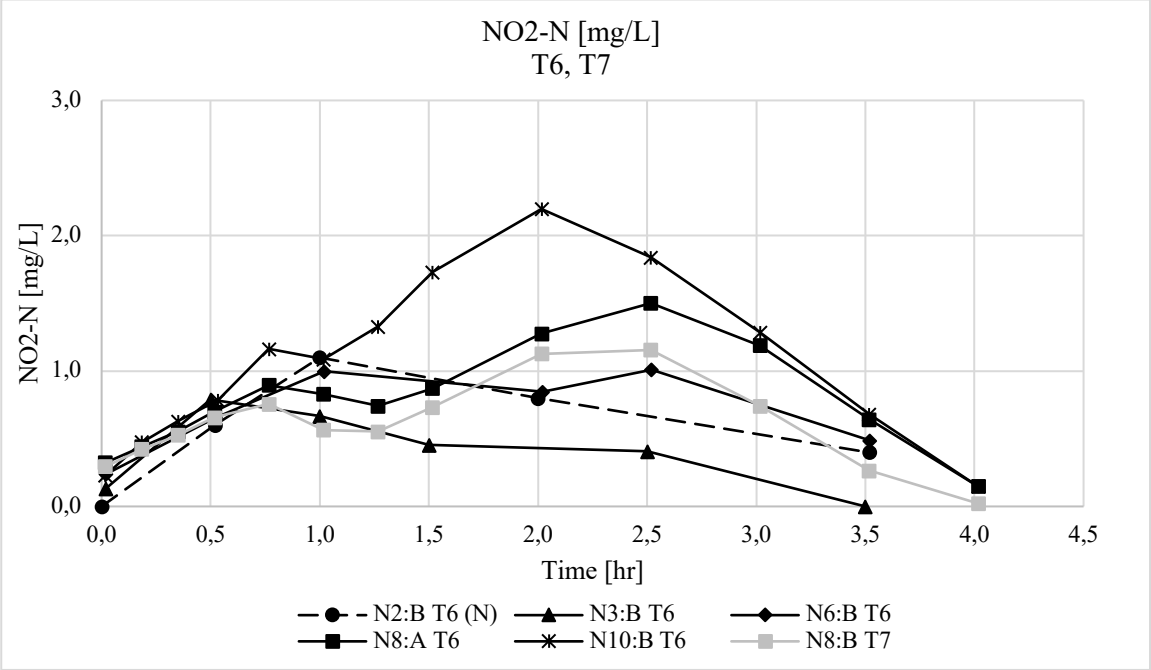


Figure A.2. Nitrite curves for fermentate.

