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Methane Emissions During Sludge Storage in Relation to the Microbial Community Composition of Sludge

A Benchmark Study and Metagenomic Analysis Concerning Different Sludge Treatment Processes

Master's thesis in Biotechnology

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Abstract

Wastewater treatment plants (WWTPs) are one of numerous actors influencing the development towards a sustainable society. In the process of wastewater treatment, sludge is formed as a by-product. During storage of finished treated sludge, it is desirable to minimise methane emissions due to the contribution of greenhouse gas emissions to climate change. In this project, the methane emissions of finished treated sewage sludge from four WWTPs employing different sludge treatment processes (mesophilic and thermophilic digestion, as well as liming) was investigated. The goal was to generate benchmark values of methane emission during sludge storage to be used in tools for estimating the climate impact of WWTPs in Sweden. Additionally, a metagenomic analysis was performed to see if any relation between the amount of emitted methane and the microbial community composition of the sludge could be found.

Benchmark values for methane emission during storage of sludge treated by mesophilic and thermophilic digestion as well as liming was successfully obtained. Furthermore, the results indicated that the implementation of thermophilic digestion conditions could potentially decrease methane emission from sludge storage. The results from the metagenomic analysis showed that a higher relative abundance of methanogens resulted in increased methane emissions and that sludge containing more diverse microbial communities were able to emit larger amounts of methane at lower temperatures. Since targeting these areas to decrease methane emission during sludge storage would negatively affect the biogas production efficiency, other alternatives to minimise methane emission from sludge storage should be explored.

Keywords: methane emissions, sewage sludge, wastewater treatment plants, sludge storage, microbial composition of sludge, metagenomic analysis, benchmark values

Preface

This study constitutes a master thesis project and was done in collaboration with Gryaab and the department of Architecture and Civil Engineering at Chalmers University of Technology. The subject of the thesis was suggested by Gryaab as a part of their work in collaboration with Swedish Water (Svenskt Vatten) to improve the estimation of, and by extension lower, the climate impact of WWTPs in Sweden.

I want to take this opportunity to express my gratitude towards everyone who has been a part of this project. To begin with, I want to thank Gryaab for the opportunity to write my master thesis at your company. I also want to thank Hammargård, Sobacken and Getterö WWTPs for participating in this study. Especially thank you to Sebastian Engström, Moshe Habagil and Mikael Herbertsson for being accommodating during our visits and for your help with the collection of samples. I would also like to thank Marie Abadikhah for your guidance during the DNA-extraction. I would like to thank my examinator Oskar Modin for sharing your knowledge within environmental biotechnology and for your support and guidance. Lastly, I want to thank my supervisor, Dag Lorick for your encouragement, patience and valuable feedback as well as for being helpful and supportive throughout the course of the project.

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List of Abbreviations

AD - Anaerobic digestion

FA - Fatty acid

GHG - Greenhouse gas

OTU- Operational taxonomic unit

RMP - Residual methane potential

SAO - Syntrophic Acetate Oxidation

TS – Total solids

VFA - Volatile fatty acid

VS – Volatile solids

WWTP - Wastewater treatment plant

1 Introduction

Wastewater treatment plants (WWTPs) are both consumers and generators of energy and they are one of numerous actors influencing the development towards a sustainable society. For example, according to the UN agency IPCC, WWTPs can be important emitters of greenhouse gases (GHGs) [1]. Swedish Water (Svenskt Vatten), an industry member organisation consisting of municipal water service companies in Sweden, is currently working towards lowering the climate impact of its members. As a part of this, they have set a goal stating that the wastewater treatment facilities included in their organisation should have a net zero climate change impact by 2030. The emission of greenhouse gases is specifically mentioned as the initial target to meet this goal [2]. Previous studies have shown that the finished treated sewage sludge releases a considerable amount of the GHG methane (CH_4) [3]. For example, the methane emission from the sludge storage at Rya WWTP in Gothenburg is the second largest contribution to the plant's carbon footprint [4].

One important aspect of reaching this goal is the use of tools for calculating the climate impact of WWTPs. This concerns both the current climate impact of WWTPs and for example how a change in a wastewater or sludge treatment process would affect the climate impact of the WWTP. One such tool for calculating the climate impact has been made through the project program Water and Sewer Technology South (VA-teknik Södra) and is used in most of the larger WWTPs in Sweden today [5]. Swedish water is now working on a new common methodology for calculating and presenting the climate impact of WWTPs, which is meant to replace the former [6]. When using these tools, data points for different emission factors are needed. Numerous wastewater treatment facilities do not record data of the methane emission from the storage of finished treated sludge. Thus, there is a need for reference data points of methane emission form sludge storage, for the tool to predict the climate impact as accurately as possible. In addition to this, the environmental permit of Gryaab from 2020 demands that the company performs an internal investigation of the emissions from the sludge management before 2025, and what measures that can be taken to decrease these [7]. It is therefore highly relevant to investigate and collect data of the methane emission from sludge storage, both at Rya WWTP and at other WWTPs companies. Methane emission data from sludge storage at WWTPs with different sludge treatment processes could be used as a basis for future decision-making processes and enable improvement of the sludge treatment process and the emissions it gives rise to.

In this project, the methane emission of sludge from four different WWTPs on the Swedish west coast in proximity to Gothenburg was investigated: (1) Rya WWTP in Gothenburg, (2) Sobacken WWTP in Borås, (3) Hammargård WWTP in Kungsbacka and (4) Getterö WWTP in Varberg. The different facilities perform different types of sludge treatments. Sobacken, Rya and Getterö WWTPs all produce biogas from the sludge by anaerobic digestion (AD) [8-10], whereas Hammargård WWTP limes the sludge in combination with a temperature increase in order to kill pathogens [11]. Both Getterö and Rya WWTP use mesophilic conditions in the digestion chamber which refers to a temperature of approximately 35 °C [9, 10], whereas

Sobacken WWTP uses thermophilic conditions, which refer to a temperature of approximately 55 °C [8].

As previously mentioned, the finished treated sludge releases a considerable amount of methane during storage. The emitted methane can be seen as an energy potential of the outgoing digestate, and this is referred to as the residual methane potential (RMP) of sludge. It is not fully understood how the RMP are correlated with parameters affecting the finished treated sludge [3]. Since microorganisms play a crucial role in anaerobic digestion, the composition of the microbial community is essential for an effective biogas production process [12] and previous research has shown that the microbial community in anaerobic digesters have a considerable effect on the production of methane [3]. Hence, by studying the microbial communities of the sludge through metagenomics, a more nuanced picture can be given to the properties of the sludge and to the different sludge treatment processes. By analysing the microbial composition of sludge, it is possible to investigate whether an increase in methane emission can be the result of the presence or relative abundance of specific species involved in the formation of methane.

1.1 Aim

The purpose of the project was to perform a benchmark study investigating the methane emission of finished treated sewage sludge from four different WWTPs. The investigated WWTPs employ different treatment processes such as thermophilic and mesophilic conditions during anaerobic digestion for production of biogas as well as of the sludge that is not anaerobically digested. The aim of the study was to generate benchmark values for methane emission during sludge storage at Swedish WWTPs which can be used in tools for estimating the climate impact of WWTPs in Sweden. The goal was also to compare the methane emission from the different WWTPs and create a foundation which can contribute to further limiting the methane emissions from sewage sludge in Swedish wastewater treatment facilities.

Furthermore, the purpose was to perform a metagenomic analysis of the composition of microbial communities between the different types of sludge treatment conditions. The goal was to identify differences and similarities in the microbial composition among the various types of WWTPs and treatment conditions, and to see if any relation between the amount of emitted methane and the presence of microorganisms involved in anaerobic digestion and methanogenesis in the different samples could be found.

1.2 Scope and Limitations

The scope of this project included several parts:

- Temperature measurement of sludge during storage at Rya WWTP to determine conditions for methane emission measurements.
- Methane emission measurements of the sludge from four different WWTPs, at the west coast of Sweden which employ different sludge treatment methods.

- Analysis of microbial communities in the collected sludge, including diversity and differences in abundance of methanogens.
- Investigation of the content of the volatile fatty acids (VFAs) formate and acetate in the collected sludge as a complement to the bioinformatic analyses.

The project is limited to the four WWTPs previously mentioned: (1) Rya WWTP in Gothenburg, (2) Sobacken WWTP in Borås, (3) Hammargård WWTP in Kungsbacka and (4) Getterö WWTP in Varberg. The project concerns finished treated, dewatered sludge from these four WWTPs. The study was conducted January-May 2022 and the temperature measurements were limited to the weather conditions at this time of the year since the sludge is stored openly outside at Rya WWTP. Furthermore, shotgun metagenome sequencing could not be performed on the sludge from Hammargård WWTP due to low DNA concentrations in this sample. Thus, the analysis of microbial communities is limited to the WWTPs which employ anaerobic digestion as a part of their sludge treatment.

2 Theory

Methane is one of the most abundant GHGs in the atmosphere. Furthermore, methane is a potent GHG and has a global warming potential 28-34 times higher compared to carbon dioxide (CO_2) over a 100-year period. If instead looking at a 20-year period, the global warming potential of methane is 84-86 times greater than that of carbon dioxide [13].

As previously mentioned, the industry member organisation Swedish Water alongside WWTPs in Sweden aim to minimise the climate impact of the wastewater treatment industry and limiting greenhouse gas emissions are specifically targeted when pursuing this [2]. At Rya WWTP, the methane emission from the sludge storage is the second largest contribution to the plant's carbon footprint [4] and studies show that the finished treated sewage sludge at WWTPs release a considerable amount of methane [3]. Sludge is formed as a by-product during the process of treating sewage water. Historically the sludge has been considered a problem, however this perception has changed in recent years and sludge is now seen as a useful resource in Sweden [14]. The sludge consists of particles removed from the wastewater, which are rich in organic matter and nutrients. Hence, it can be a resource from which nutrition can be recycled [1]. By using sludge as fertiliser in agriculture, nutrients are recycled between urban and rural areas. Thus, it can be seen as a central way of obtaining a circular bioeconomy [3]. Regardless of whether the sludge is used as fertiliser or if it is discarded, the sludge is required to go through some type of treatment [14]. The type of treatment carried out varies between different WWTPs. In the following section, the treatment of sludge is described both in general terms and more specifically for the four treatment plants whose sludge were investigated in this study.

2.1 Sludge Treatment

One considerable difference in the treatment of sludge at different WWTPs is whether the sludge is anaerobically digested**,** which is the case at WWTPs such as Rya, Getterö and Sobacken [8-10]. Anaerobic digestion is a biological process where various microorganisms decompose organic material and release biogas and other fermentation products [15]. The resulting biogas consists of 50-70 % methane, 30-50% CO_2 and trace amounts of other gases such as hydrogen sulphide (H_2S), ammonia (NH_3) and water (H_2O) [16]. Anaerobic digestion can either be mesophilic with a temperature of approximately 35 °C which is the case at Rya [9] and Getterö [10] WWTPs, or thermophilic with a temperature of approximately 55 °C which is the case at Sobacken WWTP [8]. The finished treated, digested sludge has low biological activity since the accessible biomass has been degraded. The amounts of pathogens are reduced during digestion, and the odour emission is significantly decreased [1]. Once the digested sludge has finished treatment, it consists of 70-80 % water and has the appearance of moist soil and approximately half of the original organic material remains in the sludge [9]. The process of anaerobic digestion is further described in section *2.2 Anaerobic Digestion*.

Regardless of whether the sludge is digested or not, the treatment of sludge generally includes dewatering and thickening to reduce the proportion of water in the sludge. If the sludge is anaerobically digested, it is dewatered both before and after digestion [14]. As the biogas is

formed the sludge is stabilised, and its dry matter content is reduced [1]. As a consequence of the solid matter in the sludge being converted into biogas, the sludge becomes more liquid again needs to be dewatered once more [9]. The sludge is also easier to dewater after digestion [1].

Furthermore, hygienisation of the sludge is required to enable the use of the finished treated sludge as for example fertiliser. Hygienisation refers to reducing or killing pathogens in order to prevent transmission of diseases. There are several methods for this at WWTPs, for example long term storage (6 months or longer) and exposing the organisms to harmful conditions such as the addition of chemicals, increased temperature or increased pH [17].

2.1.1 Rya WWTP

Rya WWTP is owned by Gryaab, which in turn is owned by the municipalities of Ale, Bollebygd, Gothenburg, Härryda, Kungälv, Lerum, Mölndal and Partille. Approximately 900 000 pe (person equivalents) are connected to Rya WWTP and the facility has a maximal capacity of 1 850 000 pe [4]. The wastewater treated at Rya WWTP comes from for example households, schools, industries, offices and hospitals [18]. When separated from the wastewater during the wastewater treatment process, the sludge initially consists of approximately 99% water [9]. At Rya WWTP, the sludge is thickened by the addition of polymers and the use of band gravity thickeners. Thereafter the thickened sludge is pumped into the biogas production facility. External organic material that is pumpable, such as food residues or fat from the food industry or restaurants, are digested together with the wastewater sludge [4].

At Rya WWTP, the biogas production facility consists of two larger digestion chambers (11400 m^3 each) and one smaller (4260 m^3). The three chambers are run in series [4]. The decision to run the chambers in series was made based on an investigation in 2017 where it was concluded that this mode of operation resulted in the lowest methane emission from the finished treated sludge [19]. The sludge initially enters one of the larger chambers and is then transported into the second large digestion chamber. The level of sludge in these two large digestion chambers are kept somewhat constant. From the second large digestion chamber, the sludge is pumped into the third, smaller digestion chamber. In this smaller digestion chamber, the level of sludge varies, and the chamber acts as a buffer. The average residence time of the sludge inside the digestion chambers is approximately 20 days and as previously mentioned, the digestion is mesophilic and takes place at approximately 35 °C. Stirrers and circulation pumps inside the chambers ensure the sludge is mixed. After digestion, the sludge is pumped to the dewatering equipment. Polymers are added to thicken the sludge and the sludge is dewatered using screw presses [4]. The produced biogas is upgraded, meaning that carbon dioxide is removed, and the proportion of methane is increased to 95-98 %. The upgraded gas is used as fuel in vehicles [20]. The finished treated sludge is temporarily and stored in open piles between 1-4 weeks [9]. The sludge is then transported away from the WWTP and stored for additionally 6 months as method of hygienisation. The finished treated, hygienised sludge is used as fertiliser in agriculture or as a constituent in soil production, depending on the quality of the sludge regarding heavy metals. During 2020 approximately 46% was used in agriculture [4].

2.1.2 Getterö WWTP

Getterö WWTP is run by the company VIVAB which is owed by Varberg and Falkenberg municipalities. Getterö WWTP has a capacity of 80 000 pe [21] and it treats household wastewater as well as wastewater from for example the concrete industry, car washes, painting companies and hospitals. External sludge from other WWTPs in Varberg municipality is also treated at Getterö WWTP [22]. At Getterö WWTP the sludge is initially thickened by the addition of polymers and the use of band gravity thickeners. The sludge is then lead to digestion chambers where the sludge is anaerobically digested under mesophilic conditions [10]. Getterö WWTP has four digestion chambers [23], 900 m^3 each [E-mail M Habagil 2022-04-21], set up in a master-slave system. The sludge is initially pumped into a master digestion chamber and is thereafter distributed to the remaining three digestion chambers (slaves). Sludge is recirculated from the slave digestion chambers back to the master chamber according to a time-controlled program. The purpose of the recirculation is to maintain optimal conditions in the master chamber [23]. The biogas produced at Getterö WWTP is not upgraded and used for fuel, instead it is used for heating within the WWTP. After digestion, the sludge is dewatered by centrifugation. The sludge is intermediately stored in a silo with a volume of 4800 m^3 [24]. The silo is emptied 2-3 times a week, and the sludge is then stored openly on a sludge plate for approximately 4 weeks [E-mail M Habagil 2022-04-21]. The finished treated sludge is used for production of construction soil [10].

2.1.3 Hammargård WWTP

Hammargård WWTP is owned by Kungsbacka municipality and has the capacity of 52 000 pe connected, whereas 41 000 pe are currently connected [11]. The WWTP also receives external sludge from other WWTPs in Kungsbacka [25]. First the sludge is separated from the wastewater and thickened [11], using a gravimetric sludge thickener. Sludge buffers are used to regulate the flow. The sludge is thereafter stored in an aerated [25] sludge storage chamber while external sludge is added. The sludge is further thickened by the addition of polymer and then dewatered through centrifugation. Thereafter the sludge is hygienised. This is done by adding burnt lime (calcium oxide, CaO) [11] and mixing it into the sludge [25] while the temperature of is increased to approximately 54 °C [11]. The addition of lime causes the pH to rise to approximately pH 12 and this increase in pH in combination with the temperature increase kills pathogens present in the sludge. After treatment, the sludge is temporarily stored openly in a pile before being transported to the long-term storage which is adjacent to the WWTP. At the long-term storage, the sludge is stored openly at a plate [11] in monthly batches of approximately 650 ton [25]. The sludge is stored here between 2-8 months [11], depending on the demand for fertiliser [E-mail S Engström 2022-04-26]. Before the sludge is transported away and distributed on agricultural land, the sludge is tested for salmonella and salmonella has never been detected at Hammargård WWTP. The sludge is mainly used as fertiliser on agricultural land, however depending on the quality of the sludge regarding heavy metal content, it can be used to cover landfills or be composted into soil improvement products. However, limed sludge does not compost well and only a limited involvement of limed sludge can be made to the whole compost [11].

2.1.4 Sobacken WWTP

Sobacken WWTP in Borås has the capacity of wastewater treatment for 150 000 pe [26]. At Sobacken WWTP, sludge from wastewater treatment is digested together with household and food waste as well as biological waste from companies and industries, for example residues from butchers and production waste from food industries. Additionally, dewatered undigested sludge from other smaller WWTPs in the area are transported to Sobacken WWTP and treated there as well [8]. Sludge from the wastewater treatment is pumped into sludge storages which work as buffers. These buffers are used to ensure both the correct flow and the quality of the sludge. In these storages, the sludge is mixed. To thicken the sludge, polymers are added, and the sludge is pumped into mechanical thickeners [27]. Household waste is added and mixed with the sludge [8].

The facility has two digestion chambers which are run parallelly. The sludge has a residence time of 15-20 days in the chambers, depending on the input flow. The sludge is hygienised in the digestion chamber, through intermittent input and output of sludge. The sludge is kept inside the digestion chamber for 2-6 h in a minimum of 55 °C and the input to the digestion chambers are adapted to ensure hygienisation and heat recovery. From the digestion chambers, the sludge is lead into a sludge buffer tank. The buffer is needed since the output from the digestion chamber is intermittent and the flow of the remaining process is continuous. From the buffer, the sludge is pumped to a heat exchanger and the sludge is cooled down before being led to a sludge storage chamber. The storage is equipped with mixers to avoid sedimentation during storage. This storage has vocsidizers which collects any gas emitted at this stage. The sludge is then dewatered through addition of polymers and centrifugation. After dewatering, the sludge is stored in a sludge silo. From the silo, the sludge is continuously transported away from the facility by trucks [27]. The dewatered sludge is partly used as landfill cover and partly burned at a waste incineration plant in Borås [8].

2.2 Anaerobic Digestion

As previously mentioned, anaerobic digestion (AD) is a part of the sludge treatment process at Rya, Getterö and Sobacken WWTPs [8-10]. AD is used to generate biogas and the digestate can be used as fertilisers in agriculture. Hence, it is a way to valorise organic waste while achieving a circular bioeconomy. Anaerobic digestion is a technology that is well-established [3] and due to its environmental advantages, AD is increasingly applied [28]. In recent years, the number of AD plants has increased in Europe due to its contribution towards a circular bioeconomy. In addition to sewage sludge, residues from agriculture, industries and municipal organic waste can be treated in biogas production facilities [3]. AD involves microorganisms in the breakdown of organic matter and in the case of WWTPs the organic matter consists of sludge from wastewater treatment as well as other waste such as household and food waste [28]. The anaerobic degradation of organic matter by microorganisms involves four main steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Each of these four steps are performed by separate groups of microorganisms. In certain conditions, acetate-oxidising bacteria have also been observed. These different microbial groups are metabolically linked

and typically operate in a synchronised manner [3]. The different main steps of AD are further explained below.

2.2.1 Hydrolysis

The first step in AD is hydrolysis, where extracellular enzymes such as lipases, proteases and cellulases degrade polymeric substrates such as lipids, protein and polysaccharides into their respective oligomers and monomers. The substrate accessibility strongly affects the reaction rate of this step [3]. If the substrate is recalcitrant (for example as with lignin) hydrolysis is often the rate limiting step of methane production. The hydrolysis step also depends on factors such as pH, particle size, enzyme production, diffusion and adsorption of enzymes in substrate particles. Although the hydrolytic bacteria involved in this step are phylogenetically diverse, the two phyla *Bacteroidetes* and *Firmicutes* includes most of the known species. Hydrolytic bacteria are not as sensitive to changes in environmental factors (e.g. pH or temperature) as methanogens and they also grow more rapidly than methanogens [28].

2.2.2 Acidogenesis

The second step is acidogenesis in which the oligomers and monomers formed during hydrolysis are converted into volatile fatty acids (VFAs), such as acetate, propionate, isobutyrate, valerate and isovalerate. Additionally, acidogenesis also produces products such as formate, lactate, alcohols, CO_2 and hydrogen gas (H_2) [28].

The process of acidogenesis is in general rapid and this can potentially cause accumulation of VFAs. An accumulation of VFAs affects methanogenesis negatively in two ways. Firstly, VFAs in high concentrations are inhibitory for methanogenesis. Secondly, an accumulation of VFAs cause a decrease in pH and since the optimal pH for methanogenesis is around 7-8, a lower pH may slow down or stop the methanogenesis. Additionally, the inhibitory effect of VFAs is stronger at low pH since the undissociated form of VFAs can be toxic to microorganisms, and the proportion of undissociated form of VFAs are more abundant at low pH. Some VFAs can even inhibit methanogenesis at neutral pH. Phyla which are known to contain most of the identified species of acidogenic bacteria are *Bacteroidetes, Chloroflexi, Firmicutes,* and *Proteobacteria* [28].

2.2.3 Acetogenesis

In the third step of AD, the fatty acids are further oxidised into mainly acetic acid as well as H_2 and CO_2 via acetogenesis [3]. Compounds such as acetate, formate, H_2, CO_2 and methyl compounds can be directly used by methanogens in the final step of methanogenesis. Other intermediates formed in the acidogenesis step must be further degraded by acetogens before the methanogens can utilize them to produce methane. For example, this applies to propionate, butyrate, isobutyrate, valerate, isovalerate and ethanol. The process in which these longer FA intermediate compounds are further transformed into acetate, H_2 and CO_2 is referred to as

syntrophic acetogenesis [28]. In municipal wastewater and typical AD conditions, approximately 30% of the substrates can be transformed via the propionate pathway when forming methane. Thus, fermentation of propionate via syntrophic acetogenesis is of interest when studying AD. Additionally, propionate is one of the previously mentioned VFAs able to inhibit methanogenesis at neutral pH. Due to this, syntrophic acetogenesis is often the rate limiting step in the AD process and thus of critical of importance when optimizing the conditions of AD. Some acetogens involved in propionate degradation, which are often observed in AD processes, are for example *Pelotomaculum, Smithllela,* and *Syntrophobacter*. Acetogens involved in oxidation of butyrate and other FAs are for example *Syntrophus* and *Syntrophomonas* [28].

Hydrogenotrophic methanogens utilizes H_2 to form methane. These organisms live in syntrophy with acetogens, where the hydrogenotrophic methanogens consumes H_2 released by acetogens. This syntrophic relationship ensures that the partial pressure of H_2 is low enough for the acetogenesis to become thermodynamically possible. Formate and formate acid can be seen as H_2 associated with CO_2, thus the consumption of formate also presents a syntrophic relationship which is critical for acetogenesis. The syntrophy based on H_2 and formate transfer from organisms which produces H_2 or formate to those which consumes it is referred to as interspecies H_2 transfer. It has been found that some organisms, including *Methanotrichaceae* (also referred to as *Methanosaetaceae*), can perform something referred to as direct interspecies H_2/formate transfer. This process is more rapid and has the potential to improve AD efficiency [28].

2.2.4 Methanogenesis

The final step in AD is methanogenesis. Methanogenesis is performed by a group of microorganisms called methanogens which belongs to the domain A*rchaea* [28]. Methanogens are obliged anaerobes and methane producers, thus they do not grow using fermentation of alternative electron acceptors for respiration [29].

Physiologically there are three types of methanogens: hydrogenotrophic, acetoclastic and methylotrophic. The hydrogenotrophic methanogens utilizes H_2 and formate to reduce CO_2 into methane, acetoclastic methanogens converts acetate into methane and CO_2 and methylotrophic methanogens use methyl compounds like methanol, methylsulphides or methylamines to produce methane. In general, 70 % of the methane produced in a standard municipal AD is through conversion of acetate, and the remaining proportion is from H_2 and CO_2. Methylotrophic methanogenesis merely stands for a small amount of the produced methane. Common hydrogenotrophic methanogens in AD processes includes *Methanobacterium, Methanobrevibacter, Methanoculleus, Methanospirillum*, and *Methanothermobacter.* The acetoclastic methanogens are divided into the two genera *Methanotrichaceae* and *Methanosarcina. Methanotrichaceae* only uses acetate to produce methane and has a high affinity for acetate. Because of this, it often dominates at low acetate concentrations. However, *Methanotrichaceae* has a slow growth rate. *Methanosarcina* are a facultative acetoclastic methanogen and can in addition to acetate, utilise H_2 and CO_2 for

methane production. *Methanosarcina* has a relatively high growth rate, but a lower affinity for acetate. Hence, it can dominate over *Methanotrichaceae* in conditions where acetate concentration is high [28].

All methanogens share some physiological characteristics, however they are phylogenetically diverse [30]. In addition to looking at the methanogens based on their physiological properties, you can also look at them phylogenetically. There are currently eight well-established orders of methanogens. These are associated to three primary phyla (*Euryarchaeota, Halobacterota, Thermoplasmatota),* but the exact placement is unknown. Within the phyla *Euryarchaeota*, the orders *Methanococcales, Methanopyrales* and *Methanobacteriales* are included. The phyla *Halobacterota* includes the orders *Methanomicrobiales, Methanocellales, Methanonatronarchaeales,* and *Methanosarcinales.* Within the phyla *Thermoplasmatota* the order *Methanomassiliicoccales* is included [29]. The classification of methanogens is supported by 16S rRNA gene sequencing analysis, is combination with difference in physiological properties [30]. Methanogens belonging to *Euryarchaeota* have been found to grow at various temperatures, including both thermophilic and mesophilic conditions. This group of methanogens are most often hydrogenotrophic and prefer neutral pH. Methanogens belonging to *Halobacterota* have a more diverse substrate range compared to *Euryarchaeal* methanogens and includes hydrogenotrophic, acetoclastic and methylotrophic methanogens. The methanogens belonging to *Halobacteriora* are commonly mesophilic or moderately thermophilic and are moderately acidophilic. The methanogens of the order *Methanomassiliicoccales* prefer neutral pH [29].

2.2.5 Syntrophic Acetate-Oxidising Bacteria

In certain conditions, an alternative pathway for methane production has been observed in AD processes. Syntrophic acetate-oxidising (SAO) bacteria can convert acetate into H_2 and CO_2, which in turn is converted to methane by hydrogenotrophic methanogens. Few species of acetate-oxidising bacteria have been identified, but some strains are acetate-oxidising *Reversibacter* (AOR), *Clostridium ultunense, Thermacetogenium phaeum, Tepidanaerobacter acetatoxydans, Thermotoga lettingae,* and *Syntrophaceticus schinkii*. It is believed that regular acetoclastic methanogens outcompete the SAO bacteria in most AD processes. However, this group of bacteria is potentially important for AD systems [28]. One occurring problem during AD is the inhibition of acetoclastic methanogens by for example high ammonia concentrations [3]. The presence of SAO bacteria could in these cases contribute to a continued efficient biogas production [28]. It has also been shown that the fraction of acetoclastic methanogenesis compared to hydrogenotrophic methanogenesis strongly depends on the environmental conditions, since at thermophilic digestion or at high ammonia levels, the methane formation progress mainly through acetate oxidation coupled to hydrogenotrophic methanogenesis [3].

2.2.6 Optimisation of Anaerobic Digestion

By making the AD as efficient as possible, both environmental benefits and economical profits are optimised. An efficient AD process is characterised by a high degree of digestion in combination with a high yield of biogas per reactor volume. A high degree of digestion is of significance for the nutrient level in the digestate and leads to a low RMP, which in turn decreases greenhouse gas emissions associated with storage of the digestate. It has previously been found that the efficiency of biogas production depends on numerous factors which are often interlinked. One key factor is the composition of the incoming substrate, for example sludge and household or food waste. Operational parameters also play an important role, for example organic load, hydraulic retention time, mixing, digester fluid behaviour, temperature and digester technology. Additionally, the microbial community composition in anaerobic digesters have a considerable effect on the production of methane [3] and is essential for an effective AD process [1]. There are several aspects of this effect, for example, both the microbial diversity and an active and synchronized microbial community is necessary for efficient methane production [3]**.** When attempting to increase the methane production rate, the rate limiting step of AD should be targeted since increasing the rate of the other steps will have minimal impact. For example, the rate of methanogenesis is limited by the rate of one of the previous steps of AD (hydrolysis, acidogenesis, acetogenesis). The rate limiting step varies depending on the chemical structure of the substrate and other operational parameters [28].

2.2.6.1 Factors Affecting the Microbial Community

The microbial community in a biogas production depends on parameters such as pH, ammonia levels and VFA concentration. To acquire a rapid and stable digestion, maintaining a balanced reaction rate among these steps is required. For example, as previously mentioned, hydrolysis and acidogenesis are generally faster compared to acetogenesis and methanogenesis and if there is an excess of substrate, VFAs are formed in a higher rate than they are consumed leading to an accumulation of VFAs. If the temperature changes rapidly or toxicants are present, the consumption of acids is slowed down which also causes accumulation of VFAs. The balance of reaction rates depends on the rate of the acid and H_2 consuming reactions being faster than the acid and H_2 producing steps since an accumulation of H_2 will inhibit the degradation of VFAs. Another factor is the availability of trace metals and ions which also affects the microbial community, mainly at the enzyme level, and thus in turn affects the substrate degradation efficiency [3].

