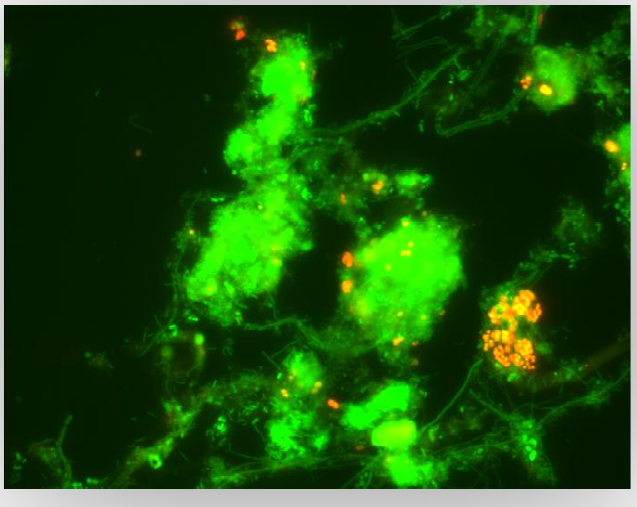
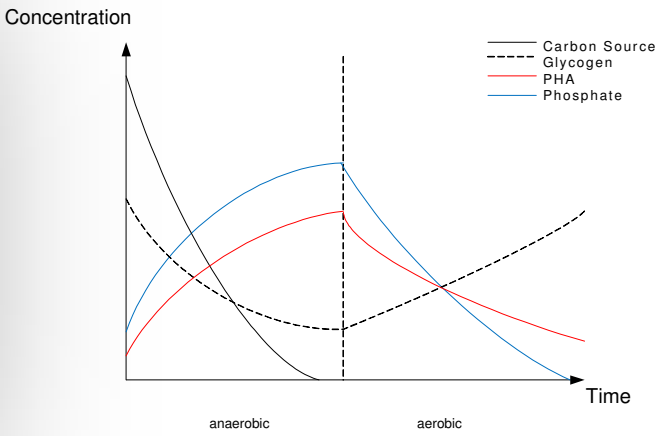
Kinetics and stoichiometry of P-release with different carbon sources in the anaerobic phase of the biological phosphorus removal process in activated sludge wastewater treatment plants





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Title

Kinetics and stoichiometry of P-release with different carbon sources in the anaerobic phase of the biological phosphorus removal process in activated sludge wastewater treatment plants

This thesis was prepared and submitted at

Department of Biotechnology, Chemistry and Environmental Engineering

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"You don't find fish in lakes and rivers anymore. You have to catch them in cans.

Towns die. Oil spills. Money talks"

Joseph Heller – Something Happened (1974)

"Everything is from water and everything comes from water."

Tales from Milet (VII century)

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Abstract

The anaerobic part of the enhanced biological phosphorus removal process is important and must be conducted in proper extracellular conditions to obtain a well-working EBPR plant. One of them is the supply of a suitable carbon source for polyphosphate accumulating organisms (PAO). The purposes of the project were to analyze kinetic and stoichiometry of anaerobic P-release process with amino acids comparing to the VFAs as carbon source, to analyze the most suitable pH conditions for AAs uptake by bacteria and to compare the bacterial community structure and its influence on the P-release process. Activated sludge from three Danish wastewater treatment plants: Aalborg West, Hjørring and Bjerringbro, was used to conduct laboratory tests on P-release.

The batch experiments conducted in the project led to the observation that amino acids can be regarded as a carbon source for the anaerobic phase of the EBPR process, however, not all of them. Among 20 amino acids glycine had the highest influence on the process. Lab tests showed that pH conditions 7-8 were the most suitable for anaerobic P-release with usage of AAs as carbon source. Besides that, it was observed high variety in the process due to microbial population structure and the period of the year.

Keywords: bio-P removal, AS WWTPs, VFAs, amino acids, P-release rate, acetic acid, pH,

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1. List of abbreviations

Abbreviation	-	Definition
AAs	-	Amino Acids
APAO	-	*Actinobacteria*-related PAO
AS	-	Activated Sludge
COD	-	Chemical Oxygen Demand
DSVI	-	Diluted Sludge Volume Index
EBPR	-	Enhanced Biological Phosphorus Removal
GAOs	-	Glycogen Accumulating Organisms
HAc	-	Acetic Acid
HLa	-	Lactic Acid
HPLC	-	High Performance Liquid Chromatography
HPr	-	Propionic Acid
N	-	Nitrogen
P	-	Phosphorus
PAOs	-	Polyphosphate Accumulating Organisms
PE	-	Population Equivalent
PHAs	-	Polyhydroxyalkanoates
PHB	-	Polyhydroxybutyrate
PHV	-	Polyhydroxyvalerate
PMF	-	Proton Motive Force
Poly-P	-	Polyphosphate
RBCOD	-	Ready Biodegradable COD
SBR	-	Sequencing Batch Reactor
SS	-	Suspended Solids
FI	-	Filamentous Index
VFAs	-	Volatile Fatty Acids
VSS	-	Volatile Suspended Solids
WWTP	-	Wastewater Treatment Plant

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2. Introduction

In the last thirty years, nutrient enrichment has been recognized as a major threat for the condition of marine ecosystems and resources. The increase of nitrogen and phosphorus concentrations in the sea is caused by anthropogenic activities, particularly discharges from: agriculture, urban wastewater treatment plants and separate discharges from industries. Nutrient enrichment entails the process of euthrophication which leads to: accelerated growth of phytoplankton, micro- and macroalgae and undesirable disturbance to the balance of water organisms. The rise of algae concentrations influence on light penetration and increase oxygen consumption in the sea. The effect of the last process results in creation of areas with oxygen depletion and with totally anaerobic conditions – "dead zones" [19]. Low oxygen concentrations on the bottom of the sea result in the release of hydrogen sulphide from the sediments which has negative impact on the benthic organisms [21]. From figure 1 it can be seen that the situation in Danish coastal waters is not satisfactory. There are many places with very low concentration (0-2 mgO_2/L) during autumn, especially around Fyn. The seawater quality could be improved by reduction in nutrient discharges and emissions. This was the main objective for Action Plan on the Aquatic Environment that was passed by the Danish Parliament in 1987 [21]. The reduction of discharges from agriculture, industry and municipal WWTP was regarded as a main strategy to improve the situation in the Sea.

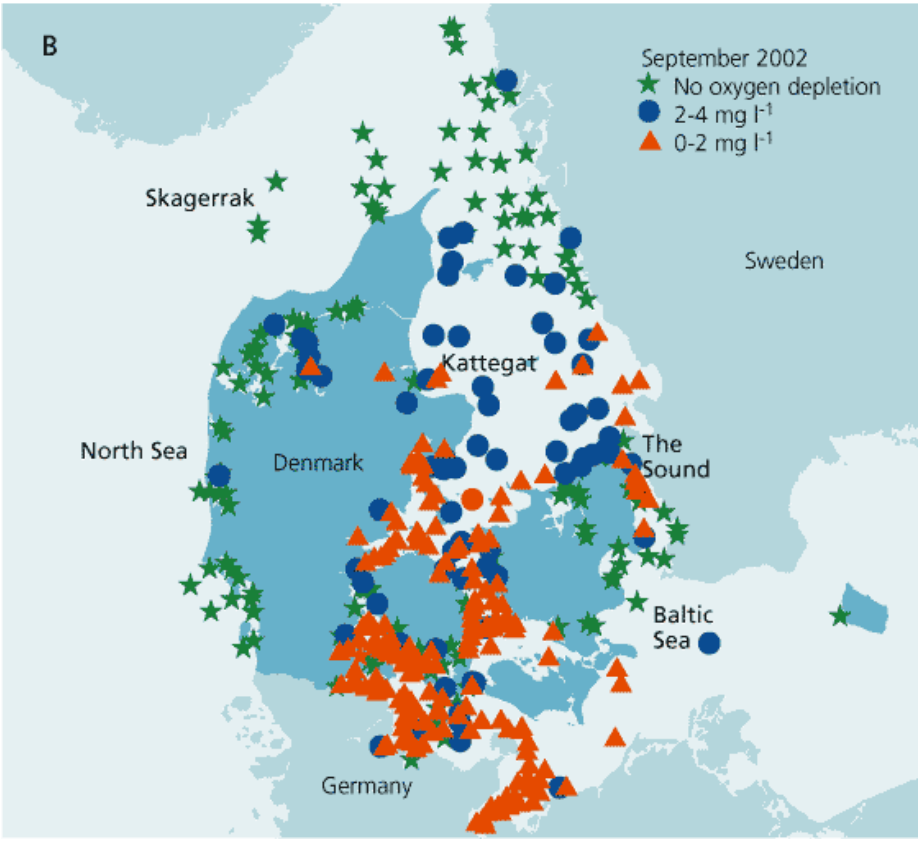

Figure 1. Distribution of oxygen concentration in Danish marine waters in September 2002 [scheme taken from 21, p.60]

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This project focuses o improvements in the removal of the macronutrient phosphorus (P) from sewage. Particular emphasis is on the kinetics of the process and looking on possible improvements. In wastewater, P is usually present in the form of organic bound P, orthophosphate, polyphosphate and it appears from detergents and from industry (e.g. fish industry). The most widely used methods to remove P from wastewater rely on either chemical or biological processes. The chemical methods consist in coagulation and flocculation processes. Usually Ca, Fe or Al cations (in the form of sulphate or chloride salts) are used to precipitate PO_4^{3-} ions. The problem of this process is that chemicals are not selective. It means that not only polyphosphate ions are tied, but also other chemical compounds, what causes bigger production of the sludge and that follow to higher cost of the process. P is removed from wastewater treatment plant with excess chemical sludge.

The second method, biological-P removal, started to be investigated before the 2nd World War, but major advances of the process were not made until the 60's. At that time, the definition of "luxury uptake of phosphorus" was used first time [13]. Later the name was changed to Enhanced Biological Phosphorus Removal. The process relies on higher (more than organisms need for their normal existence) uptake of phosphorus in the form of polyphosphate. A group of organisms (bacteria) has ability to store large amounts of poly-P are called Polyphosphate Accumulating Organisms (PAOs) [5]. This method of P-removal from wastewater has two big advantages in comparison with chemical: no salts addition for P precipitation is needed (chemicals are usually an expensive part of the treatment) and the sludge production is lower. On the other hand, a few factors must be fulfilled to achieve satisfactory effluent concentrations using bio-P removal. One of them is supply of carbon source in the anaerobic part of the process when bacteria take up substrate and release P into the ambient environment. As substrates, different carbon sources can be used: volatile fatty acids, amino acids, molasses etc [13]. From many studies it is concluded that VFAs are the most suitable carbon source for the EBPR process. It is not fully understood which group of organisms is able to uptake amino acids, but it appears that only a fraction of the microorganisms involved in EBPR process has this capability. In addition, the optimal pH conditions for AAs uptake have not been investigated completely. In order to improve our understanding of EBPR process, this project focuses on the effect of different carbon sources with main emphasis on AAs.

The efficiency of the P-removal process is strongly dependent on the composition of microorganisms in the sludge. For this reason, three WWTP (with EBPR process) were chosen for a case study: Aalborg West, Hjørring and Bjerringbro. Sludge samples were obtained from all three plants and used for lab experiments on kinetics and stoichiometry of P-release in the anaerobic phase of the EBPR process. Tests were conducted with usage of the activated sludge from aeration tanks of these WWTPs.

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2.1. Objectives

The objectives of the present study were to investigate:

- effect of different carbon sources: VFAs, AAs and mixture of VFAs and AAs on kinetics of P-release during the anaerobic phase of the EBPR process,
- effect of pH on P-release during anaerobic phase of the EBPR process with AAs as carbon source,
- short term (one week) and long term (ten months) variations in P-release kinetics in anaerobic phase of EBPR process,
- influence of the sludge microbial composition on P-release in the anaerobic phase of EBPR process in three WWTPs.

3. Theoretical Part

This part contains theoretical information about general aspects of wastewater treatment, as well specific details on the EBPR process and important factors affecting it. The intention is to provide the reader with an up-to-date knowledge on the EBPR process and to highlight research gaps.

3.1. Wastewater treatment process

Nowadays, two types of biological WWTPs are generally being used: activated sludge (AS) plants and bio-filter plants. The most popular are AS plants, because of their advantages of low exploitation costs, easiness in maintenance and building or simplicity of equipment. The AS method was invented in 1913 by Ardern and Lockett in Manchester's WWTP. It was very quickly applied for full-scale WWTPs after 1st World War [31].

Besides the biological processes, also mechanical and chemical treatment steps are included in AS plants. Usually the first step is mechanical treatment, which aims at removing all gross solids like rags and solid substances, sand, oil and suspended matter. The chemical treatment is used (only when it's needed) to adjust pH, reduce bad smells, precipitate heavy metals or phosphorus and kill pathogens (disinfection). As chemical treatment is used in many cases it can be implemented in many points of the treatment process e.g. after mechanical treatment or after settling tank [24].

The main phase of the wastewater treatment process in an AS plant is the biological part which takes place in anaerobic and afterwards aerobic/anoxic tanks. In this stage, bacteria from AS consume organic matter (in RBCOD form) and remove nutrients: nitrogen by denitrification (N_2 is released to the atmosphere) and phosphorus by the Enhanced Biological

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Phosphorus Removal process (P is accumulated in sludge which is subsequently disposed from the WWTP).

Figure 2 is a schematic illustration of the flow of wastewater in an EBPR plant. The volumes and retention times of the tanks are specifically designed according to the loading of wastewater (PE value).

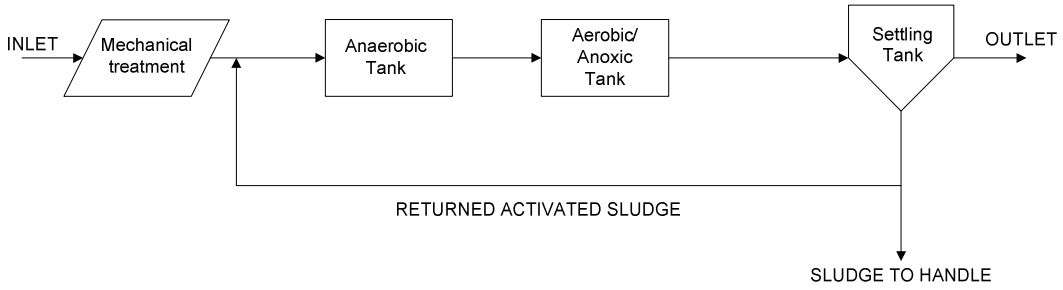

Figure 2. Scheme of continuous flow in WWTP with the EBPR process implemented [9]

3.2. Enhanced Biological Phosphorus Removal process (EBPR)

The chapter describes the mechanisms of EBPR including the optimal conditions, the microorganisms taking part in the process, and other factors which have the biggest impact on the biological P-removal.

3.2.1. Mechanism of the EBPR process

The enhanced biological phosphorus removal process was discovered accidentally by Greenburg in 1955 (Greenburg *et al.* 1955). The EBPR process relies on alternate anaerobic and aerobic/anoxic conditions in process tanks. During the first stage of the process PAOs (heterotrophs) take up substrate, which is available in the bulk liquid, under anaerobic conditions. Usually this entails VFAs; e.g. acetate or propionate, but some PAOs (probably *Actinobacteria*-related PAO - APAO) have ability to take up amino acids [5]. The microorganisms convert the substrate (only VFA, the situation with AA is unclear) to energy rich polymers called poly-hydrohyalkanoates (PHA). Principally, the energy is stored in one of two kinds of PHA: polyhydroxybutyrate (PHB) and polyhydroxyvalerate [13]. During this process P is released from the cells to the bulk liquid. The reason is that PAOs, to form PHA need energy which is obtained from breakdown of poly-P. Breakdown of poly-P molecules gives energy while releasing PO_4^{3-}, which is excreted outside the cell resulting in an increase of dissolved P-concentration in the anaerobic phase. Glycogen is used in the whole process as reducing power [12]. A schematic illustration of this part of process is shown on the figure 3a.

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In the second step of the EBPR process, PAOs take up extracellular PO_4^{3-} and restore their energy stock under aerobic conditions. PHA pools are oxidized to give energy and carbon for biomass' growth of new cells. During this phase, uptake of PO_4^{3-} (polymerization to poly-P) can be observed. In addition, the PAOs rebuild their glycogen storage. After the aerobic phase, the concentration of PO_4^{3-} in the effluent is low because around 95 % of P is kept in cells. Then sludge with a high content of P is removed as a waste from the WWTP. The sludge can be used as fertilizer, for landfill or as a raw material for production energy and heat by incineration [12, 13, 14].

The EBPR process can also be conducted in anoxic conditions (instead of aerobic). A fraction of PAOs has ability to accumulate poly-P under anoxic conditions when nitrate is present in the bulk liquid. However, in comparison P uptake rate is lower when the nitrate is used as electron acceptor instead of oxygen. Systems with anaerobic-anoxic reactors have many advantages. Denitrifying Enhanced Biological Phosphorus Removal can be made and the same carbon source can be used for both processes. This results in a reduced sludge production [13]. Also by utilizing nitrate instead of oxygen a reduced aeration demand is achieved. In the case of this process we can divide PAOs in two groups: those which can (DPAOs) and those which cannot (PAOs) use nitrate as an electron acceptor. Thus successful DEBPR process relies on composition of microorganisms in the sludge [13].

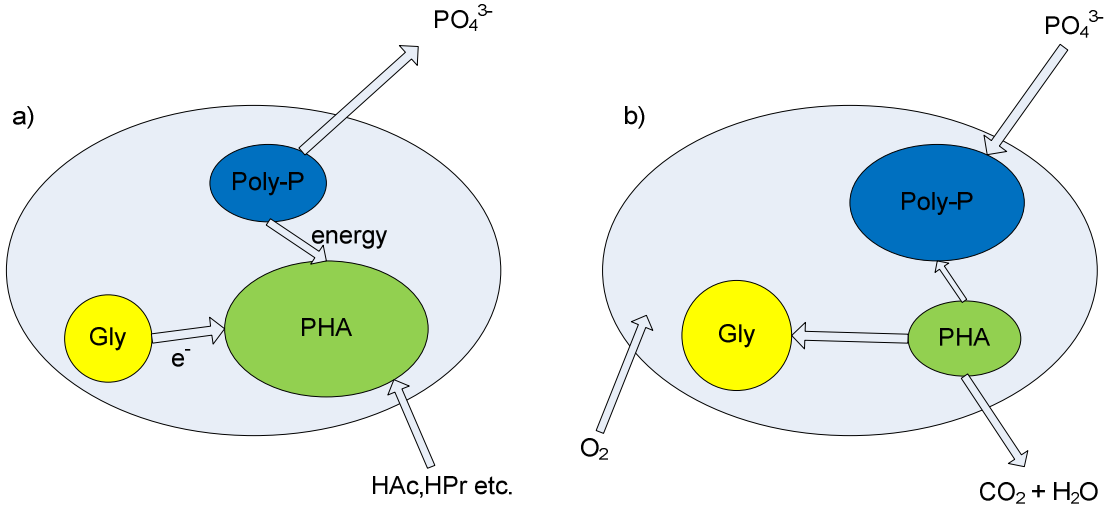

Figure 3. Metabolism of PAOs during a) anaerobic and b) aerobic phase of bio-P removal [Piotr Wiliński; 4, 14]

Figure 4 shows the typical pattern for changes of concentration of different substances during the anaerobic and aerobic phases of the bio-P removal process.

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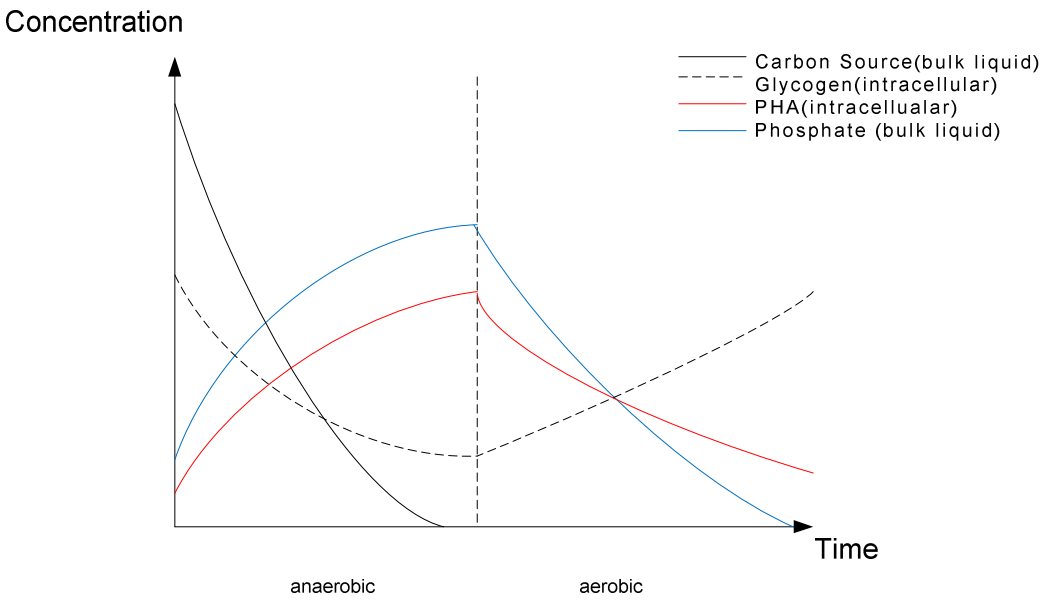

Figure 4. Typical kinetics of concentration for different chemical compounds during anaerobic and aerobic phase of bio-P removal process [Piotr Wiliński, 13]

3.2.1.1. Main chemical compounds involved in the EBPR process

During the two phases of the EBPR process in bacterial cells are occurring transitions of the storage materials and transfer of energy from one chemical compound to others. Three main storage compounds are involved in these transformations: polyphosphate, glycogen and polyhydroxyalkanoates (PHA). The chemical properties and the role of each compound are discussed in the following.

Polyphosphate (poly-P) is an anionic phosphate polymer formed from inorganic phosphates. It has energy rich bonds which are used to store energy derived from breakdown of organic substrate. The energy can be released by breaking the bonds. In the bio-P removal process, poly-P granules are created in aerobic conditions and consumed in the anaerobic phase [14].

Glycogen, which is a polymer of glucose, takes essential part in the EBPR process. The stored glycogen plays a role of maintaining the redox balance. The conversions of glycogen to acetyl-CoA and CO_2 generate reducing power (e.g. as $NADH_2$) and conversions to propionyl-CoA lead to consumption of reducing power and production of PHAs. As PAOs take up various substrates in oxidized or reduced form, glycogen plays a key role in maintaining the redox balance [28]. This polymer can be the limiting substance for the EBPR process, which has been reported in Brdjanovic *et al.* (1997a). During excess feed of acetate, anaerobic uptake of HAc stopped not because of PHA saturation or poly-P limitation, but because of glycogen exhaustion [28].