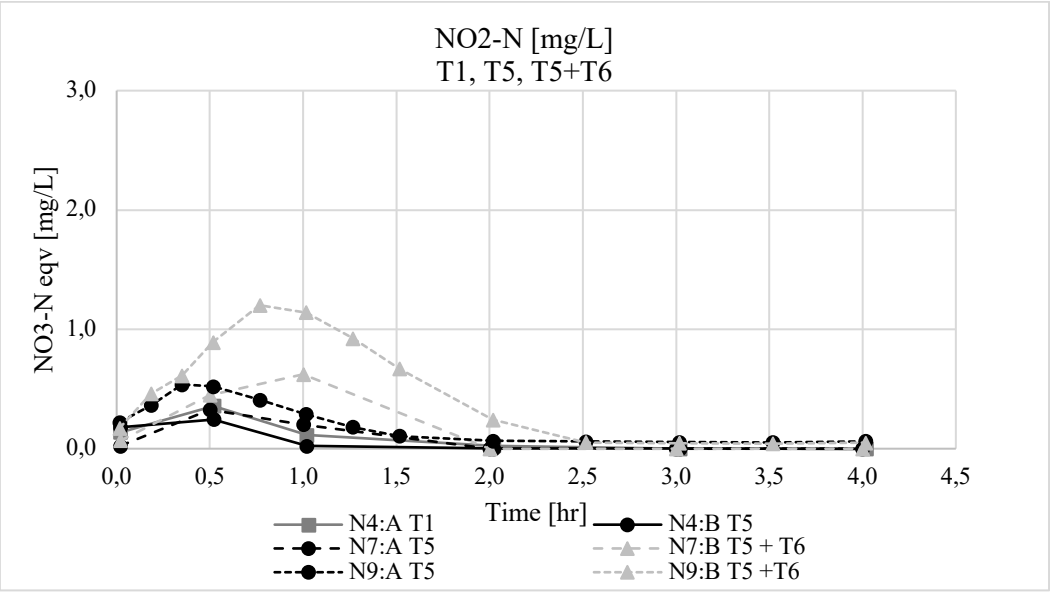
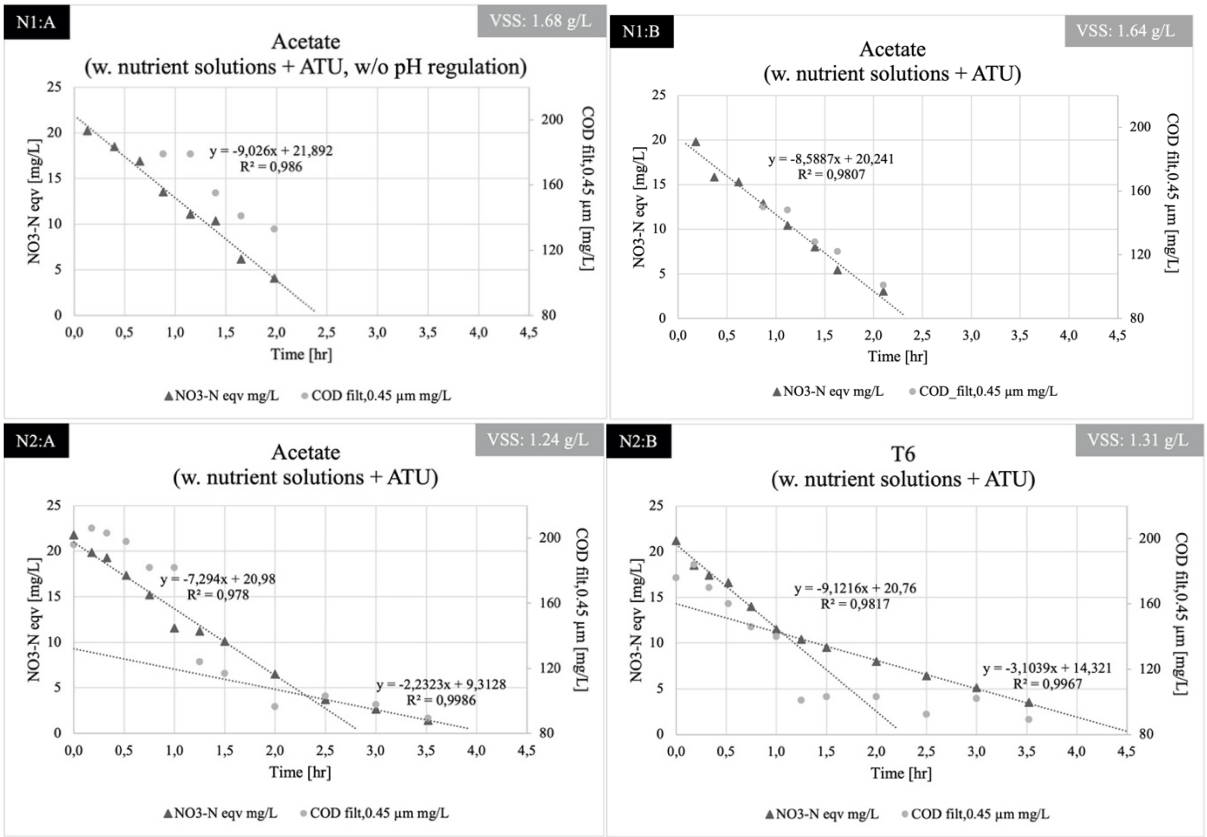
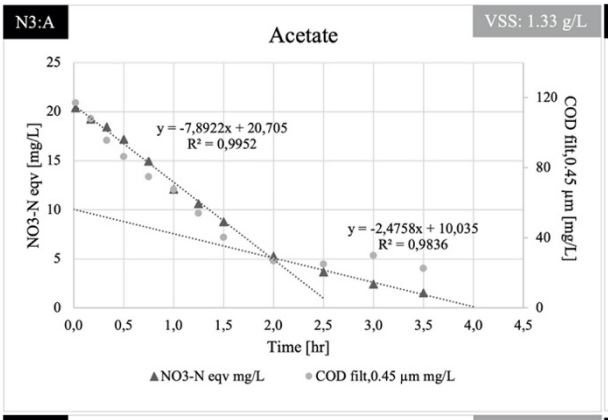
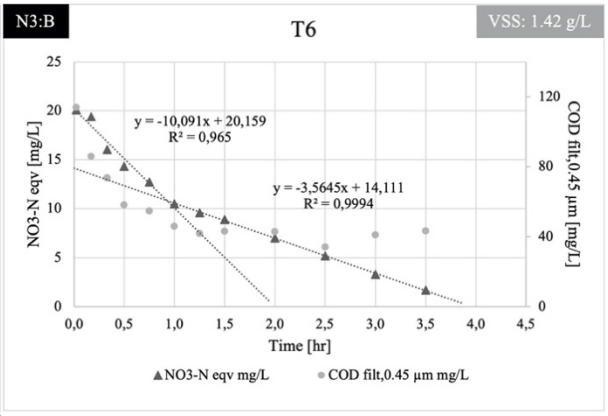


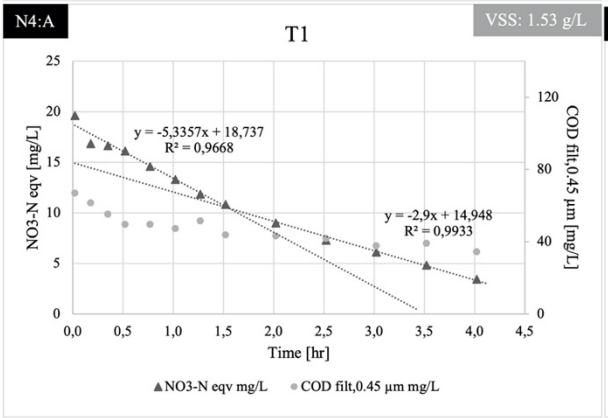
Figure A.3. Nitrite curves for tests with wastewater.