Another aspect of the microbial community composition affecting the production of methane is the microbial diversity. If a microbial community includes multiple species, at relatively equal abundance, able to perform the same function, the community is more likely to maintain this function when exposed to perturbations such as VFA accumulation or changes in digestion conditions. This since if the population of a certain species is lost due to a perturbation of the system, there is likely another species able to perform the same function that are more tolerant to the perturbation and can ensure that the function is preserved within the system [28]. Thus, a diverse microbial community is a form of insurance of its function, where different species

compensate for each other and to ensure an efficient AD process a diverse microbial community is essential [31]. Studies have also shown that digestion with diverse microbial communities result in higher methane production compared to those with lower diversity. A higher microbial diversity has also been observed in mesophilic conditions compared to thermophilic [28].

2.2.6.2 Possible Strategies to Improve Anaerobic Digestion Efficiency

There are several strategies which can be used to improve the methane production by anaerobic digestion, for example pre-treatment of the substrate to increase the accessibility of the substrate and the use of process additives such as trace metals to improve the balance of nutrients during the digestion. Another strategy is to use thermophilic operating conditions, to get enhanced degradation rates. However, the efficiency of the strategy is dependent on substrate. It has also been found that biogas production facilities with a second digestion step digesting the outgoing material from the first digestion step (a post-digester) had decreased TS in the second step [3]. This is consistent with the result of the previous investigation at Rya WWTP in 2017 where it was found that digestion chambers in series resulted in lower RMP compared parallel digestion chambers [19]. Another alternative strategy is the use of bioaugmentation which is when a specialised microbial community is added to the digestion system to improve the process function [28].

The relationship of the microbial structure and function in AD is not fully understood [3] and research are put into increasing the knowledge of the complex microbial communities which are responsible for the AD process and its interactions. According to Venkiteshwaran K. et al. [28], new knowledge within this area is crucial for the development of improved AD systems. In past decades there have been progress and key organisms influencing AD has been identified. However, a quantitative, predictable relationship between the complex microbial communities and the digester functional output is needed to improve the design and operation of anaerobic digesters for wastewater treatment and renewable energy transformation. Venkiteshwaran K. et al. specifically mentions the use of next generation sequencing technology as a method to investigate the relationship between the structure of the microbial community and the digester function, such as methane production rate [28].

2.4 Metagenomic Analyses of Microbial Communities

Microbial communities are defined as the collection of microorganisms coexisting in the same environment. As with the case of AD described above, the communities are usually complex, and it is often the cooperation within a community that is of interest when studying them. The metagenome is the collective genome of a microbial community, and it can be studied through metagenomics. Metagenomics allows the genomes of non-cultivable organisms to be studied with higher accuracy compared to microbiology and molecular methods. In metagenomics, information regarding organisms present in an environment, and their biological functions, is derived by randomly sampling DNA from the metagenome [32]. Thus, by studying the metagenome in sludge, properties of the microbial community, and thereby the properties of the sludge, can be further investigated.

There are two different approaches in metagenomics: amplicon and shotgun sequencing. In this study, shotgun metagenomics was performed. In amplicon sequencing, a selected marker region is amplified and sequenced. A commonly used marker is the 16S rRNA gene. Amplicon sequencing allows analysis of the taxonomic affiliation and estimation of abundance and diversity. Sequences which are sufficiently similar are clustered together and assumed to come from the same species. These clusters of sequences form operational taxonomic units (OTUs) which are considered putative species. In shotgun metagenomics the total amount of DNA in a sample is sequenced. This allows for characterisation of both coding and non-coding sequences, which can be used as phylogenetic markers. By analysing all genes present in the microbial community, its biological function can be analysed, as well as estimation of abundance and diversity [32].

2.4.1 Microbial Diversity

Metagenomic analyses make it possible to investigate the diversity of microbial communities. There are several ways of investigating the diversity of a microbial community. Alpha diversity investigates the diversity within a sample, i.e. at the local diversity of a community, for example in a habitat such as a specific geographical site or in a specific individual. It is also possible to look at the beta diversity, which is the diversity between samples or habitats, such as between different geographical sites or between individuals [32]. There are also several types of alpha and beta diversity: naive, phylogenetic and functional. When looking at naive alpha diversity all OTUs are considered separate entities and relationships between individual OTUs are not considered. Phylogenetic alpha diversity takes into account proximity within a phylogenetic tree. For example, if two OTUs have several branches of a phylogenetic tree in common they will contribute less to diversity compared to two OTUs which are separated from each other in the phylogenetic tree. Calculating the phylogenetic diversity requires a phylogenetic tree. Lastly, it is possible to investigate the functional alpha diversity. This diversity measurement takes into consideration the pairwise distance between OTUs. Thus, two OTUs with a small pairwise distance will contribute less to diversity compared to two OTUs with a high pairwise distance. Calculating the functional alpha diversity requires a distance matrix [33].

There are many ways to quantify the diversity of a microbial community. The most basic way to measure diversity is to look at the number of species in a sample, which is referred to as richness. However, species richness is not the only parameter that defines microbial diversity. For example, if two different communities have the same number of species, but in different abundances, the community with the most uniform distribution of species should be considered more diverse. This parameter of diversity is being considered when looking at the evenness of a sample. The evenness takes into consideration whether a few species dominate the microbial community and if there are many species that are present in low abundance. Hence, evenness attempts to quantify any unequal representation in species. There are several ways of measuring richness and evenness, for example Shannon or Simpson's diversity indices [32]. In this project the Hill index was used. Hill numbers are also called effective numbers and they are calculated using the equation

$$^{q}D = \left(\sum_{i=1}^{S} p_i^q\right)^{\frac{1}{1-q}} \quad (1)$$

where q is the diversity order, ^{q}D is the Hill number (or effective number) of order q, p_i is the relative abundance of the i^{th} OTU in a sample and S is the total number of OTUs. When q=0, the Hill number ^{0}D will be the total number of OTUs in the sample, thus a measurement of the richness. When increasing q, evenness is taken into account. When q=1, each OTU is weighted exactly according to its relative abundance, and equation (1) becomes

$$^{q}D = exp\left(-\sum_{i=1}^{S} p_i \times \ln\left(p_i\right)\right) \quad (2)$$

When q>1, more weight is given to OTUs with high relative abundance. For example, in a community with 2 highly abundant species and 8 species with low abundance, ^{q}D, will be 10 when q=0 and as q increases, ^{q}D will approach 2 [34].

When studying beta diversity, a dissimilarity matrix is often generated, containing dissimilarity values. These dissimilarity values can range from 0, corresponding to two samples having identical community composition, to 1, meaning that the two samples are completely different and have no shared taxa. When generating a dissimilarity matrix in order to look at beta diversity, pairwise Hill-based dissimilarity values can be used as well. The dissimilarity values can be considered in several ways, for example using principal coordinate analysis (PCoA) [34]. PCoA makes it possible to visualise data based on distances. PCoA generates a map which gives an understanding of which entities that are similar, and which are different depending on their placement in the map and can make it possible to identify groups or clusters. Similar to principal component analysis (PCA), PCoA uses dimensionality reduction [35]. However, instead of looking at the maximal variance as in PCA [36], PCoA considers the maximum distances [34].

Any calculated diversity indices depend on sequencing depth. For example, a higher sequencing depth will have more detected OTUs and thus a higher richness. To make the diversity of samples comparable, the data is rarefied. This means that the data is subsampled to the same sequencing depth. In this process, a sequencing depth is chosen, and fragments are randomly sampled without replacement. Thereafter, new OTU abundances are calculated for the rarefied data. Rarefication is a random process but by using a seed parameter during rarefication, the rarefication becomes reproducible [34].

3 Method

This section describes the methodology applied in this project. This includes temperature measurement of sludge storage, collection of sludge samples, methane emission measurements using eradication equipment, measurement of acetate and format content in the sludge samples and metagenomic analysis of microbial communities in the collected sludge.

3.1 Temperature Measurements

During the methane emission measurements, the aim was to imitate the conditions in which the sludge is stored at WWTPs. To further assess the conditions of the sludge storage, temperature measurements were performed on the finished treated sludge while stored openly in a pile. The temperature measurements were performed at two separate occasions. A temperature data logger was used to record the temperature and the logger was wrapped in protective plastic and attached to a steel rod. The temperature data logger was placed at a depth of approximately 1 m into the pile and the temperature was recorded every 10 s. The measurement took place during approximately 1 h in order for the temperature to stabilise.

3.2 Sample Collection

Samples of dewatered sludge were collected from Rya, Getterö, Sobacken and Hammargård WWTPs, at two separate occasions. Due to the acquired time to transport the samples from the different WWTPs to the location of measurement at Rya WWTP, the measurement could not be initiated immediately following the collection of the samples. The distance to the wastewater treatment plants varies depending on WWTP and for the results to be comparable, the samples were kept in a cooler for 1.5 h prior to the measurement. The low temperature was expected to keep the methane emission at a minimum until the measurement had been initiated, and thereby enabling all methane emission to be recorded. However, due to logistic difficulties some deviations from this setup occurred. For the metagenomic analysis, three samples of cooled sludge from each WWTP were collected and stored in the freezer.

3.3 Methane Emission Measurements

The main method for the methane emission measurements was the use of an eradication equipment from BPC Instruments called Automatic Methane Potential Test System (AMPTS®) II, which is an analytical tool for anaerobic batch fermentation testing. The AMPTS® II system consists of an incubation unit, CO_2-fixing unit and a gas flow meter unit. The incubation unit consists of 15 parallel flasks working as batch reactors. These are placed in a thermostatic water bath and each one is connected to a CO2-fixing unit containing a solution of 3M NaOH and a pH indicator (thymolphthalein). All gas that is produced during incubation passes through the CO_2-fixing unit and any CO_2 that is produced during incubation is absorbed by the solution. Once the solution is saturated with CO_2, the decrease in pH will be visible due to the

thymolphthalein and the saturated solution can be replaced with a new, unsaturated solution. The CO_2-fixing unit also retains H_2S. Each CO_2-fixing unit is connected to a gas flow meter (flow cells) and any produced methane is further transported to these. The flow cells measure the volume of gas which has passed the CO_2-fixing unit by a wet gas flow measuring device capable of recording ultra-low gas flows. This is done by utilising the principle of liquid displacement and buoyancy. The manufacturer of the AMPTS® II system guarantees that the CO_2-fixing unit has an efficiency of > 98 %. This has been confirmed during previous investigations of methane emission from sludge at Rya WWTP where the composition of the outgoing gas was analysed by gas chromatography (GC). The AMPTS® II system also removes any gas overestimation originating from inert gas in the reactor headspace.

During the experiment, the four different CO_2-fixating units became saturated and was refilled with new solution. To increase the reliability of the result, triplicates of each WWTP sample was used, resulting in 12 samples in total. Approximately 250 g of the sludge was placed in each flask and the methane emission was recorded for 30 days. With the exception of Sobacken WWTP, the sludge is stored in openly at all WWTPs, thus oxygen is available during storage. However, the sludge piles are large and in the middle of a sludge pile it is reasonable to assume that there are low levels of oxygen, more closely resembling anaerobic conditions. To imitate these conditions of sludge storage, the flasks were not emptied of oxygen after adding the sludge and no stirring equipment was applied. Additionally, the flasks were sealed, and not exposed to any additional oxygen during the measurement period. For the first measurement, the flasks were placed in a water bath with a temperature of 20 °C and in the second measurement, a temperature of 35 °C was used. These temperatures were based on temperature measurements of the sludge storage at Rya WWTP. The collected data was used to acquire figures and was analysed by calculating the average methane emission per g TS from each WWTPs' sludge sample.

Measurements of volatile solids (VS) and total solids (TS) of the sludge samples were performed by the process lab at Rya WWTP. The sludge is placed in an oven and the water of the sludge is evaporated. This was done using an IR moisture analyser at 130 °C for 30 min. The remaining mass is weighed and represents the TS, which includes VS and ash (fixed solids). When analysing VS, the samples are further exposed to a higher temperature by placing it in an ash oven at 550 °C for 2 h, which burns all organic matter. The remaining mass consists of ash and the VS are obtained by subtracting the weight of the ash form the dry weight of the sludge.

3.4 High Pressure Liquid Chromatography

To investigate the content of the VFAs acetate and formate in the different sludge samples, High Pressure Liquid Chromatography (HPLC) was performed. Duplicates of the sludge samples were diluted in 20 ml MQ water, vortexed and centrifuged. The supernatant was filtered in a 0.45 filter and frozen before performing HPLC. The samples were thawed, and a total volume of 1 ml was loaded in the HPLC, in which the samples had been diluted five times. The HPLC was performed using the Aminex HPX-87H column BIO-RAD together with a 210

nm UV detector. A solution of 1 mM acetate was used as standard, together with a previously made standard curve.

3.5 Metagenomic Analysis

Analysis of the microbial community metagenome included DNA-extraction, DNA sequencing and data analysis.

3.5.1 DNA Extraction and Sequencing

The frozen sludge samples were thawed, and DNA extraction was performed using the FastDNA® SPIN Kit for Soil by MP Biomedicals [37]. Deviations from the protocol consisted of performing the homogenisation in FastPrep two times instead of one, and the samples were put on ice for 3 min in between. This was done to ensure the samples were properly homogenised. Additionally, the binding matrix was resuspended in 60-70 µl of DES (DNase/Pyrogen-Free Water), to avoid over dilution of the purified DNA. Two standard solutions with 0 and 10 ng/ml concentration of DNA respectively were used to calculate the DNA concentration in each sample. Whole genome sequencing was done externally by Eurofins using paired-end Illumina sequencing and shotgun sequencing metagenomic data was obtained.

3.5.2 Analysis of Sequencing Data

When analysing the obtained shotgun metagenome data, a tool called SingleM was used [38]. SingleM enables determination of the relative abundances of OTUs from shotgun metagenome data without heavy reliance on reference sequence databases. SingleM can distinguish closely related species, regardless of whether those species come from lineages new to the scientific community. The SingleM tool finds 14 sequences which encodes conserved single copy marker genes. More specifically, these identified sequences are 14 short reads (approximately 60 bp) of highly conserved sections. These 14 identified sections correspond to ribosomal proteins and exist independent of taxonomy, similar to the 16s RNA gene used in amplicon sequencing. Focusing on these 14 conserved marker genes enables a variety of analyses. In contrast to 16s-analyses, the SingleM tool is independent of the copy-number variation issues. SingleM is also relative fast and scalable. The 14 single marker genes used in SingleM is generally better at differentiating closely related lineages compared to a standard 16s amplicon-based study. The input into SingleM is raw, untrimmed reads and the output is sequenced based OTUs distributed among the 14 maker genes [38]. SingleM does not manage paired end reads. Hence, the two sequencing files containing reads from different ends for samples was analysed separately. The consequence of this is that each analysis generated two results.

The 14 conserved marker genes corresponding to ribosomal proteins will in this report, from here on be referred to as protein 1-14. Full names of the 14 ribosomal proteins can be seen in

Table A.1 in Appendix A. When looking at the first protein, for example 10 OTUs could be identified among the shotgun data, whereas looking at the second, 15 OTUs could be identified. Some of the identified OTUs within these two proteins could be the same species, thus the OTUs between the proteins may be overlapping. Although in general, what is referred to as OTU1 based on one protein, is not necessarily the same putative species as OTU1 based on another marker gene.

SingleM was installed using Miniconda. Further analyses were done using the python package qdiv [39]. The qdiv package was developed for analysing results from rRNA gene amplicon sequencing or similar data and is therefore suitable to analyse the data generated from SingleM. The qdiv package can be used to rarefy and subset the data, calculate alpha and beta diversity, generate plots such as heatmap and PCoA and carry out null model analyses as well as Mantel- and Permanova statistical tests [39]. All analyses were performed in accordance with Tutorial 1 described in the qdiv documentation [34]. Additionally, independent analysis regarding the relative abundance of methanogens was performed by analysing the counts for each OTU generated by SingleM. This was done by calculating the percentage of counts which corresponded to methanogens from the count table data generated from SingleM.

The analyses in this project included generating information regarding different taxa in each sample and heatmaps, calculating and analysing naive alpha and beta diversity of order 0 and 1 (including PCoA plot) and performing Permanova tests. The heatmaps were generated to illustrate the relative abundance of different taxa in different samples. The 20 OTUs with highest relative abundance was included in each heatmap and the taxa/OTUs with highest relative abundance was defined as the OTUs with maximum relative abundance in a sample.

Permanova tests with 1000 permutations were performed to investigate whether there was a significant difference in microbial composition between the two different conditions (mesophilic and thermophilic) and the three different WWTPs (Sobacken, Rya and Getterö). The Permanova tests were based on the beta dissimilarity matrices. The average naive alpha diversity was calculated from the three replicas for each SingleM protein and WWTP, and thereafter the average naive alpha diversity of each WWTP over all 14 SingleM proteins was calculated as well.

3 Results and Discussion

The findings of this project are presented and discussed in this section. This includes temperature measurement of sludge storage, DNA extraction, methane emission measurements, measurement of acetate and format content in the sludge samples and metagenomic analysis of microbial communities in the collected sludge.

3.1 Temperature of Sludge Storage

The result of the temperature measurements performed in this project at Rya WWTP is displayed in Table 1, together with the result from previously performed temperature measurements at Rya WWTP in the study *Methane Emission from Sludge Storage at Gryaab* by Alyona Tormachen Shabsai [40]. All temperatures presented in Table 1 were measured approximately 1 m into the sludge storage pile using a temperature logger attached to a steel rod. Table 1 also shows time of measurement (month and year), storage time and the size of the sludge storage, where the two latter are approximate estimations. The size of the sludge pile is described based on how much of the sludge storage compartment that is taken up by the sludge pile (full and less than half full) and each sludge storage compartment can receive a total of 1000 ton sludge. The temperatures measured at the same time was performed at different places in the sludge pile.

Table 1. Temperature measurements 1 m into the sludge storage pile at Rya WWTP, including time of measurement (month and year), storage time and size of the sludge pile compared to the size of the sludge storage which when full contains approximately 1000 ton sludge.

Temperature [°C]	Time of measurement	Storage time	Size of sludge storage pile
24	Mars 2022	1-2 days	Less than half full
26	April 2022	1 week	Full
27	April 2022	1 week	Full
28*	October 2021	2 weeks	Full
34*	October 2021	2 weeks	Full
34*	October 2021	2 weeks	Full

*Measurements performed in the study *Methane Emission from Sludge Storage at Gryaab* by Alyona Tormachen Shabsai [40].

The data presented in Table 1 indicates that the temperature of the sludge storage pile is dependent on how long the sludge have been stored and the size of the sludge pile. Higher temperatures were obtained at larger size and when the pile had been stored a longer period of time. This indicates that the heat of the sludge storage is not only residue heat from the digestion chambers, but also the result of compost effects. The results from previous measurements performed in the study *Methane Emission from Sludge Storage at Gryaab* by Alyona Tormachen Shabsai also indicated that the temperature of the sludge storage pile is not homogenous but varies between different areas.

Based on the data in Table 1 the average temperature of the sludge storage is 28.83 °C, although it is likely that the true mean value of the temperature over a year is higher than that. The data presented in Table 1 comes from measurements performed in autumn and spring, and it is likely that the temperature of the sludge storage is higher at summer when the surrounding temperature is higher, since this would lead to an increased compost effect. It is also probable that the temperature of the stored sludge is higher deeper inside in the sludge pile. At Rya WWTP there have been previous attempts to generate a temperature profile of the sludge storage at different depths, but the weight and density of the sludge pile makes it impossible for the temperature logger and steel rod to reach deeper than 1 m into the sludge pile. If it is possible to work around this problem, a further investigation of the sludge storage temperature at longer depths would be a valuable contribution to determining the sludge storage conditions more accurately. One suggestion could be to place a temperature logger in the pile as it is formed and leave it until the sludge pile is collected and transported away from the facility. To further investigate the temperature of sludge storage, regular measurements throughout the year could be conducted, and thereby account for temperature changes of the surrounding environment. Additionally, it could be valuable to compare the temperatures of sludge storage at different WWTPs and at different storage times, for example during long-term storage.

To generate benchmark values of methane emission which resembles the actual methane emission as accurately as possible, it is important that the methane emission measurements are performed at conditions resembling the true conditions of sludge storage. According to a study performed by Swedish Water in 2020 [41], the average temperature of the storage of dewatered sludge is approximately 20 °C. However, these sludge piles were only approximately 1.5 m^3 as they were a part of pilot trials [41]. Based on the values in Table 1, the temperature is likely higher than 20 °C in many cases. By performing measurements at both 20 and 35 °C, the temperature span of Table 1 is covered, which increases the chance of obtaining benchmark values for methane emissions that resembles the true emissions as much as possible. Additionally, performing the methane emission measurements at these two temperatures makes it possible to investigate how the methane emission from finished treated sludge varies depending on temperature, especially regarding sludge treatment with mesophilic and thermophilic digestion.

3.2 Methane Emissions from Simulated Sludge Storage

As previously mentioned, the methane emission measurements were performed at two different temperatures (20 and 35 °C) to cover the temperature range of the sludge storage. For the benchmarks values to be easily adapted to the WWTPs attempting to use climate change calculation tools, the methane emission is presented in the unit [Nml/gTS].

Values for TS and VS of the sludge used for measurements at both temperatures is presented in Table 2. The TS and VS was measured from finished treated sludge collected at Hammargård, Sobacken, Getterö and Rya WWTPs. Hammargård WWTP had the lowest value of VS due to not using digestion as a part of their sludge treatment process. The sludge from Hammargård WWTP also had comparatively high TS. Rya WWTP also has a high TS value.

At the time of the measurements, only one of the two digestion chambers at Rya WWTP was running, due to maintenance. A consequence of this is a temporary lower degree of digestion in the sludge from Rya WWTP, which can be seen when comparing the value in Table 2 to the average TS in 2020 of the finished treated sludge at Rya WWTP, which was 28.4 % [4].

Table 2. Total solids (TS) and volatile solids (VS) of the sludge used in the methane emission measurements at 20 and 35 °C for Hammargård, Sobacken, Getterö and Rya WWTP.

WWTP	Measurements at 20 °C		Measurements at 35 °C	
	TS [%]	VS [%]	TS [%]	VS [%]
Hammargård	29.6	33.6	28.2	41.3
Sobacken	26.2	59.4	21.8	58.4
Getterö	21.4	68.1	23.8	65.5
Rya	29.4	64.9	32.8	58.7

Figure 1 shows the cumulative methane emissions at 20 °C. At 20 °C, the gas flow meter only recorded methane emission from the sludge digested in mesophilic conditions, i.e. from Rya and Getterö WWTP. The limed, undigested sludge from Hammargård WWTP and the sludge originating from thermophilic digestion conditions from Sobacken WWTP had no recorded methane emission during the 30 days of measurement. The equipment has previously been able to record emissions as low as 2 Nml/day. Thus, it can be assumed that the limed, undigested sludge and the sludge digested in thermophilic conditions emitted less than 2 Nml methane per day at 20 °C and any emission below this can be considered negligible.

The sludge from Rya WWTP had the highest emission, approximately the double amount compared to Getterö WWTP. As previously mentioned, only one of the two digestion chambers at Rya WWTP was running during the time of measurements, leading to a temporary lower degree of digestion in the sludge collected at Rya WWTP. This in turn generates a higher amount of methane emission form the sludge compared to during normal operating conditions. Therefore, data from previously performed methane emission measurements at Rya WWTP was analysed as well and are annotated as Rya WWTP* in Figure 1-5. This data was collected using the same measurement tools (AMPTS® II) and is an average from two replicates. Since there were no data of mass or TS of the sludge used in these previous measurements, the average value of gTS for the sludge collected at Rya WWTP for the 20 and 35 °C measurements in this project were used.

As can be seen in Figure 1, the sludge collected at Rya WWTP during normal circumstances (both digestion chambers running), had a lower methane emission compared to when only using one of the digestion chambers, which was expected. At 20 °C, the sludge collected at Rya WWTP during normal circumstances (Rya WWTP*) had approximately the same amount of emission as Getterö WWTP, indicating that mesophilically digested sludge has similar emissions independent of WWTP.

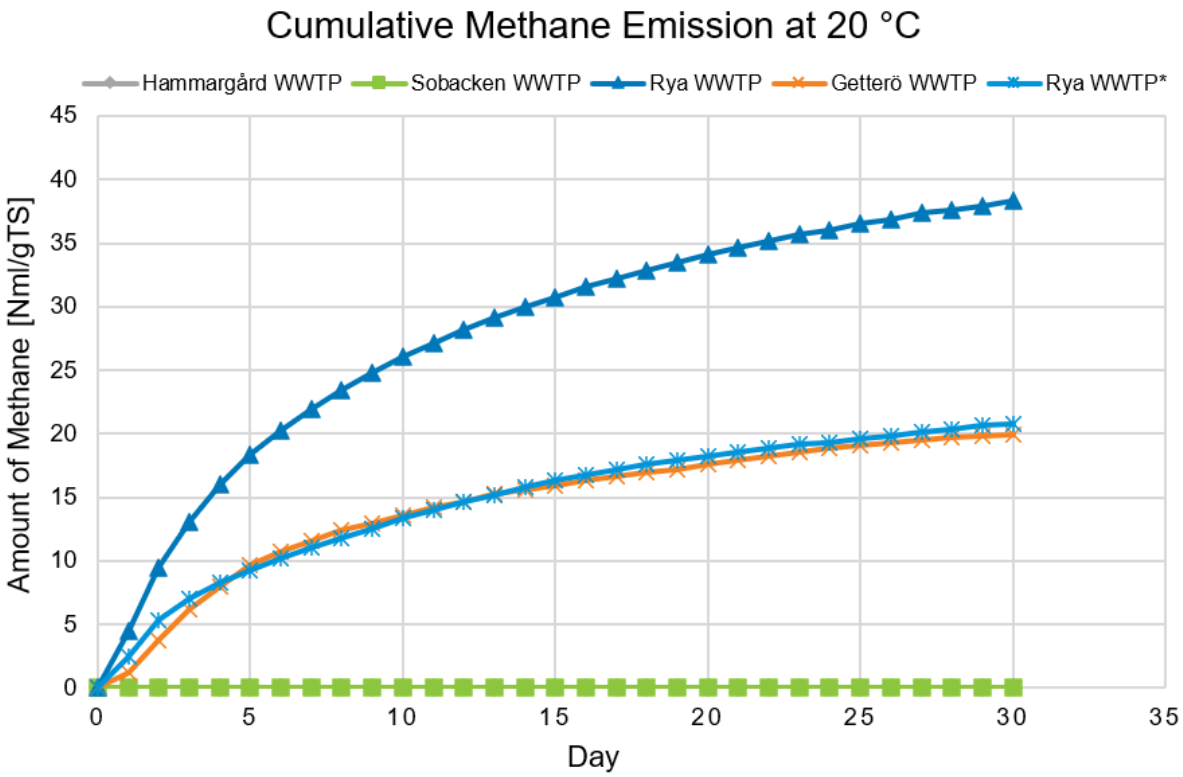

Figure 1. Cumulative amount of methane emission [Nml/gTS] at 20 °C during the measurement period of 30 days from finished treated sludge collected from Hammargård (grey), Sobacken (green), Rya WWTP (dark and light blue) and Getterö WWTP (orange). *Data form previously performed measurements of methane emission at 20 °C from sludge collected at Rya WWTP when both digestion chambers were running.

Figure 2 shows the cumulative methane emission at 35 °C. At this temperature, the gas flow meter recorded methane emission from all sludge treated with digestion, both in mesophilic and thermophilic conditions, that is from Rya, Getterö and Sobacken WWTP. In accordance with the measurement at 20 °C, the limed, undigested sludge from Hammargård WWTP had no recorded methane emission. The thermophilically digested sludge showed the lowest emission of these three samples, approximately a third as large as the emission from the sludge digested in mesophilic conditions.

At 35 °C, the sludge collected at Rya and Getterö had similar emission. The previous measurements at Rya WWTP using sludge collected when both digestion chambers were running was only performed at 20 °C. To enable comparison between the sludge samples from different WWTPs at 35 °C as well, a correction factor was calculated. The correction factor is based on the difference in emission between the sludge from Rya WWTP at 20 and 35 °C. Thus, it is assumed that the difference in emission for this previously collected data (Rya WWTP*) at 20 and 35 °C, is the same as for the measured difference in emission from the Rya WWTP sludge collected in this project.

The sludge from Getterö WWTP had a higher increase of methane emission when increasing the temperature to 35 °C compared to the sludge from Rya WWTP. This can also be seen when examining the data generated from the previous methane measurements at Rya WWTP during normal operating conditions (Rya WWTP*), since this hypothetical emission is lower.

However, the data regarding Rya WWTP* at 35 °C is based on the assumption that emission from the sludge at Rya WWTP would have the same temperature dependence at the two different measurement times and digestion operating conditions. Thus, to validate the result presented in Figure 2, new measurements at 35 °C could be performed on sludge collected at Rya WWTP when both digestion chambers are in use.