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The last discussed storage compounds are polyhydroxyalkanoates. They belong to a group of linear polyesters which serves as carbon and energy storage. In the EBPR process, PHAs are usually composed of 4, 5 or 6 carbon unit chains, but which one are exactly formed depends on substrate and environmental conditions [14]. As previously, two kind of PHAs are produced during bio-P removal: polyhydroxybutyrate (3-hydroxybutyrate [3HB]; 3-hydroxy-2-methylbutyrate [3H2MB]) and polyhydroxyvalarate (3-hydroxyvalerate [3HV]; 3-hydroxy-2-methylvalerate [3H2MV]). Two theories for explanation of PHA formation has been proposed: the Comeau-Wentzel model and the Mino model. As PHA is more reduced than HAc, it needs reducing power for formation. The first idea, the Comeau-Wentzel model, proposes that under anaerobic condition the citric acid (TCA) cycle takes place and part of HAc is oxidized to CO_2 to create reducing power in the form of NADH. In the second model (the Mino model) reducing power is obtained by degradation of glycogen to above-mentioned acetyl-CoA and oxidation to CO_2 [28].
PHAs are formed in three steps: condensation, reduction and polymerisation. The synthesis of PHAs take place when carbon source is in excess and N, P (or other essential elements e.g. Mg^{2+}) are present in non-limiting amounts [14]. The composition of PHAs formed from each VFA is different. For HAc app. 65% is 3HB and 35% is 3HV, for propionate 60% is 3HV, 30-35% 3H2MV and rest 3HB, for lactate 85-90% is 3HV and rest 3HB [30]. The situation is unclear in the case where amino acids are used as a carbon source in EBPR. There must be made more investigations if PHAs (or some other compound) are formed from AAs during the anaerobic stage of the EBPR process.

3.2.2. Polyphosphate Accumulating Organisms

The first investigations of Polyphosphate Accumulating Organisms were made around three decades ago, but still there are some mechanisms and organisms governing the process, which are not well known. The major groups of bacteria involved in the EBPR process are: *Accumulibacter phosphatis* (*Betaproteobacteria*) – Gram negative and *Actinobacteria* (APAO) – Gram positive. PAOs are small rod or oval shaped organisms with large poly-P granule pools in the aerobic conditions. They release P under anaerobic conditions.

Accumulibacter were found in Danish WWTP 4-22% of the bacteria population [5, 14, 15]. Lab scale tests have shown that, there exist correlation between significant presence of *Accumulibacter* and efficient EBPR process [15]. These bacteria are able to take up different VFAs i.e. HAc and HPr (with very similar uptake ratio) and can also take up these substrates simultaneously. In anaerobic conditions they obtain saturation point of HAc uptake usually after 3h of incubation [25]. From other carbon sources they can uptake AAs: glutamic acid and leucine and puryvate [5]. Generally it is observed that the *Accumulibacter* prefer low-molecular-weight compounds [25].

APAO are group of PAOs, for which their metabolism is not completely understood. Lab studies have shown that they have ability to uptake P (only when conditions are aerobic, not on anoxic conditions) and consume substrate in anaerobic phase, but only with AAs not

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VFAs [26]. The pathways of substrate transformation in the APAOs metabolism are not well known and a full overview of the role of APAO in the EBPR is hard to describe. Probably these organisms, opposite to *Accumulibacter*, do not form PHA during anaerobic uptake of the substrate. It is still unknown which storage compound is created in anaerobic phase for further energy demand for P-uptake in aerobic phase of the EBPR process [5, 15, 26].

The abundance analysis of these two groups of bacteria can be made by the molecular method - fluorescence *in situ* hybridization (FISH), which is described in details in chapter 4.2.5 and 9.4. in Appendix.

3.2.3. Glycogen Accumulating Organisms

There exist other organisms which have the ability to take up VFAs and store energy in the form of PHA. They are called Glycogen Accumulating Organisms (GAOs). The name was taken from the feature of the bacteria, which use glycogen (during anaerobic conditions) to make energy for uptake of carbon source, i.e. VFA and form PHAs. They are large spherical organisms arranged in pairs or tetrads. GAOs are unwelcome in WWTP from the EBPR process point of view. The reason of that is their ability to uptake HAc, HPr and some of amino acids, but opposite to PAOs without poly-P storage and PO_4^{3-} uptake from bulk liquid [14]. Therefore they compete with PAOs for substrates and if GAOs are existing in WWTPs in high quantity (present of more than 3% of the population), they can cause failure of the EBPR [5, 33]. However, there exists many types of GAOs and the substrate composition is a factor which promotes some of GAOs in the competition with PAOs for the substrate [40].
The first bacteria which were assigned to this group of organisms were *Gammaproteobacteria,* which later was called *Competibacter.* Microbiology studies have shown that it has the ability to take up HAc, but maybe unable to use HPr as a source of PHA formation. Moreover, it can compete with other organisms for substrate in anoxic conditions. Other GAOs is *Defluviicoccus (Alphaproteobacteria)* [10, 14].

3.2.4. Factors affecting the EBPR process

In this chapter, the influence of physical, chemical and general conditions during the EBPR process is discussed.

3.2.4.1. Carbon source

During the anaerobic phase, sufficient amounts of carbon source should be available for PAOs. This depends on the composition of the influent. Sometimes readily biodegradable COD in Danish sewage (depends what sort of wastewater has to be treated i.e. municipal, industrial, agricultural) is not high enough to conduct the process properly and additional

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sources of carbon must be added to run the EBPR effectively. This can be achieved by addition of molasses or other compounds, or if the situation is permanent, by building a side-stream hydrolysis tank in which hydrolysis and fermentation processes of returned sludge can produce desirable carbon source (main products of hydrolysis are VFAs: HAc and HPr in approximately ratio 3:1 [36]). To ensure sufficient carbon sources for the EBPR, it is recommended that the ratio of BOD_5:P should be at least 20:1 [13]. Furthermore, the carbon source should appear in not a complex form (short carbon chain) for facilitate bacteria assimilation of it. Several studies have focused on substrates in EBPR process and it is well known that PAO can use VFAs. It is not completely clear how other substrates such as glucose or amino acids can influence on the process. The studies were also made on substrates such as ethanol, starch but no significant EBPR effect was observed [4].

Most case studies have been made on VFAs specifically on acetic acid – CH_3COOH. There is general agreement that it is the best substrate for PAOs in EBPR process. The weight P-release/HAc-uptake ratio are in a range from 0.20 – 0.73 mgP/mgCOD-HAc [13]. It must be mentioned that these rates are pH and temperature dependent, which will be described later in this chapter. HAc has biggest influence on the EBPR, but not a whole amount of it can be assimilated by PAOs. The reason of that is competition for substrate with GAOs. However the uptake of HAc is different for those two groups of organisms. Studies determine that uptake of HAc is driven by secondary transport which requires proton motive force (PMF). PAOs generate PMF by efflux of phosphate and protons by Pit transporter (protein in cell's membrane), whereas GAOs generate PMF by efflux of protons trough ATPase and with use of the enzyme – fumarate reductase [14]. The second VFA which has big influence on bio-P process is propionic acid – C_2H_5COOH. Depending on the composition of microorganisms in the sludge and the physical conditions, P-release can be almost on the same level as with HAc, but usually it will be slower (when both kinetics and stoichiometry is taken into consideration) [11, 13]. From VFAs also lactic acid – $C_3H_6O_3$ is worth to mention. It is observed that it is taken up as fast as HAc and HPr, but the P-release is lower compared to those VFAs [13]. The example P-release/VFA uptake ratios are shown in table 1.

Table 1.Ratios of P-release/VFA uptake [Beatens,D.; 13]

Carbon source	Weight ratio of P-release/ VFA uptake [mgP/mgCOD]	Molar ratio of P-release/VFA uptake [P-mmol/C-mmol]
Acetic acid	0.37	0.77
Propionic acid	0.12	0.44
Butyric acid	0.15	0.78
Isobutyric acid	0.16	0.80
Valeric acid	0.19	1.72
Isovaleric acid	0.25	2.31

Only a few studies have focused on amino acids as carbon source for the EBPR process. Usually AAs do not appear in high concentrations in wastewater, but some wastewater contains a high amount of proteins which can be hydrolyzed to AAs [24]. The mechanism of amino acids uptake and energy storage in the bacterial cells is not well known [17]. Probably APAOs are the main group of bacteria which can take up AAs and use them for the

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P-removal process. It is not well known which amino acids have the highest impact on P-release. As well it has not been investigated what pathway of carbon transformation amino acids go trough in bacteria cells and if PAOs are able to produce PHA or some other (not known) storage compound from the AAs. Similar to VFAs, PAOs probably prefer AAs with lower carbon number in chain. However, more investigation must be done to fully understand the impact of AAs as a carbon source.

Other various organic compounds can be taken up by PAOs and used in anaerobic phase as carbon source. First of them pyruvate (acid residue of pyruvic acid) is taken up from bulk liquid even faster than HAc and HPr, but P-release rate is much lower. It is documented that puryvate is not passing through the same mechanisms with PHA formation as is the case for HAc and HPr. At first it is stored in other form, before being transformed to PHA during which P is released [4, 13].

Another organic compound which was in the early beginning considered as the most important is glucose – $C_6H_{12}O_6$. After detailed tests it was recognized that glucose as carbon source can select GAOs instead of PAOs and failure of the EBPR may be observed. As well delay effect has been observed. P-release was delayed relative to glucose uptake. Other conclusion was that glucose can be a good substrate if fermenting microorganisms are present in the sludge. They can transform glucose primarily into HAc which is easier available for PAOs [4].

3.2.4.2. pH value

The next factor which has big influence on the bio-P removal process is pH. The range from 5.5 to 8.5 is indicated as a requirement for conducting EBPR properly. However the optimum pH is 7.5 – 8 which has been shown in many independent studies [40]. Below 5.5 and above 8.5 significant slow-down or even total stoppage of P-release (anaerobic) and uptake (aerobic) is observed [13].

The pH value has an influence on the transport of carbon source into the bacterial cells and P-release during the anaerobic phase of EBPR. pH is important component of PMF (proton motive force), on which lie the responsibility of right proton and electron movement by cell membrane.
The PMF can be quantified as shown below:

$$PMF=\Delta\Psi+\Delta pH \text{ (equation 8.1; [14]) where}$$

$\Delta\Psi$ – electrical component of PMF (charge difference),
ΔpH – chemical component of PMF ($pH_{in} – pH_{out)}$ which is the difference of H^+ concentration across the cell membrane

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In order to maintain the PMF, bacterial cells maintain a higher intracellular pH than in the ambient environment. When extracellular pH is acidic ΔpH is relatively high and bacteria do not need to maintain a big increase in proton efflux (lower P-release). When extracellular pH is alkaline then cells efflux more protons to maintain ΔpH [14]. In the second case, PAOs need to use more storage poly-P to fulfil energy demands for carbon source uptake. This is the reason of greater P-release in alkaline conditions (pH 7 – 8.5).

Also important fact is acid-base equilibrium connected to pK_a (tells how large fraction of molecules are extended to dissociation) value of substrates. The value of pKa indicates the pH in which 50% of molecules are on ionized species and the rest is unionized, what can be summarized from equation 1:

$$pH = pK_a - \log\frac{[AH]}{A-} \quad \text{Equation 1.}$$

When $[AH]/[A^-]=1$ since log(1) =0, the pH at half-neutralization is numerically equal to pK_a. Conversely, when $pH = pK_a$, the concentration of AH is equal to the concentration of A^-.
The balance of ionized and unionized form of HAc can be written by the equation:

$$CH_3COOH \rightarrow CH_3COO^- + H^+ \quad \text{Equation 2.}$$

Thus, from equation 1 can be concluded that, in neutral and alkaline conditions the presence of ionized species will be close to 100%.
Besides the influence on carbon source uptake, pH is important from the PAO-GAO competition point of view. In the range of pH 6 – 7.5 when HAc or HPr is a carbon source PAOs gain competitive advantage. GAOs are less sensitive than PAOs to acidic environments so it is better to keep pH on level above 7.0. It is also worth to mention that when initial pH conditions are slightly alkaline or acidic, during the EBPR pH tends to neutralize. The reason of that is the consumption of fatty acids and intracellular pH regulations [20].

3.2.4.3. Other factors

First and most important thing is that efficacy of EBPR process depends on alternating anaerobic and aerobic (anoxic) conditions during the wastewater treatment process, which is the principle of the process. Also the time of anaerobic and aerobic phases which must be set up individually for the sludge (because of variation in microbial community structure) in every single WWTP. It has been proved that presence of nitrate in the anaerobic phase is not desired. The reason of that is then denitrifying bacteria can take some of the substrate, which originally was intended for PAOs. This can cause a lowering the EBPR process efficiency [4, 33]. Also another physical factor – temperature has significant influence on the EBPR process. A general rule is that higher temperature causes higher P-release rates and bacteria growth rates in anaerobic and aerobic phases [40]. However, if temperature increases above 30°C a slow-down of the process can be observed. Over 40°C, the process stops, probably due to bacteria decay [4]. Also in higher temperatures (20-30°C), GAOs gain a competitive advantage [16].

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4. Materials and methods

Chapter 4 describes the WWTPs used as a study case in the project as well as all methods used during lab experiments.

4.1. Wastewater Treatment Plants in project

For the case study, three treatment plants were chosen: Aalborg WWTP, Hjørring WWTP and Bjerringbro WWTP. All of them have been designed to perform biological P-removal. A detailed structure of each WWTP is described in the forthcoming sections. In chapter 4.1.4. a comparison of the three wastewater treatment plants has been made.

Figure 5. Northern and central part of Denmark with “case-cities” marked by red dots [8]

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4.1.1. Aalborg West Wastewater Treatment Plant

Aalborg West Wastewater Treatment Plant was built in 1979 and it treats sewage from the western and central part of Aalborg, Nørresundby and other small cities from the Aalborg district. Since the construction, the plant has undergone five major extensions, in order to improve the capacity and treatment performance. The last upgrade, which was finished in 2007, was implementation of side-stream hydrolysis process. The plant was designed for 330,000 PE, but currently the load is much smaller (~200,000 PE). Maximum daily flow in dry weather is 60,000 m^3/d and when it is raining up to 200,000 m^3/d. The plant uses mechanical, biological and chemical methods for treating the sewage. Produced sludge is partially sent to a sludge drying plant or used in a digester for production of gas. The plant produces 40 t/d of dewatered sludge. The treated wastewater – effluent, is discharged to Limfjorden. A detailed scheme of Aalborg WWTP is show on figure 6.

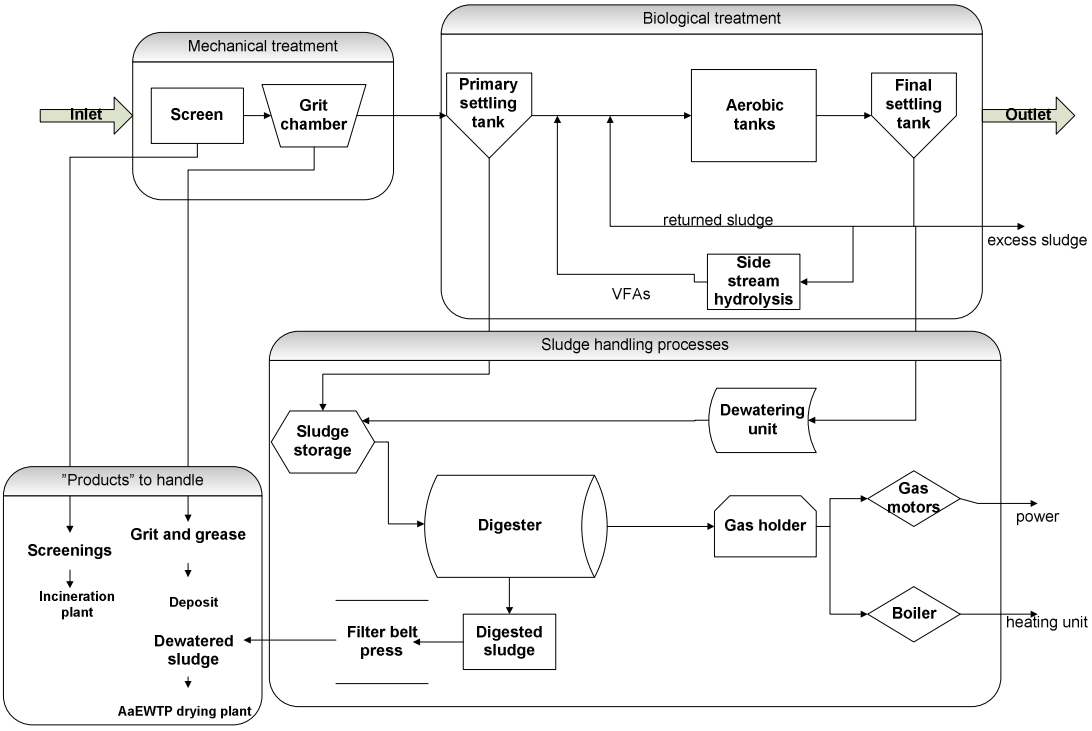

Figure 6. Scheme of Aalborg West WWTP [Piotr Wiliński]

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4.1.2. Hjørring Wastewater Treatment Plant

Hjørring Wastewater Treatment Plant is situated in Hæstrup – a small town located in the southern direction from Hjørring. In the years 1991-1994, the plant was extended to mechanical-biological-chemical treatment. The plant started working after extensions in December 1993 and in full abilities (sludge dewatering and storage) in 1994. The designed capacity of the plant is 160,000 PE, but the current load is around 100,000 PE. The daily flow is designed for: 26,400 m^3/d for dry weather and 45,000 m^3/d during rainy days. Within the year, the plant removes 3,000 t of organic matter, 75 t of N and 80 t of P from wastewater [29]. The treatment process begins with a mechanical part: screenings and grit removal. Then sewage is passed to primary settling tanks and primary sludge is pumped to anaerobic digesting tanks. The biological process is based on activated sludge process including N-removal and P-removal. The process of the bio-P removal is supplemented by simultaneous precipitation with iron salts. Primary sludge as well as excess sludge is dewatered in centrifugal separators before storage and reuse as a fertilizer [29]. On the figure 7 is shown a schematic illustration of the plant.

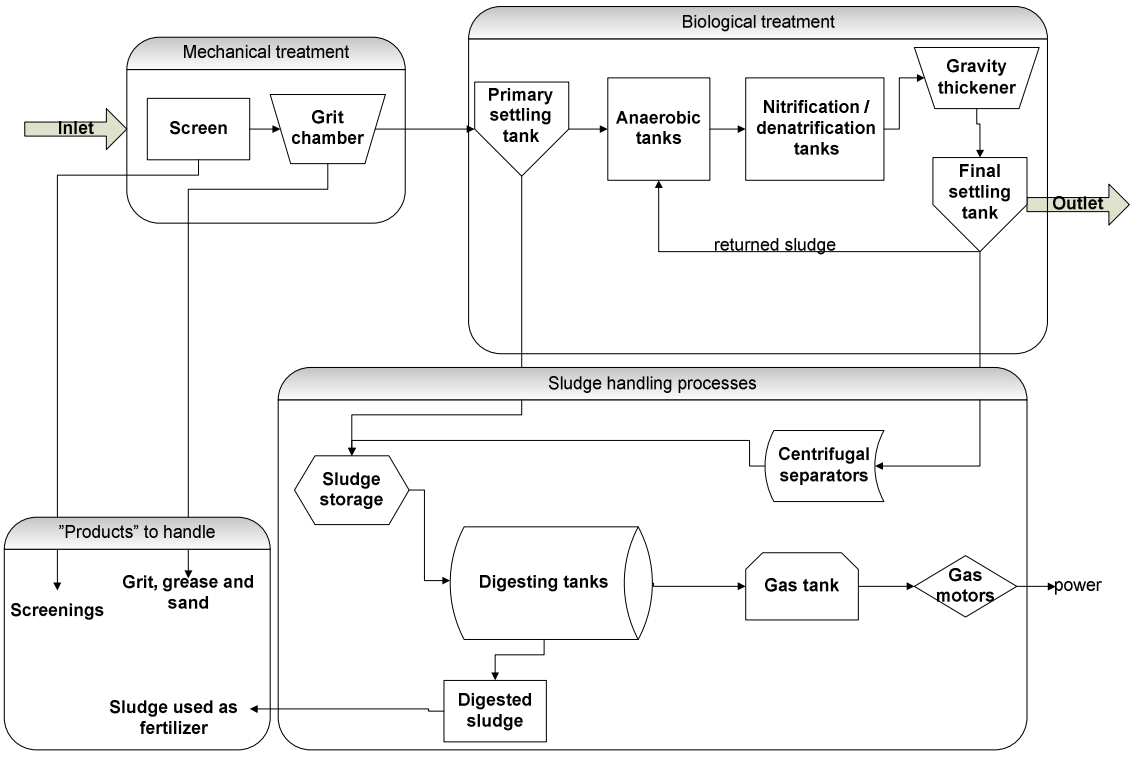

Figure 7. Scheme of Hjørring WWTP [Piotr Wiliński, 29]

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4.1.3. Bjerringbro Wastewater Treatment Plant

Bjerringbro Wastewater Treatment Plant receives wastewater from Bjerringbro Municipality. The plant was designed for 65,000 PE, but after closing down some industry (two large slaughterhouses were closed down) the actual load is around 18,500 PE. The plant has one sequencing reactors in which anaerobic and aerobic processes take place. The daily flow in to the plant is oscillating around 5,000-6,500 m^3/d and the corresponding sludge production is around 0.7-0.8 t/d. The effluent is directed to river Gudenå [31].