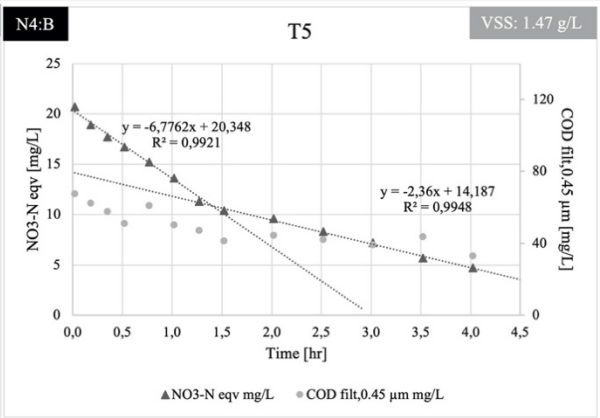
The NUR-curves for all conducted tests are presented in Figure A.4.

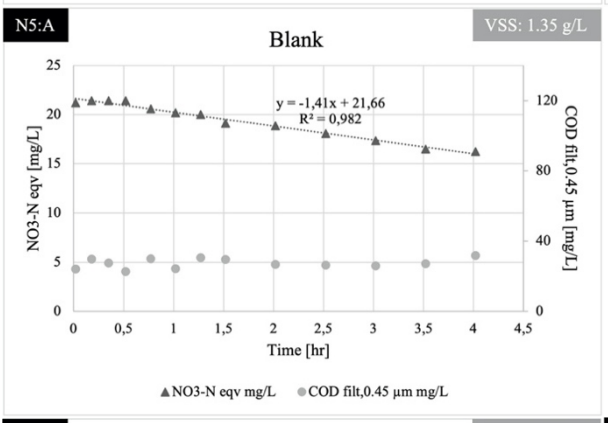


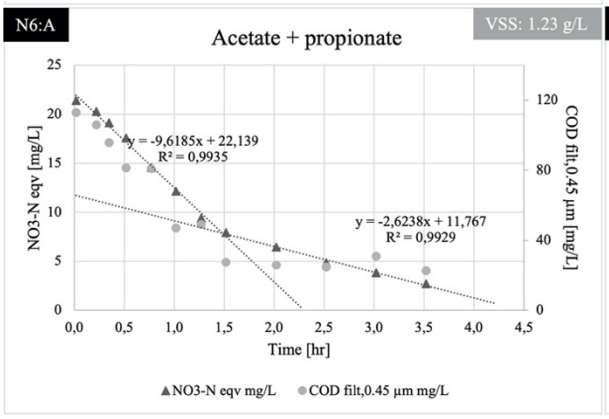

N3:A
Acetate
VSS: 1.33 g/L
y = -7,8922x + 20,705
R² = 0,9952
y = -2,4758x + 10,035
R² = 0,9836
NO3-N eqv [mg/L]
COD filt,0.45 µm [mg/L]
Time [hr]
▲NO3-N eqv mg/L
COD filt,0.45 µm mg/L

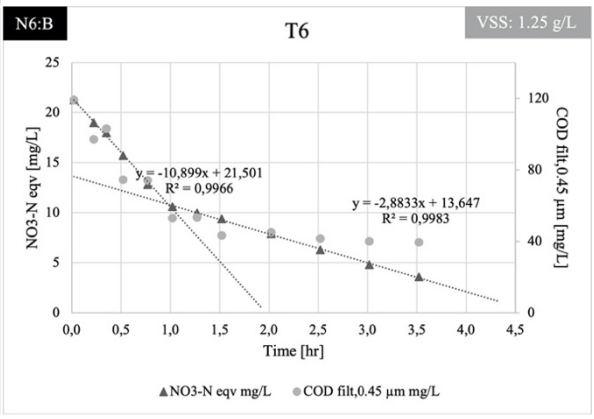

N3:B
T6
VSS: 1.42 g/L
y = -10,091x + 20,159
R² = 0,965
y = -3,5645x + 14,111
R² = 0,9994
NO3-N eqv [mg/L]
COD filt,0.45 µm [mg/L]
Time [hr]
▲NO3-N eqv mg/L
COD filt,0.45 µm mg/L


N4:A
T1
VSS: 1.53 g/L
y = -5,3357x + 18,737
R² = 0,9668
y = -2,9x + 14,948
R² = 0,9933
NO3-N eqv [mg/L]
COD filt,0.45 µm [mg/L]
Time [hr]
▲NO3-N eqv mg/L
COD filt,0.45 µm mg/L


N4:B
T5
VSS: 1.47 g/L
y = -6,7762x + 20,348
R² = 0,9921
y = -2,36x + 14,187
R² = 0,9948
NO3-N eqv [mg/L]
COD filt,0.45 µm [mg/L]
Time [hr]
▲NO3-N eqv mg/L
COD filt,0.45 µm mg/L


N5:A
Blank
VSS: 1.35 g/L
y = -1,41x + 21,66
R² = 0,982
NO3-N eqv [mg/L]
COD filt,0.45 µm [mg/L]
Time [hr]
▲NO3-N eqv mg/L
COD filt,0.45 µm mg/L


N6:A
Acetate + propionate
VSS: 1.23 g/L
y = -9,6185x + 22,139
R² = 0,9935
y = -2,6238x + 11,767
R² = 0,9929
NO3-N eqv [mg/L]
COD filt,0.45 µm [mg/L]
Time [hr]
▲NO3-N eqv mg/L
COD filt,0.45 µm mg/L