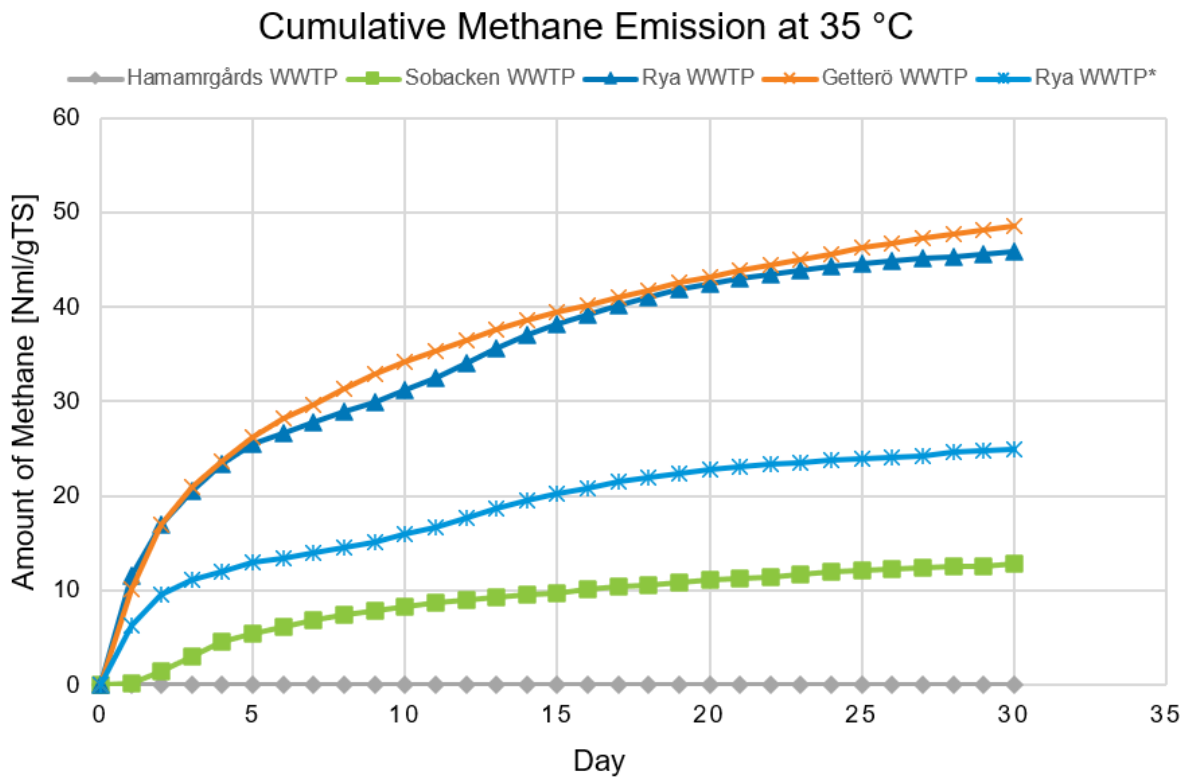

Figure 2. Cumulative amount of methane emission [Nml/gTS] at 35 °C during the measurement period of 30 days from finished treated sludge collected from Hammargård (grey), Sobacken (green), Rya WWTP (dark and light blue) and Getterö WWTP (orange). *Data based on previously performed measurements at 20 °C of methane emission from sludge collected at Rya WWTP when both digestion chambers were running, multiplied with a correction factor representing the difference between 20 and 35 °C.

As can be seen in Figure 2, the sludge from Rya and Getterö WWTP initially has approximately the same amount of methane emission in contrast to the measurements at 20 °C. After approximately 5 days, the emission from Rya WWTP became slightly lower, which can be seen as a dip in the curve in Figure 2. It is not known what could have caused this dip since any change in conditions that could affect the methane production would affect all samples, since they are placed close together in the same water bath. By observing the cumulative methane emission at 35 °C of all triplicates of each WWTP sample in Figure B.1 in Appendix B, it can be seen that this dip is observed in all triplicates of the sludge collected at Rya WWTP, indicating that the temporary decrease of methane emission is connected to the content of the sludge itself. In Appendix B, it can also be seen that while the triplicate samples collected from the same WWTPs follow each other closely in general, one of the replicates from Rya WWTP has lower emission compared to the remaining two samples from Rya WWTP. The sludge is not homogenous, and it is possible that this sample had a lower abundance of microbes compared to the remaining two or that there was a lower amount of organic material available.

It is also possible that this sample contained some type of contamination which acted inhibiting to the methane producing microorganisms.

As can be seen in Figure 1 and 2, the curves describing the cumulative methane emission flattens over time and the total amount of emitted methane is approaching a fixed value. This indicates that an estimation of the methane emission from long-term storage could be obtained by determining this fixed value through either theoretical or practical analyses.

Figure 3 and 4 shows the methane emission per day for the four WWTPs at 20 and 35 °C, respectively. The graphs clearly show how the emissions per day decreases with time, and that the emission is largest immediately following the sludge treatment. As previously mentioned, this implies that point measurements may provide an accurate estimation of long-term emissions.

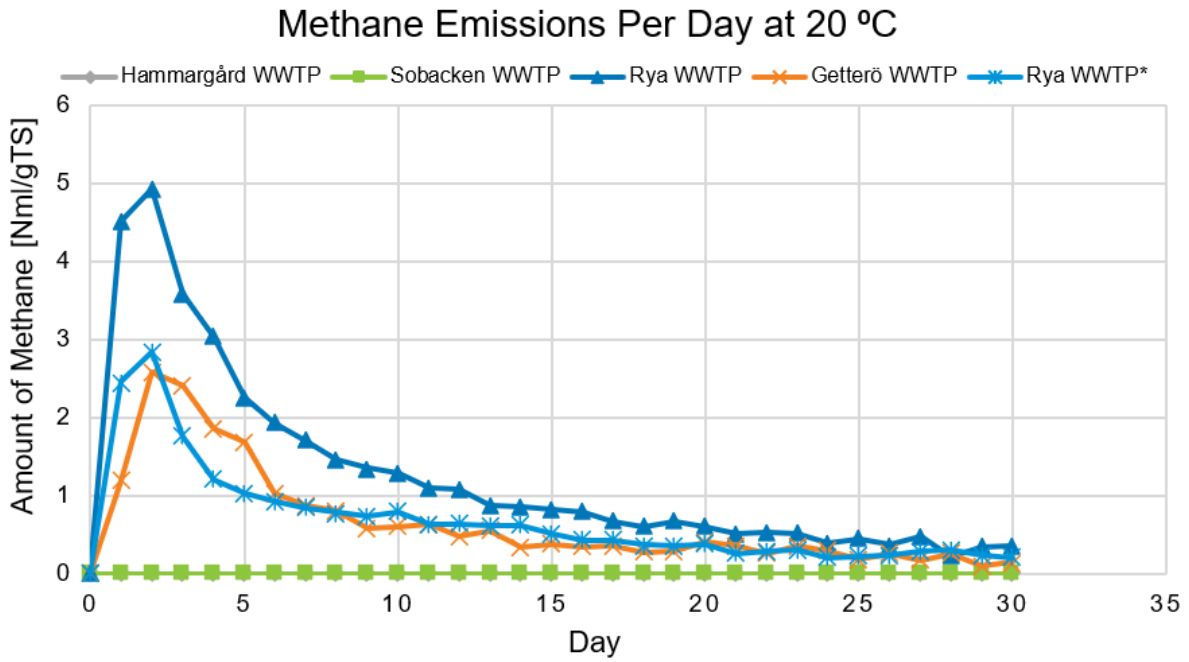

Figure 3. Methane emission per day at 20 °C [Nml/gTS] during 30 days from finished treated sludge collected at Hammargård (grey), Sobacken (green), Rya WWTP (dark and light blue) and Getterö WWTP (orange). *Data form previously performed measurements of methane emission at 20 °C from sludge collected at Rya WWTP when both digestion chambers were running.

In Figure 4 it can be seen that the thermophilically digested sludge from Sobacken WWTP require a longer amount of time to start emitting methane compared to the mesophilically digested sludge. This indicates that the microbial communities present in thermophilically digested sludge need time to adapt to the lower temperature. It is possible that some of the microorganisms which develop in the thermophilic conditions of the digestion chamber at Sobacken WWTP can still grow and produce methane at 35 °C, but 20 °C is too far from the temperature of which they prefer and thereby outside of their temperature range. The sludge might also contain microbes operating at lower temperatures, such as 35 °C, which have been in low abundance during digestion. Once adapted to the new, colder, environment these microbes would be able to grow more abundantly and start to produce more considerable amounts of methane.

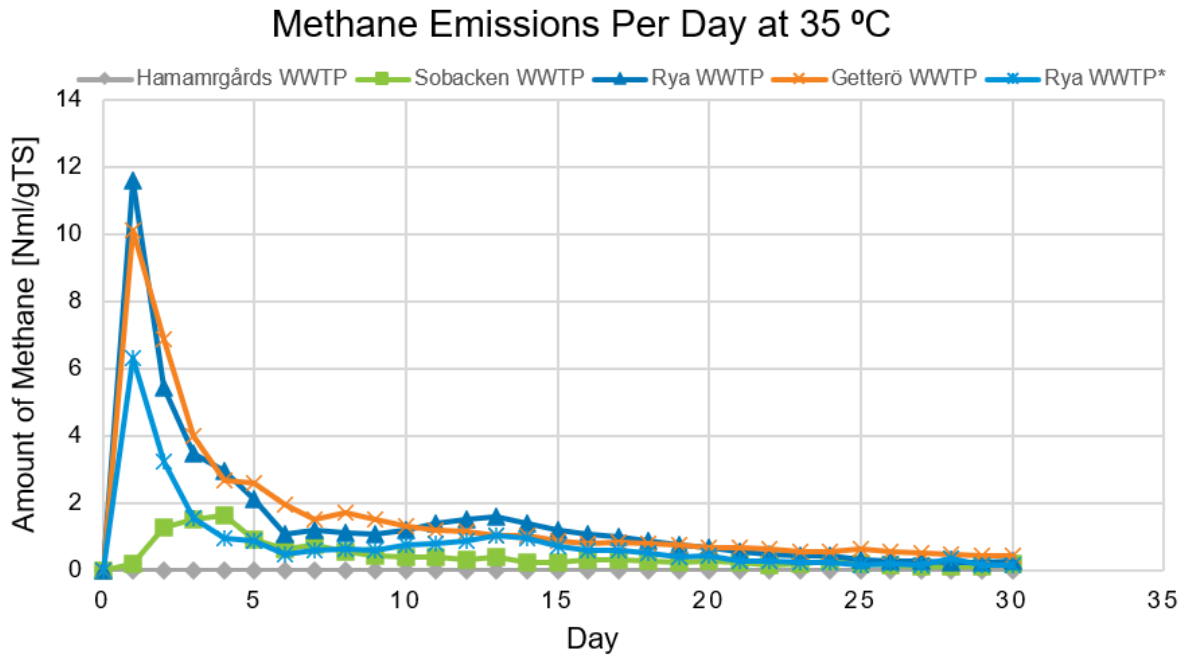

Figure 4. Methane emission per day at 35 °C [Nml/gTS] during 30 days from finished treated sludge collected at Hammargård (grey), Sobacken (green), Rya WWTP (dark and light blue) and Getterö WWTP (orange). *Data based on previously performed measurements at 20 °C of methane emission from sludge collected at Rya WWTP when both digestion chambers were running, multiplied with a correction factor representing the difference between 20 and 35 °C.

The total methane emission from each WWTP at 20 and 35 °C can be seen in Table 3. These values are the average total emission of the triplicates from each WWTP. The standard error is also included and was calculated from the standard deviation of the WWTP sample triplicates. As can be seen in Table 3, the standard error for Rya WWTP is higher compared to Getterö and Sobacken WWTP and this a consequence of the deviant sample mentioned above (see Table B.1 Appendix B).

Table 3. Total methane emission from the sludge samples collected at Hammargård, Sobacken, Rya and Getterö WWTP, as an average of triplicates. The included standard error is calculated from the standard deviation of the WWTP sample triplicates.

WWTP	Total methane emission [Nml/gTS]	
	At 20 °C	At 35 °C
Hammargård	0 ± 0	0 ± 0
Sobacken	0 ± 0	12.9 ± 0.31
Rya	38.52 ± 0.52	46.07 ± 2.01
Getterö	20.19 ± 0.30	49.02 ± 0.86
Rya*	20.83 ± 0.20	24.92 ± 0.20

*Data based on previously performed measurements at 20 °C of methane emission from sludge collected at Rya WWTP when both digestion chambers were running, multiplied with a correction factor representing the difference between 20 and 35 °C.

In Figure 5, the content of Table 3 is displayed as bar graphs. Hence, Figure 5 shows the average total methane emission from each WWTP at 20 and at 35 °C presented as bar graphs with included error bars based on the standard error.

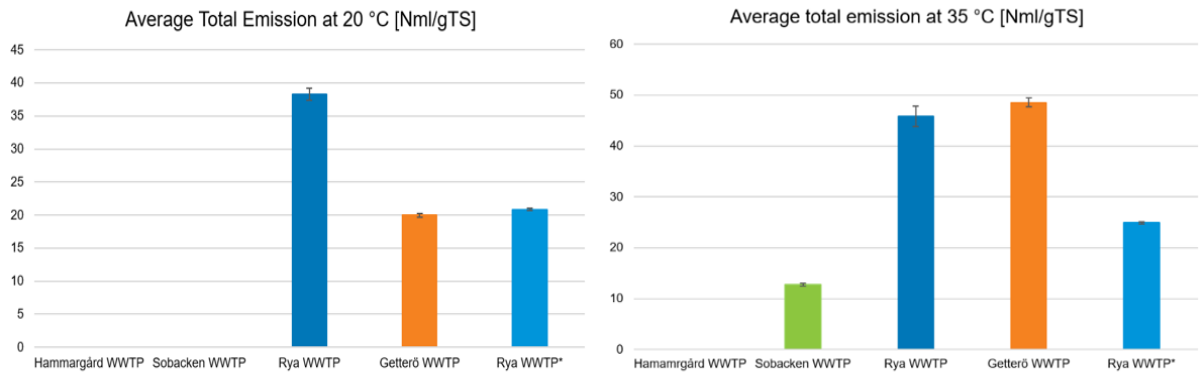

Figure 5. Average total methane emission from sludge collected at Hammargård (grey), Sobacken (green), Rya WWTP (dark and light blue) and Getterö WWTP (orange), including error bars based on the standard error over WWTP sample replicates. *Data based on previously performed measurements at 20 °C of methane emission from sludge collected at Rya WWTP when both digestion chambers were running, multiplied with a correction factor representing the difference between 20 and 35 °C.

The results presented in Table a and Figure 5 confirms that the measurement conditions, and thereby the sludge storage conditions, more closely resembles the mesophilic digestion conditions compared to the thermophilic. Thus, the microorganisms present in the mesophilically treated sludge is better adapted to the sludge storage conditions compared to the microorganisms from the thermophilically digested sludge and thereby able to grow and produce methane. All digested sludge showed increased methane emissions at higher temperature, indicating that the methane emission during sludge storage is dependent on the temperature of the sludge storage. Especially Getterö and Sobacken WWTP showed a considerable increase in methane emission at higher temperatures.

The absence of methane emission from the thermophilically digested sludge at 20 °C indicates that any methanogens present after digestion could not grow at this temperature. At 35 °C emission was recorded, but the emission was not as high as for the mesophilically treated sludge. This indicates that the thermophilic sludge contains microbes able to produce methane at temperatures ranging from 35 to 55 °C. As described in section *3.2 Sample Collection*, the sludge samples were kept in a cooler for 1.5 h prior to the measurement to keep the methane emission at a minimum until the measurement had been initiated. However, the sludge collected at Sobacken WWTP was kept at room temperature for an unknown amount of time (at maximum a couple of hours) before being put into the cooler due to logistic difficulties. Hence, it is possible that methane emission occurred during this time, causing a lower emission of methane during the measurement.

As previously mentioned, the limed, undigested sludge emitted no methane at neither 20 °C or 35 °C. This indicates that the lime and temperature increase used to hygienise the sludge is effective, and that there are no living microorganisms present able to produce methane.

As described above, the mesophilic sludge from Rya and Getterö WWTP had the highest amount of methane emission during the simulated sludge storage due to the measurement conditions more closely resembling mesophilic digestion conditions. There was however a considerable difference in amount of emission between the sludge from Rya and Getterö WWTP at 20 °C, but not at 35 °C. When comparing these measurements to the previously performed measurements at Rya WWTP, with sludge collected during normal operating

conditions (Rya WWTP*), this is likely due to the sludge from Rya WWTP having a lower degree of digestion. However, there are other factors that could contribute to a difference in emission. One factor could be a difference in microbial community composition where the sludge from Getterö WWTP contain less microorganisms adapted to lower temperatures. Other factors could be substrate composition (i.e. composition of incoming wastewater, proportion of household and food waste during digestion) or the set up and process of digestion, i.e. chambers in series at Rya WWTP compared to a master-slave system at Getterö WWTP. Naturally, factors like these also affect the thermophilically digested sludge and the mentioned factors would also affect each other. By including sludge samples from more WWTPs, in particular WWTPs using thermophilic digestion, the credibility of the methane emission data would be improved. A larger number of different WWTP sludge samples would give an indication to whether the amount of methane emission is primarily the result of different digestion temperatures or other differences, such as treatment process or substrate composition.

The results from the methane emission measurements indicates that thermophilic digestion could be preferable over mesophilic digestion with regards to methane emission during sludge storage. This is in accordance with thermophilic operating conditions being mentioned as a strategy is to obtain enhanced degradation rates and improve anaerobic digestion efficiency [3]. Another advantage of thermophilic digestion is that long-term storage is not required since the sludge is hygienised during digestion [27]. Thermophilic digestion requires more heat compared to mesophilic conditions, which could potentially increase the climate impact of the digestion. However, there is currently a surplus of heat in Gothenburg municipality [42] and if implementing thermophilic digestion at Rya WWTP, no heat would have to be originally produced, making this potential increase in climate impact negligible. Although there are additional aspects which need to be addressed when considering a transition from mesophilic digestion to thermophilic digestion. For example. the sludge needs to be digested in an enclosed container for 2-6 h in order to be hygienised [27]. Today at Rya WWTP, the sludge is continuously pumped into the digestion chamber and for the flow in and out of the digestion chamber to be intermittent, the process will have to be altered by for example the addition of a buffer tank. Thus, the environmental impact of such alterations would have to be weighed against the impact of lower methane emission during sludge storage. Additionally, previous studies have shown that thermophilically digested sludge has higher emissions of the GHG nitrous oxide (N_2O) compared to mesophilically digested sludge [43]. This suggests that there exists a trade-off between methane and N_2O emissions when comparing mesophilic and thermophilic digestion regarding emissions during sludge storage. Since N_2O is an even more potent GHG compared to methane [44], it could be more desirable to minimise these emissions. However, further investigations comparing N_2O and methane emissions, considering both potency and emitted gas volume, would have to be performed to examine whether thermophilic or mesophilic digestion would be the most environmentally advantageous option.

3.2.1 Benchmark Values for Methane Emission During Sludge Storage

Benchmark values for methane emission during sludge storage can be seen in Table 4. These values are based on the data collected during the methane emission measurements presented in Figure 1-5 and Table 3. To not overestimate the emissions from sludge treated with mesophilic AD, the previously performed measurements of methane emissions at Rya WWTP of sludge collected during normal operating conditions was used (Rya WWTP*). Since the time of sludge storage varies between different WWTPs, values for storage of sludge during 2, 7, 14 and 30 days were generated. The presented values in Table 4 are an average of the measurements at the two temperatures (20 and 35 °C) since the temperature of the sludge storage is estimated to be within this range. The values for mesophilic digestion conditions are also an average of the values for Rya* and Getterö WWTP.

Table 4. Benchmark values for methane emission during storage of sludge in 2, 7, 14 and 30 days for sludge treated through mesophilic and thermophilic digestion as well as liming.

Methane emission during sludge storage [Nml/gTS]				
Sludge treatment process	Time of sludge storage			
	2 days	7 days	14 days	30 days
Thermophilic digestion	0.7197	3.424	4.776	6.383
Mesophilic digestion	8.889	16.56	22.38	28.57
Liming	0	0	0	0

As indicated above, these values are estimations. In order for the benchmark values to be more accurate and reliable in future attempts to generate benchmark values, sludge samples form more WWTPs (especially WWTPs using thermophilic digestion) as well as data regarding conditions of different types of sludge storage, should be included. However, the use of calculation tools estimating the climate impact of any process will always be an approximation and the values presented in Table 4 are suitable to use until more reliable data becomes available. The ideal case would be for every WWTP in Sweden to record data of their individual sludge storage methane emissions, in order to estimate the climate impact of each WWTP as accurately as possible. However, until this is feasible, the use of benchmark values which estimates the methane emissions during sludge storage will be valuable. Using the benchmark values generated in this project when estimating the climate impact of WWTPs is likely to give a better estimation of a WWTPs climate impact compared to not using any data of methane emissions during sludge storage at all.

3.3 Acetate and Formate Content

The results from the HPLC investigating the content of the VFAs acetate and formate can be seen in Table 5. Acetate was found in all samples except the sample from thermophilic conditions (Sobacken WWTP). The sludge from Rya WWTP had approximately twice as high acetate content (mM/g sludge) compared to Getterö WWTP, and Hammargård WWTP had the highest content of acetate. Formate was only found in the sludge from Rya WWTP.

Table 5. Amount of acetate and formate [mM/g sludge] in the sludge samples Hammargård, Sobacken, Rya and Getterö WWTP. The presented value is an average of duplicates with included standard error, calculated from the standard deviation of the duplicates.

WWTP	Acetate content [mM/g sludge]	Formate content [mM/g sludge]
Hammargård	0.428 ± 0.0660	0 ± 0
Sobacken	0 ± 0	0 ± 0
Rya	0.335 ± 0.0287	0.262 ± 0.0256
Getterö	0.156 ± 0.0241	0 ± 0

The presence of acetate and formate in the sludge samples say something about what type of microorganisms that have dominated the sludge during its treatment, and the condition and resources available to the microorganisms when initiating the methane emission measurement. The presence of acetate in the sludge from Hammargård WWTP indicates that requirements and conditions for methane production exists, and that the absence of methene emissions is not likely a consequence of VFA shortage. However, a shortage of VFAs could be an explanation for the absence of methane emission of the sludge from Sobacken WWTP. Since neither acetate nor formate was present in the sludge, acetoclastic and hydrogenotrophic methanogens cannot produce methane until additional VFAs are formed. Assuming the formate and acetate content was similar in the sludge used in the measurement at 35 °C, it is possible that the microorganisms producing acetate and formate (e.g. acetogens) at Sobacken WWTP require thermophilic conditions, which would explain why there were some methane emissions at 35 °C but none at 20 °C.

As mentioned in section *2.2.3 Syntropic Acetogenesis*, the consumption of formate is essential during methane production through hydrogenotrophic methanogens, since formate is connected to the H_2 consumption and thus critical for the acetogenesis step of AD. The relatively high content of formate in the sludge form Rya WWTP could be explained by a lower abundance of hydrogenotrophic methanogens, and the microbial community in the sludge from Rya WWTP being dominated by acetoclastic methanogens. Another explanation could be that only one of the two digestion chambers at Rya WWTP was running when the sludge samples were collected, and it is possible that the VFA content would be different during normal circumstances.

As previously mentioned, a solution of 1 mM acetate was used as standard in the HPLC. The result showed that the standard solution had a concentration of 0.977 mM, which indicates that the detected concentrations are reliable. As previously mentioned, the sludge is not homogenous and there can be parts of sludge with higher and lower amounts of FAs. Additionally, the HPLC was only performed on the sludge used in the methane emission measurement at 20 °C and the result from investigating the acetate and formate content of the sludge would be more reliable if the sludge used for the measurement at 35 °C was analysed too. Continued investigations of FA content related to methane emission of sludge could include analysis of additional FAs, in particular propionate since a considerable proportion (30 %) of substrates are transformed via the propionate pathway during AD of municipal wastewater [28]. Continued investigations could also include measurements of the VFA content both prior to and after the methane emission measurements since this would give an indication

to what type of acetogenesis and methanogenesis processes that are taking place during sludge storage for the different types of sludge.

3.4 DNA Concentration

The final concentrations of DNA obtained in each WWTP sludge sample after performing DNA extraction can be seen in Table 6. The presented values are averages of the triplicate samples from each WWTP. The DNA concentration of the limed, undigested sludge from Hammargård WWTP was approximately 74 ng/µl in average, which was assessed to be too low to be able to sequence the DNA of this sample. The remaining samples was successfully sequenced.

Table 6. Average DNA concentration extracted from each sludge sample collected at Hammargård, Sobacken, Getterö and Rya WWTPs.

WWTP	Average DNA concentration [ng/µl]
Hammargård	< 0.1
Sobacken	41
Getterö	35
Rya	37

As previously mentioned, Hammargård WWTP uses liming to hygienise the sludge. The increase in pH caused by the lime kills microorganisms present in the sludge. The DNA of dead microorganisms can be extracted and sequenced using whole genome sequencing. However, the high pH leads to breakdown of the DNA [45]. This explains why the DNA extraction resulted in such low DNA concentrations for the limed sludge.

3.5 Analysis of Microbial Community Composition

The result of the metagenomic analysis includes the Permanova tests result, heatmaps representing differences in abundance of different microbial taxa, relative abundance of methanogens and other microbes involved in the AD process as well as quantification of the microbial diversity. The analysis of diversity includes alpha and beta diversity of diversity order 0 (presence or absence of OTUs) and 1 (OTUs weighted according to their relative abundance). The alpha diversity profile is presented by a graph showing the dependence of alpha diversity of the diversity order *q*, and the beta diversity is considered in a PCoA plot. As previously mentioned, due to SingleM does not managing paired end reads, each analysis generated two separated results: one for the forward sequences data and one for the reversed sequenced data. All results presented in this section are from the forward sequenced data, and the results from the reversed sequenced data can be seen in Appendix C-H.

3.5.1 Permanova test

The result of the Permanova test showed that there was a significant difference (p-value<0.05) in microbial composition between the two conditions (mesophilic and thermophilic) and

between the three WWTPs (Sobacken, Getter and Rya). The test was performed using dissimilarity matrices from the naive beta diversity calculations and both the diversity order of q=0 and q=1 was tested. Due to this, one test was made for each SingleM protein separately. The generated p-values when comparing mesophilic and thermophilic digestion conditions for each SingleM protein, for both q=0 and q=1 can be seen in Appendix C, Table C.1. The generated p-values when comparing Sobacken, Rya and Getterö WWTP for each SingleM protein, for both q=0 and q=1 can be seen in Appendix C, Table C.2.

3.5.2 Heatmaps

Figure 6 shows a heatmap of the 20 OTUs of highest relative abundance when examining SingleM protein 1. The X-axis represents the nine samples which were investigated in this project (three replicas each from Sobacken, Rya and Getterö WWTP) and the Y-axis presents OTUs with displayed taxonomic levels, if these could be identified by SingleM. Heatmaps for all proteins 1-14, can be seen in Figure D.1-D.28 in Appendix D.

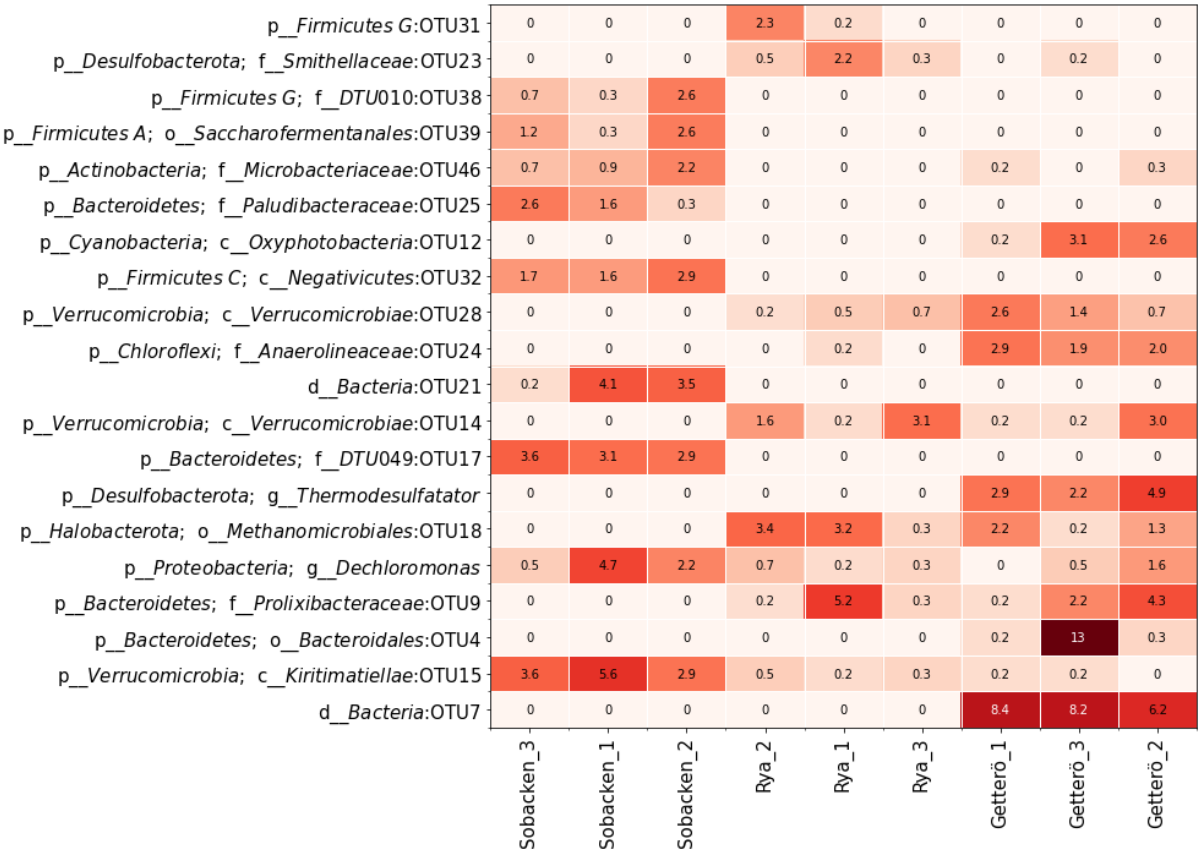

Figure 6. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 1. The X-axis shows the nine samples (three replicas each of Sobacken, Rya and Getterö WWTP) and the Y-axis presents identified OTUs with displayed taxonomic levels if these could be identified by SingleM.

In general, the heatmaps showed that the three triplicates from each WWTP sludge sample had similar taxonomic composition. Some OTUs were only identified in one of the WWTPs, some in two. In accordance with the PCoA plots presented below (see Figure 7 and Appendix H), the

two mesophilic sample triplicates more often had similar composition, i.e., high or low abundance of the same OTUs, whereas the thermophilic samples differed. Although, differences between the two mesophilic sludge samples are occurring too. This is also in accordance with the result of the Permanova tests described above and the PCoA plots described below.