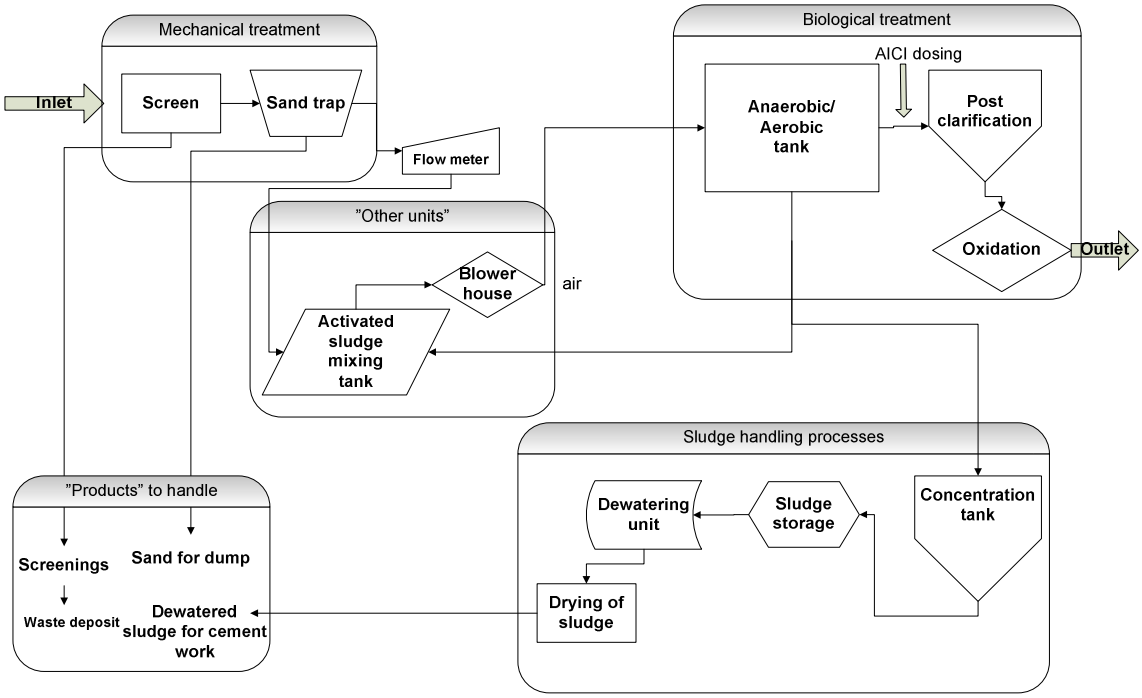

Figure 8. Scheme of Bjerringbro WWTP [Piotr Wiliński, 31]

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4.1.4. A comparison of Aalborg West, Hjørring and Bjerringbro WWTPs

There are significant differences between case plants. First one is capacity of the plants. Aalborg West has actual capacity 200,000 PE, which is twice higher than Hjørring and ten times higher than Bjerringbro. As it was mentioned previously, all treatment plants conduct the EBPR process. However, it is worth to mention that in Aalborg West WWTP is implemented BioDenipho [38] process and Hjørring WWTP is recirculating plant. Bjerringbro WWTP works in principle of sequencing batch reactor (SBR) with one tank where conditions are anaerobic or aerobic depending on the phase of wastewater treatment [31].

The most important factor which is taken into consideration is the microbial community distribution (in case of this project PAOs were taken into comparison). There is significant difference in PAOs community between Aalborg West and Hjørring compared to Bjerringbro WWTP. The presence of these organisms in first two WWTPs is twice or even triple higher than in the third plant. Moreover, distribution of *Accumulibacter* and APAO is very variable in all three plants. However, the highest abundance of *Accumulibacter* was found in Aalborg West and of APAO in Hjørring WWTP. It is also worth to notice that the fraction of PAOs involved in P-release and uptake can be very diversed in each single treatment plant. Kong *et al.* (2004) made research on three Danish treatment plants (Aalborg West, Egaa and Skagen) and found that participation of *Accumulibacter* in EBPR process can be from 15 – 95 % of population. Calculations made from *Den Mikrobiologiske Database* [10, 27] are shown in table 2. Detailed data of bacterial community structure in years 2006-2008 are shown in tables 16 and 17 in chapter 9.6. of Appendix. Comparison of these data with lab tests can be important to formulate proper conclusions for objectives posted in this project.

Table 2. Comparison of three WWTPs

WWTP / Properties	Aalborg West	Hjørring	Bjerringbro	Comments
PE designed/PE actual	330,000/195,000	160,000/100,000	65,000/18,200	
Bio – P process	+	+	+	
Side-stream hydrolysis	+	-	-	
Configuration	Biodenipho	recirculating	Biodenipho	
Industrial wastewater	30%	30%	23%	
Microbial population				
Accumulibacter	4.6 (8)*	3.1 (10)*	2.7 (4)*	*mean values from FISH data from years 2006-2008. In brackets are numbers of data taken to calculations
APAO	7.8 (8)*	11.8 (10)*	3.7 (4)*	

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4.2. Experimental investigations

In this chapter are described methods used for lab measurements in the project. There were conducted seven main lab experiments:

- P-release and C-consumption experiment with different VFAs as a carbon source,
- P-release experiment with different amino acids as a carbon source,
- P-release experiment with amino acids as a carbon source in different pH conditions,
- P-release kinetics in relation to stoichiometry of amino acid added to the reactor as carbon source
- P-release with mixture of acetate and different amino acids as a carbon source,
- P-release rates with 20 single amino acids ,
- short and long-term experiment with HAc as a carbon source with activated sludge from Aalborg West WWTP.

Following subchapters describe each of five methods used for these experiments.

4.2.1. General experimental conditions

All experiments, were conducted at a steady temperature of 15°C +/- 0.5°C. The temperature was maintained by a thermo-stated water bath. This temperature was chosen to imitate *in situ* conditions in a WWTP. pH values measured on the beginning of experiment were around 8 +/- 0.5. Experiments, where the full capacity (chapter 5.5) of P-release has to be obtained, were conducted at lab temperature (~25°C). Anaerobic reactors were 500 mL bottles capped by rubber caps. The sludge samples were taken from aerobic tanks from WWTPs used in the project (Aalborg West, Hjørring and Bjerringbro). Every time new sludge was taken, SS and VSS values were determined by method written in chap. 4.2.4. Before the experiments were started, the activated sludge was properly prepared. At first, it was pre-aerated (ca. 30~40 minutes) by pumping air into the reactors and then the conditions were switched to anaerobic by purging N_2 ca. 10~15 minutes. Next N_2 was flushed into headspace of the bottles to ensure anaerobic conditions. Then bottles were closed by rubber caps. The carbon sources (VFAs or AAs) were added in the quantity of 300 mg COD/L. Amino acids were added as 10 mL of stock solutions to 390 mL of the sludge (or 5 mL to 195 mL sludge). Volatile fatty acids were added in pure form. Throughout the experiments (around 160 RPM in anaerobic phase and 250 RPM during pre-aeration), the reactors were stirred in order to avoid settlement of the sludge. Supernatant samples were taken regularly (at time intervals of 30-45 min) by a syringe and then used for measurements of P-release or substrate uptake. A schematic illustration of the experiment is shown on figure 10.

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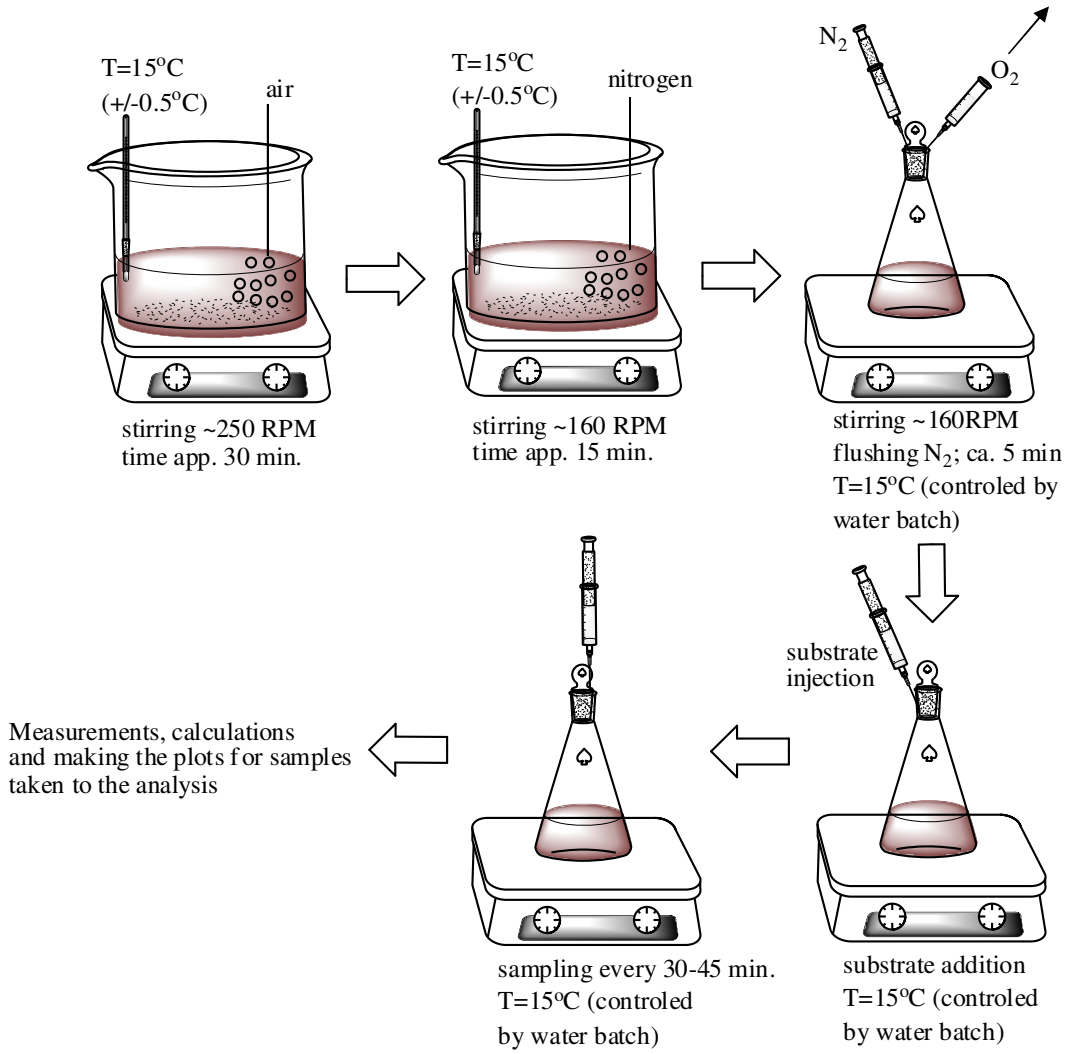

Figure 10. Scheme of lab experiments

4.2.1.1. pH adjustment

In experiments with different pH values in reactors, the pH was adjusted by adding 1 M HCl to obtain acidic environment or 1 M NaOH to achieve alkaline conditions. Acid or base were added after purging N_2 in to bottles. Then a second addition (if needed) was made after one hour to be ensure that pH value was adjusted to the right level.

4.2.2. Ortho-P assay

Ortho-P assay is method based on chemical reaction which cause a change of the color in the sample (from transparent into blue). Ammonium molybdate and potassium antimonyl tartrate react in acid medium with orthophosphate to form a heteropoly acid – phosphomolybdic acid – that is reduced to intensely colored molybdenum blue by ascorbic acid. The absorbance of

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molybdenum blue at 880 nm is proportional to the orthophosphate concentration [6].

Samples of supernatant were taken at regular intervals and at first centrifuged 3 minutes with 4200 RPM. Next, samples were filtered on glass fiber filters Advantec ® GC-50, 25 mm diameter and 0.45µm pore size. Suitable dilution (deionized water) was made if needed (from 5 to 100 times) [6] and P-concentration was measured on spectrophotometer HACH DR2500® after mixing the sample with reagents and settling for 15 minutes. Measurements were made in accordance to EN ISO 6878:2004 – Determination of phosphorus – ammonium molybdate spectrometric method [22].

4.2.3. Analysis of VFA consumption

VFA concentrations were measured using ion exclusion chromatography and suppressed conductivity detection. This method ensures efficient separation and detection of low weigh molecular aliphatic organic acids. It is based on retention time of flowing substances onto a Ionpac ICE-AS6 ion-exclusion column. It is covered by special gel substances which enable exclusion of ions between eluent and column. Samples are dissolved in eluent fluid and then transported onto column. Donnan membrane, which is located around the column, is permeable only for undissociated forms of acids. Eluent fluid was heptafluorobutyric acid (HFBA 0.4mM; flow rate – 1mL/min) and regenerant (solution used to restore the activity of an ion-exclusion column) tetrabutylammonium hydroxide (TBAOH 5mM).

After preparation, samples were put into an auto-sampler. Standard curve for formic, acetic, lactic and propanoic acid (2.5; 5; 10; 25; 50 and 100 mg/L) was prepared and these samples were set before samples from VFAs measurement [5, 18].

4.2.4.Suspended and volatile solids determination

Suspended solids were measured in well-mixed samples. 10 mL of sludge was filtered on glass fiber filters Advantec ® GA-55, 47 mm (pore size 0.6 µm) and then put into an aluminium box. The box with filter was then put in an oven at 105 °C over 2 hours and dried to constant weight. The increase of weight represents suspended solids. Volatile solids were measured by igniting the residue (from previous drying) to constant weight at 550 °C over 1 hour. The remaining solids represent the fixed total suspended solids while the weight lost on ignition is the volatile solids [6]. All SS and VSS measurements were made as triplicates. Next, the mean value of three was calculated to obtain precise result. Measurements were made in accordance to EN 872:2005 - Determination of suspended solids – method by filtration through glass fibre filters [22].

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4.2.5. Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* Hybridization (FISH) is a molecular method used for identification and enumeration of specific microbial organisms among others. A probe, specific to a particular RNA sequence is labeled with a fluorescent dye (usually cyanine or its derivatives) and introduced to the sample of interest (e.g. activated sludge). The probe consists of an oligonucleotide possessing 15 – 20 base pairs specific to 16S ribosomal RNA sequence. After hybridization to a specific sequence, the probe can be detected by fluorescence microscope. Usually a set of two gene probes is applied – the first one stains the background population (most of microorganisms) and the second one is specific to those possessing a particular feature (i.e. PAOs). FISH allows for identification and enumeration of a particular group of microorganisms. It may be a species or a group possessing particular characteristics or belonging to a given phylum [2, 5].

Samples for FISH analysis were taken into two test-tubes: one for Gram positive bacteria and another for Gram negative. For Gram positive 2.5 mL of sludge was fixed with 2.5 mL of ethanol (96%) stored 3h in a fridge and afterwards in a freezer until measurement of the bacteria population. Gram negative bacteria were fixed in the same ratio but instead of ethanol PFA (4 % paraformaldehyde) has been used. Then sample was kept 3 h in fridge. Afterwards has been washed in tap water three times and at the end storage in ethanol/water/PBS (ratio 10:9:1) solution in the freezer until measurement.

The detailed procedure of this method is described in Appendix, chapter 9.4.

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5. Results from laboratory experiments

This section contains the results obtained from the laboratory experiments. The results from tests are shown in tables and on figures. A general discussion about the obtained results and their interpretation is located in chapter 6.

5.1. P-release and C-consumption experiment with different VFAs as a carbon source

The test was made to obtain P-release rates and reaction stoichiometry for HAc, HPr and HLa and to see, how activated sludge from three WWTPs behave with different VFAs.

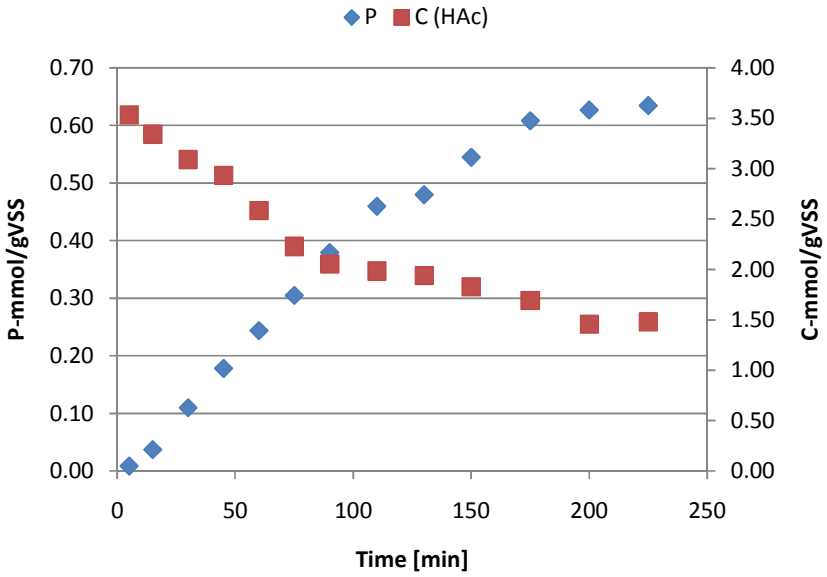

Figure 11. P-release and HAc uptake during 4 hour experiment – Aalborg West

Figure 11 shows an example of P-release and C-uptake (in this case HAc) obtained during set up lab experiments with usage of Aalborg West activated sludge. Afterwards the same experiments were conducted for three WWTPs. The kinetic results are shown in figure 12. In table 3 are placed stoichiometry calculations for each WWTP.

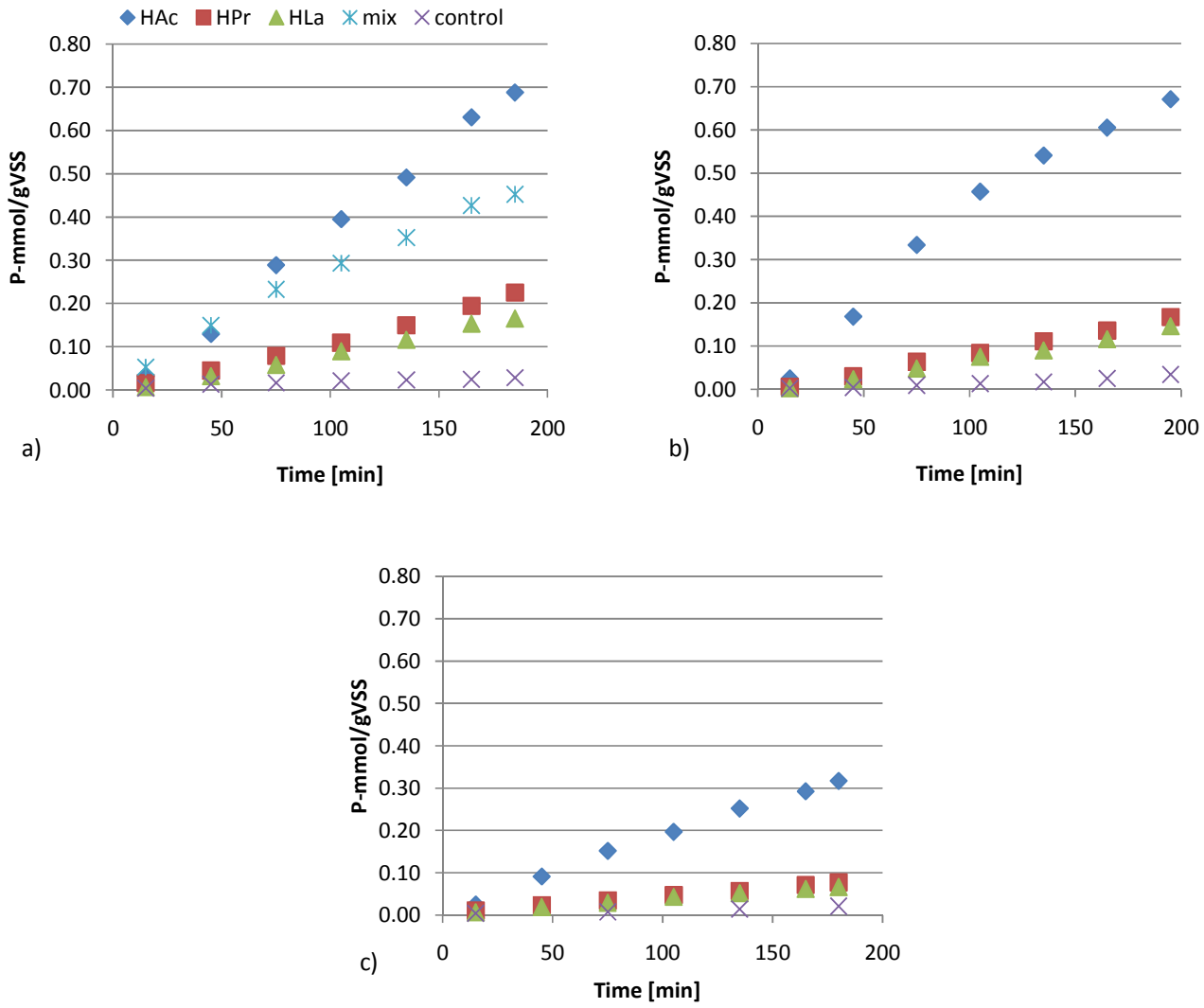

Figure 12. P-release for different VFAs in: a) Aalborg West, b) Hjørring and c) Bjerringbro

On figure 12 can be seen that the fastest P-release is obtained accordingly in reactors with HAc, HPr and HLa. The results for Aalborg West and Hjørring look comparable and outcomes from Bjerringbro are lower. In Aalborg West high P-release rate was also obtained with the mix of three VFAs.

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Table 3.Kinetics and stoichiometry data of P-release with VFAs from three WWTPs

WWTP	VFA	P-release rate [P-mmol/(gVSS*h)]	% of maximum P-release rate	VFA uptake [C-mmol/(gVSS*h)]	P-release/VFA uptake [P-mmol/C-mmol]
Aalborg West	Acetic Acid	0.230	100	0.340	0.680
	Propionic Acid	0.072	31.3	0.132	0.550
	Lactic Acid	0.060	26.1	0.096	0.630
	Mix 100 mg COD of HAc, HPr, HLa	0.138	60.0	HAc=0.072 HPr=0.100 HLa=0.042	0.640
	No substrate	0.006	2.6	n.a	n.a.
Hjørring	Acetic Acid	0.216	100	0.596	0.360
	Propionic Acid	0.054	25.0	0.216	0.250
	Lactic Acid	0.048	22.2	0.276	0.171
	No substrate	0.012	5.5	n.a	n.a.
Bjerringbro	Acetic Acid	0.102	100	0.276	0.380
	Propionic Acid	0.024	23.5	0.060	0.400
	Lactic Acid	0.024	23.5	n.a	n.a.
	No substrate	0.006	5.9	n.a	n.a.

Results in table 3 show, that in all cases P-release rate is higher when VFAs were added compared to control sample without substrates. The number of P-mmol released to C-mmol uptaken in Aalborg West is higher, compared to the other two plants. As well attention must be paid on numbers obtained when mix of substrates were used in Aalborg West compared to reactors with single VFAs. In this case P-release is higher than in HPr and HLa reactor but lower than in HAc. That can suggest some preference of bacteria to take up the different carbon source.

5.2. P-release experiment with different amino acids as a carbon source

The aim of the experiment was to investigate P-release process with AAs as a carbon source. This experiment was conducted with addition of six different amino acids: alanine, cysteine, glycine, glutamic acid, serine and valine to the six single reactors. Detailed procedure for this experiment is shown in Appendix in chapter 9.1.1.