N6:B
T6
VSS: 1.25 g/L
y = -10,899x + 21,501
R² = 0,9966
y = -2,8833x + 13,647
R² = 0,9983
NO3-N eqv [mg/L]
COD filt,0.45 µm [mg/L]
Time [hr]
▲NO3-N eqv mg/L
COD filt,0.45 µm mg/L

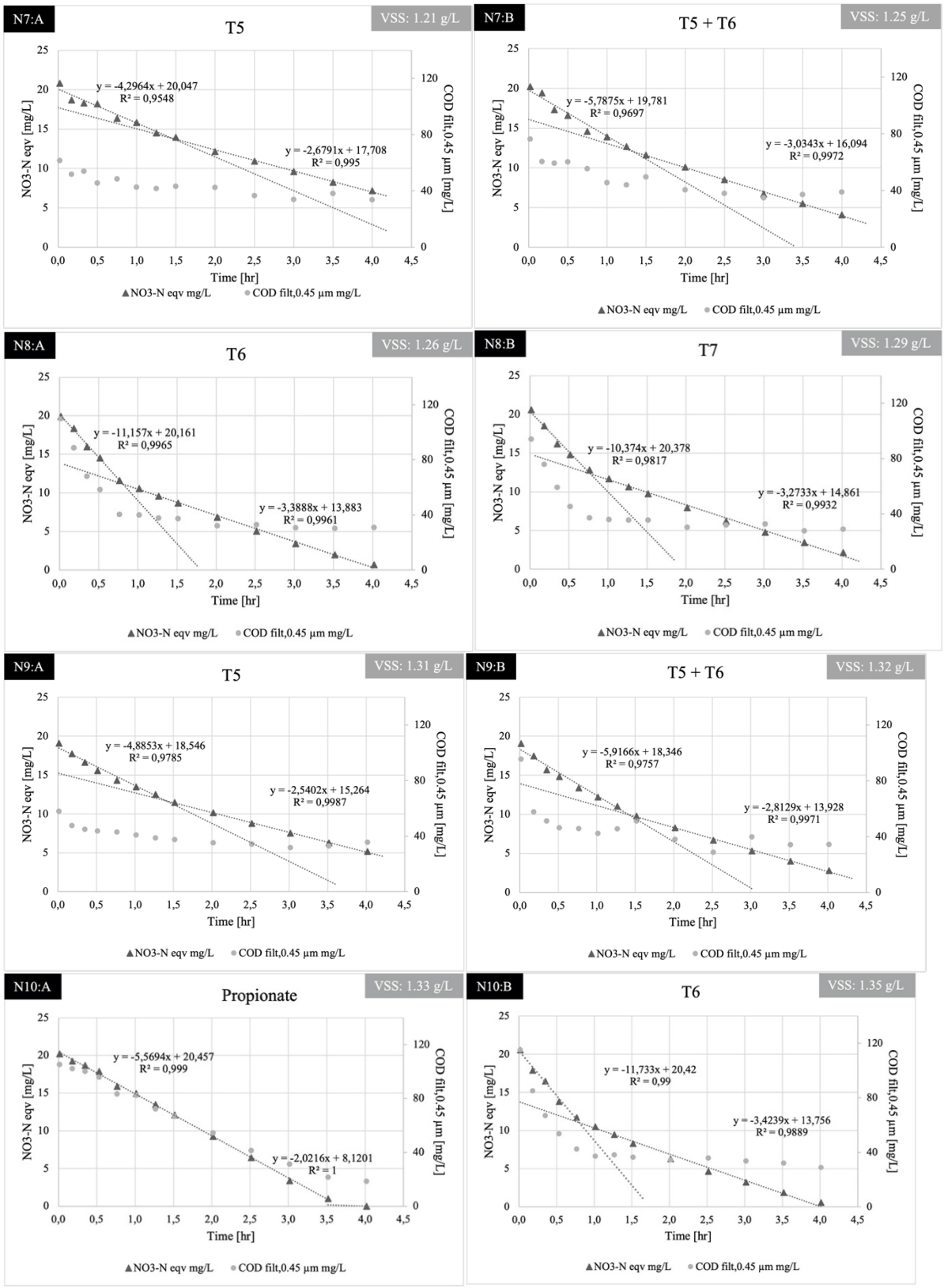


Figure A.4. NUR curves for all conducted tests.

The total SCOD and all VFA-C measurements for test N10:B with fermentate T6 as substrates are presented in Figure A.5.