When looking further into the OTUs identified in the heatmaps, several of them coincides with the context of AD of sewage sludge. For instance, OTUs included in the heatmaps represented microorganisms commonly found in soil, water or the human microbiota, for example the phyla *Verrucomicrobia* [46], *Acidobacteria* [47], *Firmicutes* and *Bacteriodetes* [48]. Belonging to the phylum *Acidobacteria* and is the class *Aminicentantia*, and species of this class have been found to be anaerobic digestors of organic matter [49]. The phylum *Synergistota* was also included in the heatmaps, and it has previously been found to be significant contributors in AD [50]. A class of *Firmicutes* included in several heatmaps was *Clorstridia*. This class is relevant to the AD process since many of its species produce FAs [51].

Another OTU included in the heatmaps which is known to participate in AD, is the family *Lachnospiraceae* of the order *Eubacteriales*. This family is known for fermenting polysaccharides into FAs such as butyrate and acetate [52]. *Burkholderiaceae* of the phylum *Pseudomonadota* was also included in several heatmaps and this family of bacteria are acetate assimilators [53]. Another occurring family was the *Anaerolineaceae* within the phyla *Chloroflexi* which has previously been shown to cooperate with *Methanotrichaceae* in AD pathways concerning acetate [54]. The genus *Acetomicrobium* of the phyla *Synergisetes* was also identified in the heatmaps as present in the samples from Sobacken WWTP. It has previously been isolated from thermophilic AD of sludge [55] and has also been found to ferment glucose via formation of acetate, CO_2 and H_2 [56]. Additionally, the family *Dethiobacteraceae* of the phyla *Firmicutes* was found among the OTUs of the heatmaps, as present in the thermophilic samples from Sobacken WWTP. *Dethiobacteraceae* has previously been found to perform SAO and replacing acetoclastic methanogenesis in thermophilic digestion [57].

3.5.3 Relative Abundance of Methanogens

The values presented in Table 6-11 are the average of the triplicates from each WWTP, and the standard errors were calculated from the standard deviation within each triplicate. Counts from all 14 SingleM proteins were included when calculating the percentage. As with the investigation of FA content, the DNA extraction and sequencing was only performed on sludge collected for the 20 ºC measurement, and the microbial community composition is assumed to be similar in the sludge used for the measurement at 35 °C. However, the sludge is not homogenous and there might be differences depending on time of sampling. The results would be more reliable if more samples over a longer time period had been included, and any further investigations could advantageously include this.

Table 6 shows the relative abundance of methanogens in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard errors. Table E.12 in Appendix E shows the corresponding results from the reversed sequenced data. As can be seen in Table 6, Rya WWTP had the highest relative abundance of methanogens, followed by Getterö WWTP. Sobacken WWTP had the lowest relative abundance of methanogens. Thus, the relative abundance of methanogens in each WWTP's sludge sample match the results of the methane emission measurements at 20 °C presented in Table 3, where Rya WWTP had the highest methane emission followed by Getterö WWTP and then Sobacken WWTP which had no emission at this temperature. Sobacken WWTP also had the lowest emission at the 35 °C measurement. Thus, the results indicates that the relative abundance of methanogens influences the amount of emitted methane during sludge storage. To increase the reliability of this result, metagenomic analysis of sludge used in methane emission measurements at both 20 and 35 °C, as well as of sludge collected during normal operating conditions at Rya WWTP would have to be performed. Thus, more investigations are needed in order to establish any definitive relation between the amount of emitted methane and the relative abundance of methanogens.

Table 6. Average relative abundance of methanogens over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of methanogens [%]
Sobacken	2.74 ±1.56
Rya	6.13 ± 0.19
Getterö	4.79 ±0.17

Methanogens from each of the three phylum *Euryarchaeota*, *Halobacteriora* and *Thermoplasmatota* was identified and among the these were hydrogenotrophic, acetoclastic and methylotrophic methanogens. Only one genus of methylotrophic methanogens was identified in among the samples, the *Methanomethylovorans*. This genus of methanogens was in low abundance (< 0.05 %, see Table E.1 and E.9 in Appendix E) compared to the identified hydrogenotrophic and acetoclastic methanogens. This is consistent with methylotrophic methanogens only accounting for a small amount of the produced methane in a standard municipal AD [28]. Among hydrogenotrophic methanogens several genera were identified, including *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus* and *Methanothermobacter*. Several OTUs of the family *Methanobacteriaceae* was identified as well, but no genus was identified within this family. Among the acetoclastic methanogens the *Methanotrichaceae,* was identified. Additionally, several OTUs of the family *Methanosarcina* was identified. As previously mentioned, the *Methanosarcina* are facultative acetoclastic methanogen and can in addition to acetate, utilise H_2 and CO_2 for methane production [28]. Other identified methanogens were of the family *Methanoregulaceae.* This family uses H_2 and CO_2 for methane production but requires acetate for growth [58]. Neither *Methanosarcina* nor *Methanoregulaceae* were included in the hydrogenotrophic or acetoclastic relative abundances presented in Table 7 and 8, but the relative abundances of these can be seen in Table E.2 and E.3 as well as E.10 and E.11 in Appendix E.

Table 7 shows the relative abundance of the hydrogenotrophic methanogens mentioned above, including standard errors. Table E.13 in Appendix E shows the corresponding results from the reversed sequenced data. Based on the values presented in Table 7, Sobacken WWTP has the highest relative abundance of hydrogenotrophic methanogens and Rya WWTP has the lowest. *Methanothermobacter* is a thermophilic hydrogenotrophic methanogen [59], and this was found to have a higher relative abundance at Sobacken WWTP compared to Rya and Getterö (see Table E.4 and E.14 in Appendix E), which is in accordance with the thermophilic conditions during AD at Sobacken WWTP. Thus, the relative abundance of *Methanothermobacter* in the sludge from Sobacken WWTP could contribute to the higher relative abundance of hydrogenotrophic methanogens in these samples compared to the samples from Rya and Getterö WWTP.

Table 7. Average relative abundance of hydrogenotrophic methanogens over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of hydrogenotrophic methanogens [%]
Sobacken	0.32 ± 0.13
Rya	0.18 ± 0.03
Getterö	0.23 ±0.007

As previously mentioned, formate is connected to the H_2 consumption and essential for hydrogenotrophic methanogens. Thus, an absence of formate can be an indication that all formate has been consumed during the AD process by hydrogenotrophic methanogens and a presence of formate could indicate low hydrogenotrophic activity. The only WWTP which contained formate was Rya WWTP (see Table 5), and as can be seen in Table 7, this is also the WWTP with the lowest relative abundance of hydrogenotrophic methanogens.

The relative abundance of acetoclastic methanogens in each WWTP sludge sample can be seen in Table 8, including standard errors. Table E.15 in Appendix E shows the corresponding results from the reversed sequenced data. Rya WWTP has the highest relative abundance, followed by Getterö WWTP, and Sobacken WWTP has a low relative abundance of acetoclastic methanogens in comparison. As previously mentioned, approximately 70 % of the methane produced in a standard municipal AD is in general through conversion of acetate [28]. In the mesophilically digested sludge, this is consistent with the relative abundance of acetoclastic methanogens being higher compared to the hydrogenotrophic methanogens.

Table 8. Average relative abundance of acetoclastic methanogens over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of acetoclastic methanogens (*Methanotrichaceae)* [%]
Sobacken	0.21 ±0.021
Rya	2.13 ± 0.29
Getterö	2.07 ±0.30

As previously mentioned, *Methanotrichaceae* can perform direct interspecies H_2/formate transfer which can improve the AD efficiency [28], and the higher abundance of *Methanotrichaceae* could be a contributing factor to the higher methane emissions from the mesophilically digested sludge.

The relative abundance of *Methanotrichaceae* was higher compared to *Methanosarcina* (see Table 8 as well as E.8, E.10 and E.15) which is in accordance with low acetate concentrations, since *Methanotrichaceae* has a high affinity for acetate, and it often dominates over *Methanosarcina* at low acetate concentrations [28]. The high relative abundance of acetoclastic methanogens in combination with the presence of acetate in the sludge from Rya and Getterö WWTP (see Table 5 and 8) presents good condition for methane production and is an explanation of the higher methane emissions from the mesophilically treated sludge compared to the thermophilically treated sludge. In the same way, the absence of formate in combination with the higher relative abundance of hydrogenotrophic methanogens in the sludge form Sobacken WWTP could explain the lower emission from this sludge, both at 20 and 35 ºC.

3.5.4 Relative Abundance of Acetogens and Syntrophic-Oxidising Bacteria

The relative abundance of other microbes involved in other stages of the AD process, such as hydrolysis, acidogenesis and acetogenesis, were investigated as well. Among the phyla mentioned in section *2.2 Anaerobic Digestion*, the phyla *Bacteroidetes, Firmicutes, Chloroflexi* and *Proteobacteria* was identified. The relative abundance of these phyla in each WWTP sludge sample triplicate were investigated, see Table E.5-E.8 and E.16-E.19 in Appendix E. Additionally, the genus *Syntrophus*, the order *Syntrophobacterales* belonging to the class *Syntrophobacteria* and the family *Smithellaceae* to which the genus *Smithella* belongs was identified. *Smithella* and *Syntrophobacter* is of particular interest since these are involved in acetogenesis, specifically propionate degradation. As previously mentioned, syntrophic acetogenesis is often the rate limiting step in AD processes and approximately 30% of the substrates can be transformed via the propionate pathway during typical AD of municipal wastewater [28].

The relative abundance of *Smithellaceae* and *Syntrophobacterales* in the different WWTP sludge samples can be seen in Table 9. Table E.20 in Appendix E shows the corresponding results from the reversed sequenced data.

Table 9. Average relative abundance of acetogens involved in propionate degradation over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Smithellaceae* [%]	Relative abundance of *Syntrophobacterales* [%]
Sobacken	0.054 ±0.032	0.0040 ±0.020
Rya	1.37 ± 0.15	0.055 ± 0.024
Getterö	2.63±0.18	0.10±0.017

A considerable difference in relative abundance of *Smithellaceae* and *Syntrophobacterales* can be seen in Table 9 between the mesophilically and thermophilically digested sludge. This could be an indication that the digestion at Sobacken WWTP contains substrates with less long FA intermediate compounds such as propionate, and therefore do not need to go through syntropic acetogenesis, at least not through the propionate pathway. It is also possible that these organisms are sensitive to the elevated temperatures of the thermophilic conditions.

The genus *Syntrophus* is also involved in acetogenesis, but in the oxidation of butyrate and other FAs. The sludge from Sobacken WWTP had the lowest relative abundance of this genus too, see Table 10. Table E.21 in Appendix E shows the corresponding results from the reversed sequenced data.

Table 10. Average relative abundance of acetogenic genus *Syntrophus* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Syntrophus* [%]
Sobacken	0 ±0
Rya	0.023 ± 0.013
Getterö	0.082±0.016

Regarding the presence of SAO bacteria, none of the species mentioned in section *2.2.5 Syntrophic Acetate-Oxidising Bacteria* was identified. However, several OTUs of the order *Thermoanaerobacterales* was identified, and these could include the SAO species *Thermacetogenium phaeum* since it belongs to this order. As previously mentioned, the SAO bacteria family *Dethiobacteraceae* was also found. Table 11 shows the relative abundance of *Thermoanaerobacterales* and *Dethiobacteraceae* in the different WWTP sludge samples. As can be seen in Table 11, the sludge from Sobacken WWTP has a higher relative abundance of SAO bacteria. When examining the reversed sequenced data, the higher relative abundance of *Thermoanaerobacterales* and *Dethiobacteraceae* in the sludge from Sobacken WWTP was more prominent, see Table E.22 in Appendix E.

Table 11. Average relative abundance of SAO bacteria over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Thermoanaerobacterales* [%]	Relative abundance of *Dethiobacteraceae* [%]
Sobacken	0.051 ± 0.016	0.080 ± 0.030
Rya	0.031 ± 0.010	0 ± 0
Getterö	0.0061± 0.0061	0± 0

A higher relative abundance of SAO bacteria in the sludge from Sobacken WWTP could be related to the low relative abundance of acetoclastic methanogens in this sludge, since the SAO bacteria are able to convert acetate into H_2 and CO_2, which in turn is converted to methane by hydrogenotrophic methanogens [28]. This is in accordance with previous mentioned studies where it been shown that the methane formation progress mainly through acetate oxidation coupled to hydrogenotrophic methanogenesis at thermophilic digestion [3]. Additionally, the order of *Thermoanaerobacterales* is in general adapted to elevated temperatures [60] and might

therefor be more adapted to the thermophilic environment at Sobacken WWTP compared to acetoclastic methanogens. Furthermore, *Dethiobacteraceae* has previously been found to replace acetoclastic methanogenesis in thermophilic digestion [57].

3.5.5 Quantification of Microbial Diversity

The result regarding quantification of naive alpha and beta diversity is presented in this section. The quantification of the naive alpha diversity includes Hills numbers and alpha diversity profile plots. The quantification of beta diversity is visualised in PCoA plots.

3.5.5.1 Naive Alpha Diversity

The average naive alpha diversity of the three replicas for each protein and WWTP can be seen in Table 12 with corresponding standard errors. The naive alpha diversity was calculated for diversity order q=0 and q=1. The standard errors were calculated from the standard deviation of all 14 SingleM proteins and the three samples of each WWTP. The average naive alpha diversity based on the revered sequenced data can be seen in Table F.1 in Appendix F.

As can be seen in Table 12, Rya WWTP had the highest diversity for q=0, thus the highest species richness, followed by Sobacken WWTP and lastly Getterö WWTP which had the lowest richness. Thus, the results show that Rya WWTP has the most species, Sobacken WWTP the second highest and Getterö WWTP the least number of species. The naive alpha diversity for q=1 follows the same pattern as q=0, meaning highest at Rya WWTP, and lowest at Getterö WWTP. Thus, Rya WWTP also had the most uniform distribution of species when each OTU is weighted exactly according to its relative abundance.

Table 12. Naive alpha diversity of the sludge samples from Sobacken, Rya and Getterö WWTP. The presented values are averages over all SingleM proteins and the three replicas of each WWTP and standard error is included.

WWTP	Naive alpha diversity	
	q=0	q=1
Sobacken	147.12 ± 2.51	107.62 ± 3.64
Rya	160.36 ± 2.73	125.48 ± 3.23
Getterö	136.14 ± 2.87	90.82 ± 3.01

Figure 7 shows the naive alpha diversity profile of SingleM protein 1. The plot shows how Hills diversity number ${}^{q}D$ depend on the diversity order q for the three WWTPs: Sobacken (blue), Rya (red) and Getterö (yellow). The diversity number decreases as the diversity order increases and more wight is given to the relative abundance of each OTU. Hence, there are certain species dominating the microbial communities. In accordance with the data in Table 12, Rya WWTP has the highest diversity followed by Sobacken and lastly Getterö. Alpha diversity plots for SingleM protein 1-14 can be seen in Figure G.1-G.28 in Appendix G.

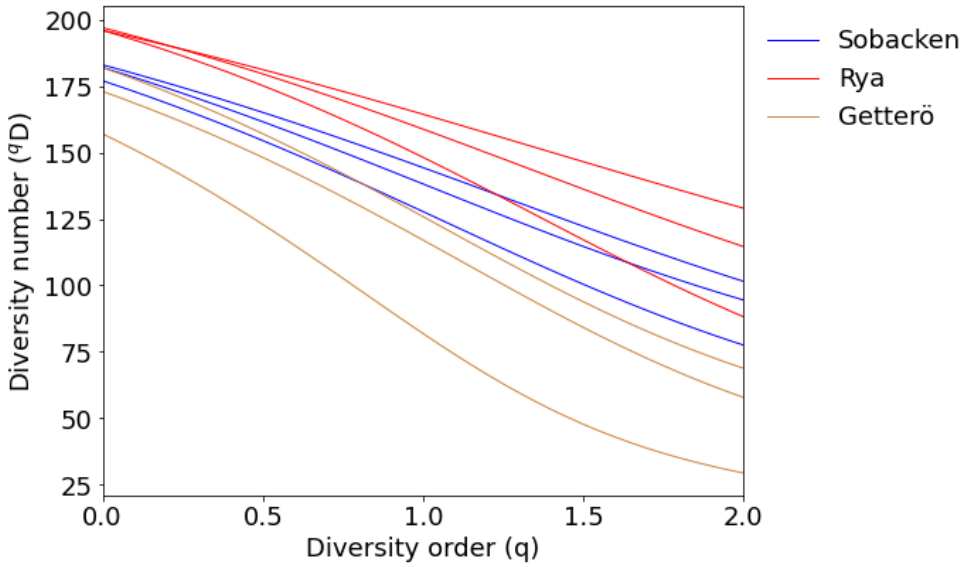

Figure 7. Naive alpha diversity profile for SingleM protein 1. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for the three WWTPs Sobacken (blue), Rya (red) and Getterö (yellow).

The collected sludge samples do not necessarily represent the true diversity of the microbial communities, since the sludge is not homogenous. To increase the credibility of the results, more samples could be collected and sequenced. Additionally, the phylogenetic and functional diversity could be examined to nuance the results and make any connections between diversity and methane emissions more reliable. Furthermore, it is possible that the microbial diversity of the sludge at Rya WWTP also is affected by only one of the digestion chambers at Rya WWTP running during the sample collection.

As previously mentioned, the microbial diversity of the sludge influences the methane production, and a higher diversity means that a function of the microbial community is more likely to sustain when exposed to perturbations. This is in accordance with the sludge from Rya WWTP being able to emit higher amounts of methane at lower temperatures compared to Getterö WWTP, indicating that Rya WWTP containing microorganisms more adapted to lower temperatures. When examining the methane emission from the previous measurements at Rya WWTP using sludge collected during normal operating conditions, the connection between a higher microbial diversity at Rya WWTP and higher methane emissions from sludge storage cannot be made. However, it cannot be assumed that the microbial diversity of the sludge at Rya WWTP is independent on the changed operating condition. When only using one digestion chamber, it is likely that the microbial diversity is higher. The incoming sludge will contain a variety of microbial communities, and with only one digestion chamber there is less time for the microbes to outcompete each other, and a few species are therefore less likely to dominate. This can be seen in the previously performed investigation of the microbial diversity at Rya WWTP by Alyona Tormachen Shabsai, where the first digestion chamber in the series had a higher alpha diversity compared to digestion chamber two and three [40].

To investigate the relationship between the microbial diversity and the amount of emitted methane further, investigations regarding the microbial diversity at Rya WWTP during normal operating conditions and at Getterö WWTP in combination with methane emission

measurements could be performed. If future investigations would show a relation between a higher microbial diversity and a lower methane emission during sludge storage, that could have other explanations. As mentioned in section *2.2.6.1 Factors Affecting the Microbial Community*, previous studies have shown that digestion with diverse microbial communities result in higher methane production, compared to those with lower diversity [28]. A higher methane production will result in a lower RMP which in turn is related to lower methane emission during sludge storage.

Previous research has also found that thermophilically digested sludge has less diverse microbial communities than mesophilically digested sludge [28]. This is the case in this study when comparing Rya and Sobacken WWTP, but it does not concur with the results regarding Getterö WWTP. To further investigate this matter, sludge from more WWTPs practising thermophilic and mesophilic digestion as a part of their sludge treatment would have to be analysed.

3.5.5.2 Naive Beta Diversity

Naive beta diversity dissimilarity matrices were obtained for all samples. The dissimilarity matrices were used to generate a PCoA plot for each SingleM protein, displaying the similarity in microbial community composition between the nine samples (three replicates of each of the three WWTPs). This was done for diversity order q=0 and q=1. Figure 8 shows the PCoA plot of q=0 for SingleM protein 1. All PCoA plots for protein 1-14 and diversity order q=0 and q=1 can be seen in Figure H.1-H.28 in Appendix H.

As can be seen in Figure 8, the microbial communities of the three replicates from each WWTP sludge sample resembles each other and forms separate clusters. This was the case for all PCoA plots, see Figure H.1-H.28 in Appendix H. However, the clusters were in general more strongly prominent in the graphs showing q=0 compared to q=1. This was expected since the replicates of each WWTP sample showed similar microbial composition in the heatmaps.

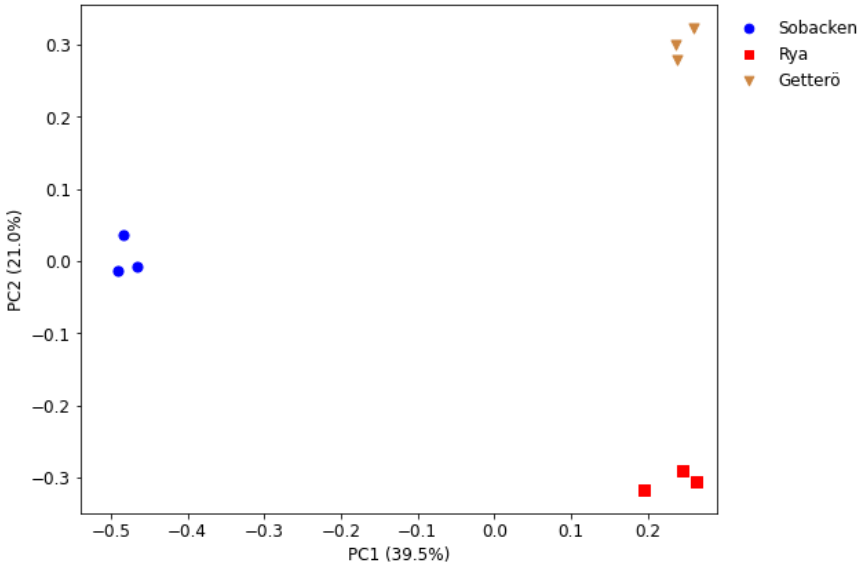

Figure 8. PCoA plot for SingleM protein 1 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order q=0.

In PCoA, the longest distance is explained by the X-axis, and in general for all SingleM proteins, the sludge samples from the two mesophilic WWTPs had similar positions in the direction of the X-axis. This indicates that the mesophilic sludge samples have greater similarity in microbial composition compared to the thermophilic one. This was expected since the microorganisms in the two mesophilically digested sludge samples would have to tolerate temperature within the same ranges. This is also in accordance with the generated heatmaps, see Figure 6 and Figure D.1-D.28 in Appendix D. The three clusters are at approximately the same distance of each other indicating a difference in the microbial community composition, which was also seen from the Permanova tests where it was found to be a significant difference in the microbial composition of the three WWTPs.

3.5.6 Microbial Community Composition in Relation to Methane Emissions

The results presented above shows a possible relation of a higher methane emission during sludge storage to a higher relative abundance of methanogens and a higher microbial diversity. However, a higher diversity and abundance of methanogens are also qualities contributing to a higher production of methane during digestion, which is something desirable. Thus, the results indicate the existence a trade-off between maximising the methane production during digestion and minimising the methane emission of the finished treated sludge.

As previously mentioned, an efficient AD process, low RMP and a high degree of digestion decreases methane emissions associated with storage of the digestate. Therefore, instead of targeting the methane emission from sludge storage by attempting to decrease the relative abundance of methanogens or the diversity of the microbial communities in the sludge, it would be more efficient to enhance the degree of digestion by targeting these areas. One possible option to achieve this could be through bioaugmentation of species involved in the AD process. Such species could for example be methanogens, acetogens, SAO bacteria or species able to perform direct interspecies H_2/formate transfer. This could both enhance the diversity and target the rate limiting steps of AD to make the digestion more efficient and thus decreasing the RMP. Microorganisms involved in hydrolysis or extracellular enzymes such as lipases, proteases, and cellulases could be added to increase the substrate availability of subsequent steps of AD. Another alternative could be to change the structure of the process, for example as previously mentioned by using thermophilic digestion conditions or a post digester.

Another strategy to minimise the methane emissions at sludge storage could be to extend the sludge treatment process to include treatment with a compound inhibiting further methane production. The results from this study showed that liming of sludge prevents methane emissions at both 20 and 35 °C and could therefore be an alternative. However, the climate impact of the lime would have to be taken into consideration. For example, it might not be possible to use the sludge for soil production in the same extent since limed sludge does not compost well. The climate impact of manufacturing of lime needs to be taken into consideration as well as any impact of the lime during fertilisation on agricultural land.

Other alternatives which are being explored is treatment of the sludge by addition of urea, natural cooling or coverage with organic material for increased methane oxidation [41]. If these treatments prove to be effective, the trade-off between maximising the methane production during digestion and minimising the methane emission of the finished treated sludge could be avoided.

5 Conclusion

The result of the methane emission measurement showed that the limed sludge had no methane emission at neither 20 nor 35 °C. The thermophilically digested sludge digested sludge emitted methane at 35 °C, but not at 20 °C. The sludge treated by mesophilic digestion emitted methane at both 20 and 35 °C and the emission increased at the higher temperature. At 35 °C the mesophilically digested sludge emitted higher amounts of methane compared to the thermophilically digested sludge. Since this study only included sludge sample from four WWTPs, further investigations of sludge from additional WWTPs are needed to confirm the findings of this study. However, the results generated in this project can be used as a foundation for further research within this area.

Benchmark values for methane emission during storage of sludge for 2, 7, 14 and 30 days, for sludge treated through mesophilic and thermophilic digestion as well as liming was successfully obtained and can be used in tools for estimating the climate impact of WWTPs in Sweden. In extension, the benchmark values could be a step on the way towards a net zero climate impact of Swedish WWTPs and reaching the goal set by Swedish Waters. The accuracy and reliability of the benchmark values could be further improved by including a larger number of WWTPs in future investigations. However, the use of calculation tools for estimating the climate impact of any process is an approximation in itself, and the benchmark values generated in this project are suitable to use until more reliable data becomes available.

The results from the metagenomic analysis showed that there was a significant difference (p-value<0.05) in microbial composition between the two conditions (mesophilic and thermophilic) and between the three WWTPs (Sobacken, Getterö and Rya). The results also showed that the samples from mesophilic digestion conditions had higher similarity to each other than to the samples from thermophilic digestion conditions.

The aim of the study was also to investigate whether there is any relation between the amount of emitted methane and the presence of microorganisms involved in anaerobic digestion and methanogenesis. It was found that a higher abundance of methanogens resulted in increased methane emissions. Additionally, the results showed that the type of methanogens varied depending on digestion condition. A higher relative abundance of hydrogenotrophic and acetoclastic methanogens was found in thermophilically and mesophilically digested sludge receptively. The results also indicated that sludge containing more diverse microbial communities are able to produce methane more efficiently at lower temperatures. Since targeting the diversity or abundance of methanogens when attempting to decrease methane emission during sludge storage would negatively affect the biogas production efficiency, other alternatives to minimise methane emission from sludge storage should be explored. For example, the use of thermophilic digestion instead of mesophilic, optimising the biogas production during digestion to lower the RMP, or treatment of the sludge after digestion in order to inhibit further production of methane.

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Appendix A

Table A.1. shows the ribosomal proteins corresponding to the SingleM proteins annotated protein 1-14 in this project.

Table A.1. Proteins corresponding to SingleM proteins 1-14.

Protein number	SingleM Ribosomal protein
Protein 1	Ribosomal protein L2 rplB
Protein 2	Ribosomal protein L3 rplC
Protein 3	Ribosomal protein L5 rplE
Protein 4	Ribosomal protein L6 rplF
Protein 5	Ribosomal protein L11 rplK
Protein 6	Ribosomal protein L14b L23e rplN
Protein 7	Ribosomal protein L16 L10E rplP
Protein 8	Ribosomal protein S2 rpsB
Protein 9	Ribosomal protein S5
Protein 10	Ribosomal protein S7
Protein 11	Ribosomal protein S10 rpsJ
Protein 12	Ribosomal protein S12 S23
Protein 13	Ribosomal protein S15P S13e
Protein 14	Ribosomal protein S19 rps S

Appendix B

Figure B.1 shows the cumulative methane emission recorded at 35 °C for all sludge samples, i.e. the triplicates of each WWTP.

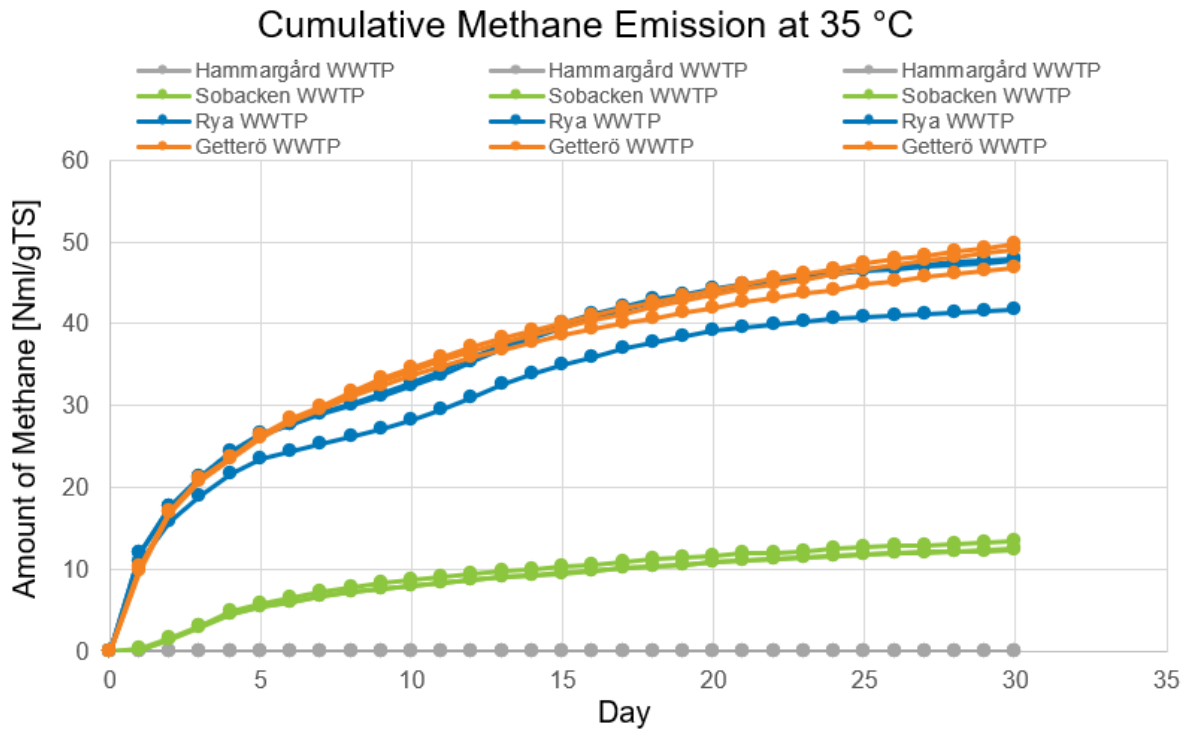

Figure B.1. Cumulative methane emission [Nml/gTS] at 35 °C during the measurement period of 30 days from triplicate samples from finished treated sludge collected from Hammargård (grey), Sobacken (green), Rya WWTP (blue) and Getterö WWTP (orange).