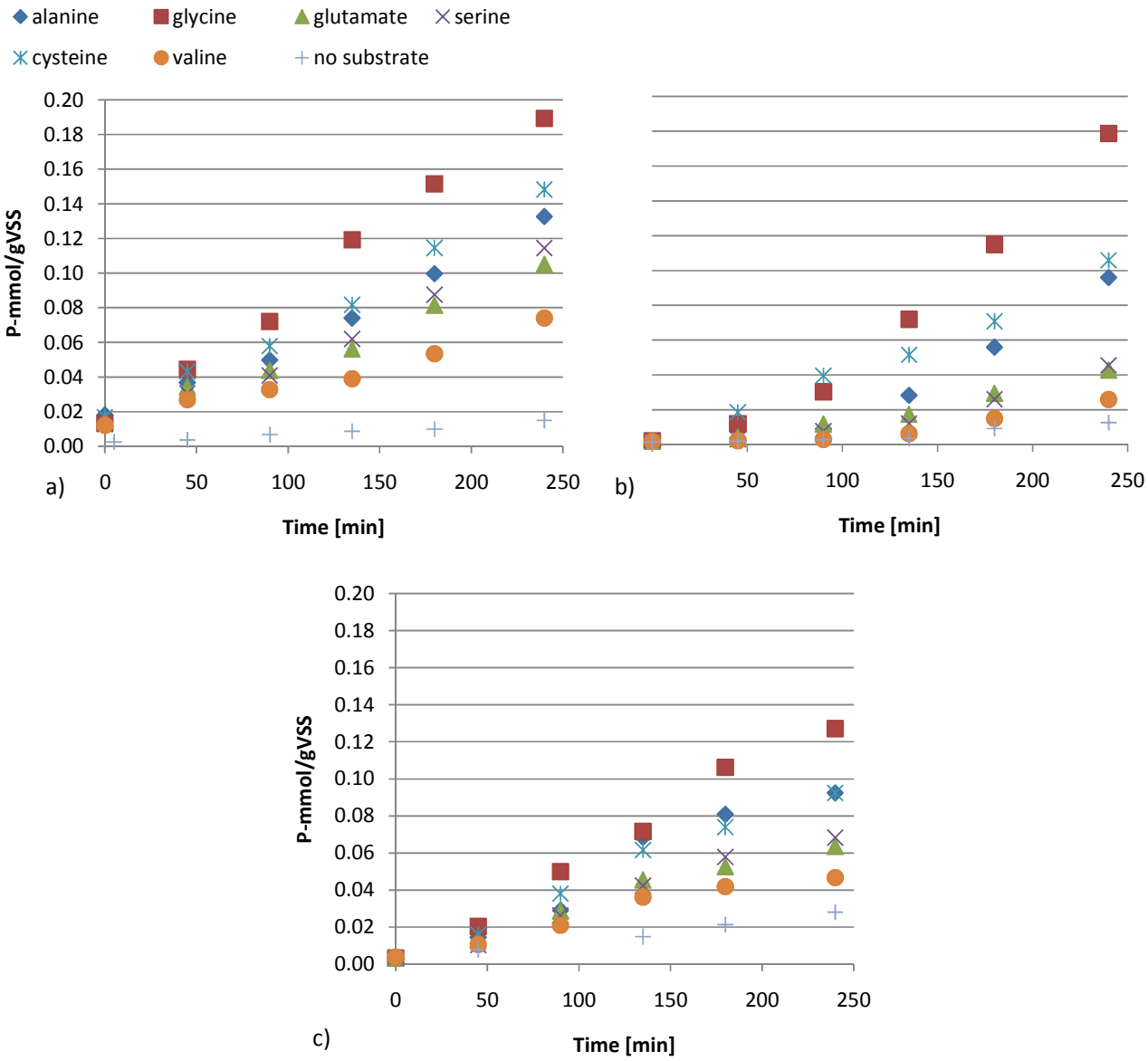

Figure 13. P-release versus time for 6 chosen amino acids in a) Aalborg West, b) Hjørring and c) Bjerringbro (01.2009)

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In all three cases glycine gave the highest P-release rate. The P-release rates in Bjerringbro were the lowest from three investigated WWTPs.

Table 4. Rates of P-release for six different amino acids in three WWTPs

WWTP	**Aalborg West**	**Hjørring**	**Bjerringbro**
Amino acid	**Release rate [P-mmol/(gVSS*h)]**		
Alanine	0.030	0.030	0.024
Glycine	0.048	0.054	0.036
Glutamate	0.024	0.012	0.018
Serine	0.024	0.012	0.018
Cysteine	0.030	0.024	0.024
Valine	0.012	0.006	0.012
No substrate	0.003	0.004	0.006

As well as in a case with VFAs, it can be seen, that addition of amino acid results in higher P-release rate than the control with no substrate (table 4). Common pattern is shown in all three sludge that glycine, cysteine and alanine yielded the highest rates. Figure 13 shows, the same as in the VFAs case, that P-release rates in Aalborg West and Hjørring were comparable and smaller in Bjerringbro.

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5.3. P-release experiment with amino acids as a carbon source at different pH conditions

The aim of this experiment was to investigate, if pH had an effect on P-release, when AAs were used as a carbon source. From previous test (chapter 5.2) three amino acids which cause highest P-release rate were chosen (glycine, cysteine and alanine) as a carbon source to conduct pH experiment. Before substrate was added to the reactors pH conditions were changed in the range from 5 to 10 with the step of 1 according to the procedure written in chap. 4.2.1.1. In Appendix 9.1.2.1. are attached figures of P-release in Aalborg West in the case of three substrates and different pH. The figures from Hjørring and Bjerringbro are enclosed on a CD. Figures 14-16 show combined column figures on which can be seen P-release rates expressed in P-mmol/(gVSS*h) depending on pH value and substrate added in all three WWTPs.

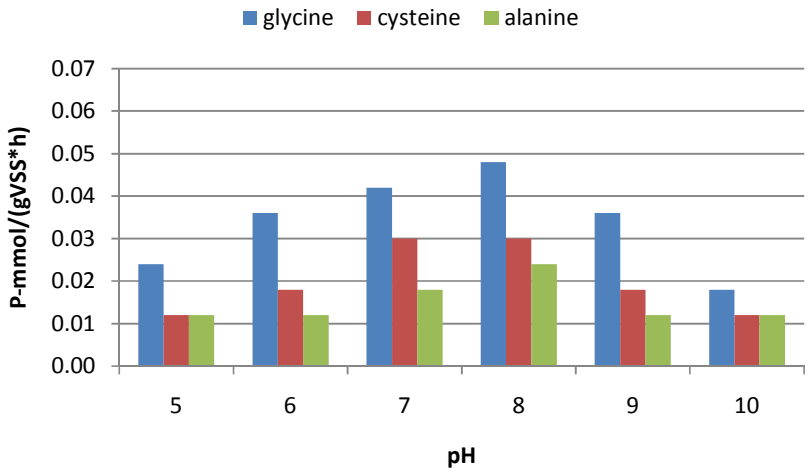

Figure 14. P-release rates versus pH in Aalborg West WWTP

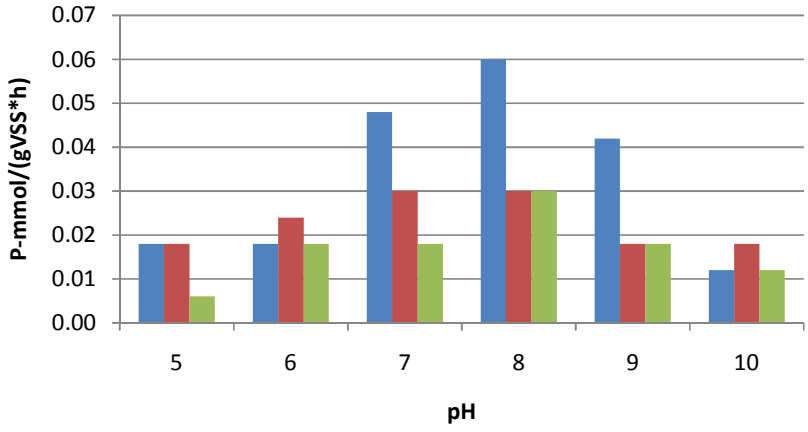

Figure 15. P-release rates versus pH in Hjørring WWTP

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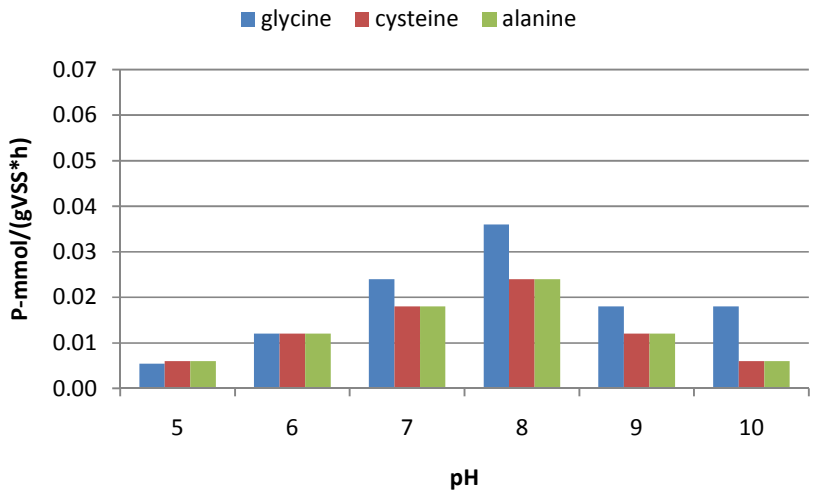

Figure 16. P-release rates versus pH in Bjerringbro WWTP

Results from figures 14-16 show two same tendencies for all three WWTPs. At first, on each figure the highest P-release rate is for glycine. For each amino acid can be seen same P-release tendency due to pH conditions. In all cases the lowest release was for the most alkaline (pH 10) and afterwards the most acidic (pH 5) conditions. The highest P-release was accordingly to reactor with pH 8, than 7.

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5.4. P-release kinetics in relation to stoichiometry of amino acid added to the reactor as carbon source

In connection with lack of the result of C-consumption (HPLC defect) in P-release experiment with usage of AAs, this experiment was conducted to obtain stoichiometry for P-release with glycine as carbon source. Four reactors were prepared with addition of limited amount of glycine of 0, 10, 20 and 30 mg COD/L. The difference in P-release after a certain period was assumed to be related to different glycine additions. The experiment was made on sludge from Aalborg West WWTP.

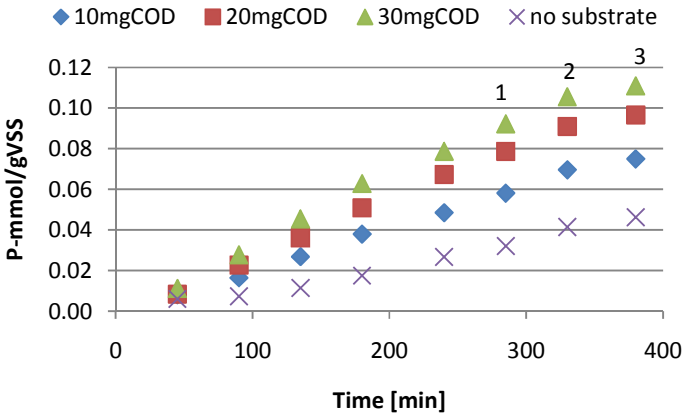

Figure 17. P-release kinetics in four reactors: three with different amounts of carbon source (glycine) and control reactor without substrate in Aalborg West WWTP

P-released for this experiment was calculated for the last three points for each curve. As was mentioned before, there was made assumption that differences in the PO_4^{3-} concentration in reactors are caused by addition of glycine. Than for each reactor (10, 20 and 30 mgCOD) and for each point (1, 2, 3) concentration in supernatant is calculated as a difference of concentration for reactor with glycine and with no substrate:

$$\text{Release} = C_P(WS)-C_P(NS)\ [\text{P-mmol/gVSS}] \qquad \text{Equation 3.}$$

$C_P(WS)$ – concentration of PO_4^{3-} in supernatant in reactor with substrate,
$C_P(NS)$ – concentration of PO_4^{3-} in supernatant in reactor with no substrate.

Table 5. Comparison of three reactors with different addition of glycine

Reactor	C-mmol/L	$C_P(WS)-C_P(NS)=$			mean [P-mmol/gVSS]	[C-mmol/gVSS]	Release [P-mmol/C-mmol]
10 mgCOD	0.277	1) 0.026	2)0.028	3)0.029	0.028	0.081	0.340
20 mgCOD	0.554	1) 0.047	2)0.049	3)0.050	0.049	0.162	0.301
30 mgCOD	0.831	1) 0.060	2)0.064	3)0.065	0.063	0.242	0.261

From table 5 it can be seen that stoichiometry experiment gave average value of 0.300 P-mmol/C-mmol ratio for P-release (mean of three reactors) with usage of glycine.

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5.5. P-release with mixture of acetate and different amino acids as a carbon source

The aim of experiment was to look on the sludge behavior with mix of carbon source: VFAs and AAs. Besides experiments were made in lab temperature (~25°C) to accelerate the process and obtain overview about full capacity of P-release with different carbon sources. Lab investigations were divided on three parts and are shown in forthcoming sections.

5.5.1. P-release maximum with amino acids and casamino acids

The test was conducted to get the knowledge about maximum P-released with three chosen amino acids (glycine, cysteine, alanine) and casamino acids compare to P-release with HAc.

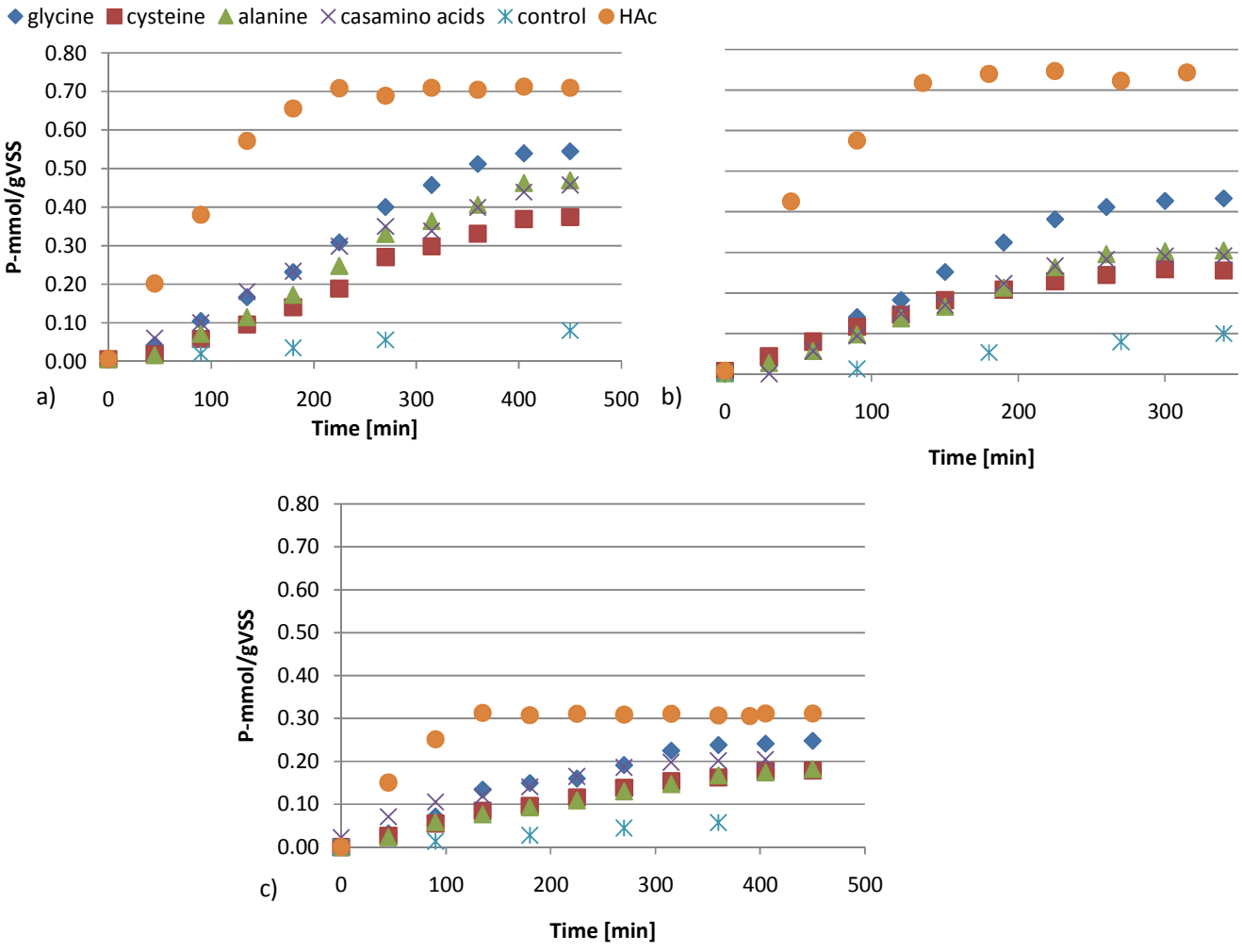

Figure 18. P-release with amino acids compare to HAc in a) Aalborg West, b) Hjørring and c) Bjerringbro

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From the results gathered on figure 18 it can be seen that higher and comparable P-release were obtained in Aalborg West and Hjørring in relation to Bjerringbro. As in the previous investigations with amino acids, it is shown that highest P-release is in reactors with glycine and then accordingly with casamino acids, alanine, cysteine. In the case of casamino acids the P background was subtracted (0.06 P-mmol/gVSS) from the final results. The comparison of obtained results with P-release with HAc is shown in table 6.

Table 6. Maximum P-release capacity in reactor with amino acids compare to P-release in reactor with HAc expressed in percent for all three WWTPs

WWTP	Glycine/HAc [%]	Cysteine/HAc [%]	Alanine/HAc [%]	Casamino acids/HAc [%]
Aalborg West	66.6	45.8	57.5	63.2
Hjørring	55.7	33.3	39.2	45.2
Bjerringbro	79.3	57.1	58.1	84.6

5.5.2. P-release with mix of HAc and single amino acids

The aim of this experiment was to look how the P-release process looks, when as a carbon source are delivered both VFAs (HAc) and AAs (glycine, cysteine, alanine).

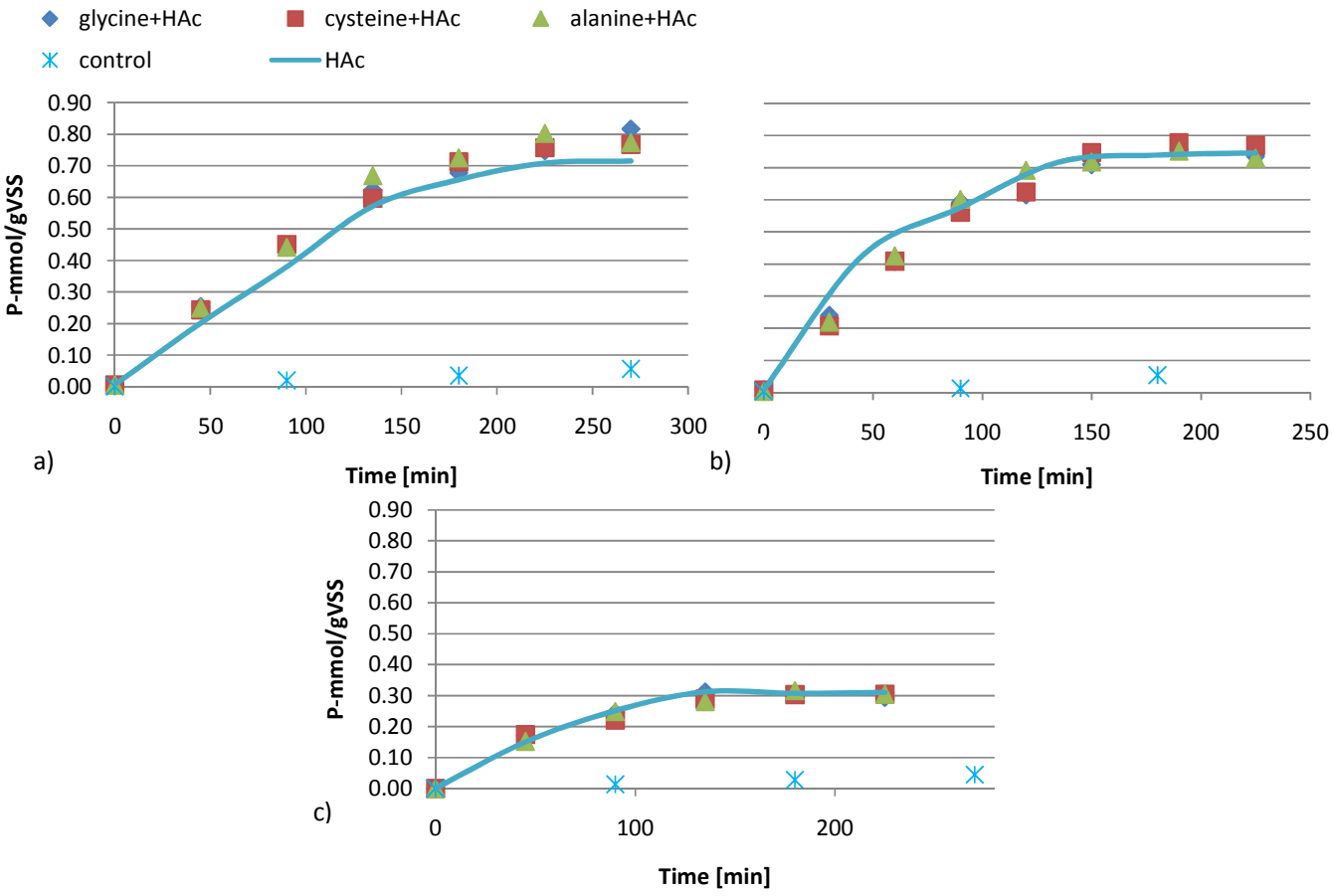

Figure 19. P-release with mix of HAc with AAs in a) Aalborg West, b) Hjørring and c) Bjerringbro

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On the figure 19 for all three WWTPs P-release is finished on the certain level. However for Aalborg West and Hjørring is much higher than for Bjerringbro. Moreover in Aalborg West and Hjørring it can be observed slightly – around 10-15% higher P-release level compare to reactor with only HAc, what was not noticed in Bjerringbro. The maximum P-release is obtained faster than in experiment with single AAs.

5.5.3. P-release with reverse addition of HAc and glycine as carbon source

The aim of the experiment was to investigate how PAO release P, when carbon sources were added on reverse way. Into the first reactor was added HAc and to second glycine. After time, to the reactor one was added glycine and to the reactor two HAc, to look if it will cause the additional P-release.