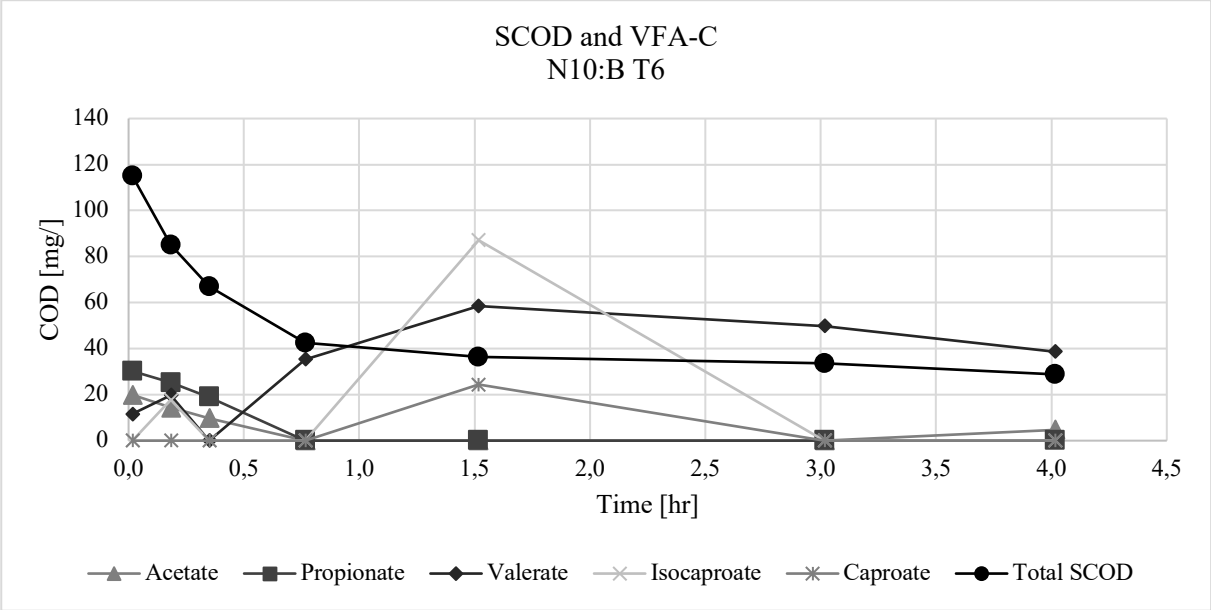


Figure A.5. SCOD and VFA-C curves in test N10:B with fermentate T6 as substrate.

Appendix C

Results: Tables

Table A.9. Carbon sources, SCOD concentrations, VSS concentrations, start ratios of SCOD/VSS- and SCOD/NO3-N-ratios in reactor A and B in all tests.

	Reactor A					Reactor B				
	Carbon source	**SCOD**	**VSS**	**SCOD/ VSS**	**SCOD/ NO3-N eqv**	**Carbon source**	**SCOD**	**VSS**	**SCOD/ VSS**	**SCOD/ NO3-N eqv**
Unit		**mg/L**	**g/L**	**mg/mg**	**mg/mg**		**mg/L**	**g/L**	**mg/g**	**mg/mg**
N1	Acetate	110*	1.68	0.07*	5.50*	Acetate	110*	1.64	0.08*	5.50*
N2	Acetate	123**	1.24	0.10	5.64	T6	115**	1.31	0.09	5.42
N3	Acetate	117	1.33	0.09	5.74	T6	114	1.42	0.08	5.68
N4	T1	66.9	1.53	0.04	3.41	T5	67.4	1.47	0.05	3.25
N5	Blank	24.2	1.35	0.02	1.14	-	-	-	-	-
N6	Acetate + propio-nate	113	1.23	0.09	5.28	T6	119	1.25	0.10	5.58
N7	T5	61.6	1.21	0.05	2.96	T5 + T6	76.3	1.25	0.06	3.77
N8	T6	110	1.26	0.09	5.52	T7	94.1	1.29	0.07	4.57
N9	T5	57.8	1.31	0.04	3.03	T5 + T6	95.6	1.32	0.07	5.02
N10	Propio-nate	105	1.33	0.08	5.20	T6	115	1.35	0.09	5.60
P1	Acetate	110*	1.46	0.08	-	Acetate	110*	1.50	0.07	-
P2	Acetate	118	1.50	0.08	-	T6	110	1.53	0.07	-
P3						Blank	23.3	1.38		-

* Assumed and calculated based on concentration of substrate solution (~220 mg SCOD/L)
** Measured before addition of nutrient solution and nitrate addition

Table A.10. SCOD and VFA-COD, fermentate T6 and T7, November and December 2020.

T6	SCOD [mg/L]	VFA-COD [mg/L]	VFA %
11-18-2020	8100	7392	91%
11-26-2020	7875	7219	92%
12-02-2020	7395	6661	90%
Average:	**7790**	**7091**	**91%**
T7	SCOD	VFA-COD	
11-18-2020	6925	5454	79%
11-26-2020	6975	5401	77%
12-02-2020	7650	6007	79%
Average:	**7183**	**5621**	**78%**

Table A.11. VFA composition, fermentate T6 and T7, November and December 2020.

T6	Hac	HPr	Hbu-iso	Hbu	Hval-iso	Hval	Hcap-iso	Hcap
11-18-2020	24%	36%	4%	14%	6%	9%	3%	3%
11-26-2020	24%	35%	4%	15%	6%	9%	4%	3%
12-02-2020	24%	33%	4%	16%	7%	10%	4%	3%
Average:	**24%**	**35%**	**4%**	**15%**	**6%**	**10%**	**3%**	**3%**
T7	Hac	HPr	Hbu-iso	Hbu	Hval-iso	Hval	Hcap-iso	Hcap
11-18-2020	27%	37%	3%	15%	6%	9%	1%	2%
11-26-2020	25%	34%	3%	18%	7%	10%	1%	2%
12-02-2020	26%	34%	3%	17%	7%	10%	1%	3%
Average:	**26%**	**35%**	**3%**	**16%**	**6%**	**10%**	**1%**	**2%**

Table A.12. Prepared substrates with fermentate T6 and T7.