Appendix C

Table C.1 shows the p-values from the Permanova tests of each SingleM protein and for diversity order q=0 and q=1, when evaluating whether there was a significant difference between the microbial community composition of thermophilically and mesophilically digested sludge, for both the forward sequenced data and the reversed sequenced data.

Table C.1. Resulting p-values from the Permanova tests with 1000 permutations comparing mesophilic and thermophilic digestion conditions for each SingleM protein. The p-values of the forward and reversed sequenced data and the diversity order q=0 and q=1 is shown.

SingelM protein	p-values			
	Forward sequenced data		Reversed sequenced data	
	q=0	q=1	q=0	q=1
Protein 1	0.010989	0.016983	0.014985	0.009990
Protein 2	0.008991	0.010989	0.011988	0.004995
Protein 3	0.016983	0.003996	0.017982	0.005994
Protein 4	0.014985	0.011988	0.006993	0.016983
Protein 5	0.011988	0.011988	0.008991	0.010989
Protein 6	0.000999	0.005994	0.012987	0.011988
Protein 7	0.011988	0.014985	0.006993	0.010989
Protein 8	0.008991	0.009990	0.010989	0.012987
Protein 9	0.006993	0.008991	0.011988	0.003996
Protein 10	0.013986	0.008991	0.015984	0.017982
Protein 11	0.007992	0.010989	0.004995	0.007992
Protein 12	0.013986	0.010989	0.005994	0.016983
Protein 13	0.006993	0.011988	0.011988	0.003996
Protein 14	0.009990	0.011988	0.005994	0.002997

Table C.2 shows the p-values from the Permanova tests of each SingleM protein and for diversity order q=0 and q=1, when evaluating whether there was a significant difference between sludge collected at the three WWTPs (Rya, Sobacken and Getterö WWTP), for both the forward sequenced data and the reversed sequenced data.

Table C2. Resulting p-values from the Permanova tests with 1000 permutations comparing Sobacken, Rya and Getterö WWTP for each SingleM protein. The p-values of the forward and reversed sequenced data and the diversity order q=0 and q=1 is shown.

SingelM protein	p-values			
	Forward sequenced data		Reversed sequenced data	
	q=0	q=1	q=0	q=1
Protein 1	0.003996	0.006993	0.006993	0.000999
Protein 2	0.004995	0.000999	0.005994	0.003996
Protein 3	0.003996	0.003996	0.004995	0.000999
Protein 4	0.004995	0.003996	0.002997	0.004995
Protein 5	0.005994	0.003996	0.002997	0.002997
Protein 6	0.004995	0.003996	0.001998	0.004995
Protein 7	0.002997	0.003996	0.004995	0.008991
Protein 8	0.003996	0.001998	0.000999	0.003996
Protein 9	0.006993	0.001998	0.003996	0.002997
Protein 10	0.007992	0.003996	0.004995	0.003996
Protein 11	0.004995	0.002997	0.002997	0.002997
Protein 12	0.007992	0.004995	0.003996	0.004995
Protein 13	0.005994	0.004995	0.002997	0.004995
Protein 14	0.002997	0.006993	0.005994	0.004995

Appendix D

Appendix D contains all heatmaps generated in this study, for SingleM proteins 1-14 for both the forward and reversed sequenced data. Figure D.1-D.14 shows the heatmaps generated from the forward sequenced data for SingleM protein 1-14 respectively, and Figure D.15-D.28 shows the heatmaps generated from the reversed sequenced data for SingleM protein 1-14 respectively.

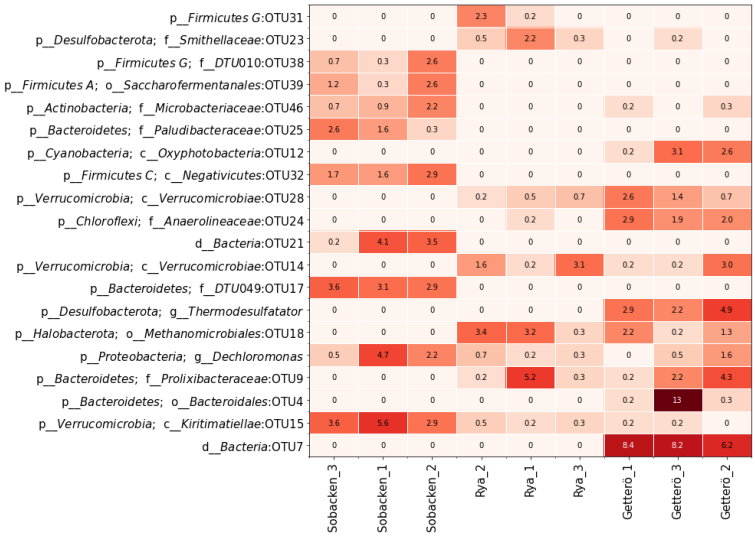

Figure D.1. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 1.

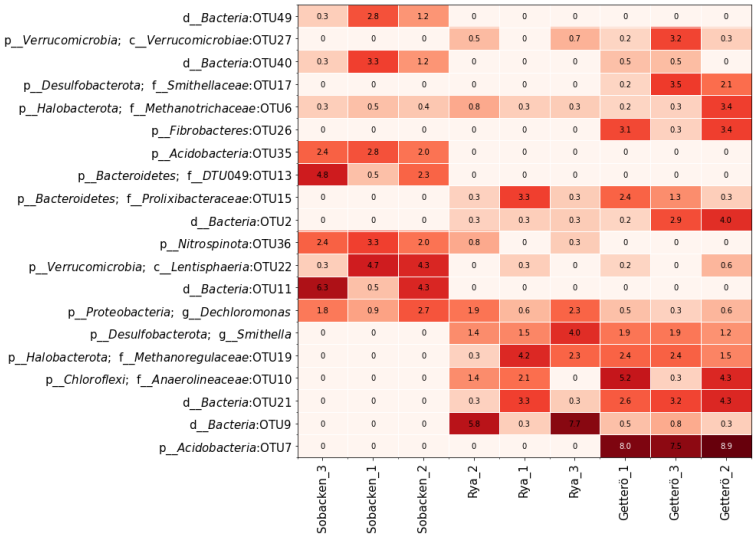

Figure D.2. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 2.

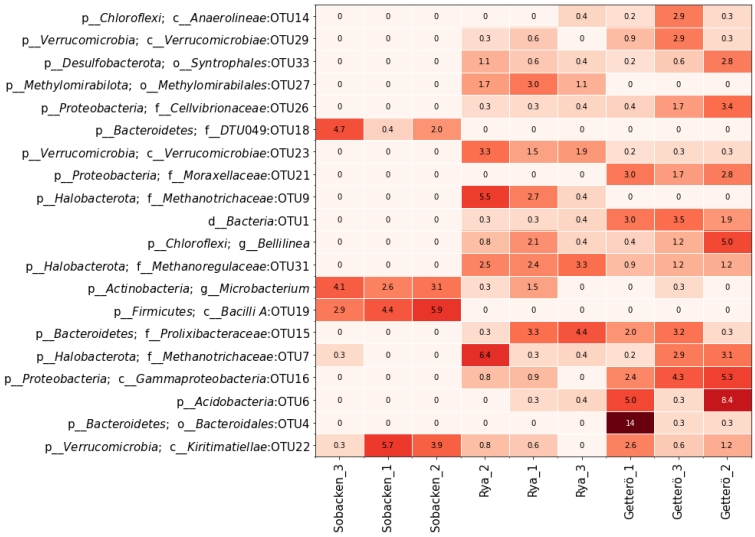

Figure D.3. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 3.

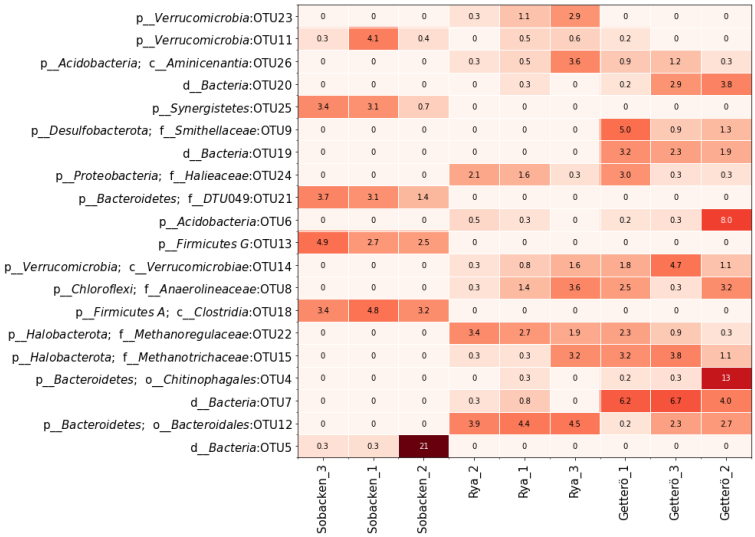

Figure D.4. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 4.

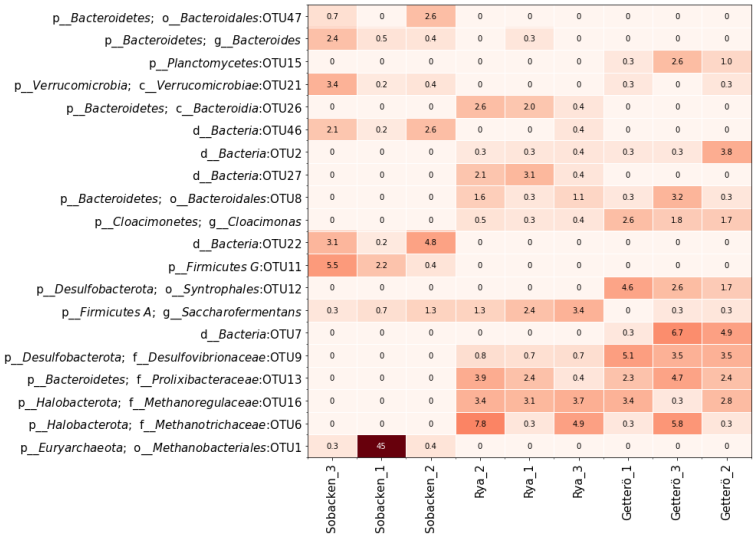

Figure D.5. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 5.

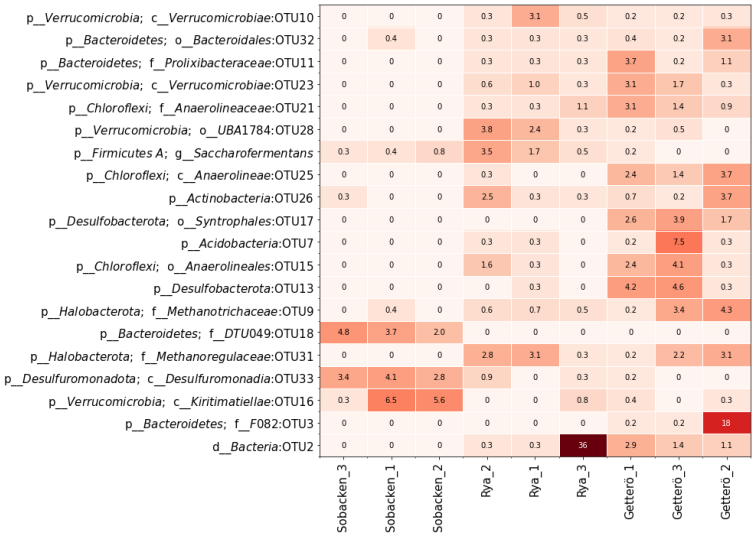

Figure D.6. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 6.

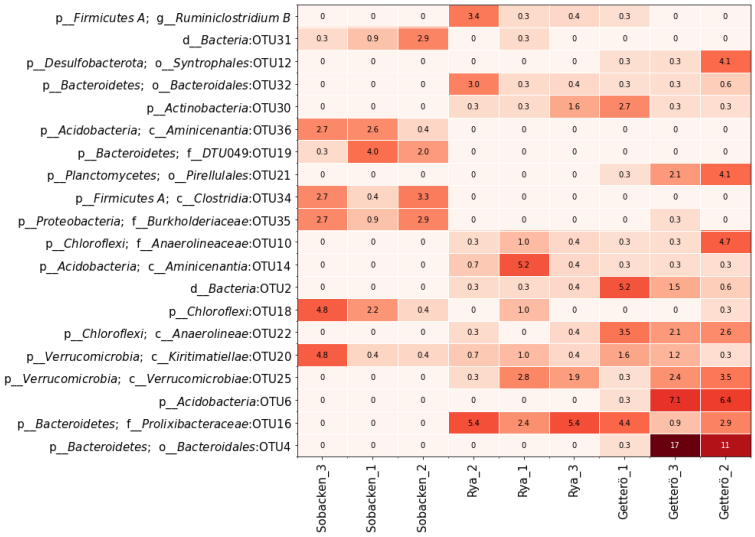

Figure D.7. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 7.

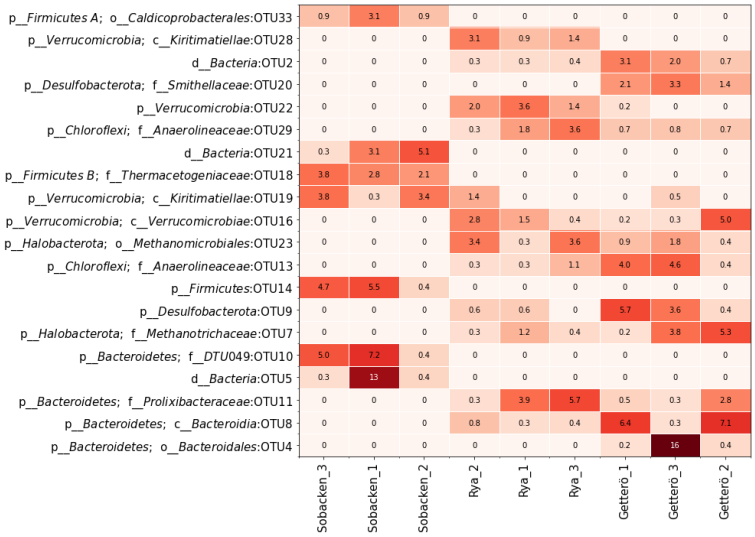

Figure D.8. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 8.

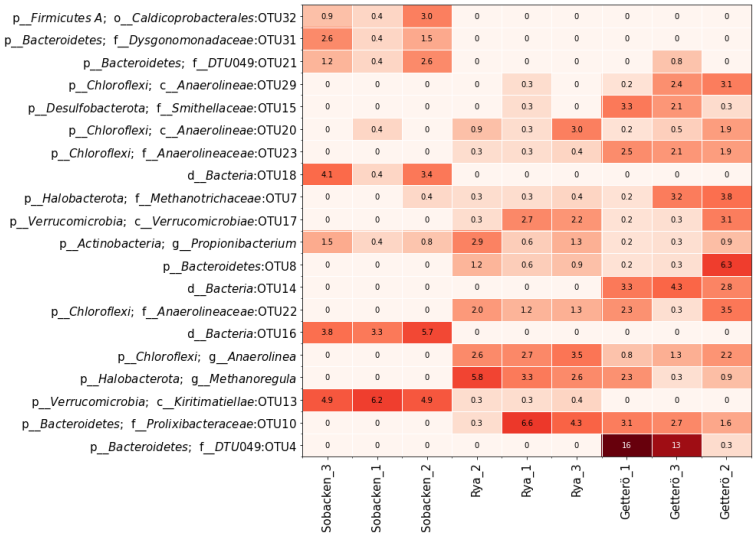

Figure D.9. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 9.

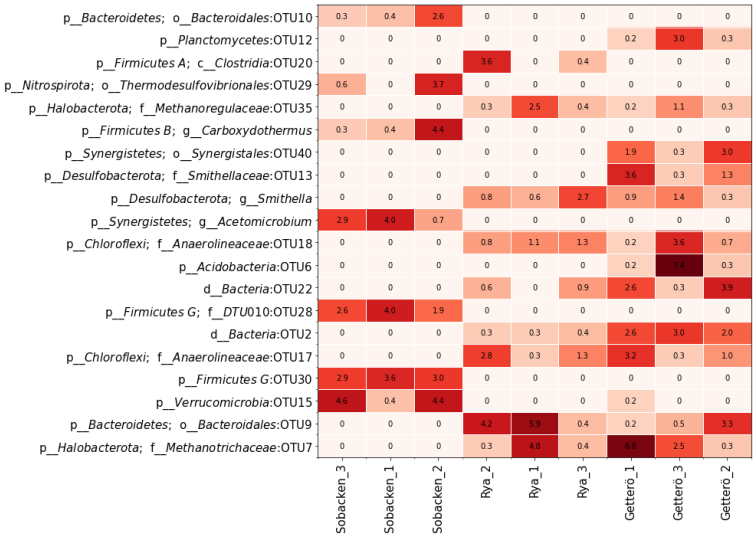

Figure D.10. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 10.

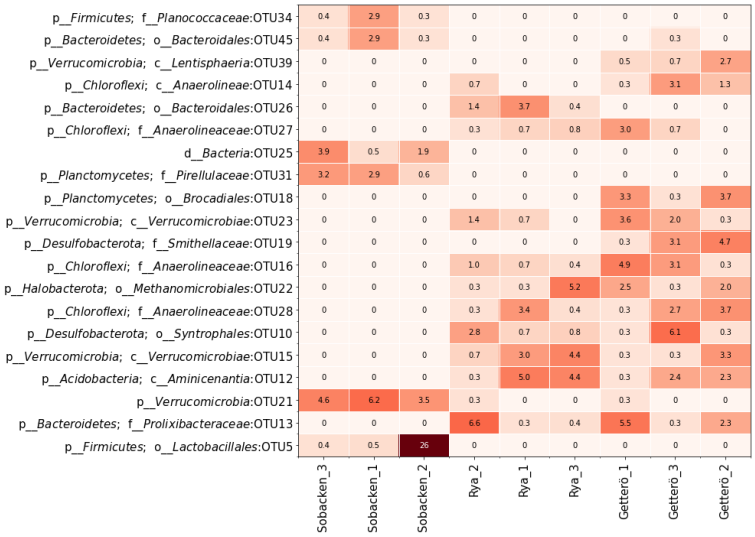

Figure D.11. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 11.

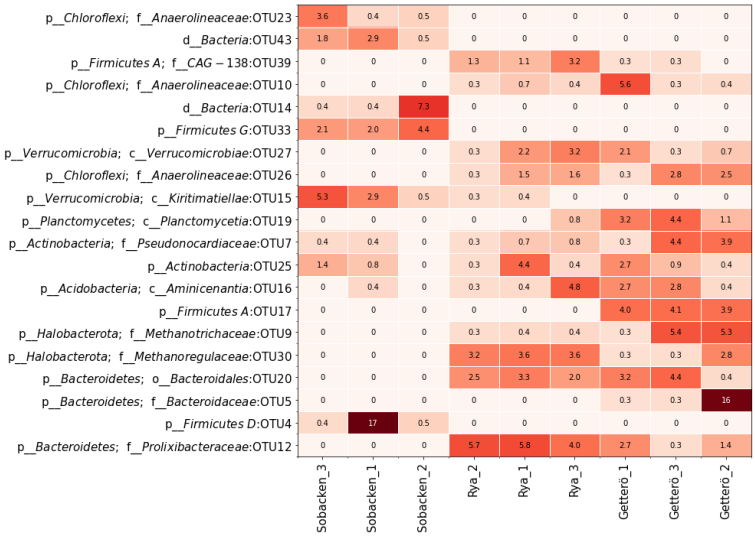

Figure D.12. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 12.

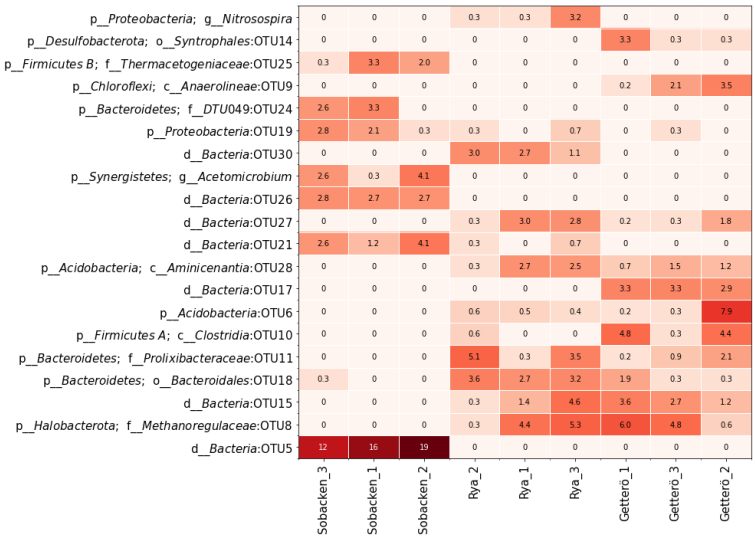

Figure D.13. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 13.

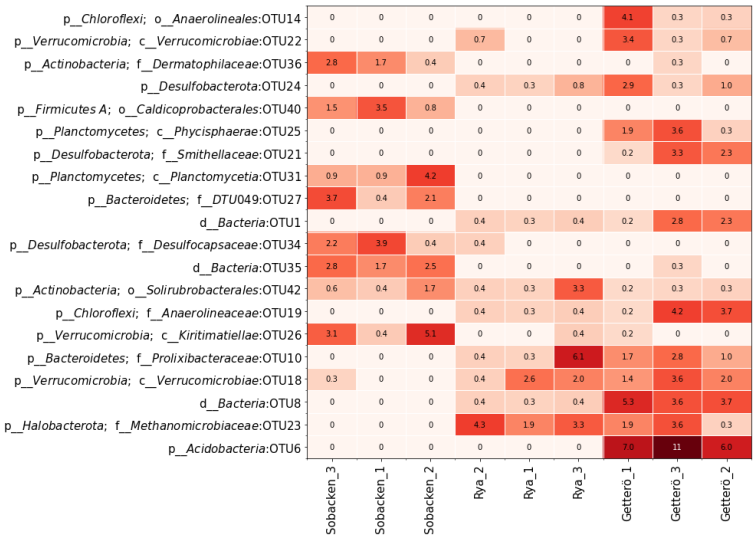

Figure D.14. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 14.

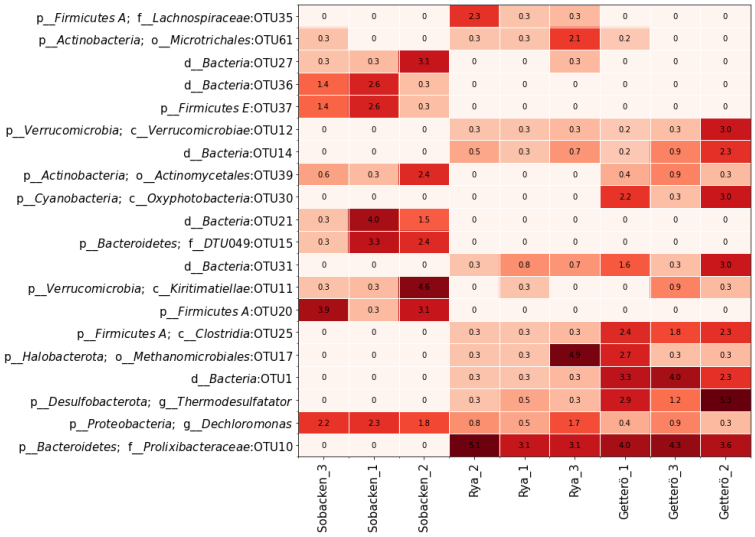

Figure D.15. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 1.

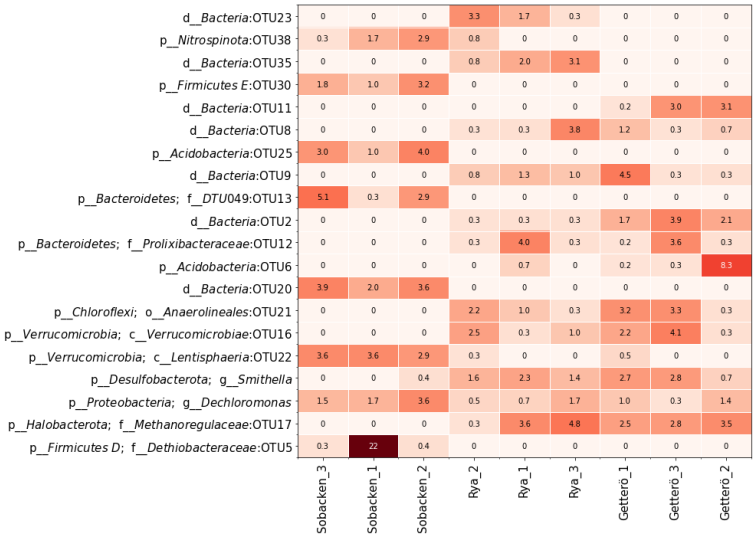

Figure D.16. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 2.

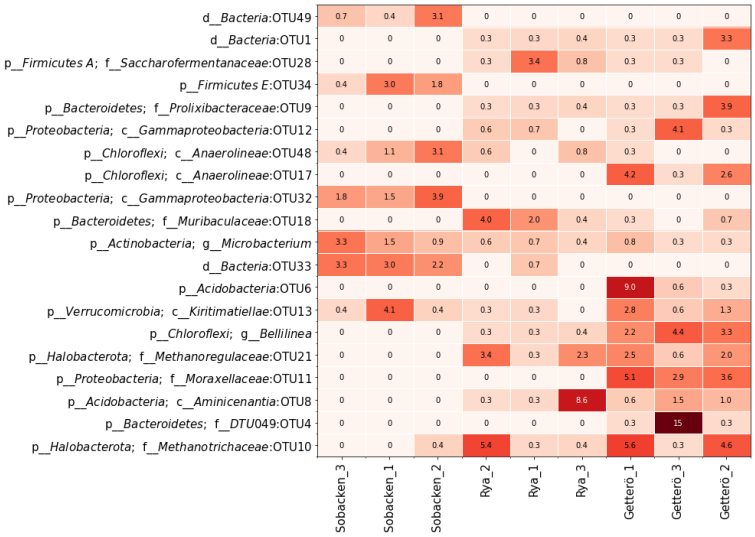

Figure D.17. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 3.

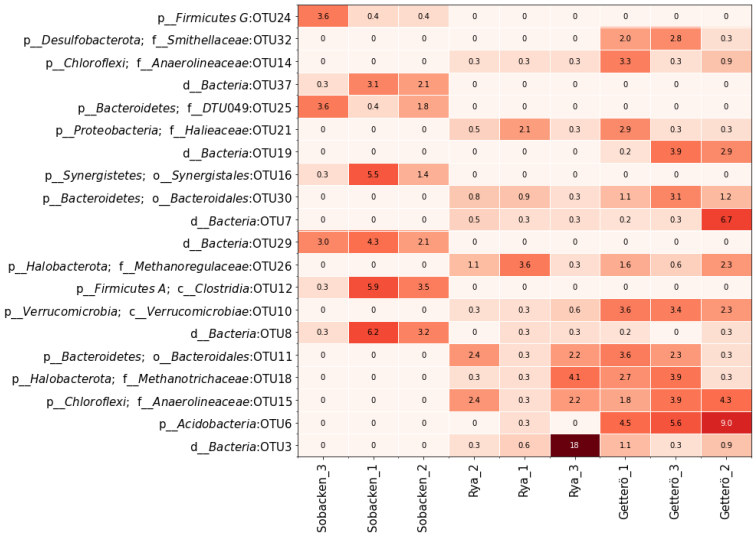

Figure D.18. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 4.

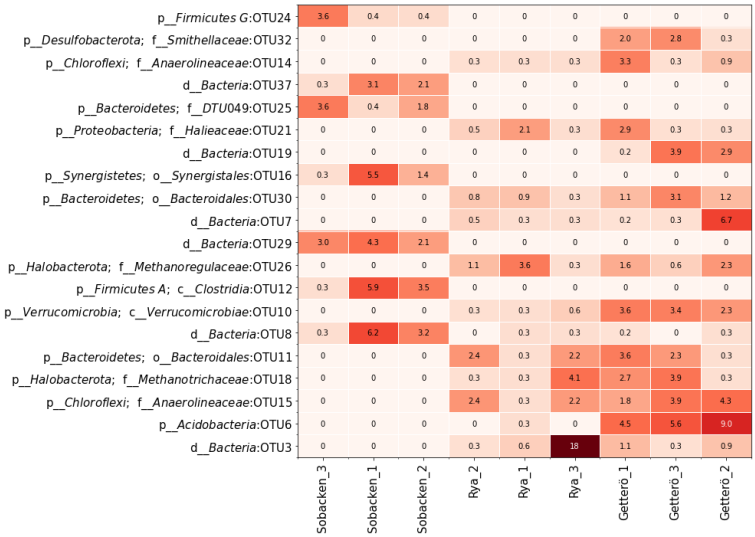

Figure D.19. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 5.

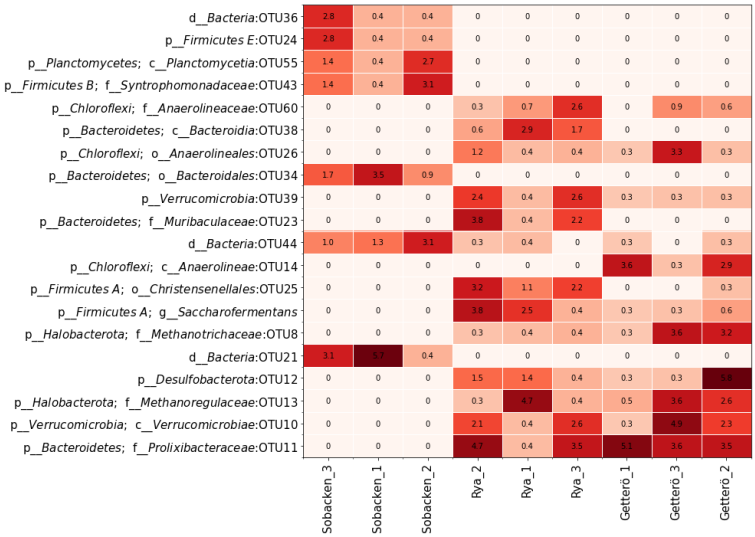

Figure D.20. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 6.