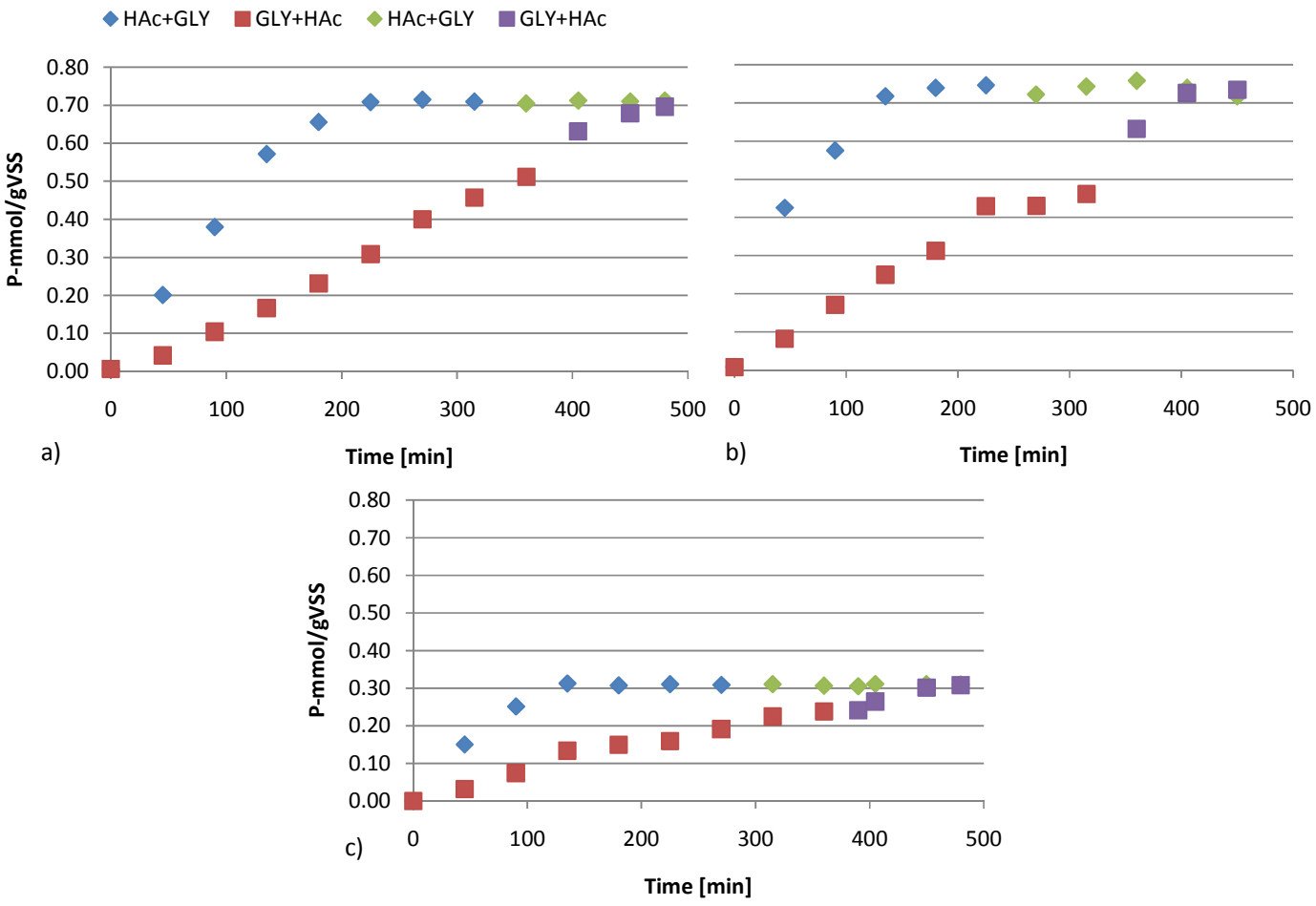

Figure 20. P-release with reverse addition of HAc and glycine in a) Aalborg West, b) Hjørring and c) Bjerringbro

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P-release in all three WWTPs finished at the certain level, no matter in which order substrates (HAc, glycine) were added. As previous, the P-release in Aalborg West and Hjørring was comparable and outstanding from Bjerringbro results.

5.5.4. FISH - bacteria quantification

FISH analysis was made to get exact and actual numbers of PAOs in the bacteria community and is shown in table 7. Numbers mean % of specified group of bacteria in the whole population of bacteria in the sludge used for experiments in section 5.5.

Table 7. Bacterial community diverse in sludge used for experiment

WWTP / **Groups of organisms**	Aalborg West	Hjørring	Bjerringbro
Accumulibacter	3.6	2.0	2.5
Actinobacteria type1	6.0	5.2	1.5
Actinobacteria type2	4.5	11.4	2.5
GAOs	0.5	1.5	0.5
Defluviiicoccus vanus	0.5	0.9	1.1

The diversity of bacteria is similar to the values in table 2. The highest number of *Accumulibacter* is in Aalborg West and APAO is in Hjørring.

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5.6. P-release rates with 20 single amino acids

The aim of experiment was to check how different sludge communities behave with each of 20 single amino acids and control reactor without carbon source. It was made due to very limited number of results on this aspect. Experiments were made to obtain the P-release rate and full capacity of P-release (which in some cases was not obtained probably due to insufficient incubation time). Tests were made in lab temperature ~25°C to accelerate the process. Figures 21-23 shows the results for each WWTP.

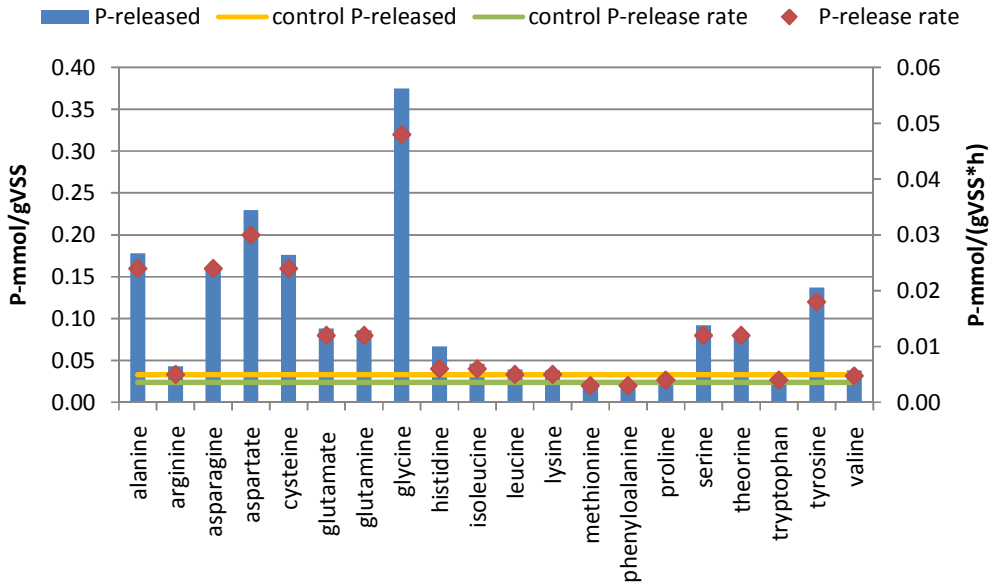

Fig 21. P-released and P-release rate with usage of 20 single amino acids in Hjørring WWTP (07.2009)

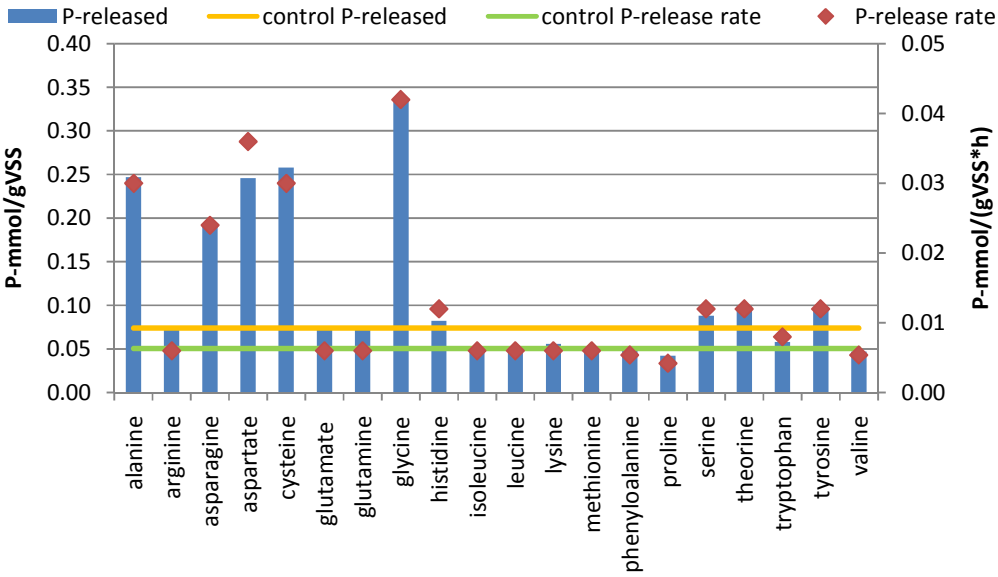

Fig 22. P-released and P-release rate with usage of 20 single amino acids in Aalborg West WWTP (07.2009)

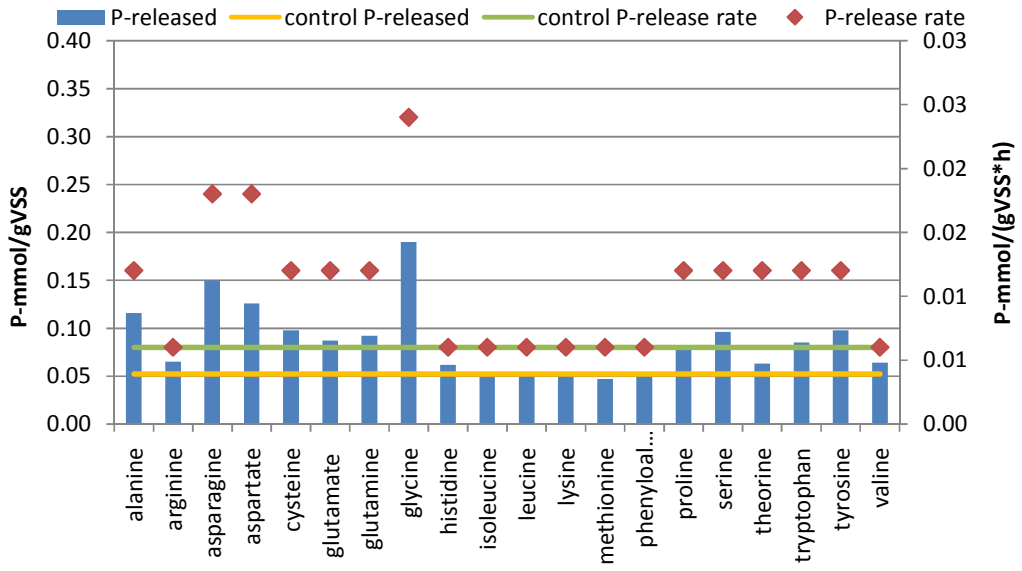

Fig 23. P-released and P-release rate with usage of 20 single amino acids in Bjerringbro WWTP (07.2009)

From the three figures above can be seen that only some amino acids caused a P-release. As in the previous experiments, results from Aalborg West and Hjørring are quite comparable to each other and a little different from Bjerringbro outcomes. In all three cases glycine caused highest P-release rate and P-released after 8h of experiment what corresponds to previous results.

By taking the P-release rate and the total amount of P released can be seen, that these results correspond well. The same trend was observed in all three case WWTPs and is shown on figure below.

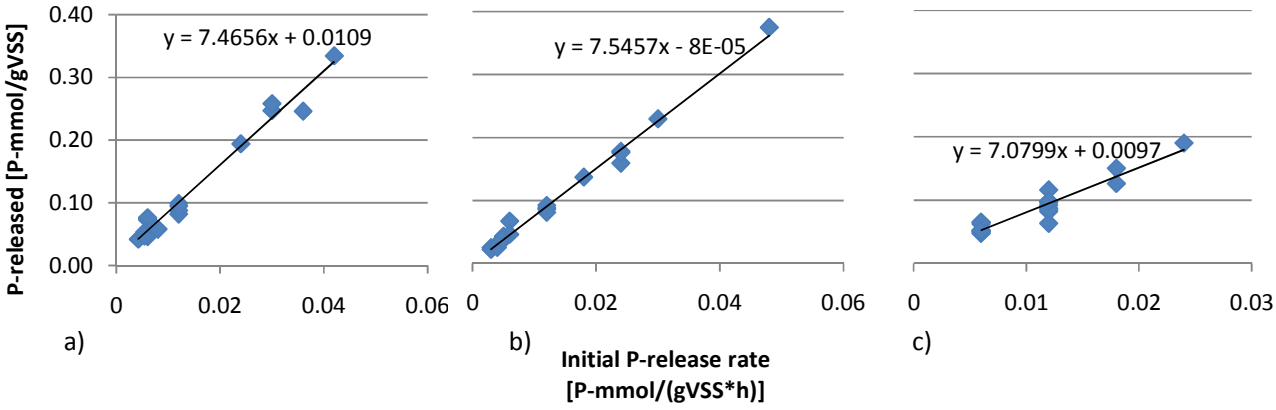

Figure 24. Initial P-release rate versus total P-released in a) Aalborg West, b) Hjørring and c) Bjerringbro

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5.6.1. FISH - bacteria quantification

Besides P-release batch test, FISH analysis was made to obtain precise quantification of microorganisms involved in the process (PAO).

Table 8. Bacterial community diverse in sludge used for experiment

WWTP / **Groups of organisms**	Aalborg West	Hjørring	Bjerringbro
Accumulibacter	3.2	2.4	2.8
Actinobacteria type1	4.0	4.8	2.7
Actinobacteria type2	3.6	9.7	2.0
GAOs	0.5	1.7	0.5
Defluviiicoccus vanus	0.8	1.0	0.5

The highest APAO community (bacteria considered to be responsible of AAs uptake) was in Hjørring WWTP. Moreover *Actinobacteria* type 2 was greatly outstanding in this WWTP. In Bjerringbro population of APAO was the lowest from all three cases. *Accumulibacter* population was the highest in Aalborg West WWTP.

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5.7. Variations in P-release during short and long-term experiment with HAc as a carbon source in Aalborg West Wastewater Treatment Plant

This experiment was conducted to get overview how the P-release process is changing during the time. At first short term variations were measured during 5 days of one week. Besides during the eternal time of the project, the P-release rate was measured once a week with some exceptions. The experiment was conducted in standardized conditions, with usage of HAc in quantity of 300 mg COD/L and in 15°C. P-release was measured during 3 hours in the anaerobic phase of the EBPR on sludge from Aalborg West WWTP.

5.7.1. Short term – daily variations

The short time experiment was made during five days in November 2008. The P-release rate was measured every day of those five days in 2 reactors. Every day, fresh activated sludge sample was brought from Aalborg West WWTP.

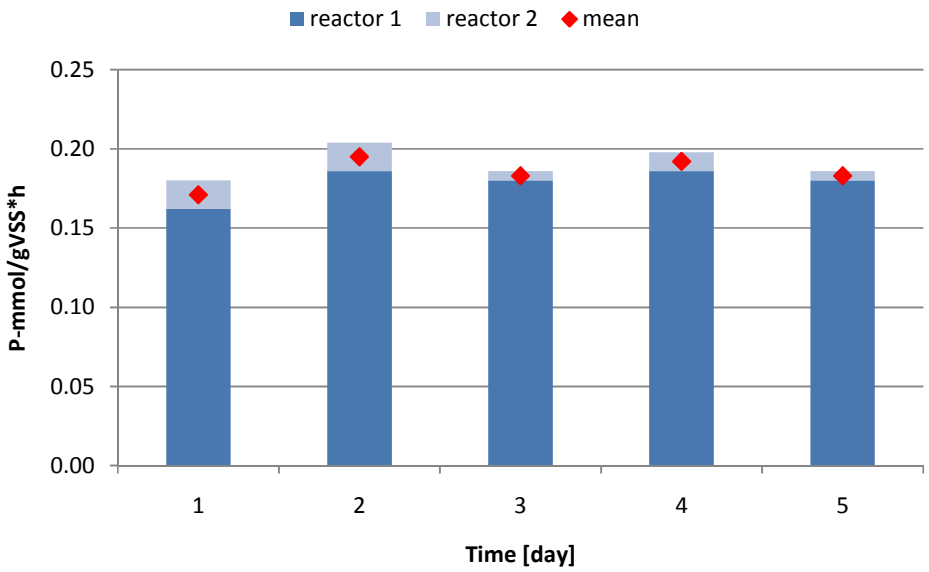

Figure 25. Comparison of P-release rates for 5 days of measurements (Aalborg West)

Table 9. Results from daily variations in 5 days experiment

Date	Reactor 1 P-mmol/(gVSS*h)	Reactor 2 P-mmol/(gVSS*h)	Mean of 2 reactors P-mmol/(gVSS*h)	VSS [g/L]
24.11.2008	0.180	0.162	0.171	3.14
25.11.2008	0.204	0.186	0.195	2.80
26.11.2008	0.186	0.180	0.183	3.05
27.11.2008	0.198	0.186	0.192	3.21
28.11.2008	0.186	0.180	0.183	3.11

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From the results it can be seen than the P-release was very similar and daily variations were not significant. Median for these data is 0.186 P-mmol/(gVSS*h), mean 0.185 P-mmol/(gVSS*h), standard deviation 0.011 P-mmol/(gVSS*h) and coefficient of variation C_v= 6.05%. VSS were measured every time new sample of sludge was brought from WWTP. Mean for VSS from week measurements was 3.06 gVSS/L.

5.7.2. Long term – ten months variations

The long term experiment was conducted during the entire time of project work. P-release of activated sludge from Aalborg West WWTP was measured as far as possible once every single week. SS and VSS were measured every time new sludge was obtained.

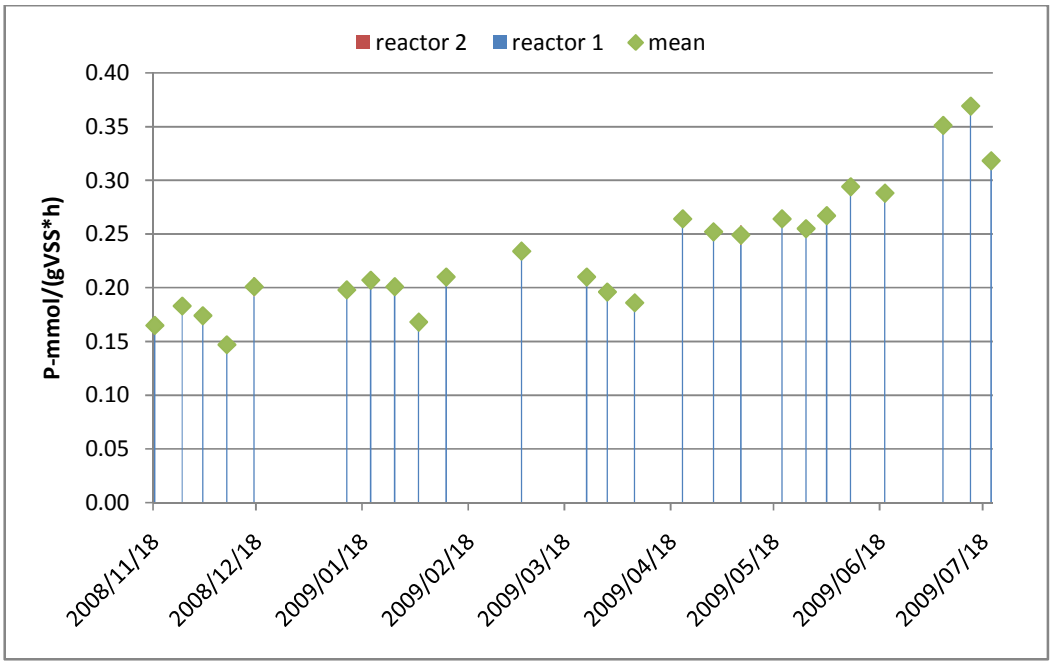

Figure 26. Comparison of P-release rate for long term experiment measurements (Aalborg West)

On figure 26 is shown P-release rate for long term variations experiment. The range of P-release rate during ten months experiment was 0.174-0.372 P-mmol/(gVSS*h). Median for these data is 0.210 P-mmol/(gVSS*h), mean 0.234 P-mmol/(gVSS*h), standard deviation 0.0578 P-mmol/(gVSS*h) and coefficient of variation C_v=27.5%. Mean value of VSS is 3.18 g/L.

Detailed data for this experiment are placed in Appendix 9.7.

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6. Discussion and summary of the results

Three main lab techniques – anaerobic P-release test, C-consumption measured by the HPLC method and FISH analysis – were used to compare the ability of three types of activated sludge to maintain anaerobic phase of EBPR process. The purpose of the study was to explore the effect of carbon source on the anaerobic stage of the bio-P removal process and to investigate the correlation between reaction rates and stoichiometry and the microbial community. In order to get an overview about sludge behaviours seven different lab experiment were conducted. In the following, a discussion of the results of each type has been made.