Substrate	Unit	N2:B T6	N3:B T6	N6:B T6	N8:A T6	N8:B T7	N10:B T6	P2:B T6
COD*	mg/L	328	294	360	342	304	336	317
$COD_{filt,0.45\ \mu m}$	mg/L	249	231	248	242	215	219	228
$COD_{filt,0.45\ \mu m}$/ COD	*%*	*76 %*	*79 %*	*69 %*	*71 %*	*71 %*	*65 %*	*72 %*
PO_4^{3+}-P	mg/L	3.72	3.33	3.9	3.7	3.52	3.68	3.54
NH_4^+-N	mg/L	11.5	9.27	11.8	9.8	8.87	8.8	9.86
Addition of fermentate	mL	25	25	24.5	24.5	24.5	24.5	25

* Reflect the $COD_{filt,18.5\ \mu m}$ in the fermentate

Table A.13. Prepared substrates with influent wastewater (T1), filtered wastewater (T5) and filtered wastewater with addition of fermentate (T5+T6).

	Unit	N4:A T1	N4:B T5	% T5/T1	N7:A T5	N7:B T5+T6	% T5+T6/ T5	N9:A T5	N9:B T5+T6	% T5+T6/ T5
COD	mg/L	557	312	*56 %*	329	386	*117 %*	261	307	*118 %*
$COD_{filt,0.45\ \mu m}$	mg/L	112*	124*	*111%*	132	195	*148 %*	129	194	*150 %*
PO_4^{3+}-P	mg/L	-	-	-	3.53	4.18	*118 %*	4.17	4.86	*117 %*
NH_4^+-N	mg/L	37.6	39.4	*105 %*	37.6	40.8	*109%*	41.5	42.7	*103 %*
$COD_{filt,0.45\ \mu m}$/ PO_4^{3+}-P	mg/mg				37	47	*127 %*	31	40	*129%*
$COD_{filt,0.45\ \mu m}$/ NH_4^+-N	mg/mg				3.5	4.8	*137 %*	3.1	4.5	*145%*

** Filtered with vacuum filter

Table A.14. Data of filtered fermented primary sludge.

Test	Date	Filtered fermented primary sludge	Temp °C	pH	$COD_{filt,18.5\ \mu m}$ mg/L	$COD_{filt,0.45\ \mu m}$ mg/L	NO3-N mg/L	NO2-N mg/L	PO4-P mg/L	NH4-N mg/L
N2	03-16-2021	T6	17.4	5.4	13120*	9960	-	-	150	480
N3	03-18-2021	T6	16.7	5.4	11760*	9360	-	-	140	390
P2	03-22-2021	T6	-	-	12680*	9120*	-	-	142*	394*
N6	04-06-2021	T6	19.3	5.3	14690*	10120*	-	-	159*	482*
N7	04-08-2021	T6	19.9	-	14180	8940	-	-	143	404
N8	04-19-2021	T6	20.0	5.5	13960*	9880*	-	-	151*	400*
N8	04-19-2021	T7	20.5	5.5	12410*	8780*	-	-	144*	362*
N9	04-21-2021	T6	23.2	5.2	12640	9720	0.56	0	140	391
N10	04-29-2021	T6	21.7	5.2	13920	10160	0.2	0	154	382

* Calculated from substrate measurements

Table A.15. Data of wastewater, T1 and T5.

Test	Date	Wastewater	Characterisation	Temp °C	pH	COD mg/L	$COD_{filt,0.45\ \mu m}$ mg/L	NO3-N mg/L	NO2-N mg/L	PO4-P mg/L	P-TOT mg/L	NH4-N mg/L
N4	03-24-2021	T1	X	< 5	7.7	557	112*	-	-	-	6.8	37.6
N4	03-24-2021	T5	X	< 5	7.8	312	124*	-	-	-	5.4	39.4
N7	04-08-2021	T5	-	< 5	-	329	132	-	-	3.53	5.3	37.6
N9	04-21-2021	T5	X	< 5	7.8	261	129/118*	0.258	0	4.17	-	41.5

* Filtrated with vacuum filter

Table A.16. COD characterisation of influent wastewater (T1), 24-03-2021.

Sample: Influent wastewater (T1)				
Type of sample: 24 hour sample				
Date characterisation: 24-03-2021				
Volume of filtered wastewater (mL)	**Filter (µm)**	**COD (mg/L)**	**COD (mg/L)**	
30	0.1	103	103	**<0,1 µm**
30	0.45	112	9	**0,1-0,45 µm**
30	1.6	171	59	**0,45-1,6 µm**
30	5	181	10	**1,6-5 µm**
30	10	266	85	**5-10 µm**
30	40	337	71	**10-40 µm**
30	60	369	32	**40-60 µm**
30	100	373	188	**>60 µm**
	Tot	557	557	**Tot**

Table A.17. COD characterisation of filtered wastewater (T5), 24-03-2021.