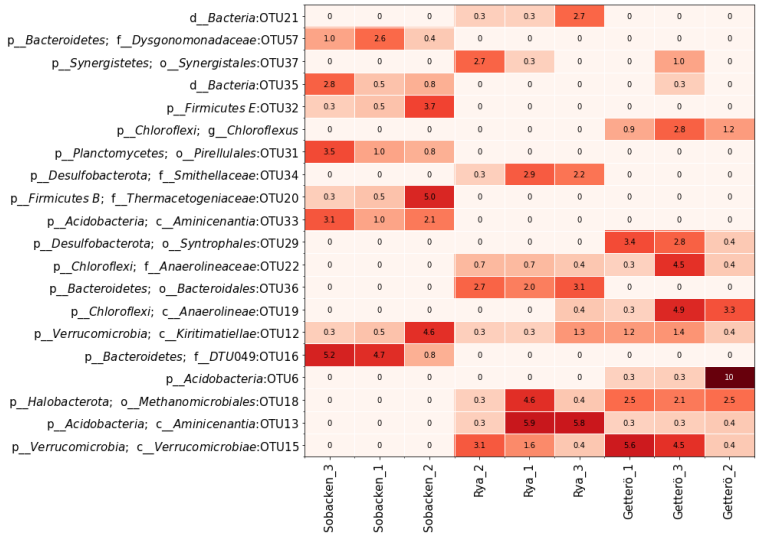

Figure D.21. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 7.

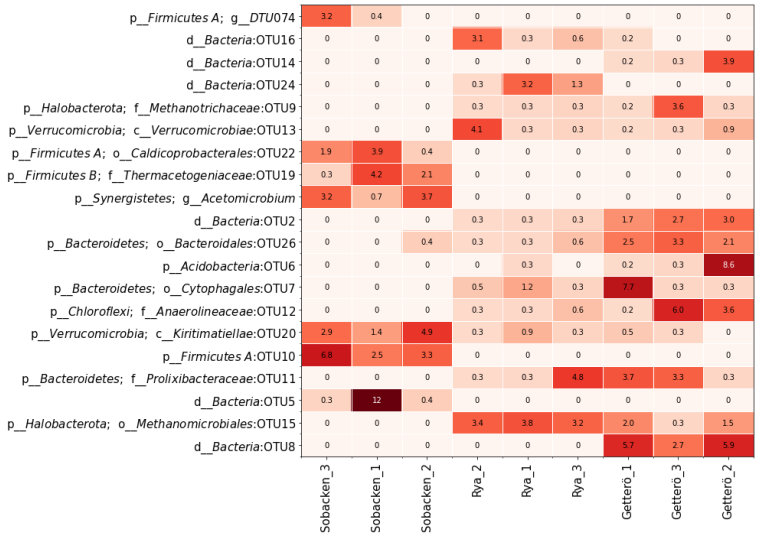

Figure D.22. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 8.

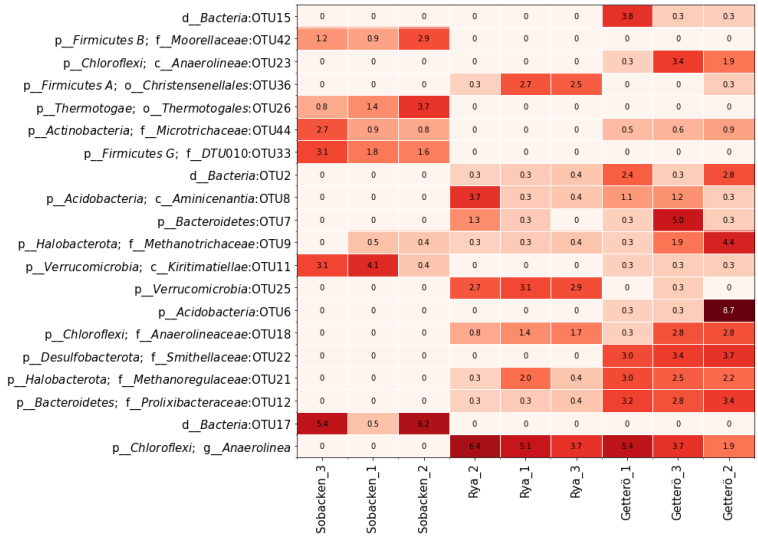

Figure D.23. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 9.

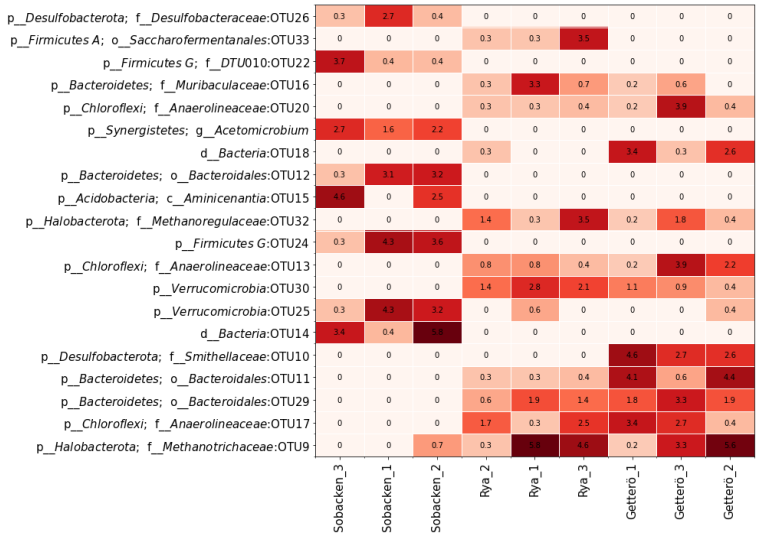

Figure D.24. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 10.

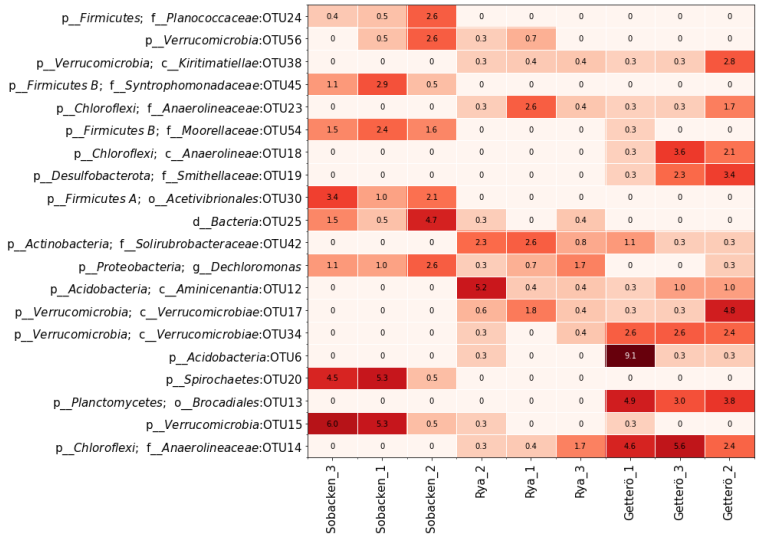

Figure D.25. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 11.

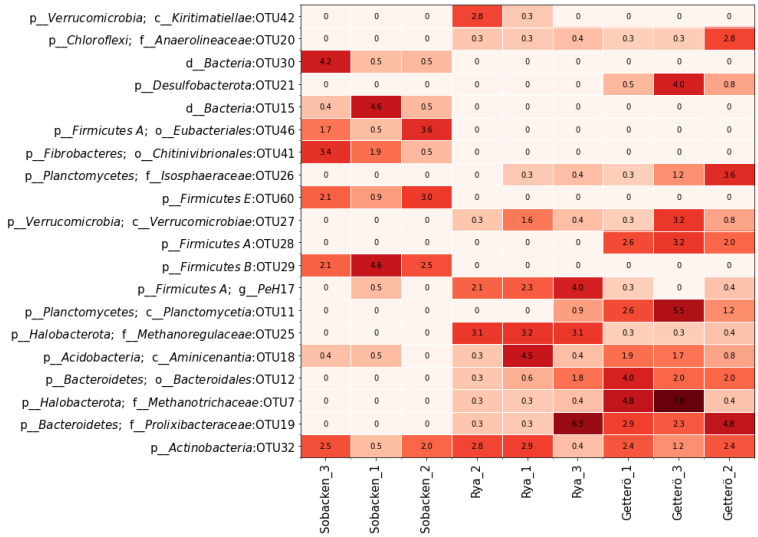

Figure D.26. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 12.

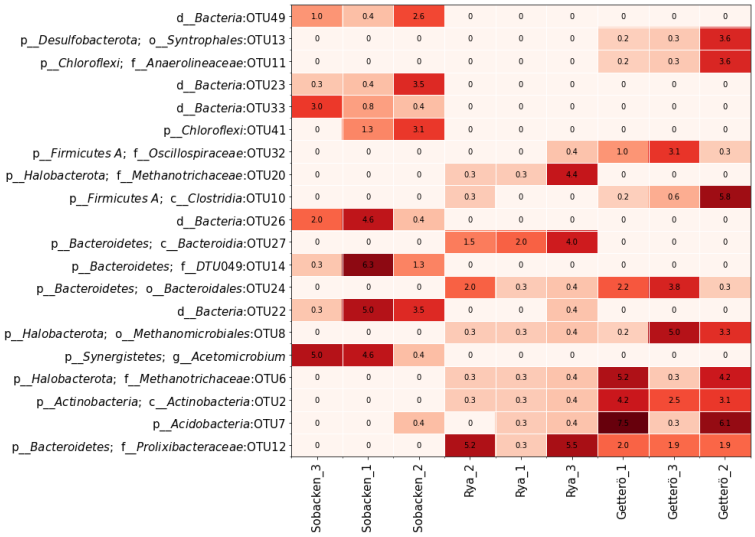

Figure D.27. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 13.

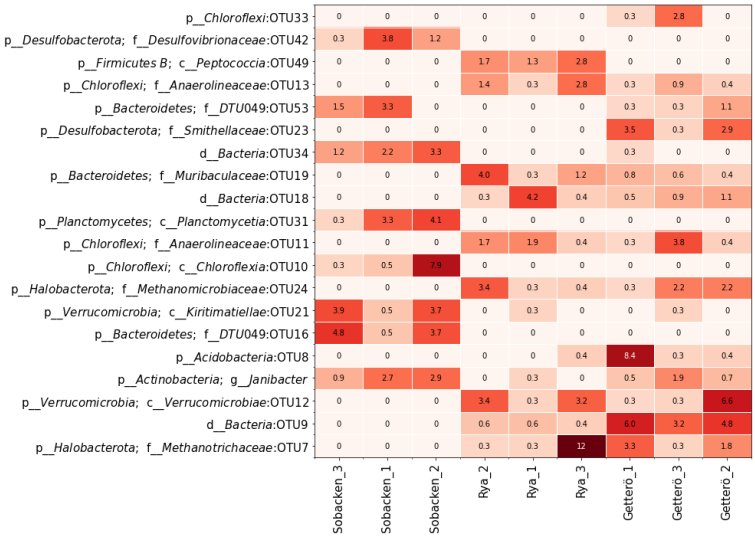

Figure D.28. Heatmap displaying the 20 OTUs with highest abundance for SingleM protein 14.

Appendix E

Table E.1-E8 shows the result of the forward sequenced data from investigating the relative abundance of different microorganisms involved in AD.

Table E.1. Average relative abundance of methylotrophic methanogens over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of methylotrophic methanogens (*Methanomethylovorans)* [%]
Sobacken	0.018 ± 0.018
Rya	0 ± 0
Getterö	0.013 ±0.0068

Table E.2. Average relative abundance of the genus *Methanosarcina* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Methanosarcina* [%]
Sobacken	0.057 ± 0.031
Rya	0.0069 ± 0.0069
Getterö	0.014±0.014

Table E.3. Average relative abundance of *Methanoregulaceae* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Methanoregulaceae* [%]
Sobacken	0.031 ± 0.019
Rya	2.08 ± 0.20
Getterö	1.32 ±0.19

Table E.4. Average relative abundance of *Methanothermobacter* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Methanothermobacter* [%]
Sobacken	0.32 ± 0.13
Rya	0 ± 0
Getterö	0.034 ±0.0082

Table E.5. Average relative abundance of *Bacteroidetes* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Bacteroidetes* [%]
Sobacken	7.77 ±1.02
Rya	10.30 ± 0.37
Getterö	14.82 ±0.21

Table E.6. Average relative abundance of *Firmicutes* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Firmicutes* [%]
Sobacken	29.42 ±0.53
Rya	16.67 ± 0.71
Getterö	7.08 ±0.26

Table E.7. Average relative abundance of *Chloroflexi* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Chloroflexi* [%]
Sobacken	3.01 ±0.20
Rya	9.55 ± 0.20
Getterö	14.28 ±0.47

Table E.8. Average relative abundance of *Proteobacteria* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Proteobacteria* [%]
Sobacken	14.88 ±0.40
Rya	14.39 ± 0.70
Getterö	8.88 ± 0.81

Table E.9-E22 shows the result of the reversed sequenced data from investigating the relative abundance of different microorganisms involved in AD.

Table E.9. Average relative abundance of methylotrophic methanogens over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of methylotrophic methanogens (*Methanomethylovorans)* [%]
Sobacken	0.018 ± 0.009
Rya	0.0076 ± 0.0076
Getterö	0.0061 ± 0.0061

Table E.10. Average relative abundance of the genus *Methanosarcina* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Methanosarcina* [%]
Sobacken	0.034 ± 0.021
Rya	0 ± 0
Getterö	0.018 ± 0.018

Table E.11. Average relative abundance of *Methanoregulaceae* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Methanoregulaceae* [%]
Sobacken	0.029 ± 0.017
Rya	1.43 ± 0.25
Getterö	1.17 ±0.12

Table E.12. Average relative abundance of methanogens over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of methanogens [%]
Sobacken	1.33 ±0.09
Rya	5.91 ± 0.72
Getterö	4.95 ±0.13

Table E.13. Average relative abundance of hydrogenotrophic methanogens over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of hydrogenotrophic methanogens [%]
Sobacken	0.48 ± 0.096
Rya	0.16 ± 0.032
Getterö	0.34 ±0.014

Table E.14. Average relative abundance of *Methanothermobacter* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Methanothermobacter* [%]
Sobacken	0.42 ± 0.066
Rya	0 ± 0
Getterö	0.0061 ± 0.0061

Table E.15. Average relative abundance of acetoclastic methanogens *(Methanotrichaceae)* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of acetoclastic methanogens (*Methanotrichaceae)* [%]
Sobacken	0.21 ±0.042
Rya	2.41 ± 0.54
Getterö	2.21 ±0.070

Table E.16. Average relative abundance of *Bacteroidetes* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Bacteroidetes* [%]
Sobacken	7.56 ±0.46
Rya	9.45 ± 0.37
Getterö	11.97 ±0.68

Table E.17. Average relative abundance of *Firmicutes* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Firmicutes* [%]
Sobacken	29.05 ±0.37
Rya	17.42 ± 0.26
Getterö	7.32 ±0.25

Table E.18. Average relative abundance of *Chloroflexi* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Chloroflexi* [%]
Sobacken	2.61 ±0.25
Rya	9.97 ± 0.52
Getterö	14.37 ±0.82

Table E.19. Average relative abundance of *Proteobacteria* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Proteobacteria* [%]
Sobacken	15.60 ±0.31
Rya	15.15 ± 0.81
Getterö	9.51 ±0.15

Table E.20. Average relative abundance of acetogens involved in propionate degradation over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Smithella* [%]	Relative abundance of *Syntrophobacter* [%]
Sobacken	0.099 ± 0.020	0.0097 ± 0.0097
Rya	1.11 ± 0.22	0.053 ± 0.019
Getterö	2.23±0.17	0.049 ± 0.020

Table E.21. Average relative abundance of acetogenic genus *Syntrophus* over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Syntrophus* [%]
Sobacken	0.029 ± 0.017
Rya	0.053 ± 0.017
Getterö	0.064 ± 0.012

Table E.22. Average relative abundance of SAO bacteria over all 14 SingleM proteins in each WWTP sludge sample (Sobacken, Rya and Getterö WWTPs), including standard error.

WWTP	Relative abundance of *Thermoanaerobacterales* [%]	Relative abundance of *Dethiobacteraceae* [%]
Sobacken	0.13 ± 0.018	0.78 ± 0.65
Rya	0.016 ± 0.0083	0 ± 0
Getterö	0.027 ± 0.014	0± 0

Appendix F

Table F.1 shows the naive alpha diversity for the microbial communities found in the sludge samples from Sobacken, Rya and Getterö WWTP, based on the reversed sequenced data.

Table F.1. Naive alpha diversity of the sludge samples from Sobacken, Rya and Getterö WWTP. The presented values are averages over all SingleM proteins and the three replicas of each WWTP and standard error is included.

WWTP	Naive alpha diversity	
	q=0	q=1
Sobacken	149.69 ± 2.69	115.32 ± 2.41
Rya	159.57 ± 3.22	128.28± 3.03
Getterö	142.52 ± 2.34	102.06 ± 2.03

Appendix G

Figure G.1-G.14 shows the naive alpha diversity profile for SingleM proteins 1-14 generated from the forward sequenced data and Figure G.15-G.28 shows the naive alpha diversity profile for SingleM proteins 1-14 generated from the reversed sequenced data.

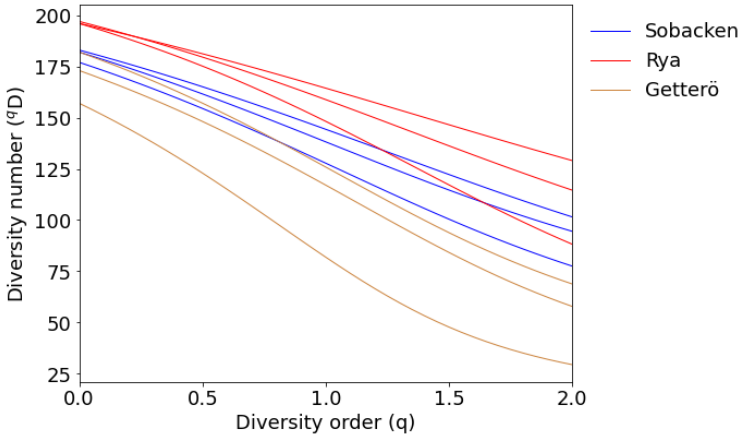

Figure G.1. Naive alpha diversity profile for SingleM protein 1. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

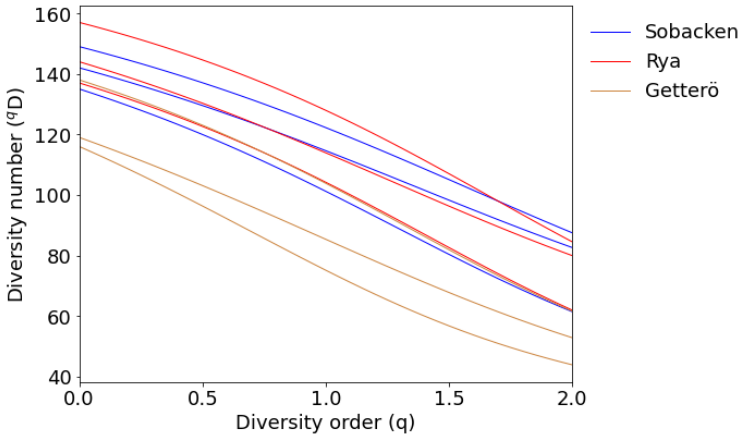

Figure G.2. Naive alpha diversity profile for SingleM protein 2. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

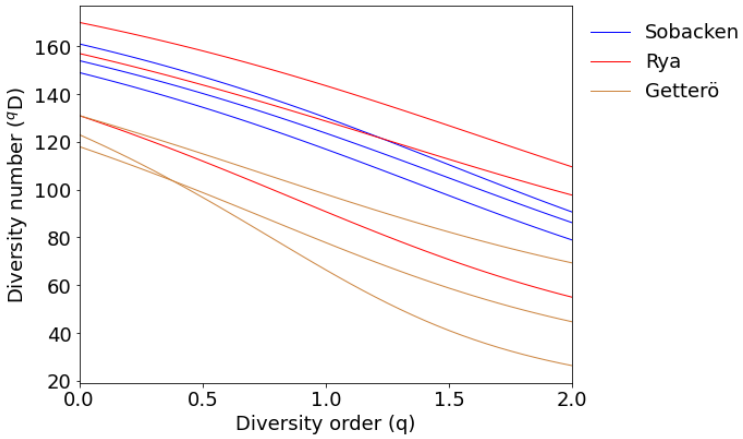

Figure G.3. Naive alpha diversity profile for SingleM protein 3. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

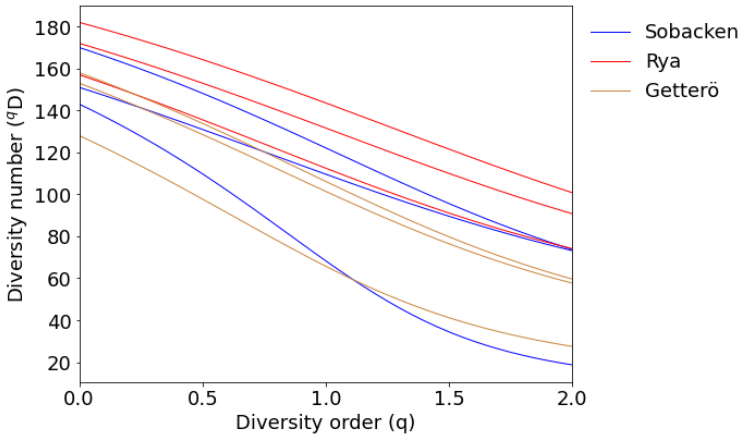

Figure G.4. Naive alpha diversity profile for SingleM protein 4. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

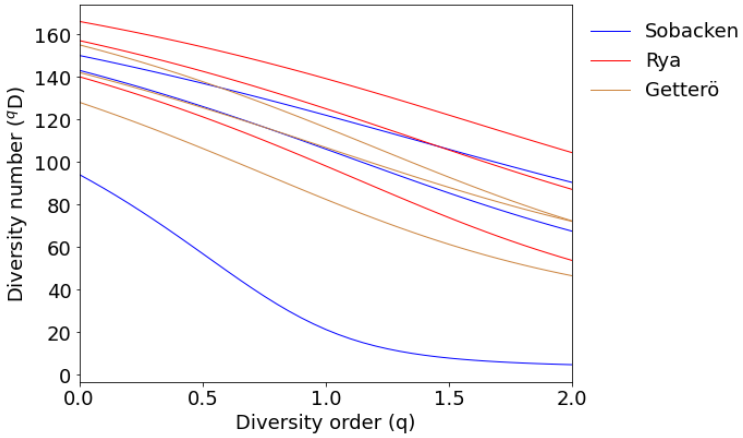

Figure G.5. Naive alpha diversity profile for SingleM protein 5. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

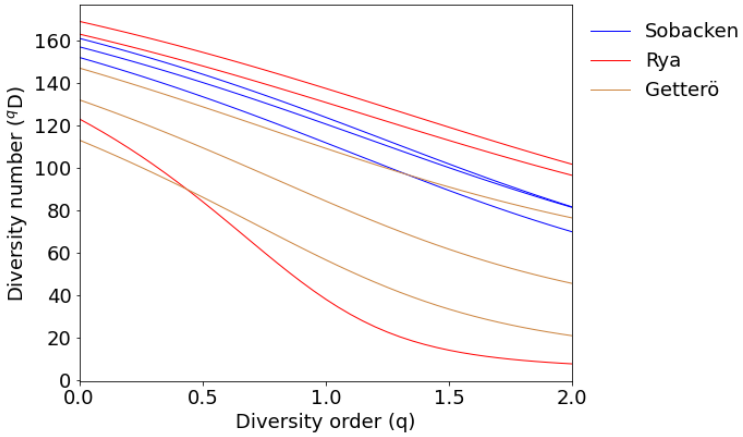

Figure G.6. Naive alpha diversity profile for SingleM protein 6. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

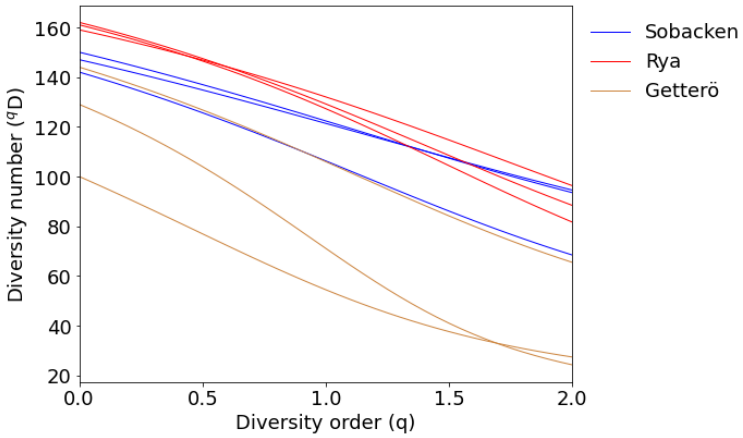

Figure G.7. Naive alpha diversity profile for SingleM protein 7. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

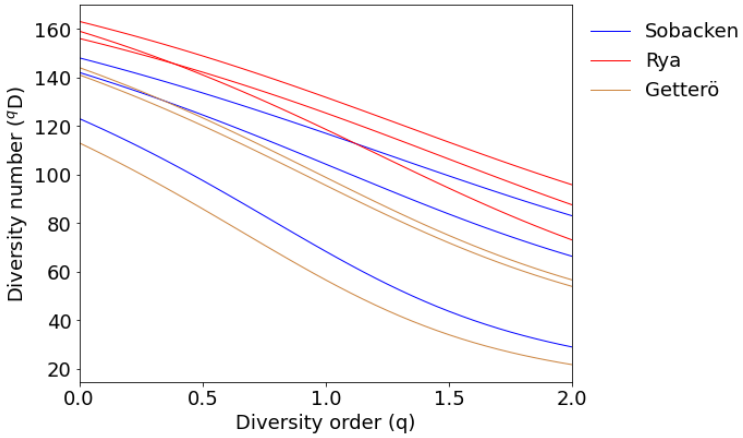

Figure G.8. Naive alpha diversity profile for SingleM protein 8. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

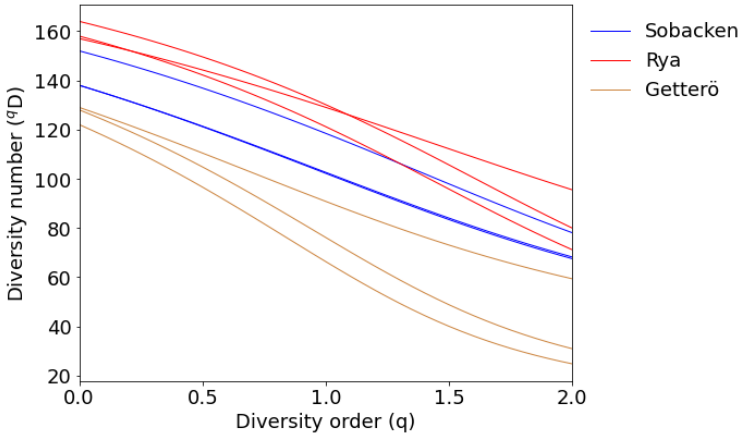

Figure G.9. Naive alpha diversity profile for SingleM protein 9. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

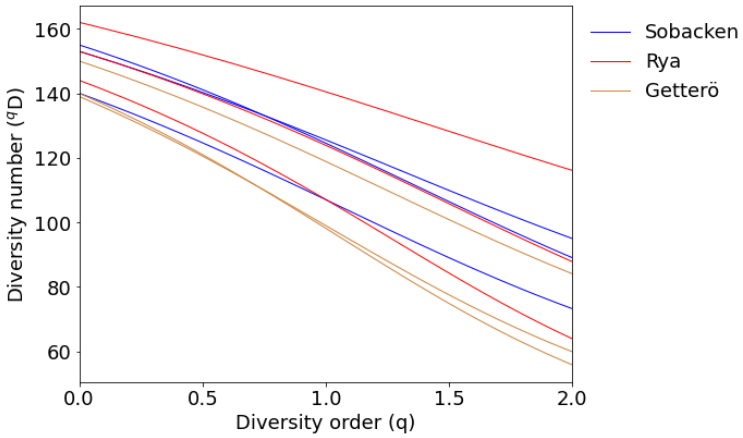

Figure G.10. Naive alpha diversity profile for SingleM protein 10. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

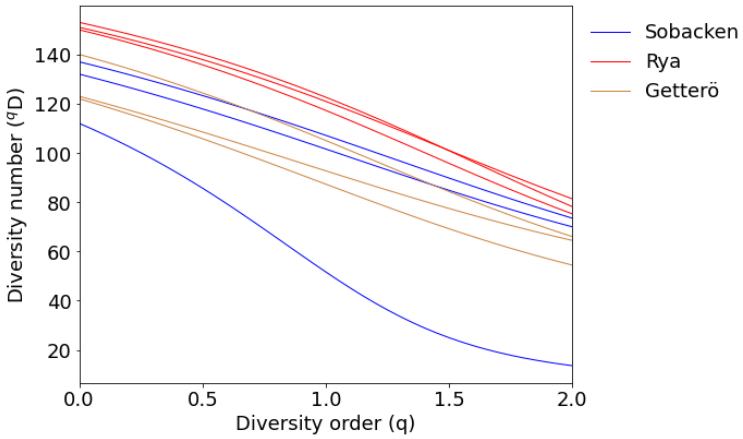

Figure G.11. Naive alpha diversity profile for SingleM protein 11. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