6.1. P-release and C-consumption experiment with different VFAs as a carbon source

The experiment with different VFAs shows what was confirmed in many previous studies that acetic acid is the most effective carbon source from EBPR point of view. From the three VFAs used in experiment: HAc, HPr and HLa the highest P-release was obtained in reactors with usage of HAc in all three WWTPs and next accordingly with HPr and HLa. As can be seen from the results, P-release rate with HPr as carbon source were around: 25% for Hjørring and Bjerringbro and 31% for Aalborg West WWTP value of P-release rate with HAc as carbon source. That is comparable with results obtained by Tykesson *et al.* 2006 [32] where this proportion was ca. 30%. The release/uptake ratio expressed in P-mmol/C-mmol is reported to be 0.72 for HAc and 0.46 for HPr by Pijuan 2004 [16] and 0.77 for HAc and 0.44 for HPr by Abu-Ghararah *et al.* 1991 [41]. Actually, results obtained from this study are in the same range in Aalborg West: HAc 0.68 P-mmol/C-mmol and HPr 0.55 P-mmol/C-mmol. However ratios of 0.36 P-mmol/C-mmol for HAc and 0.25 P-mmol/C-mmol for HPr in Hjørring and 0.38 P-mmol/C-mmol for HAc and 0.40 P-mmol/C-mmol for HPr in Bjerringbro seem to be slightly different. When we compare P-release rates in Aalborg West and Hjørring they look quite similar. P-release for HAc was 0.230 in Aalborg West and 0.216 P-mmol/(gVSS*h) in Hjørring and for HPr accordingly 0.072 and 0.054 P-mmol/(gVSS*h). Nevertheless, when we look on uptake of VFA (table 3) it is higher in Hjørring. It can be explained by microbial community structure. The difference of VFA uptake is probably caused by higher abundance of GAOs (table 16 and 17, chapter 9.6 in Appendix) in bacterial community, ca. 3% in Hjørring and 0.5% in Aalborg West [10, 27]. GAOs are able to take up carbon source in anaerobic conditions, but without release of P. This probably caused a difference in release/uptake ratio in these two WWTPs, even though what was above mentioned P-release is on the same level. Looking on the results from this experiment, it is easy to see distinction between Bjerringbro and rest two WWTPs. P-release in Bjerringbro was much lower (0.102 P-mmol/(gVSS*h) for HAc and 0.024 P-mmol/(gVSS*h) for HPr) than in Aalborg West and Hjørring. It can be explained by lower general number of PAOs (*Accumulibacter* and APAO) in Bjerringbro WWTP. The abundance of PAO in Bjerringbro is around 6.5% compared to 12.5% in Aalborg West and 15% in Hjørring. In summary, it seems

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that Aalborg West microbial community has the highest ability to conduct anaerobic P-release the fastest and most efficiency way using VFAs, what corresponds to the highest number of *Accumulibacter* in this WWTP. Additionally, in Aalborg West P-release was also conducted for mix of three investigated VFAs. What can be seen on fig. 12a P-release is lower than in a case of HAc. That can suggest that when mixture of VFAs was injected to the reactor, PAOs take all three VFAs, not only HAc (see table 3, p.33). The composition of substrates and their relation play important role in the process, what was in depth shown by Lopez-Vasquez *et al.* 2008 [11].

6.2. P-release experiment with different amino acids as a carbon source

Similar experiments as with VFAs were conducted with usage of six chosen amino acids – alanine, cysteine, glycine, glutamic acid, serine and valine. Based on the investigations, it can be concluded, that P-release rates, in the case of amino acids, are much slower than in the case of VFAs. In each investigated WWTP the highest P-release rate was obtained in reactors with glycine, cysteine and alanine accordingly (figure 13 and table 4). From these three AAs, PAOs were able to release P the fastest with usage of glycine. It seems that as HAc is the best among the VFAs, glycine is the best AA for bacteria to conduct anaerobic phase of the EBPR. However P-release rates in reactors with glycine were around 21% for Aalborg West, 25% for Hjørring and 35% Bjerringbro of values obtained for HAc as carbon source. In this group of six amino acids, the ones with highest P-release rate, have the simplest structure without long carbon chains. That can lead to the conclusion that bacteria prefer low chain molecules in the case of AAs the same as in the case of VFAs (Pijuan 2004, [16]).

6.3. P-release experiment with amino acids as a carbon source at different pH conditions

From available information for the author, there are no studies on the influence of pH on the anaerobic P-release with AAs carbon source. This experiment has led to the observation that the highest P-release was at 7-8 pH conditions. The same trend was observed both for all WWTPs and all three AAs used in this test. These values seem comparable with the literature results obtained with VFAs. Schuler *et al.* 2002 [23] noticed that respectively values of pH 8 and 6.9 were the best among 5.2 - 9.5 for anaerobic P-release, likewise Felipe *et al.* 2001 [42] who notice the best efficiency at pH 6.5 - 8 and value of pH 7.5 was noticed by Lopez-Vasquez *et al.* 2008 [11]. Obtained results reveal that substrate uptake in case of AAs is similar to VFAs. Moreover, it means that, APAO which are primarily linked with the P-release process with usage of AAs [26], behave similar to *Accumulibacter* mainly connected with conducting the process with VFAs uptake [25]. In strongly acidic and alkaline conditions carbon source cannot be taken up so efficiently by PAOs as in neutral conditions. The observable effect of this phenomenon is slower P-release. The reason of that are higher energy demands for uptake of carbon source [23].

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6.4. P-release kinetics in relation to stoichiometry of amino acid added to the reactor as carbon source

This experiment was conducted to check approximately P-release/AA-uptake ratio for glycine. What can be concluded the ratio varied around 0.30 P-mmol/C-mmol (average for 3 reactors, table 5, p.38). This value corresponds to the numbers obtained by Mielczarek 2007 [5]. The results from his project show ratio around 0.34 P-mmol/C-mmol for Hjørring and 0.30 P-mmol/C-mmol for Aalborg East WWTP. These ratios are admitted to differ in each WWTP as it can be seen in a case of VFAs, what is depended on microbial structure of the sludge. However comparison of this value to ratios for propionic acid and lactic acid which have close P-release rate to glycine (0.072 HPr, 0.060 HLa and 0.050 P-mmol/(gVSS*h) for glycine) seems much differently. These VFAs have accordingly values 0.55 and 0.63 P-mmol/C-mmol.

6.5. P-release with mix of acetate and different amino acids as a carbon source

6.5.1. P-release maximum with amino acids and casamino acids

This experiment was conducted to understand the nature of PAOs and their behavior with mix of carbon source supplied for the process. At first from figure 18 can be seen that maximum P-released with single amino acids is lower than in the case of HAc only. What is also visible from these figures, glycine is the AA for which the highest P-release is reached. Casamino acids also seem to be an efficient carbon source, but as it was mentioned before, these results may be disturbed by P background which is made by casamino acids. Table 7 shows that for amino acids released P can vary and reach 35-80% of P-released with HAc as carbon source.

6.5.2. P-release with mix of HAc and single amino acids

According to the figure 19 it can be seen the same tendency was observed in each WWTP. Namely P-released with three different mixes of carbon source (HAc+glycine, HAc+cysteine and HAc+alanine) gave the similar level in each plant separately. However, in Aalborg West and Hjørring a bit higher (~10-15%) P-release was observed for reactors with HAc and AA compared to reactor with only HAc. In Bjerringbro this dependency was not noticed. This can cooperate with results obtained by Mielczarek 2007 [5], where also higher (up to 25%) P- release was noticed in some cases of mix HAc with AA. This can imply inclusion of some group of PAOs when carbon source is the mix of VFAs and AAs.

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6.5.3. P-release with reverse addition of HAc and glycine as carbon source

An interesting observation was made in experiments with reverse addition of HAc and glycine which is shown on fig. 20. When HAc was added first, P-release obtained a certain level and then after addition of glycine no P-release or very insignificant P-release was observed. In the case of second reactor where at first glycine was added, can be seen also some certain P-release, but afterwards and addition of HAc again P is released. It is finished on the same level as in first reactor. The results were expected to be different due to previous experiment where a little bit higher P-release was obtained in reactors with mix of HAc and AAs compare to single HAc. The additional release was observed in 2nd reactor, but in 1st was not. This observation was made in all three WWTPs. The results can be explained on few hypothetical assumptions by author. One can be presence of some specified group of *Accumulibacter* (or APAO which could utilize HAc), which in presence of HAc is able to conduct anaerobic phase of EBPR and release all P from the cells. After addition of AA this group is excluded from the process and do not utilize substrate and release P any longer. In case when glycine is first substrate, they can use it and get maximum release. Afterwards and addition of HAc this group do not take substrate anymore and further release is obtained by the other *Accumulibacter*. The second hypothesis state on knowledge from literature that *Accumulibacter* is able to take some AAs [25]. This experiment can prove that presence of this specific group in *Accumulibacter* community is quite significant and that they have a high influence of AAs uptake. The third conclusion is that experiment should be conducted longer time that maybe not all P was released especially when we look on 2nd reactor (glycine+HAc addition in this order). However, when results from 1st reactor are analyzed it can be stated that time was sufficient, because of no longer P-release.

6.6. P-release rates with 20 single amino acids

However previous experiments, with AAs (chapter 5.2.), were made with six of them, verification of all 20 single AAs and their ability to be carbon source in EBPR process was measured. As figures 21-23 shows not all amino acids gave P-release effect. In all three cases (three plants) isolucine, leucine, lysine, methionine and phenylalanine did not give P-release effect compared to the control reactor. The highest rates were accordingly in reactors with: glycine, aspartic acid, cysteine, asparagine and alanine. Only in Bjerringbro this order was disturbed and cysteine with asparagine had reverse order in this WWTP (fig.23). For serine, tyrosine, theorine, glutamine, glutamic acid P-release effect was observed and for histidine and valine very marginal effect appeared. These result confirmed results obtained by Sun Tao 2007 [4]. For Assens WWTP she found the highest P-release with: glycine, alanine, serine, aspartic acid and glutamic acid (cysteine and asparagine were not investigated). For Arla WWTP, the results were a bit different and the highest P-release was obtained for: serine, theorine, tyrosine, alanine, glycine however all rates were on very low level (P-release rates from 0.004-0.010 P-mmol/(gVSS*h). Generally, from this experiment can be concluded, that low chain AAs (C_1-C_4) are more suitable for APAOs to release P in anaerobic part of the

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EBPR process. Moreover long carbon chains (e.g. phenylalanine) or ring structure (e.g. histidine, tryptophan) influence on lowering efficiency of the anaerobic P-release (table 12) [5]. The reason is probably longer time needed for hydrolyse these compound and demand of microorganisms which can conduct breakdown of AAs on compounds with more simply structure. As well is not completely understood if AAs are directly utilized by APAOs or products of hydrolysis are e.g. taken up by *Accumulibacter* [4].

The linear correspondence was observed between initial P-release rate and P-released in all three cases (fig. 24). This observation leads to conclusion that P-release tests do not need to be conducted for a long time. It can be helpful in creation of standard test for WWTP's ability to conduct bio-P removal process.

FISH analysis has shown that the highest number of APAO (in total type one and 2) appeared in Hjørring WWTP. However the number of *Actinobacteria type 1* was similar in Aalborg West and Hjørring. As the results of P-release were similar in these two plants, it can imply that this group bacteria was responsible for the P-release process.

6.7. Variations in P-release during short and long-term experiment with HAc as a carbon source in Aalborg West Wastewater Treatment Plant

During the entire time of the project short term and long term variations in P-release were monitored in Aalborg West. Results are shown for daily variations on figure 25. It can be easily noticed that daily differences during the week are not significant ($C_v = 6.05\%$) and have probably no real influence on the entire EBPR process. However, in the space of months, there were observed higher fluctuations ($C_v = 27.5\%$). It can be seen on figure 26 that P-release rates were getting higher summer and spring compare to winter. It is probably strongly connected with changes in bacterial community during the year. Even though the experiment was always conducted in same temperature (15°C) and substrate addition (300 mg COD/L added as HAc) conditions, it can be state that sludge from warmer months has higher ability to conduct anaerobic phase of EBPR at high rate. It can be connected to abundance of PAOs in bacterial community which tends to be higher during warmer periods of the year [5]. As can be seen on fig. 39 in Appendix, changes in bacterial community during the year are noticeable. Especially the trend of higher abundance of RPAO during warm months can be noticed. It is the presumable reason of higher P-release rates obtained in long term experiment during the summer.

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6.8. Summary

Table 10. Summary results from the study case

WWTP / Characteristic	Aalborg West	Hjørring	Bjerringbro	Comments
General characteristics				
PE designed/PE actual	330,000/195,000	160,000/100,000	65,000/18,200	
Bio – P process	+	+	+	
Side-stream hydrolysis	+	-	-	
Configuration	Biodenipho	recirculating	Biodenipho (SBR)	
Industrial wastewater	30%	30%	23%	
Group of organisms	**Microbial population**			
Accumulibacter	1) 3.6 2) 3.2	1) 2.0 2) 2.4	1) 2.5 2) 2.8	1)05.2009 2)07.2009
Actinobacteria type1	1) 6.0 2) 4.0	1) 5.2 2) 4.8	1) 1.5 2) 2.7	
Actinobacteria type2	1) 4.5 2) 3.6	1) 11.4 2) 9.7	1) 2.5 2) 2.0	
GAOs	1) 0.5 2) 0.5	1) 1.5 2) 1.7	1) 0.5 2) 0.5	
Chemical compound	**Kinetics and stoichiometry of anaerobic P-release**			
VFAs: HAc	1) 0.230 2) 0.680	1) 0.216 2) 0.360	1) 0.102 2) 0.380	1)P-release [P-mmol/gVSS*h] 2) P-release/VFA uptake ratio [P-mol/C-mmol]
HPr	1) 0.072 2) 0.550	1) 0.054 2) 0.250	1) 0.024 2) 0.400	
HLa	1) 0.060 2) 0.630	1) 0.048 2) 0.171	1) 0.024	
AAs: Glycine	0.048	0.054	0.036	P-release [P-mmol/gVSS*h]
Alanine	0.030	0.030	0.024	
Cysteine	0.030	0.024	0.024	
Amino acids with the highest P-release rates	alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, serine, tyrosine, theorine, valine			

The obtained results show that anaerobic P-release occurred with usage of amino acids by PAOs. That can imply that in full-scale WWTPs, where exist high concentration of proteins (can be hydrolyzed to AAs) in the influent (e.g. fish industry) the anaerobic phase of the EBPR process can be conducted. However the P-release kinetic is slower than in the case of VFAs and it can cause slow-down of the process.

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7. Conclusions

The project has led to the following conclusions:

- acetic acid among different volatile fatty acids and glycine among tested amino acids gave the highest P-release rate in the anaerobic phase of EBPR process,

- PAOs were able to release maximum 35-80% of P with amino acids compared to P-release with acetic acid, what can imply that these substrates are taken by different groups of bacteria. It also imply that not all carbon sources are suitable for EBPR process and cause the same P-release efficiency,

- among 20 single AAs, P can be released only with group of them (see table 10 in Summary-chapter 6.8),

- the highest P-release with usage of amino acids occurred in pH 8 and 7. These results are comparable to literature results with volatile fatty acids,

- short term variations (one week) on potential P-release rates were not significant and probably have no influence on the EBPR process. However there are existing seasonal variations in the P-release process which are connected with microbial population variations during the year. The observed variations are important and were in the range from 0.147 to 0.372 P-mmol/(gVSS*h).

All in all, this study showed that importance of AAs as carbon source in the EBPR process cannot be omitted. The results demonstrate that AAs – along with the VFAs – are important substrates. Standardized tests for the P-release capacity should not only focus on VFAs but preferably also include AAs. Among the AAs, glycine could be used as a possible model carbon source, in standard P-release test. However, future investigations must be made on analysis of consumption of amino acids and formation of PHA by PAOs, what would give the full overview about this carbon source.

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8. Literature

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9. Appendix

9.1. Procedures of lab experiments

9.1.1. P-release experiment with different carbon sources (VFAs, AAs)

Almost all experiments were made following to procedure below:

- activated sludge was pre-aerated during ca. 40 min and kept in water bath in 15°C, stirring was made by magnetic plate and stirrers (~250RPM),
- after pre-aeration conditions were changed to anaerobic by purging N_2 gas into bulk liquid by ca. 15 min. Stirring was kept on 150RPM,
- next N_2 was flushed into the head space of reactor to get rid of O_2,
- after those preparation substrates were added to reactors,
- sampling was made every 30-45 minutes. P and C consumption were measured.

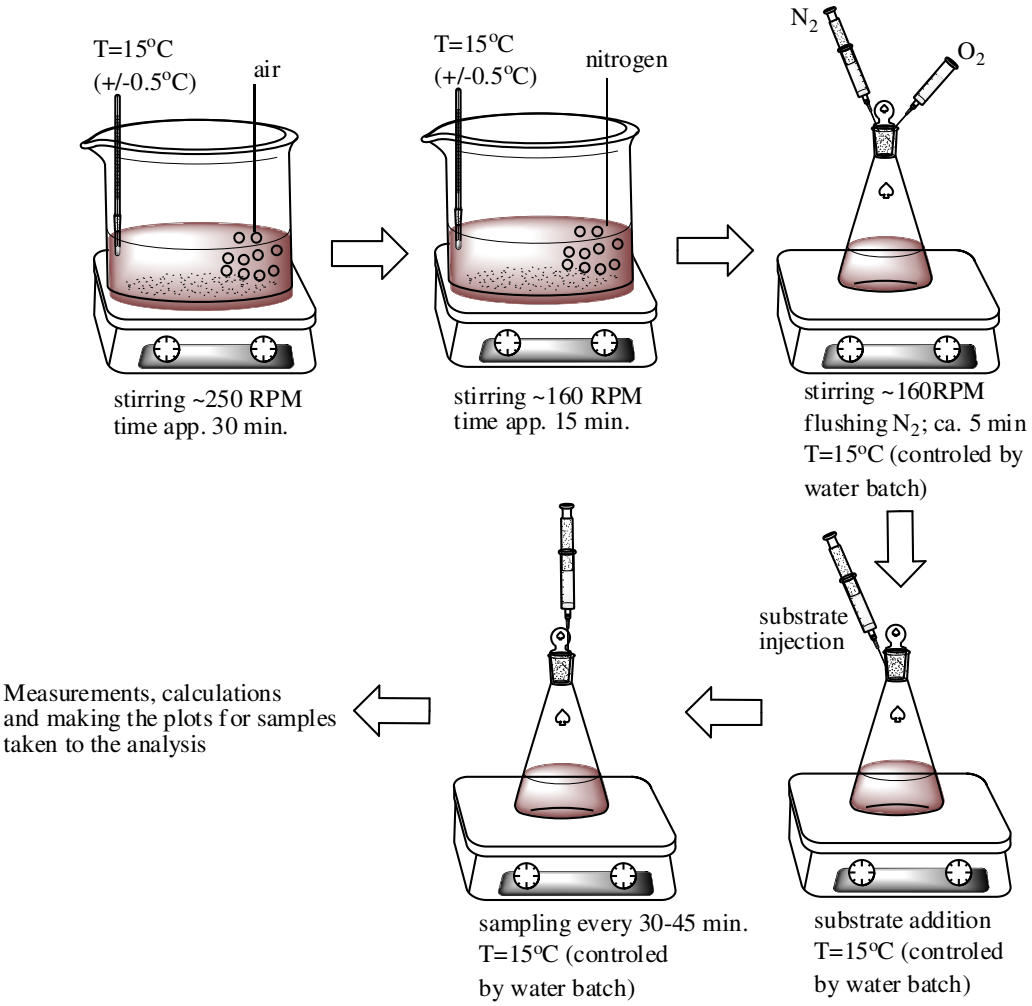

Figure 27. General experiment set up and conditions

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9.1.2. P-release experiment with pH adjustment and AAs as carbon source

Procedure of this experiment was identical as 9.1.1. Only difference was before adding the substrate pH conditions were change in the reactors by adding 1M HCl to obtain acidic conditions in reactor or 1M NaOH for alkaline conditions.

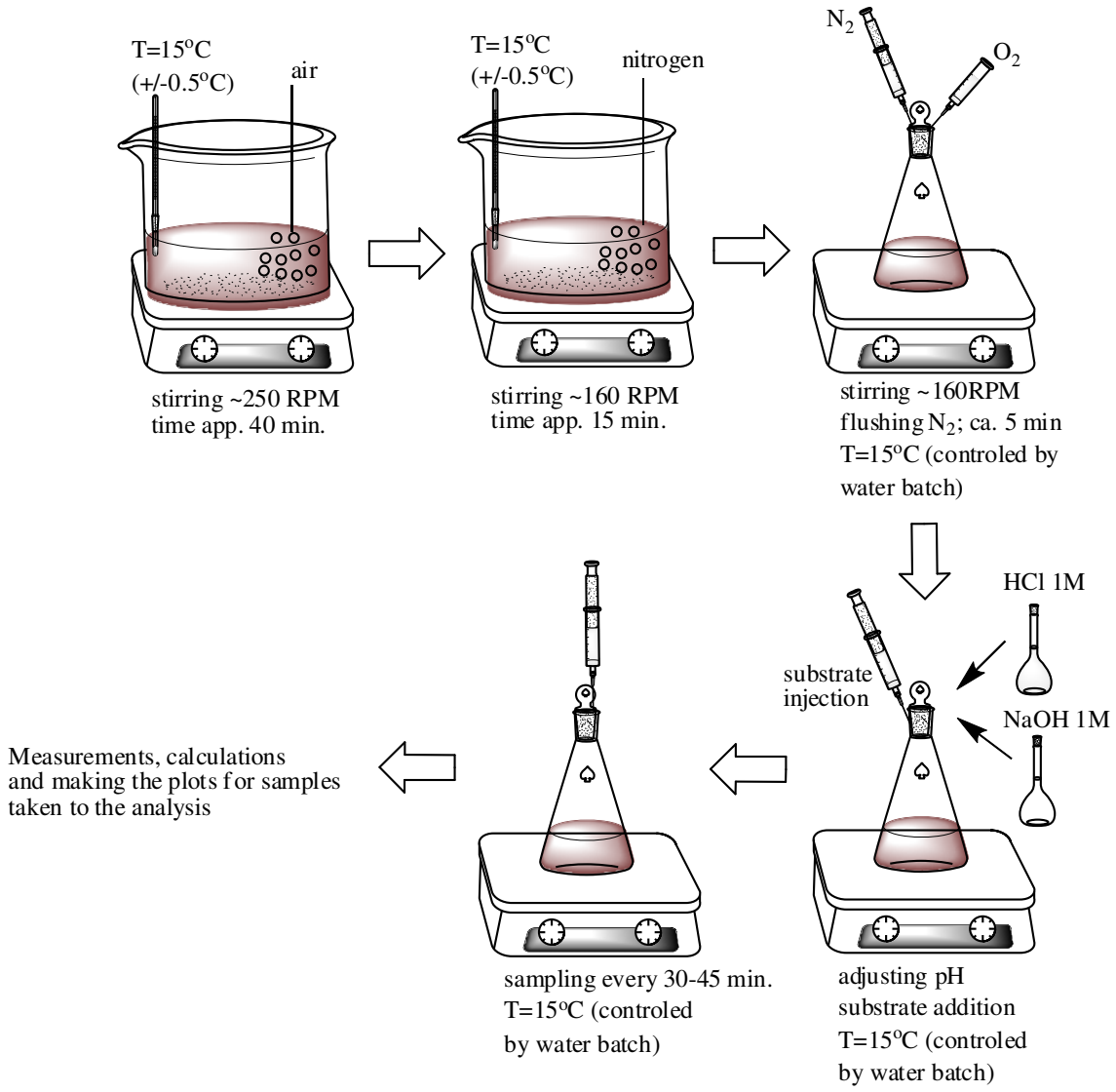

Figure 28. General experiment set up and conditions in experiment with different pH values in reactors

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9.1.2.1. Results from the experiment with different pH conditions