Sample: Filtered wastewater (T5)				
Type of sample: 24 hour sample				
Date characterisation: 24-03-2021				
Volume of filtered wastewater (mL)	**Filter (µm)**	**COD (mg/L)**	**COD (mg/L)**	
30	0.1	121	121	**<0,1 µm**
30	0.45	124	3	**0,1-0,45 µm**
30	1.6	158	34	**0,45-1,6 µm**
30	5	192	34	**1,6-5 µm**
30	10	260	68	**5-10 µm**
30	40	291	0	**10-40 µm**
30	60	289	-2	**40-60 µm**
30	100	296	23	**>60 µm**
	Tot	312	312	**Tot**

pH

The initial pH after addition of substrate are presented, as well as the max, min and average pH during pH regulation. First value before pH regulation started is omitted from the average, to get a more representative value of how well the pH was regulated. The max and min value represent the max and min pH in the reactor during regulation, in order to give a picture of the fluctuations in pH aside from the standard deviation.

Table A.18. Initial pH after addition of substrate, average pH values during pH regulation, and max and min pH value during regulation.

	Reactor A				**Reactor B**			
	Initial pH after addition of substrate	**Average pH during test**	**Max pH during test**	**Min pH during test**	**Initial pH after addition of substrate**	**Average pH during test**	**Max pH during test**	**Min pH during test**
N1	7.44	7.05±0.56*	7.44	6.65	6.66	7.07±0.24	7.70	6.68
N2	5.97	7.07±0.09	7.22	6.95	5.93	7.06±0.08	7.24	6.97
N3	7.87	7.10±0.09	7.32	6.95	7.18	7.09±0.07	7.30	6.97
N4	7.70	7.05±0.03	7.15	7.00	7.73	7.04±0.04	7.22	7.00
N5	7.69	7.03±0.04	7.15	6.93				
N6	6.68	7.06±0.07	7.32	6.98	7.14	7.05±0.08	7.42	6.97
N7	7.89	7.04±0.12	7.11	6.98	7.85	7.04±0.12	7.15	6.99
N8	6.90	7.04±0.07	7.22	6.90	6.94	7.05±0.09	7.35	6.94
N9	7.78	7.02±0.05	7.20	6.93	7.77	7.00±0.17	7.15	6.93
N10	5.83	7.01±0.10	7.30	6.79	6.88	7.02±0.06	7.20	6.86
P1	7.45	7.55±0.07*	7.62	7.45	7.54	7.00±0.07	7.11	6.85
P2	7.78	7.08±0.07	7.27	6.99	7.18	7.02±0.06	7.11	6.91
P3					7.94	7.02±0.05	7.17	6.92

* No pH regulation, average value during test

Temperature

Table A.19. Min, max and average temperature during tests.

	Reactor A			Reactor B		
	Average temp during test	**Max temp during test**	**Min temp during test**	**Average temp during test**	**Max temp during test**	**Min temp during test**
N1	19.85±0.29 °C	20.4 °C	19.6 °C	19.85±0.15 °C	20.1 °C	19.6 °C
N2	20.00±0 °C	20.0 °C	20 °C	20.00±0 °C	20.0 °C	20.0 °C
N3	19.96±0.13 °C	20.0 °C	19.6 °C	19.95±0.16 °C	20.0 °C	19.5 °C
N4	19.69±0.50 °C	19.9 °C	17.8 °C	19.79±0.47 °C	20.0 °C	18 °C
N5	19.85±0.20 °C	20.2 °C	19.8 °C			
N6	19.77±0.18 °C	19.9 °C	19.4 °C	19.83±0.15 °C	19.9 °C	19.5 °C
N7	19.90±0.00 °C	19.9 °C	19.9 °C	19.83±0.05 °C	19.9 °C	19.8 °C
N8	19.73±0.21 °C	20.0 °C	19.4 °C	19.75±0.20 °C	20.0 °C	19.5 °C
N9	19.93±0.08 °C	20.0 °C	19.8 °C	20.09±0.03 °C	20.1 °C	20.0 °C
N10	19.90±0.30 °C	20.4 °C	19.0 °C	20.08±0.21 °C	20.4 °C	19.7 °C
P1	20.00±0 °C	20.0 °C	20.0 °C	20.00±0 °C	20.0 °C	20.0 °C
P2	19.91±0.04 °C	20.0 °C	19.9 °C	20.00±0 °C	20.0 °C	20.0 °C
P3				19.92±0.20 °C	20.1 °C	19.8 °C

Table A.20. Average values of concentration of different compounds in anoxic sludge and aerobic sludge, as well as corresponding washed and aerated sludge, with and without ATU addition.

		Anoxic sludge		**Washed and aerated sludge (no ATU)**		**Washed and aerated sludge (with ATU)**	
		Average	*Stdav*	*Average*	*Stdav*	*Average*	*Stdav*
TCOD	*mg/L*	3913.33	310.09	3785.71	226.60	4041.25	
SCOD	*mg/L*	39.09	6.32	24.17	4.09	55.78	5.47
NO_3^--N	*mg/L*	0.68	1.07	0.52	0.15	0.24	9.72
NO_2^--N	*mg/L*	0.15	0.19	0.0	0.0	0.0	0.05
PO_4^{3-}-P	*mg/L*	1.85	0.46	0.17	0.18	0.32	1.81
NH_4^+-N	*mg/L*	7.02	2.13	0.14	0.08	0.93	4.03
MLSS	*mg/L*	3279	210	3187	181	1534	1770
MLVSS	*mg/L*	2640	177	2550	163	1245	1435
		Aerobic sludge		**Washed and aerated sludge (no ATU)**		**Washed and aerated sludge (with ATU)**	
		Average	*Stdav*	*Average*	*Stdav*	*Average*	*Stdav*
TCOD	*mg/L*	2850.0	360.62	4175.00	273.08	3795.0	
SCOD	*mg/L*	45.10	2.12	35.43	15.71	60.95	