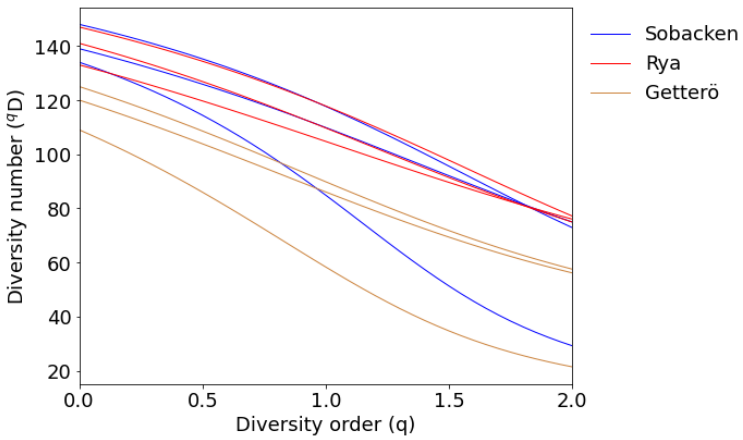

Figure G.12. Naive alpha diversity profile for SingleM protein 12. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

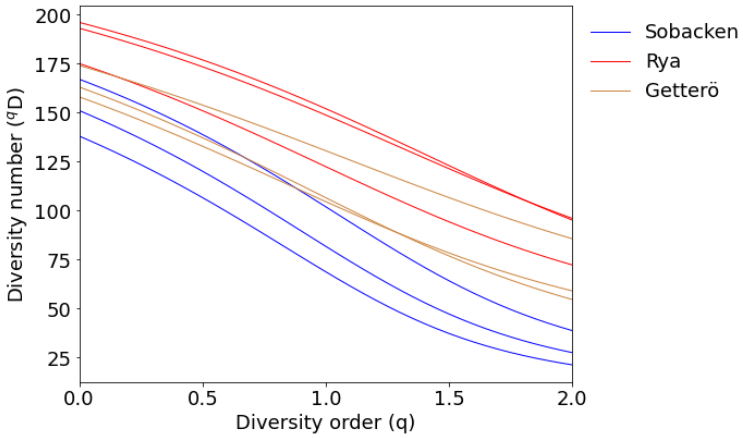

Figure G.13. Naive alpha diversity profile for SingleM protein 13. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

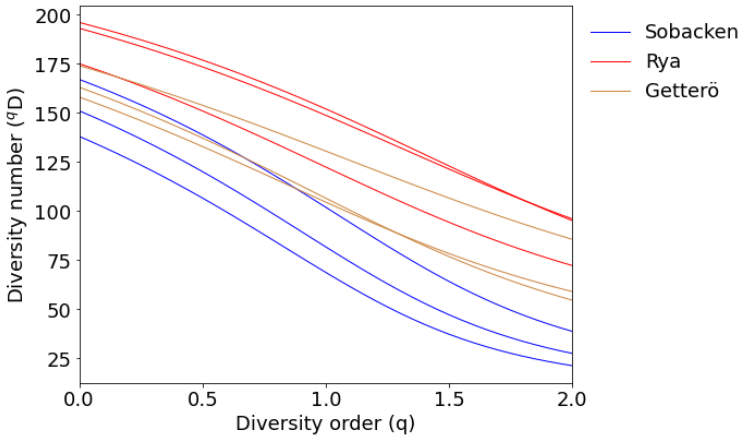

Figure G.14. Naive alpha diversity profile for SingleM protein 14. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

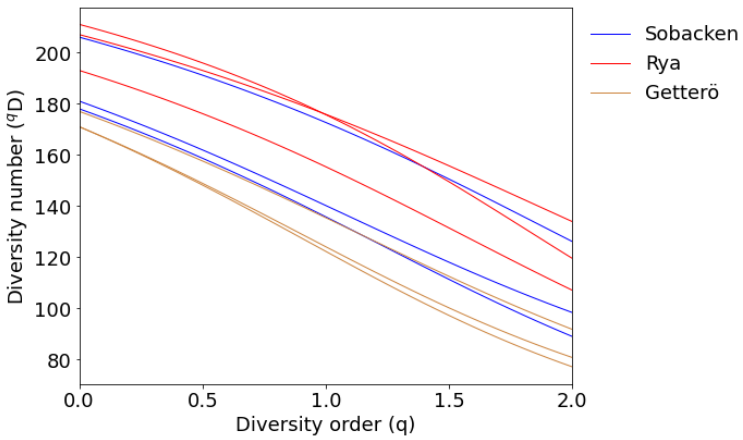

Figure G.15. Naive alpha diversity profile for SingleM protein 1. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

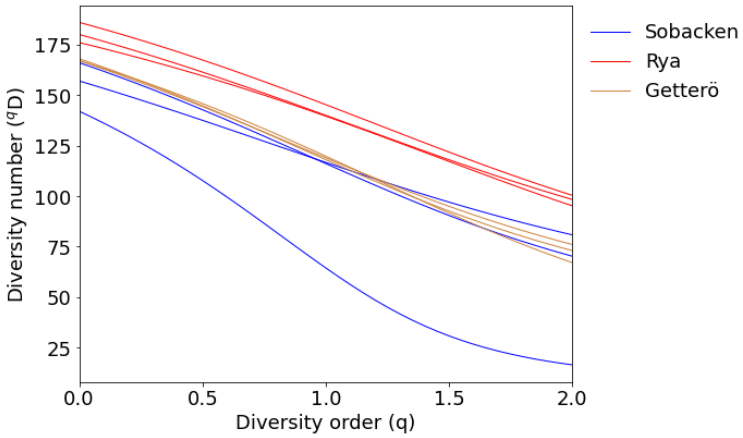

Figure G.16. Naive alpha diversity profile for SingleM protein 2. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

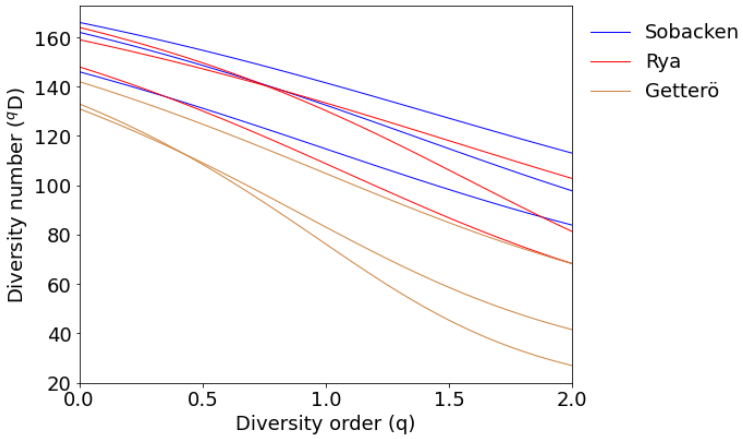

Figure G.17. Naive alpha diversity profile for SingleM protein 3. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

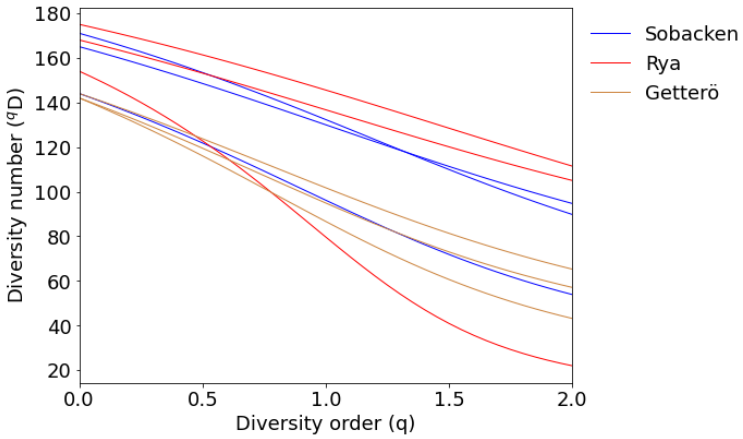

Figure G.18. Naive alpha diversity profile for SingleM protein 4. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

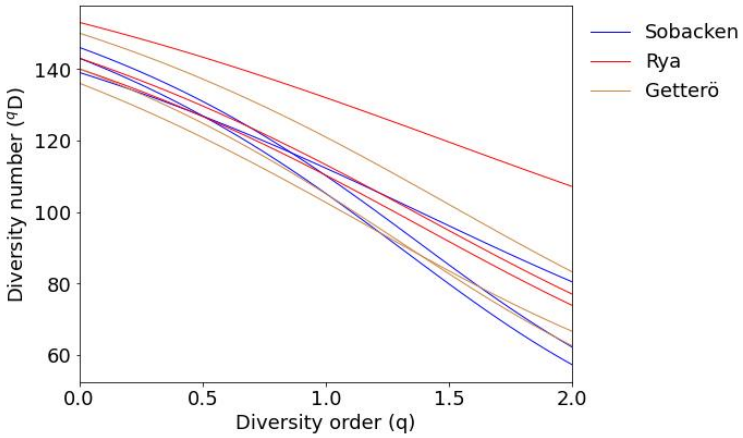

Figure G.19. Naive alpha diversity profile for SingleM protein 5. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

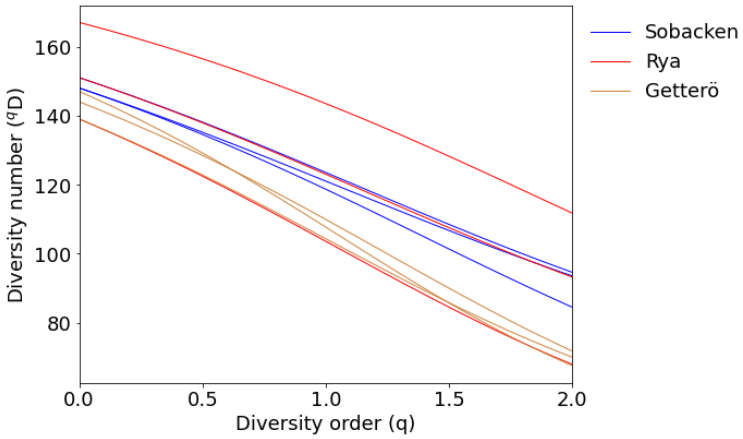

Figure G.20. Naive alpha diversity profile for SingleM protein 6. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

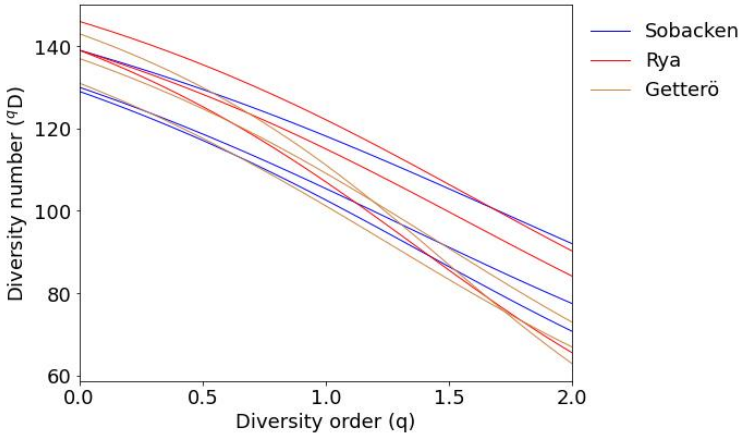

Figure G.21. Naive alpha diversity profile for SingleM protein 7. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

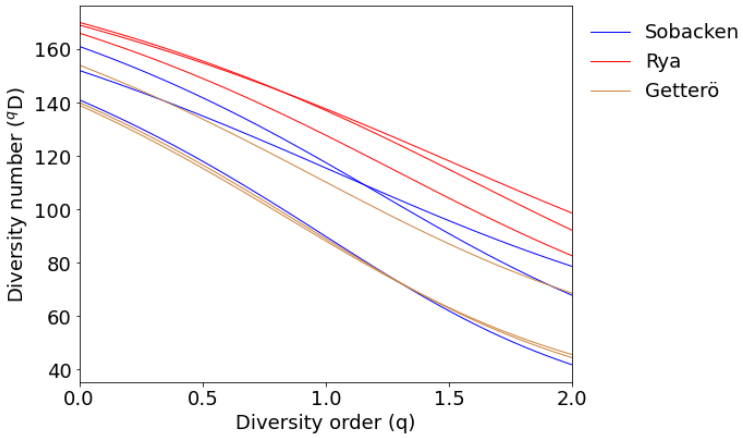

Figure G.22. Naive alpha diversity profile for SingleM protein 8. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

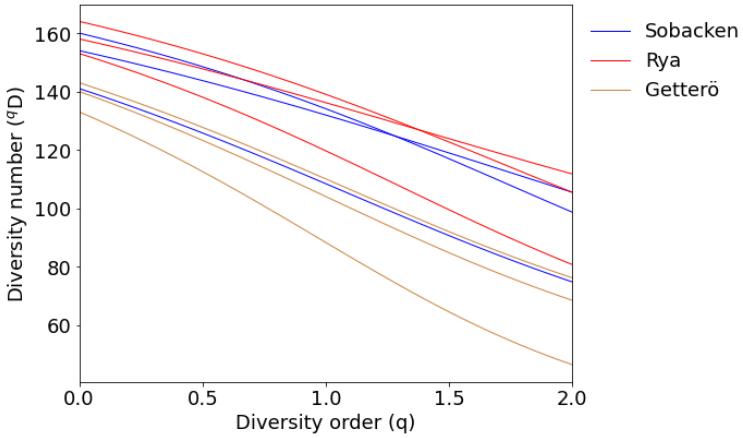

Figure G.23. Naive alpha diversity profile for SingleM protein 9. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

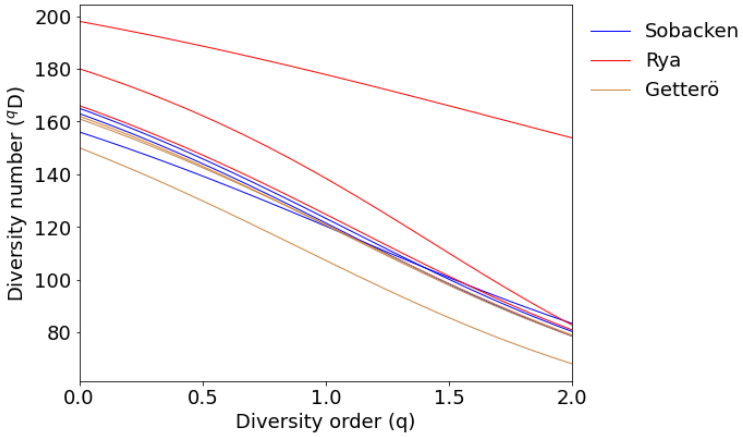

Figure G.24. Naive alpha diversity profile for SingleM protein 10. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

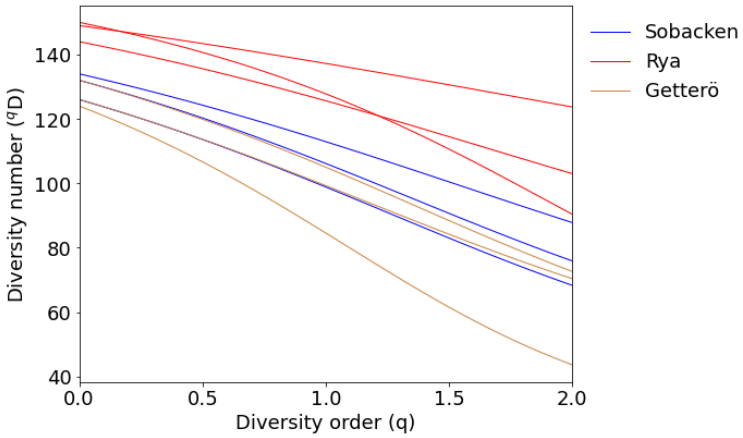

Figure G.25. Naive alpha diversity profile for SingleM protein 11. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

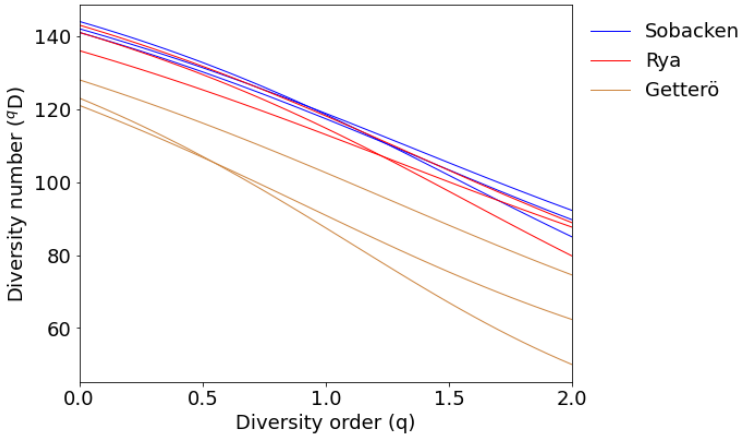

Figure G.26. Naive alpha diversity profile for SingleM protein 12. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

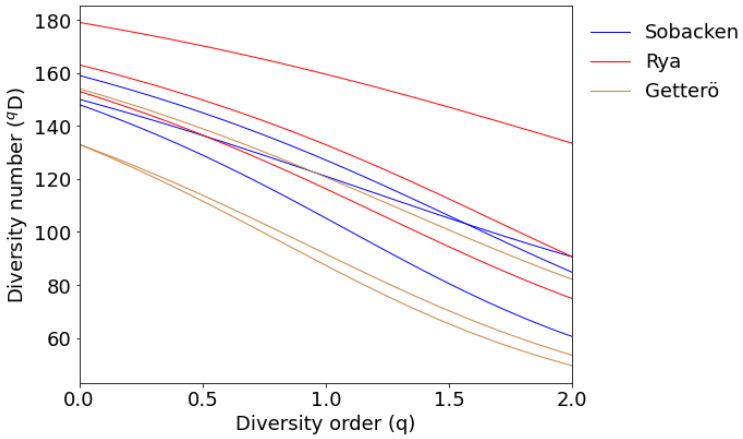

Figure G.2. Naive alpha diversity profile for SingleM protein 13. The plot shows the dependence of Hills diversity number ^qD of the diversity order q for Sobacken, Rya and Getterö WWTP.

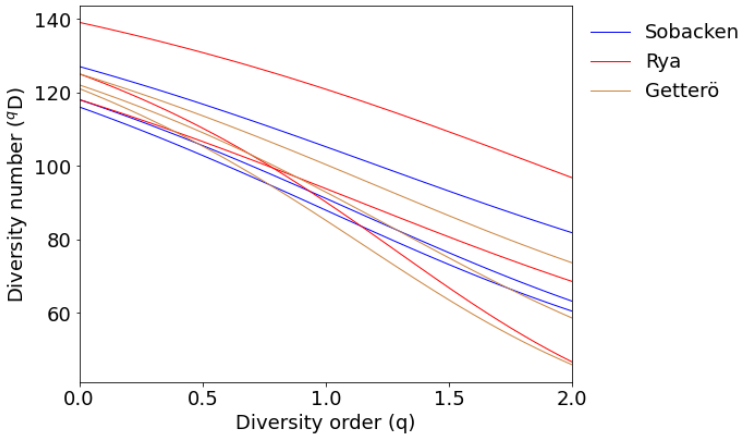

Figure G.28. Naive alpha diversity profile for SingleM protein 14. The plot shows the dependence of Hills diversity number ${}^{q}D$ of the diversity order q for Sobacken, Rya and Getterö WWTP.

Appendix H

Figure H.1-H.28 shows the PCoA plots for diversity order *q*=0 and *q*=1 for Single M protein 1-14. Figure H.1-H.14 is based on the forward sequenced data and Figure H.15-H.28 is based on the reversed sequenced data.

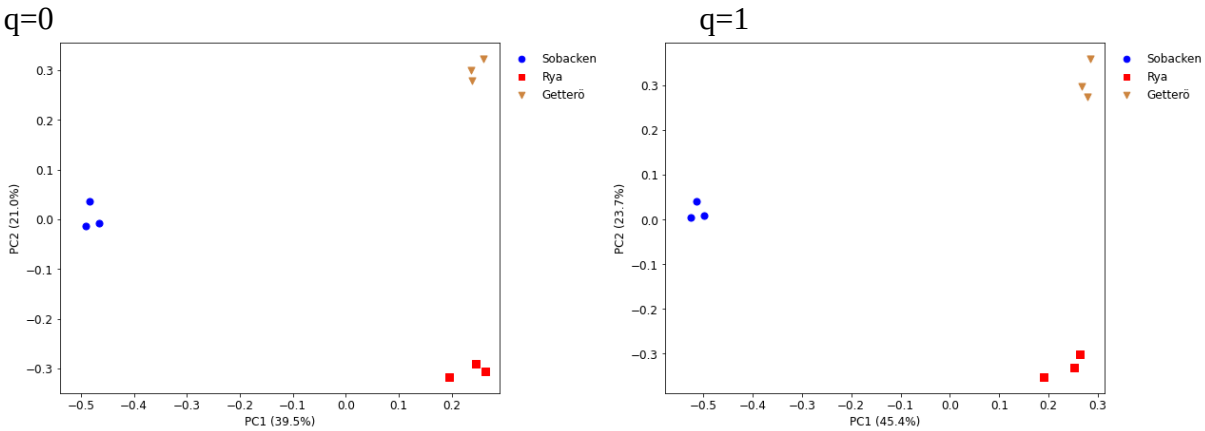

Figure H.1. PCoA plot for SingleM protein 1 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

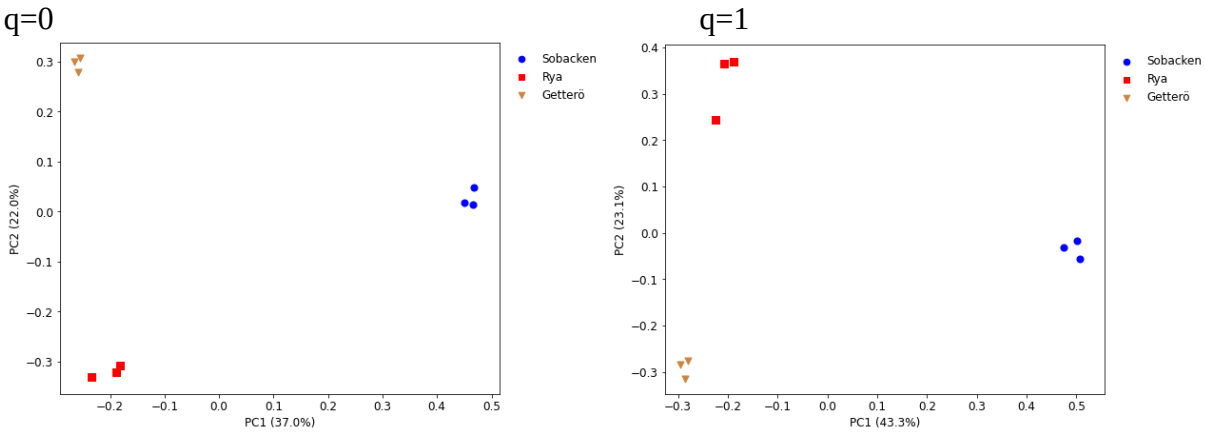

Figure H.2. PCoA plot for SingleM protein 2 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

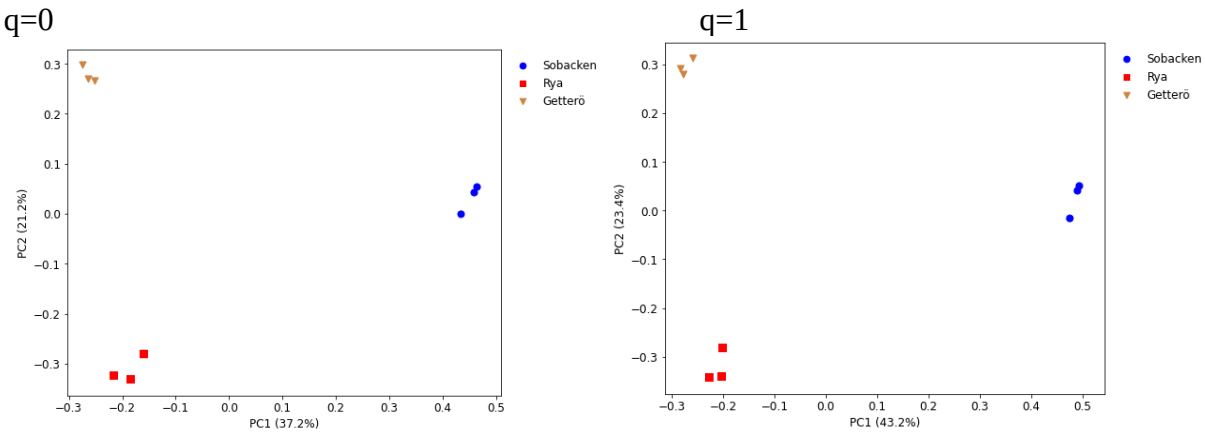

Figure H.3. PCoA plot for SingleM protein 3 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

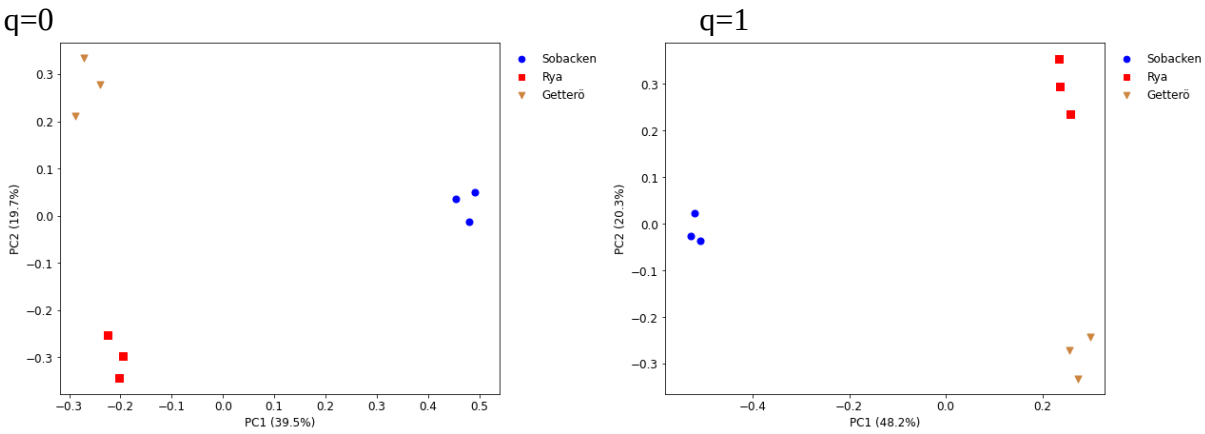

Figure H.4. PCoA plot for SingleM protein 4 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

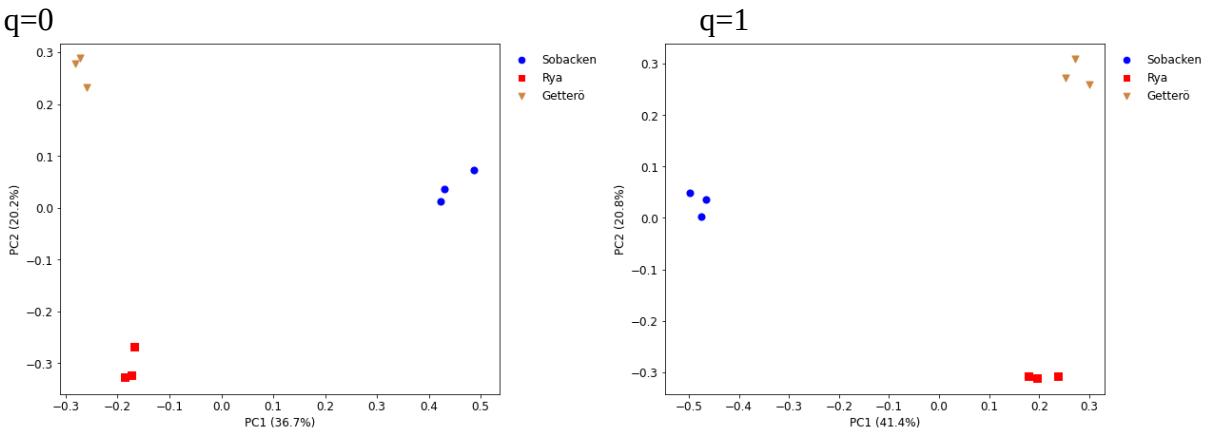

Figure H.5. PCoA plot for SingleM protein 5 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

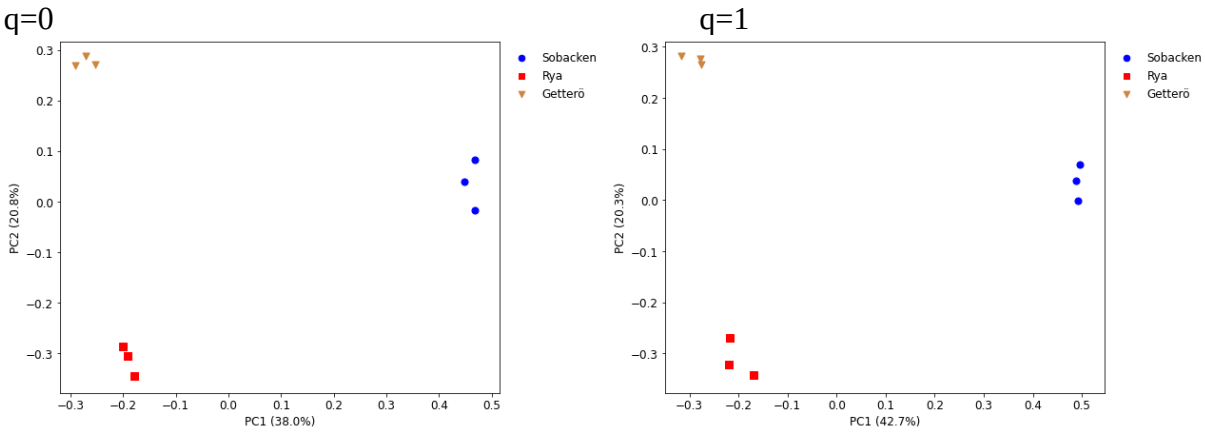

Figure H.6. PCoA plot for SingleM protein 6 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