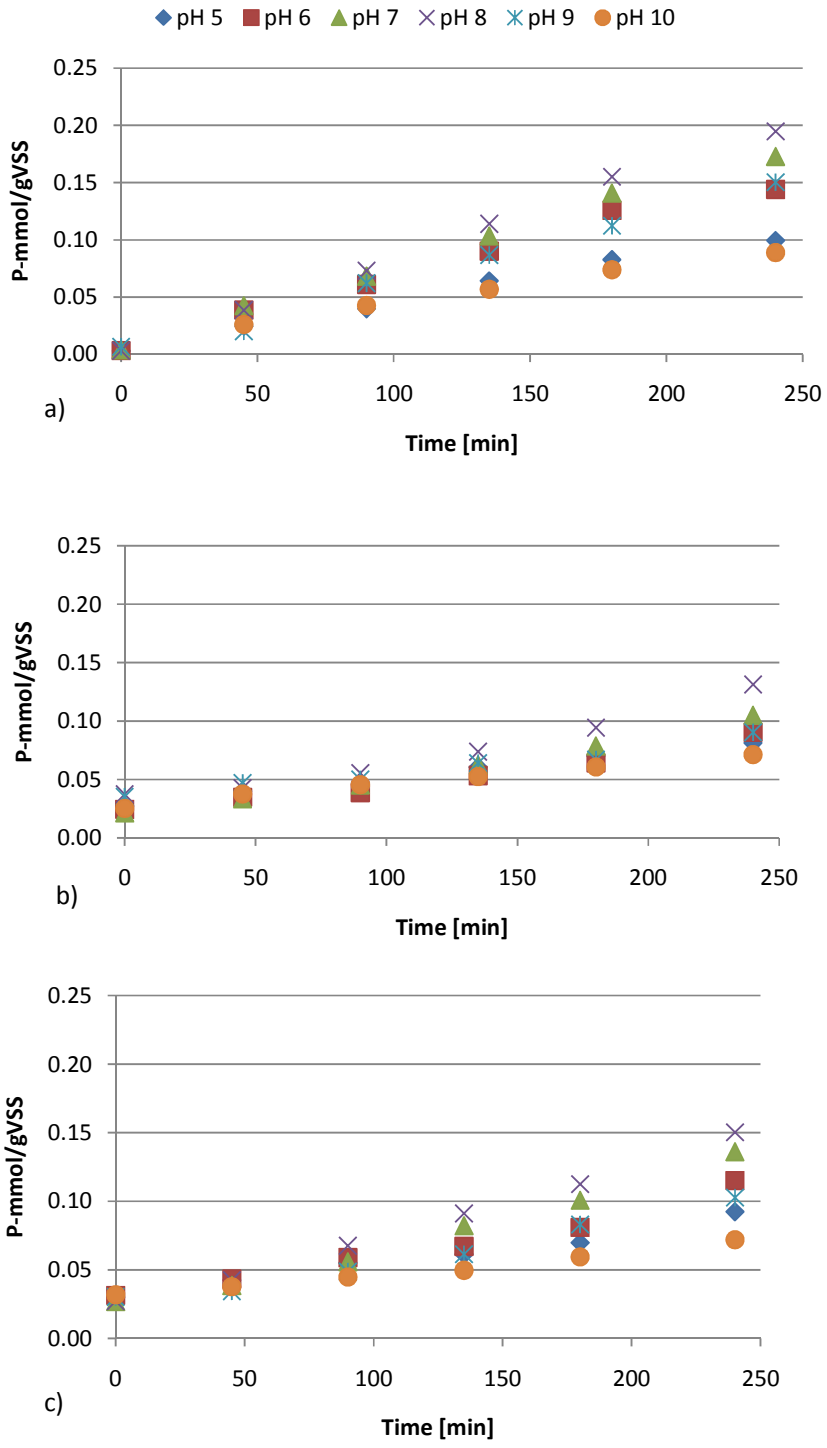

Figure 29. P-release in reactors with different pH conditions in Aalborg West WWTP and a) glycine, b) cysteine, c) alanine as a carbon source

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9.2. Procedures of calculations

9.2.1. SS and VSS calculations

$$SS=\frac{(B-A)\times 1000}{V}\ [g/L]\ \text{Equation 4.}$$

$$VSS=SS-\frac{(C-A)\times 1000}{V}\ [g/L]\ \text{Equation 5. where}$$

SS – suspended solids

VSS – volatile suspended solids

V – volume of the sample [mL]

A – weight of container with filter [g]

B – weight of sample after 2 hours drying in 105°C [g]

C – weight of sample after 1 hour ignition in 550°C [g]

9.2.2. P-release rate

Weight P-release rate

P-release rate was calculated from trend line of linear stage of P-release curve. The formula for calculation the rate is:

$$R=y\times t\ [mgP/gVSS*h]\ \text{Equation 6. where}$$

R – P-release rate [mgP/gVSS*h]

y – slope of trend line [mgP/gVSS]

t – time for calculation of release rate [h]

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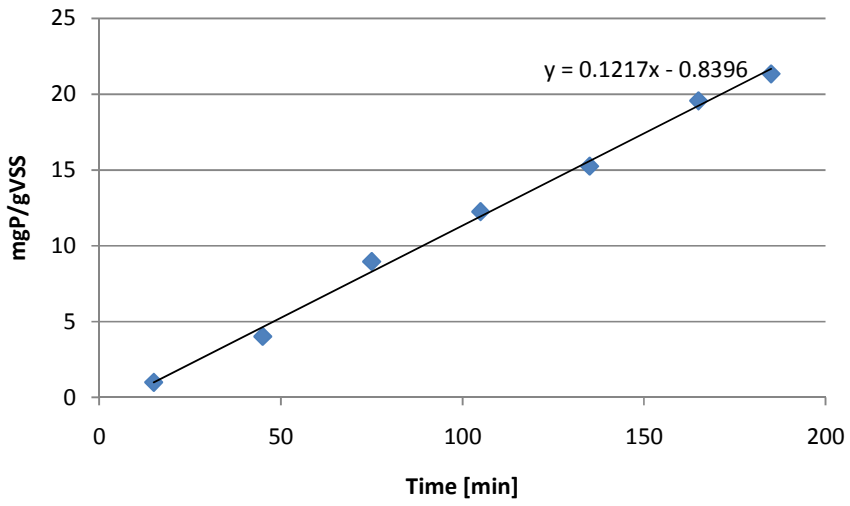

Figure 30. Example of curve for P-release rate calculation

Molar P- release rate

P-release was calculated on the same way as in the case of weight release. The figure with P-mmol/gVSS versus time was prepared.

R= $y \times t$ [mgP/gVSS*h] Equation 7. where

R – P-release rate [P-mmol/gVSS*h]

y – slope of trend line [P-mmol/gVSS]

t – time for calculation of release rate [h]

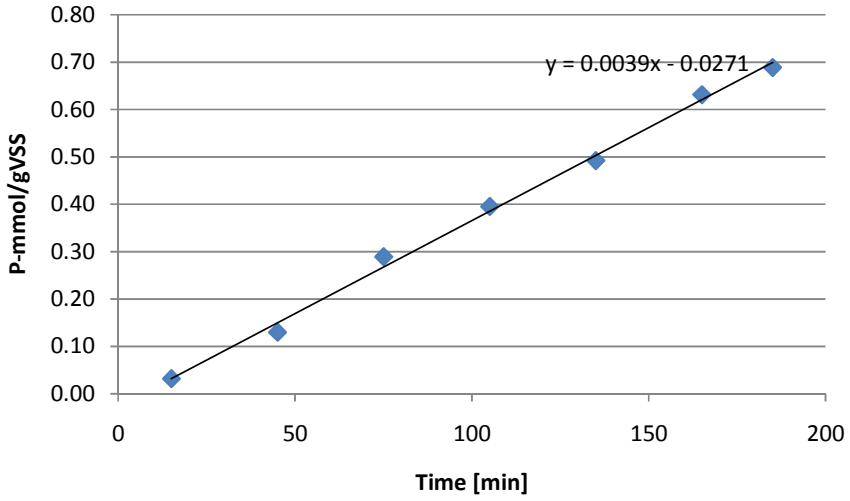

Figure 31. Example of curve for P-release rate calculation

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9.2.3. Volatile fatty acids consumption

Consumption measured as weight

Consumption rates were calculated from figures of VFA uptake versus time.

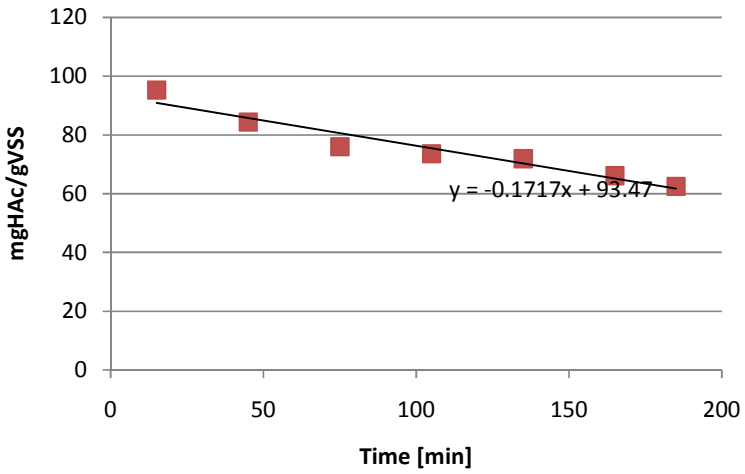

Figure 32. Example of curve of substrate uptake (HAc)

$R = y \times t$ [mgHAc/gVSS*h] Equation 8. where

R – substrate uptake rate [mgHAc/gVSS*h]

y – slope of trend line [mgHAc/gVSS]

t – time for calculation of uptake rate [h]

Consumption measured in moles

$R = y \times t$ [C-mmol/gVSS*h] Equation 9. where

R – substrate uptake rate [C-mmol/gVSS*h]

y – slope of trend line [C-mmol/gVSS]

t – time for calculation of uptake rate [h]

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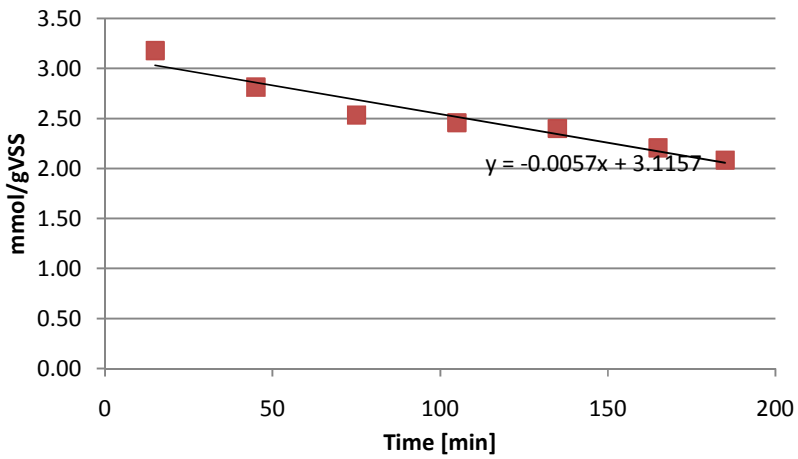
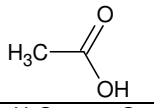
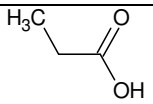
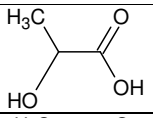
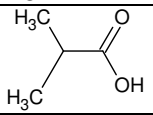


Figure 33. Example of curve of substrate uptake (HAc)

9.3. Chemical properties of VFAs and amino acids used during experiments

9.3.1. Volatile fatty acids

Table 11. Basic properties of volatile fatty acids used in experiment

Volatile fatty acid	Molecular formula	Structural formula [9]	pK_a value	Molar mass [g/mol]	[gCOD/ g substance]
Acetic	CH_3COOH		4.76	60.05	1.07/0.78*
Propionic	C_2H_5COOH		4.86	74.08	1.51
Lactic	$C_3H_6O_3$		3.08	90.08	1.07
Isobutyric	$C_4H_8O_2$		4.83	88.10	1.82

*instead acetic acid sodium acetate was used in experiments

<u>COD calculations:</u>
Acetic (sodium acetate)
$CH_3COONa + 2O_2 \rightarrow 2CO_2 + H_2O + NaOH$ COD= 2*32 = 64 [gCOD* mol^{-1}]

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Lactic acid
$C_3H_6O_3 + 3O_2 \rightarrow 3CO_2 + 3H_2O$ COD= 3*32 = 96 [gCOD* mol^{-1}]

$$\frac{96\ gCOD/mol}{90.08 g/mol} = 1.07\ gCOD/g[lactic\ acid]$$

Isobutyric acid
$C_4H_8O_2 + 5O_2 \rightarrow 4CO_2 + 4H_2O$ COD= 5*32 = 160 [gCOD* mol^{-1}]

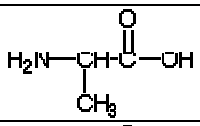
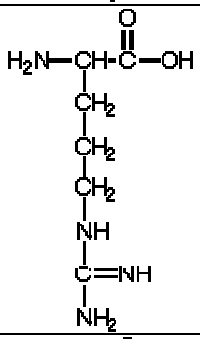
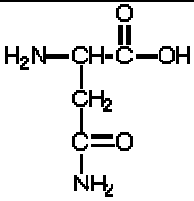
$$\frac{160\ gCOD/mol}{88.10 g/mol} = 1.82\ gCOD/g[butyric\ acid]$$

Propionic acid
$C_3H_6O_2 + 3.5O_2 \rightarrow 3CO_2 + 3H_2O$ COD= 3.5*32 = 112 [gCOD* mol^{-1}]

$$\frac{112\ gCOD/mol}{74.08 g/mol} = 1.51\ gCOD/g[propanoic\ acid]$$

9.3.2. Amino acids

Table 12. Basic properties of amino acids used in experiment

Amino acid	Molecular formula	Structural formula [9]	pK_a value 1) α-COOH 2) α-NH_3^+	Molar weight [g/mol]	[gCOD/ g substance]
Alanine	$C_3H_7NO_2$		1)2.35 2)9.87	74.04	1.35
Arginine	$C_6H_{14}N_4O_2$		1)1.82 2)8.99	174.20	1.56
Asparagine	$C_4H_8N_2O_3$		1)2.14 2)8.72	132.12	1.09

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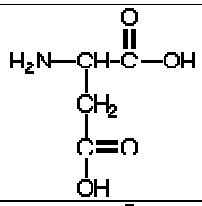
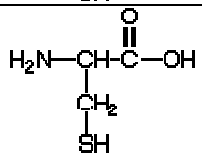
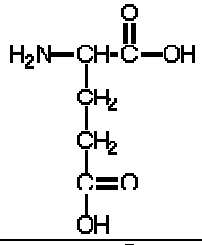
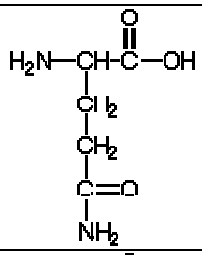
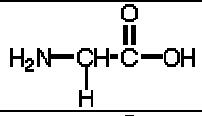
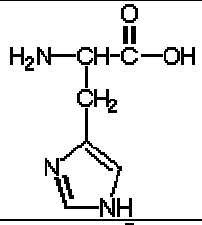
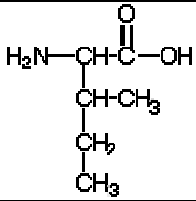
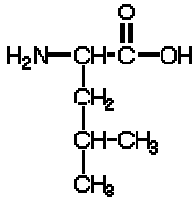
Aspartate	$C_4H_7NO_4$		1)1.99 2)9.90	133	1.09
Cysteine	$C_3H_7NO_2S$		1)1.92 2)10.70	121.16	1.26
Glutamate	$C_5H_9NO_4$		1)2.10 2)9.13	147.13	1.14
Glutamine	$C_5H_{10}N_2O_3$		1)2.17 2)9.13	146.14	1.31
Glycine	$C_2H_5NO_2$		1)2.35 2)9.78	75.07	0.96
Histidine	$C_6H_9N_3O_2$		1)1.80 2)9.33	155.15	1.50
Isoleucine	$C_6H_{13}NO_2$		1)2.32 2)9.76	131.17	2.01
Leucine	$C_6H_{13}NO_2$		1)2.33 2)9.74	131.17	2.01

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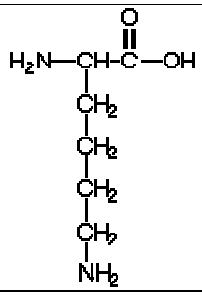
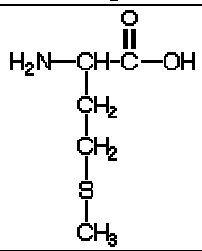
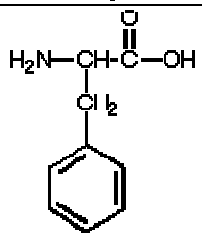
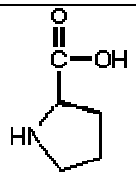
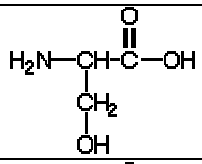
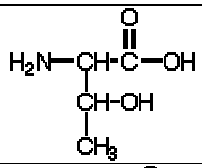
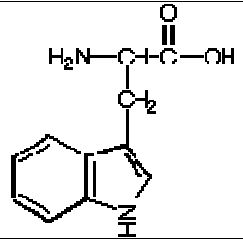
Lysine	$C_6H_{14}N_2O_2$		1)2.16 2)9.06	146.19	1.86
Methionine	$C_5H_{11}NO_2S$		1)2.13 2)9.28	149.21	1.66
Phenylalanine	$C_9H_{11}NO_2$		1)2.20 2)9.31	165.19	2.08
Proline	$C_5H_9NO_2$		1)1.95 2)10.64	115.13	1.74
Serine	$C_3H_7NO_3$		1)2.19 2)9.21	105.09	1.07
Theorine	$C_4H_9NO_3$		1)2.09 2)9.10	119.12	1.28
Tryptophan	$C_{11}H_{12}N_2O_2$		1)2.46 2)9.10	204.23	2.04

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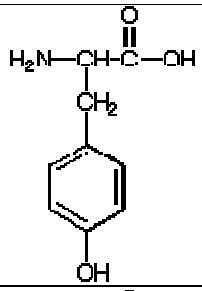
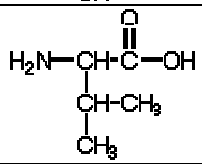
Tyrosine	$C_9H_{11}NO_3$		1)2.20 2)9.21	181.19	1.81
Valine	$C_5H_{11}NO_2$		1)2.29 2)9.74	117.15	1.84

COD calculations:

Alanine

$C_3H_7NO_2 + 3.75\ O_2 \rightarrow 3\ CO_2 + 3.5\ H_2O + 0.5\ N_2$ COD= 3.75*32 = 120 [gCOD* mol^{-1}]

$$\frac{120\ gCOD/mol}{89.09\ g/mol} = 1.35\ gCOD/g[alanine]$$

Arginine

$C_6H_{14}N_4O_2 + 8.5O_2 \rightarrow 6\ CO_2 + 7H_2O + 2N_2$ COD= 8.5*32 = 272 [gCOD* mol^{-1}]

$$\frac{272\ gCOD/mol}{174.20\ g/mol} = 1.56\ gCOD/g[arginine]$$

Asparagine

$C_4H_8N_2O_3 + 4.5\ O_2 \rightarrow 4CO_2 + 4H_2O + N_2$ COD= 4.5*32 = 144 [gCOD* mol^{-1}]

$$\frac{144\ gCOD/mol}{132.12\ g/mol} = 1.09\ gCOD/g[asparagine]$$

Aspartate

$C_4H_7NO_2 + 4.5\ O_2 \rightarrow 4\ CO_2 + 3.5\ H_2O + 0.5\ N_2$ COD= 4.5*32 = 120 [gCOD* mol^{-1}]

$$\frac{144\ gCOD/mol}{133\ g/mol} = 1.09\ gCOD/g[aspartic\ acid]$$

Cysteine

$C_3H_7NO_2S + 4.75\ O_2 \rightarrow 3\ CO_2 + 3.5\ H_2O + 0.5\ N_2 + SO_2$

COD= 4.75*32 = 136 [gCOD* mol^{-1}]

$$\frac{136\ gCOD/mol}{121.16\ g/mol} = 1.26\ gCOD/g[cysteine]$$

Glutamate

$C_5H_9NO_4 + 5.25\ O_2 \rightarrow 5\ CO_2 + 4.5\ H_2O + 0.5\ N_2$ COD= 5.25*32 = 168 [gCOD* mol^{-1}]

$$\frac{168\ gCOD/mol}{147.13\ g/mol} = 1.14\ gCOD/g[glutamic\ acid]$$

Glutamine

$C_5H_{10}N_2O_3 + 6\ O_2 \rightarrow 5\ CO_2 + 5\ H_2O + N_2$ COD= 6*32 = 192 [gCOD* mol^{-1}]

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$$\frac{192\ gCOD/mol}{146.14\ g/mol} = 1.31\ gCOD/g[glutamine]$$

Glycine

$C_2H_5NO_2 + 2.25\ O_2 \rightarrow 2\ CO_2 + 2.5\ H_2O + 0.5\ N_2$ COD= 2.25*32 = 72 [gCOD* mol^{-1}]

$$\frac{72\ gCOD/mol}{75g/mol} = 0.96\ gCOD/g[glycine]$$

Histidine

$C_6H_9N_3O_2 + 7.25\ O_2 \rightarrow 6\ CO_2 + 4.5\ H_2O + 1.5\ N_2$ COD= 7.25*32 = 232 [gCOD* mol^{-1}]

$$\frac{232\ gCOD/mol}{155.15g/mol} = 1.50\ gCOD/g[Histidine]$$

Isoleucine

$C_6H_{13}NO_2 + 8.25\ O_2 \rightarrow 6\ CO_2 + 6.5\ H_2O + 0.5\ N_2$ COD= 8.25*32 = 264 [gCOD* mol^{-1}]

$$\frac{264\ gCOD/mol}{131.17g/mol} = 2.01\ gCOD/g[isoleucine]$$

Leucine

$C_6H_{13}NO_2 + 8.25\ O_2 \rightarrow 6\ CO_2 + 6.5\ H_2O + 0.5\ N_2$ COD= 8.25*32 = 264 [gCOD* mol^{-1}]

$$\frac{264\ gCOD/mol}{131.17g/mol} = 2.01\ gCOD/g[leucine]$$

Lysine

$C_6H_{14}N_2O_2 + 8.5\ O_2 \rightarrow 6\ CO_2 + 7\ H_2O + N_2$ COD= 8.5*32 = 272 [gCOD* mol^{-1}]

$$\frac{272\ gCOD/mol}{146.19\ g/mol} = 1.86\ gCOD/g[lysine]$$

Methionine

$C_5H_{11}NO_2S + 7.75\ O_2 \rightarrow 5\ CO_2 + 5.5\ H_2O + 0.5\ N_2 + SO_2$

COD= 7.75*32 = 248 [gCOD* mol^{-1}]

$$\frac{248\ gCOD/mol}{149.21g/mol} = 1.66\ gCOD/g[met\square ionine]$$

Phenylalanine

$C_9H_{11}NO_2 + 10.75\ O_2 \rightarrow 9\ CO_2 + 5.5\ H_2O + 0.5\ N_2$ COD= 10.75*32 = 344 [gCOD* mol^{-1}]

$$\frac{344\ gCOD/mol}{165.19g/mol} = 2.08\ gCOD/g[p\square enylalanine]$$

Proline

$C_5H_9NO_2 + 6.25\ O_2 \rightarrow 5\ CO_2 + 4.5\ H_2O + 0.5\ N_2$ COD= 6.25*32 = 200 [gCOD* mol^{-1}]

$$\frac{200\ gCOD/mol}{115.13\ g/mol} = 1.74\ gCOD/g[proline]$$

Serine

$C_3H_7NO_3 + 3.25\ O_2 \rightarrow 3\ CO_2 + 3.5\ H_2O + 0.5\ N_2$ COD= 3.25*32 = 112 [gCOD* mol^{-1}]