NO_3^--N	*mg/L*			0.90	0.27	0.42	
NO_2^--N	*mg/L*			0.0	0.0		
PO_4^{3-}-P	*mg/L*			0.11	0.06	0.0	
NH_4^+-N	*mg/L*	0.81		0.23	0.10	0.06	
MLSS	*mg/L*	2348	148	3554	124	3459	
MLVSS	*mg/L*	2819	72	2874	92	2841	

Table A.21. MLSS and MLVSS concentration in the washed and aerated sludges, first and final samples, anoxic and aerobic sludges.

		Reactor A				Reactor B				
		Washed aerated sludge	**First sample**	**Final sample**	**$MLVSS_{final}$/ $MLVSS_{first}$**	**Washed aerated sludge**	**First sample**	**Final sample**	**$MLVSS_{final}$/ $MLVSS_{first}$**	**Anox/ aerob**
N1	MLSS [g/L]	4.08	2.01	1.69		4.06	1.97	1.53		3.91
	MLVSS [g/L]	3.30	1.68	1.42	85 %	3.26	1.64	1.28	78 %	3.19
	MLVSS [%]	81 %	83 %	84 %		80 %	83 %	84 %		81 %
N2	MLSS [g/L]	2.95	1.49	1.11		3.18	1.56	1.31		3.20
	MLVSS [g/L]	2.41	1.24	0.94	76 %	2.56	1.31	1.10	84 %	2.55
	MLVSS [%]	82 %	83 %	84 %		81 %	83 %	85 %		80 %
N3	MLSS [g/L]	3.33	1.62	1.05		3.42	1.74	1.52		3.50
	MLVSS [g/L]	2.64	1.33	0.91	68 %	2.76	1.42	1.28	90 %	2.82
	MLVSS [%]	80 %	82 %	87 %		81 %	82 %	84 %		81 %
N4	MLSS [g/L]	3.49	1.85	1.83		3.37	1.75	1.70		3.61
	MLVSS [g/L]	2.79	1.53	1.52	99 %	2.74	1.47	1.41	96 %	2.91
	MLVSS [%]	80 %	83 %	83 %		81%	84 %	83 %		81 %
N5	MLSS [g/L]	3.26	1.64	1.57						3.34
	MLVSS [g/L]	2.66	1.35	1.31	97 %					2.74
	MLVSS [%]	82 %	82 %	83 %						82 %
N6	MLSS [g/L]	3.06	1.51	1.13		2.92	1.53	1.22		3.12
	MLVSS [g/L]	2.43	1.23	0.93	76 %	2.33	1.25	1.02	82 %	2.50
	MLVSS [%]	80 %	82 %	83 %		80%	82%	84 %		80 %
N7	MLSS [g/L]	2.88	1.51	1.48		2.92	1.54	1.49		3.00
	MLVSS [g/L]	2.29	1.21	1.20	99 %	2.33	1.25	1.19	96 %	2.44
	MLVSS [%]	80 %	81 %	81 %		80%	81%	80 %		81 %
N8	MLSS [g/L]	3.09	1.61	1.49		3.07	1.64	1.50		3.18
	MLVSS [g/L]	2.40	1.26	1.23	98 %	2.41	1.29	1.25	97 %	2.53
	MLVSS [%]	78 %	78 %	83 %		78%	79%	83 %		80 %
N9	MLSS [g/L]	3.12	1.60	1.59		3.14	1.63	1.60		3.20
	MLVSS [g/L]	2.51	1.31	1.29	98 %	2.52	1.32	1.30	98 %	2.54

	MLVSS [%]	80 %	82 %	81 %		80 %	81 %	81 %		79 %
N10	MLSS [g/L]	3.24	1.64	1.42		3.25	1.67	1.56		3.26
	MLVSS [g/L]	2.58	1.33	1.18	89 %	2.63	1.35	1.24	92 %	2.62
	MLVSS [%]	80 %	81 %	83 %		81 %	81 %	80 %		80 %
P1	MLSS [g/L]	3.54	1.70	1.73		3.38	1.73	1.68		3.53
	MLVSS [g/L]	2.90	1.46	1.49	102 %	2.78	1.50	1.47	98 %	2.91
	MLVSS [%]	82 %	86 %	86 %		82 %	87 %	87 %		83 %
P2	MLSS [g/L]	3.62	1.79	1.79		3.65	1.83	1.80		3.63
	MLVSS [g/L]	2.94	1.50	1.45	97 %	2.94	1.53	1.48	97 %	2.87
	MLVSS [%]	81 %	84 %	82 %		81 %	84 %	82 %		79 %
P3	MLSS [g/L]					3.39	1.64	1.56		3.42
	MLVSS [g/L]					2.74	1.38	1.31	95 %	2.77
	MLVSS [%]					81 %	84 %	84 %		81 %