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$$\frac{112\ gCOD/mol}{105 g/mol} = 1.07\ gCOD/g[serine]$$

Theorine

$C_4H_9NO_3 + 4.75\ O_2 \rightarrow 4\ CO_2 + 4.5\ H_2O + 0.5\ N_2$ COD= 4.75*32 = 152 [gCOD* mol^{-1}]

$$\frac{152\ gCOD/mol}{119.12 g/mol} = 1.28\ gCOD/g[theorine]$$

Tryptophan

$C_{11}H_{12}N_2O_2 + 13\ O_2 \rightarrow 11\ CO_2 + 6\ H_2O + N_2$ COD= 13*32 = 416 [gCOD* mol^{-1}]

$$\frac{416\ gCOD/mol}{204.23 g/mol} = 2.04\ gCOD/g[typtop\square an]$$

Tyrosine

$C_9H_{11}NO_3 + 10.25\ O_2 \rightarrow 9\ CO_2 + 5.5\ H_2O + 0.5\ N_2$ COD= 10.25*32 = 328 [gCOD* mol^{-1}]

$$\frac{328\ gCOD/mol}{181.19 g/mol} = 1.81\ gCOD/g[tyrosine]$$

Valine

$C_5H_{11}NO_2 + 6.75\ O_2 \rightarrow 5\ CO_2 + 5.5\ H_2O + 0.5\ N_2$

COD= 6.75*32 = 216 [gCOD* mol^{-1}]

$$\frac{216\ gCOD/mol}{117.15\ g/mol} = 1.84\ gCOD/g[valine]$$

Table 13. Amino acid stock solutions

Amino acids	[gCOD/g substance]	Solubility [g/L] at 25°C	Addition to reactor [mg]* 300mgCOD/L
Alanine	1.35	166.5	74.05
Arginine	1.56	150	76.92
Asparagine	1.09	35.3	110.09
Aspartate	1.09	7.78	110.09
Cysteine	1.26	v.s.	95.24
Glutamate	1.14	8.6	100.80
Glutamine	1.31	25	91.60
Glycine	0.96	250	124.99
Histidine	1.50	41.9	80.00
Isoleucine	2.01	41.2	59.70
Leucine	2.01	24.3	59.70
Lysine	1.86	v.s.	64.52
Methionine	1.66	33.1	72.29
Phenylalanine	2.08	29.7	57.69
Proline	1.74	1623	68.97
Serine	1.07	50.23	112.15
Theorine	1.28	v.s.	93.75
Tryptophan	2.04	11.4	58.82
Tyrosine	1.81	0.45	66.30
Valine	1.84	88.5	65.22

*reactor has 500mL volume, AS was added in quantity of 390mL and AA stock solution 10 mL to obtain 300 mgCOD/L of substance in reactor; v.s.-very soluble

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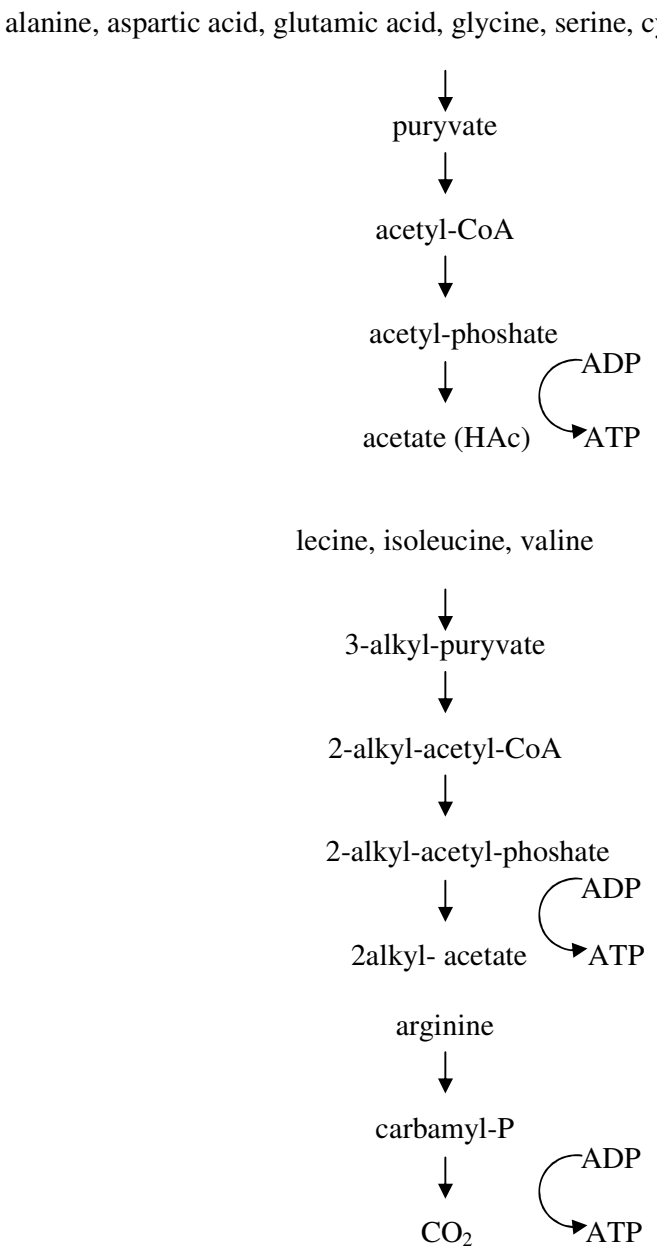
Casamino acids were also used in the lab work. They are mix of 19 amino acids (tryptophan is not included) and created during hydrolysis of casein.

9.3.2.1. Pathways of amino acids decomposition



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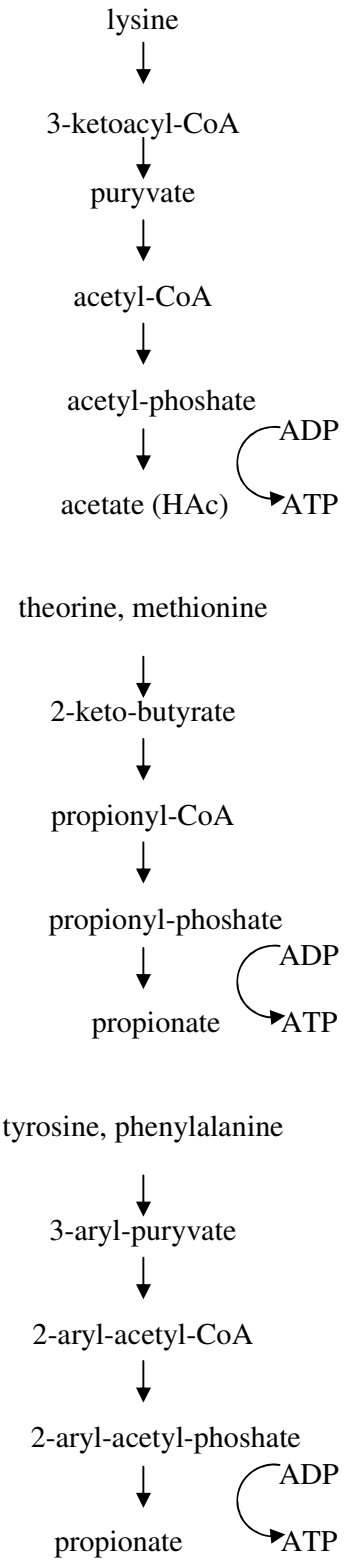

Figure 34. Pathways of AAs decomposition [43, 44]

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9.4. Detailed procedure of Fluorescence *in situ* Hybridization (FISH)

Whole procedure of FISH can be divided on 5 main parts: fixation of samples, preparation, hybridization, washing and visualization.

Fixation of samples is made to storage the samples of Gram-positive and Gram-negative bacteria and prepare them for further step - hybridization. The aim of fixation is to: permeabilize cell walls, protect RNA from cellular RNAses and maintenance of cell integrity and morphology. Fixation is made with usage of ethanol (96%) for Gram-positive bacteria and paraformaldehyde (8%) for Gram-negative.

Next process of preparation is applied for make FISH on slide or on filter (depends on outcome). Samples are filtered or put on slide and dehydrated [34].

Afterwards, the most important step - hybridization takes place. Oligonucleotides labeled with fluorescent dyes are introduced into the permeabilized cells. The process is conducted in dark and at the temperature of approximately 46°C. The probes anneal to 16s ribosomal RNA, which they are designed to be complementary with [2].

Washing out, by special buffer, non-hybridized oligonucleotide probes is next part of FISH analysis. This gives assurance from disturbation of FISH image by background fluorescence [34].

Last part of analysis is visualisation and counting. Samples are visualized on fluorescence microscope equipped with proper light filter appropriate for the fluorescent dye coupled to the applied gene probe [34].

Table 14. Names of the probes used for FISH analysis

Probe name	**Target**	**Sequence 5' – 3'**	**Reference**
PAO462	Most *Accumulibacter*	CCGTCATCTACWCAGGG TATTAAC	Crocetti et al. (2000)
PAO651	Most *Accumulibacter*	CCCTCTGCCAAACTCCAG	
PAO846	Most *Accumulibacter*	GTTAGCTACGGCACTAAA AGG	
PAOmix	Most *Accumulibacter*	PAO462, PAO651 and PAO846	
Actino-221 Type1	*Actinobacteria*— potential PAOs	CGCAGGTCCATCCCAGAC	Kong et al. (2005)
Actino-658 Type2	*Actinobacteria*— potential PAOs	TCCGGTCTCCCCTACCAT	
GAOQ989	Some *Competibacter*	TTCCCCGGATGTCAAGGC	Crocetti et al. (2002)
GB_G2	Some *Competibacter*	TTCCCCAGATGTCAAGGC	Kong et al., 2002
GAO mix	*Competibacter*	GAOQ989 and GB_G2	
TFO_DF218	*Defluviicoccus*-related organisms (cluster 1)	GAAGCCTTTGCCCCTCAG	Wong et al., (2004)
TFO_DF618	*Defluviicoccus*-related organisms (cluster 1)	GCCTCACTTGTCTAACCG	
Defluviicocus	*Defluviicoccus*-related organisms (cluster 1)	TFO_DF218 and TFO_DF618	

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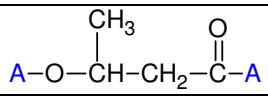
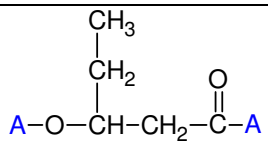
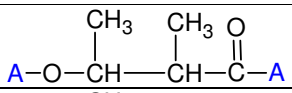
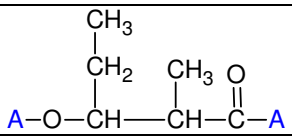
9.5. Polyhydroxyalkanoates produced by PAOs and GAOs

PAOs and GAOs, in anaerobic phase of EBPR, produce polyhydroxyavalerate and polyhydroxybutyrate depending on which substrate is available to uptake. In table 15 are shown chemical units used to production of PHAs.

Table 15. Structure of hydroxyalkanoates used for PHA formation [14,28]

Name of PHA unit	Structural formula of unit[9]	Precursors
3-hydroxybutyrate (3HB)		2 acetyl-CoA
3-hydroxyvalerate (3HV)		1 acetyl-CoA 1propionylo-CoA
3-hydroxy-2-methylbutyrate (3H2MB)		1 acetyl-CoA 1propionylo-CoA
3-hydroxy-2-methylvalerate (3H2MV)		2 propionylo-CoA

9.6. Aalborg West, Hjørring and Bjerringbro WWTPs on photographs

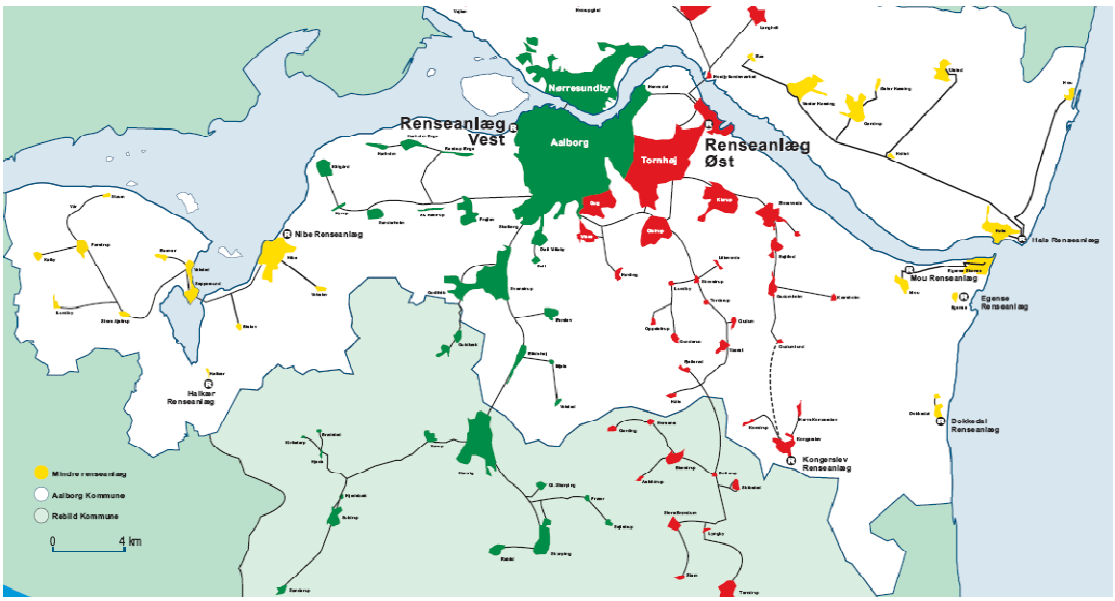


Figure 35. Catchment area for Aalborg WWTP (marked on green colour) [7]

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Figure 36. Bird's-eye view of Aalborg West WWTP [8]

Figure 37. Bird's-eye view of Hjørring WWTP [8]

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Figure 38. Bird's-eye view of Bjerringbro WWTP [8]

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9.7. Microbial community data from years 2006-2008 in case WWTPs

Table 16. Comparison of microbiological data from Aalborg WWTP, Hjørring WWTP and Bjerringbro WWTP for the years 2006 and 2007 [10]

WWTP / Properties	Aalborg West						Hjørring						Bjerringbro					
Date / **Group of organisms**	***2.2006***	***8.2006***	***2.2007***	***5.2007***	***8.2007***	***11.2007***	***2.2006***	***8.2006***	***2.2007***	***5.2007***	***8.2007***	***11.2007***	***2.2006***	***8.2006***	***2.2007***	***5.2007***	***8.2007***	***11.2007***
Ammonium oxidizing bacteria (%)	8.20	10.50	8.70	-	8.50	-	8.70	7.50	7.90	7.70	7.20	9.50	6.90	8.90	8.30	-	9.80	-
Nitrate oxidizing bacteria (%)	7.50	5.10	6.60	-	6.80	-	7.20	5.80	5.40	5.60	7.60	4.70	6.40	6.90	5.10	-	3.40	-
PAOs (%)	13.04	15.30	9.90	-	11.50	-	15.00	27.70	13.30	13.30	10.90	12.80	5.50	3.60	7.90	-	8.70	-
Accumulibacter	3.14	4.20	3.20	-	3.00	-	2.60	2.20	1.90	1.90	2.70	2.80	2.50	2.60	2.40	-	3.30	-
Actinobacteria T1	6.60	5.30	3.90	-	4.20	-	4.80	11.60	4.80	4.80	4.30	4.80	2.50	0.50	3.90	-	3.10	-
Actinobacteria T2	3.30	5.80	2.80	-	4.30	-	7.60	13.90	6.60	6.60	3.90	5.20	0.50	0.50	1.60	-	2.30	-
GAOs (%)	1.00	1.00	1.00	-	1.00	-	5.30	1.50	2.50	2.50	1.50	1.50	0.50	0.50	1.50	-	0.50	-
DSVI	-	100	114	-	99	-	-	77	76	73	71	60	-	67	46	-	74	-
FI	-	2.5	2.5	-	2.5	-	-	2.0	2.5	2.0	2.0	2.5	-	2.5	2.5	-	2.5	-

Table 17. Comparison of microbiological data from Aalborg WWTP, Hjørring WWTP and Bjerringbro WWTP for the year 2008 [27]

WWTP / Properties	Aalborg West				Hjørring				Bjerringbro
Date / **Group of Organisms**	***1.2008***	***5.2008***	***8.2008***	***11.2008***	***1.2008***	***5.2008***	***8.2008***	***11.2008***	
Ammonium oxidizing bacteria (%)	4.1	3.4	3.9	3.6	4.2	2.9	3.4	3.5	***No data available from this period***
Nitrosospira	1.8	2.2	1.5	1.6	2.2	2.6	2.4	2.3	
Nitrosomonas	0.8	0.7	1.6	0.8	0.5	0.5	0.5	0.5	
Nitrate oxidizing bacteria (%) *Nitrospira*	2.5	2.9	1.8	2.2	5.6	5.3	4.8	4.9	
Nitrospira T1	0.5	0.0	0.5	0.5	3.4	3.9	3.2	2.8	
Nitrospira T2	0.5	0.5	0.5	0.5	2.1	1.7	2.5	1.8	
PAOs (%)	12.4	12.4	12.3	12.1	14.1	13.2	13.5	14.1	
Accumulibacter	5.5	5.9	6.1	5.7	4.1	4.4	4.2	4.5	
Actinobacteria T1	3.2	3.3	2.8	2.5	5.2	4.7	4.4	4.9	
Actinobacteria T2	3.7	3.2	3.4	3.9	4.8	4.1	4.9	4.7	
GAOs (%)	0.5	0.5	0.5	0.5	2.8	3.5	1.9	3.7	

`

9.8. Detailed data from long term variations in P-release experiment

Table 18. Data from long term experiment for P-release in Aalborg West

	Date	Rate1	Rate2	Mean	VSS
	yy-mm-dd	P-mmol/gVSS*h	P-mmol/gVSS*h	P-mmol/gVSS*h	g/L
1	2008-11-18	0.162	0.168	0.165	3.27
2	2008-11-26	0.180	0.186	0.183	3.05
3	2008-12-02	0.174	0.174	0.174	3.25
4	2008-12-09	0.144	0.150	0.147	3.10
5	2008-12-17	0.192	0.210	0.201	2.89
6	2009-01-13	0.198	0.198	0.198	2.65
7	2009-01-20	0.204	0.210	0.207	2.93
8	2009-01-27	0.198	0.204	0.201	3.03
9	2009-02-03	0.168	0.168	0.168	3.75
10	2009-02-11	0.210	0.210	0.210	2.62
11	2009-03-05	0.234	0.234	0.234	2.80
12	2009-03-24	0.210	0.210	0.210	3.70
13	2009-03-30	0.196	0.196	0.196	3.40
14	2009-04-07	0.180	0.192	0.186	3.58
15	2009-04-21	0.264	0.264	0.264	3.10
16	2009-04-30	0.246	0.258	0.252	3.13
17	2009-05-08	0.240	0.258	0.249	3.02
18	2009-05-20	0.264	0.264	0.264	3.21
19	2009-05-27	0.252	0.258	0.255	3.15
20	2009-06-02	0.264	0.270	0.267	3.32
21	2009-06-09	0.294	0.294	0.294	3.4
23	2009-06-19	0.288	0.288	0.288	3.32
24	2009-07-06	0.348	0.354	0.351	3.24
25	2009-07-14	0.366	0.372	0.369	3.24
26	2009-07-20	0.312	0.324	0.318	3.4

Statistic for these data: median 0.210 P-mmol/(gVSS*h), mean 0.234 P-mmol/(gVSS*h), standard deviation 0.0578 P-mmol/(gVSS*h) and coefficient of variation C_v=27.5%.

`

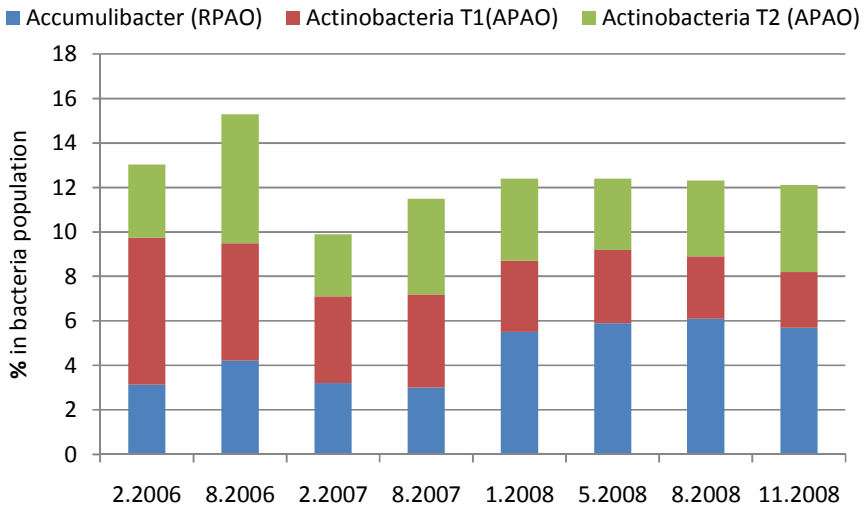

Figure 39. Variations in PAOs community in the space of time in Aalborg West WWTP [10, 27]

9.9. Note about results from lab experiments

All lab results obtained from entire project's period were collected in MS Office Excel' files and are placed on CD included to the project.