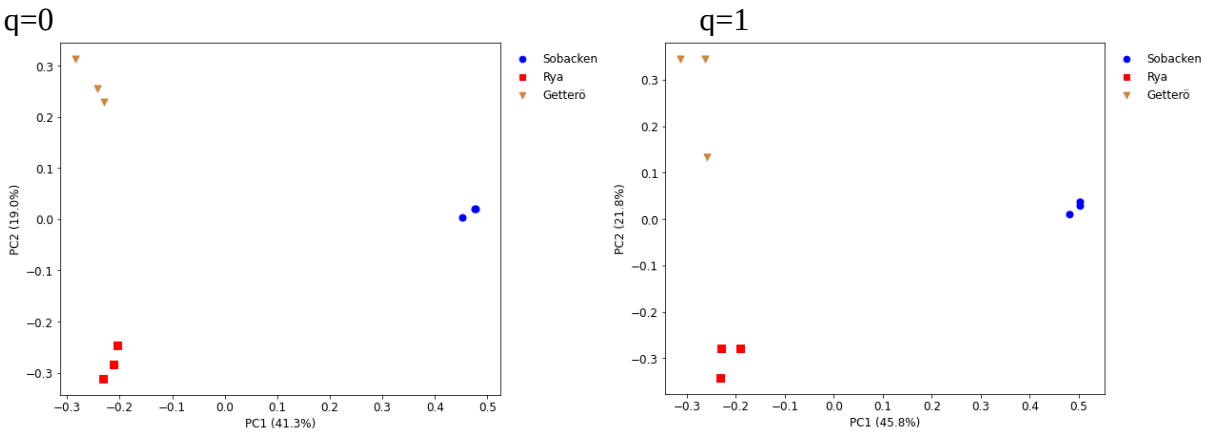

Figure H.7. PCoA plot for SingleM protein 7 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

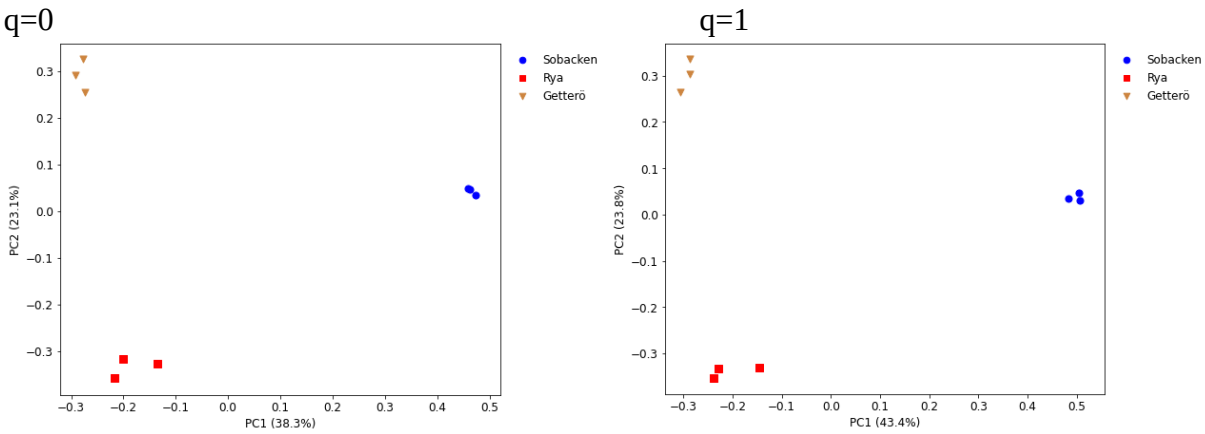

Figure H.8. PCoA plot for SingleM protein 8 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

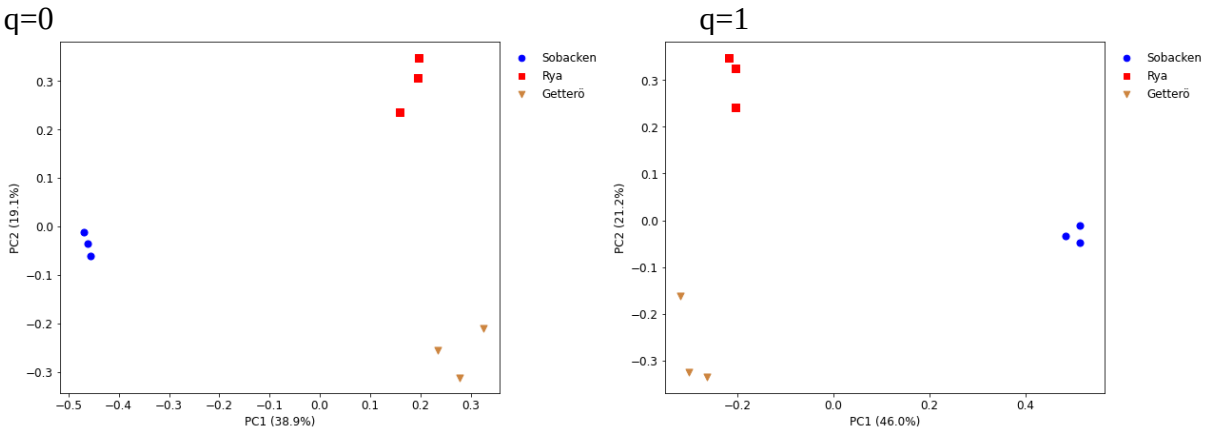

Figure H.9. PCoA plot for SingleM protein 9 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

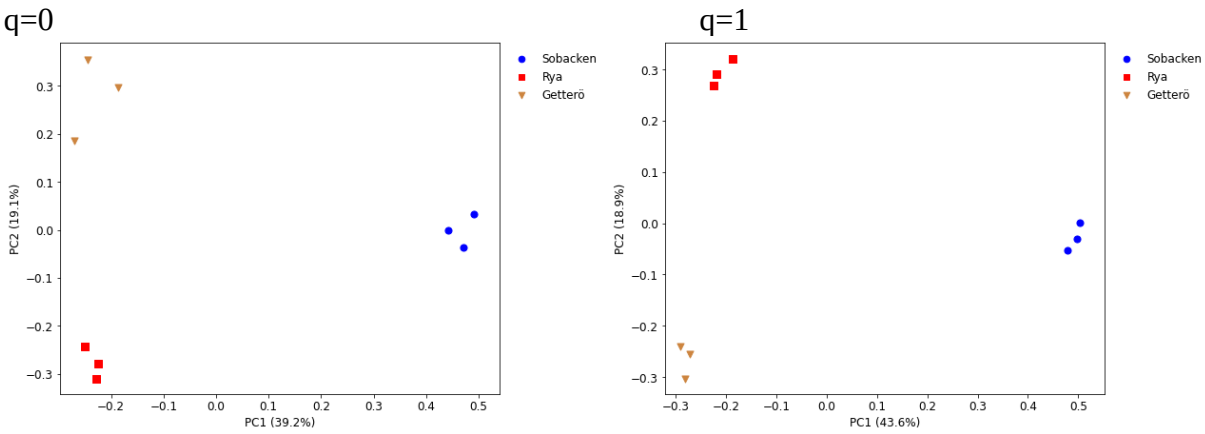

Figure H.10. PCoA plot for SingleM protein 10 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

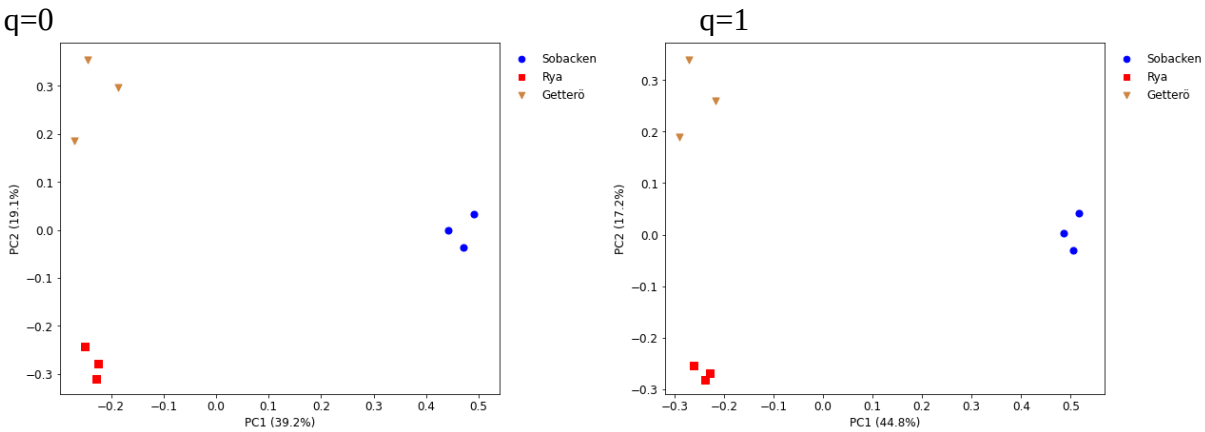

Figure H.11. PCoA plot for SingleM protein 11 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

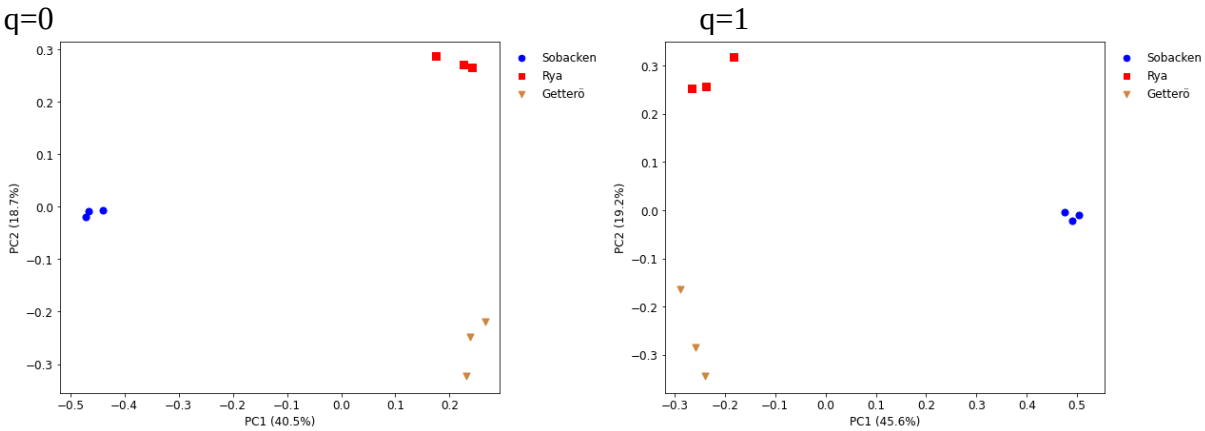

Figure H.12. PCoA plot for SingleM protein 12 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

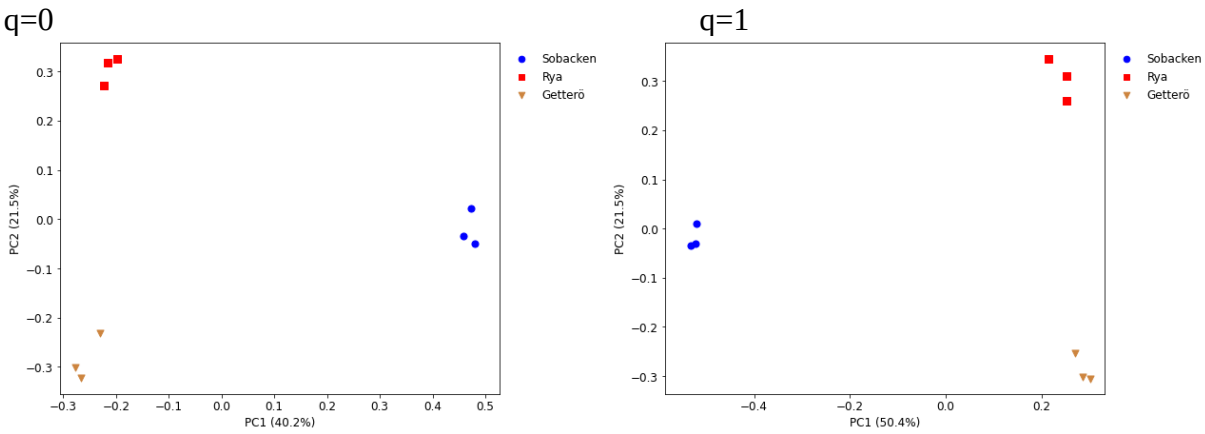

Figure H.13. PCoA plot for SingleM protein 13 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

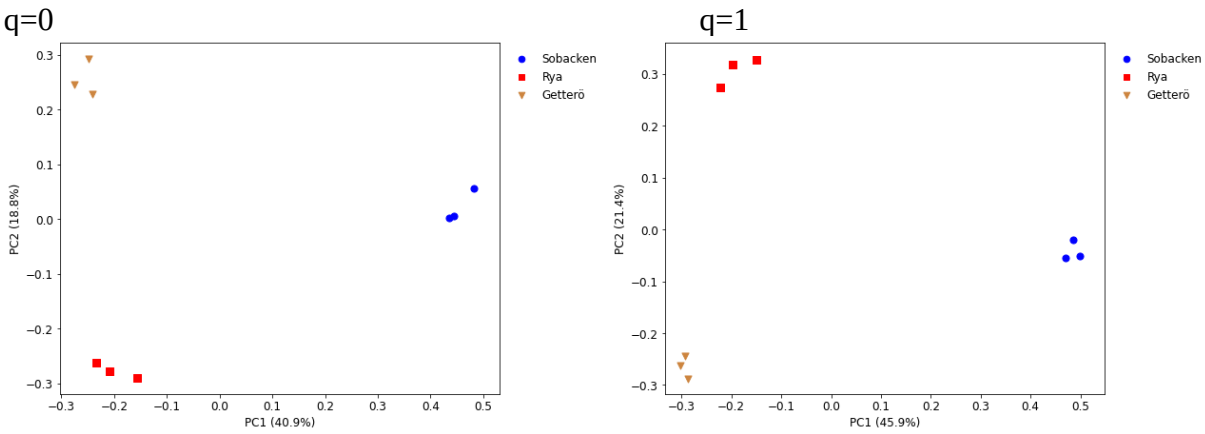

Figure H.14. PCoA plot for SingleM protein 14 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

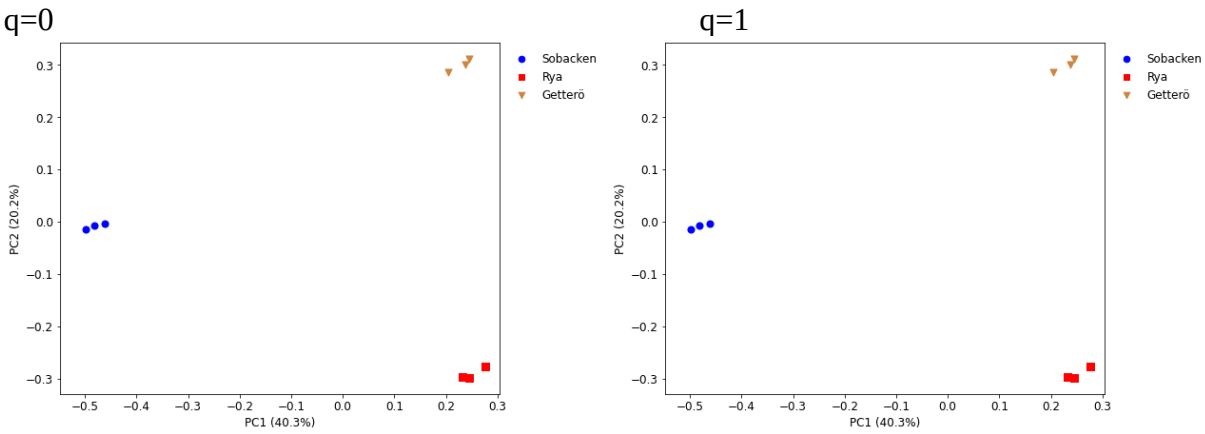

Figure H.15. PCoA plot for SingleM protein 1 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

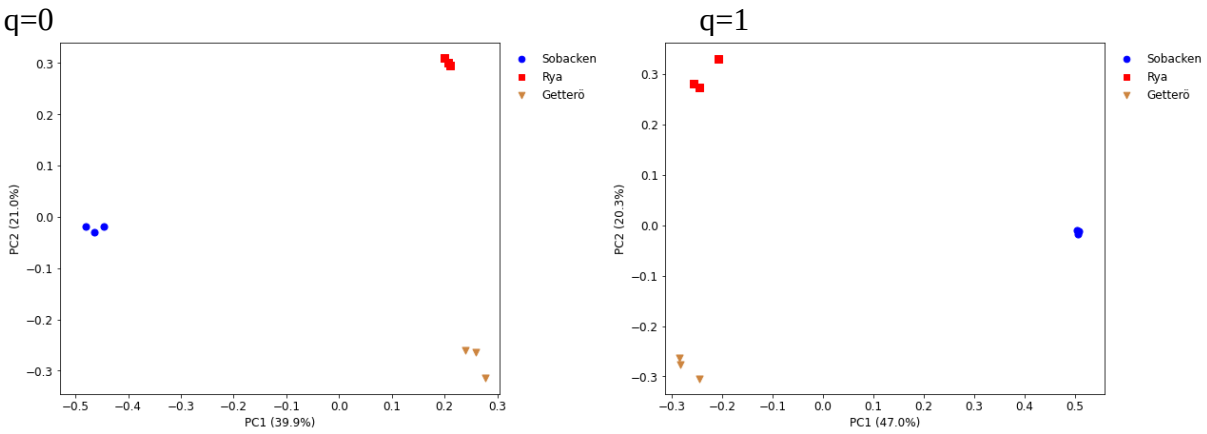

Figure H.16. PCoA plot for SingleM protein 2 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

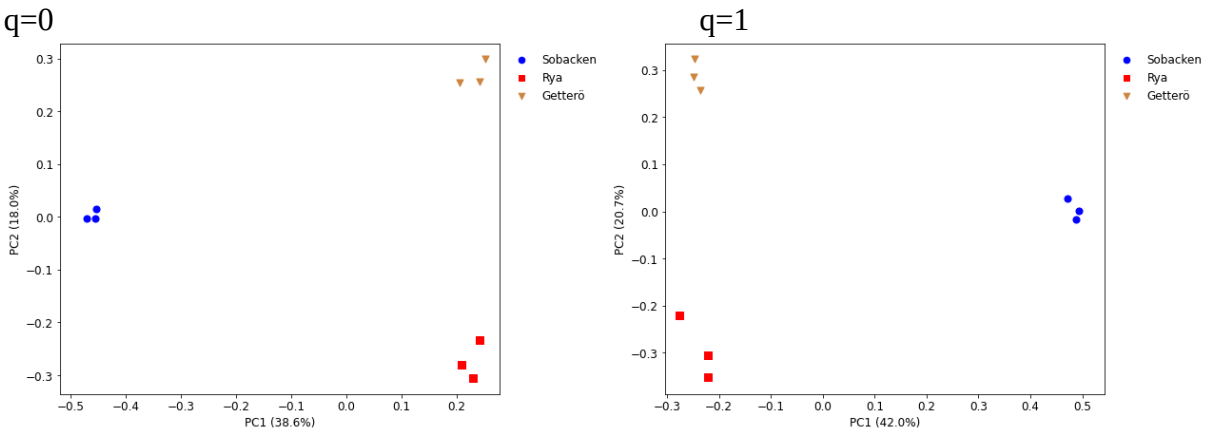

Figure H.17. PCoA plot for SingleM protein 3 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

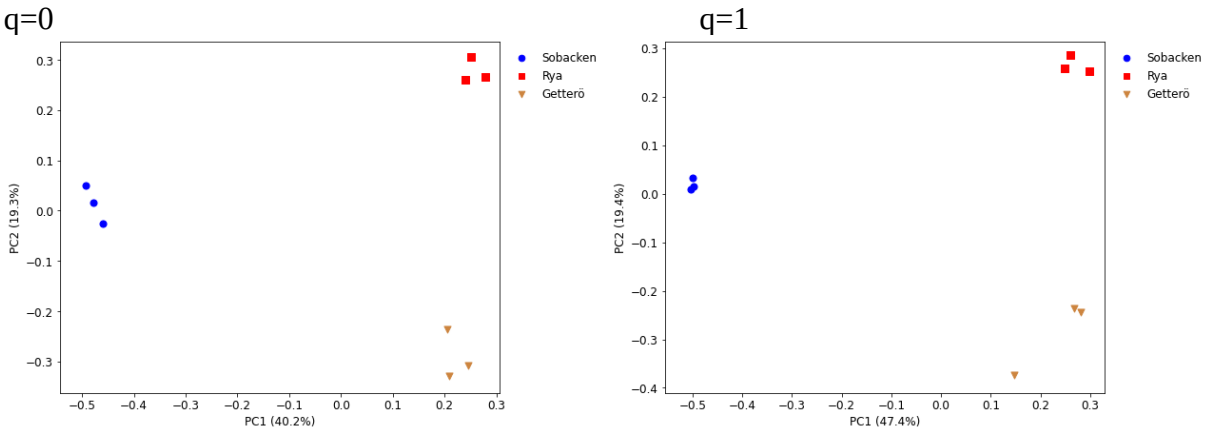

Figure H.18. PCoA plot for SingleM protein 4 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

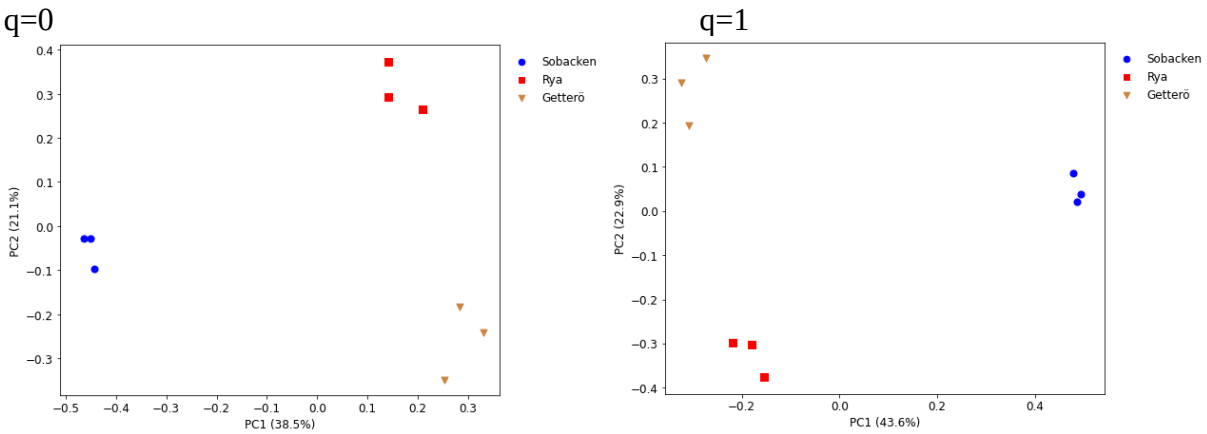

Figure H.19. PCoA plot for SingleM protein 5 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

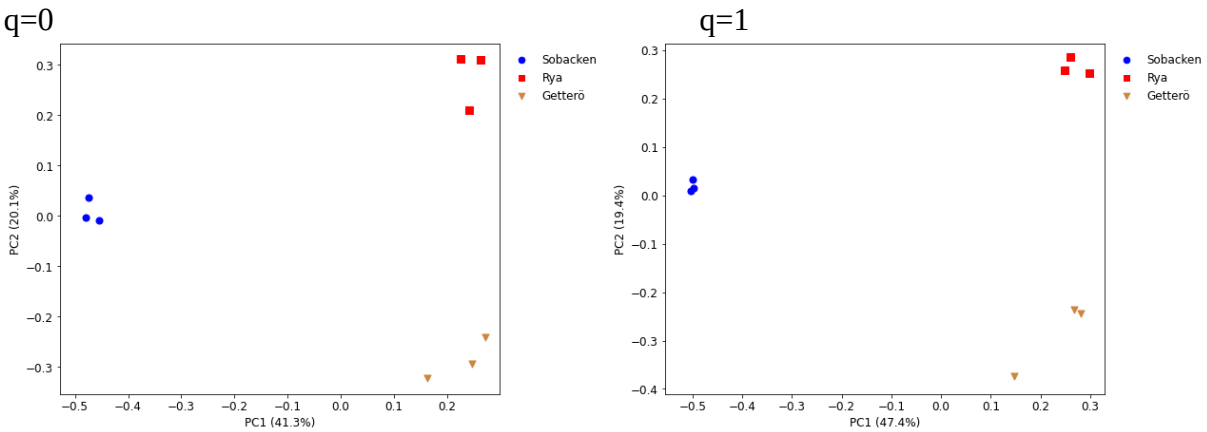

Figure H.20. PCoA plot for SingleM protein 6 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

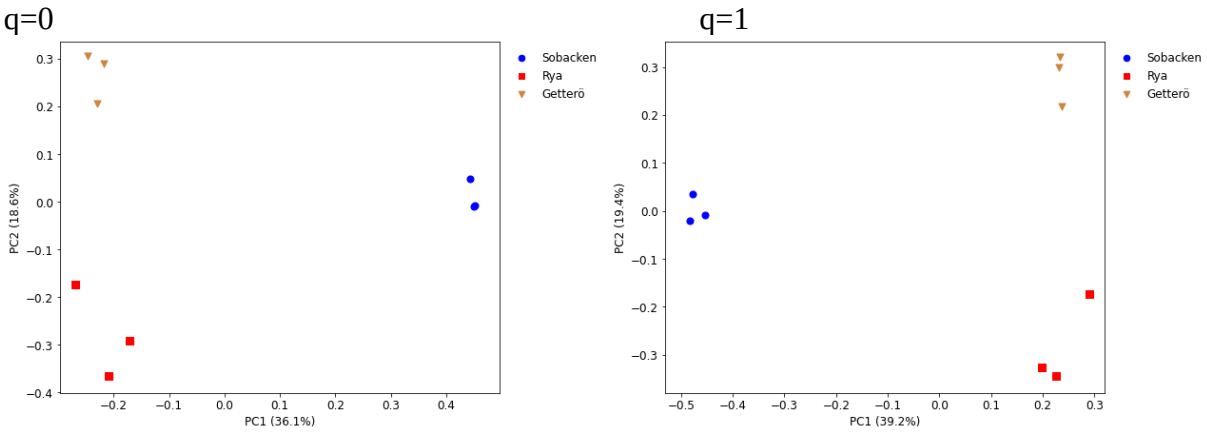

Figure H.21. PCoA plot for SingleM protein 7 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

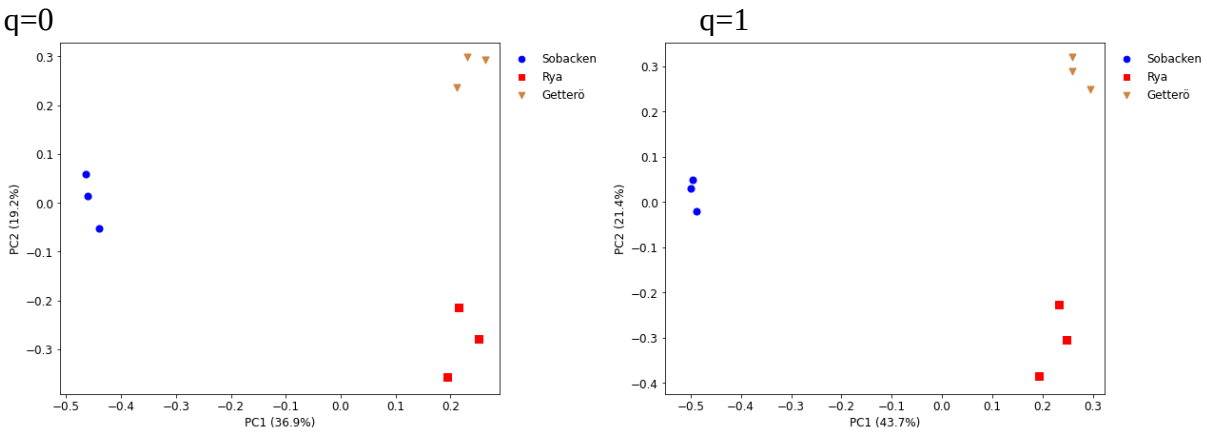

Figure H.22. PCoA plot for SingleM protein 8 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

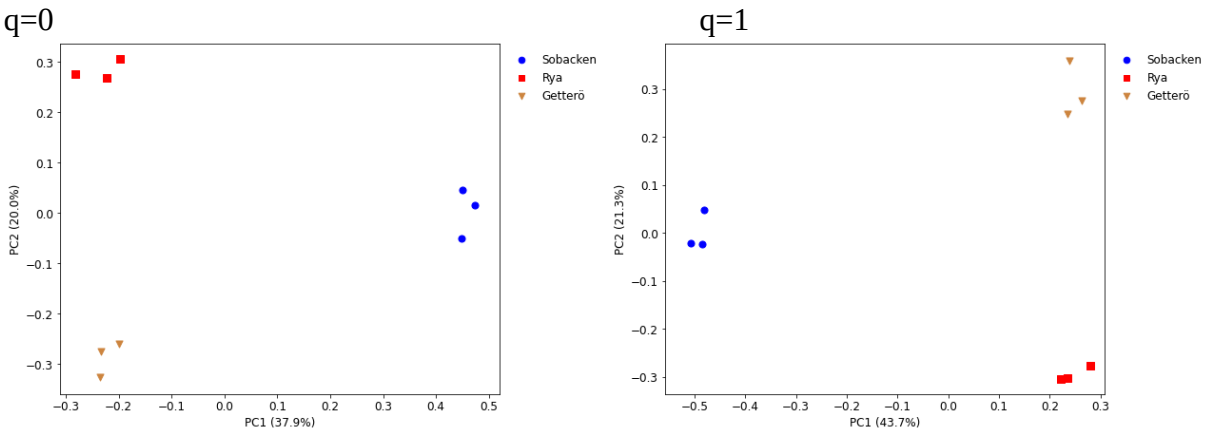

Figure H.23. PCoA plot for SingleM protein 9 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

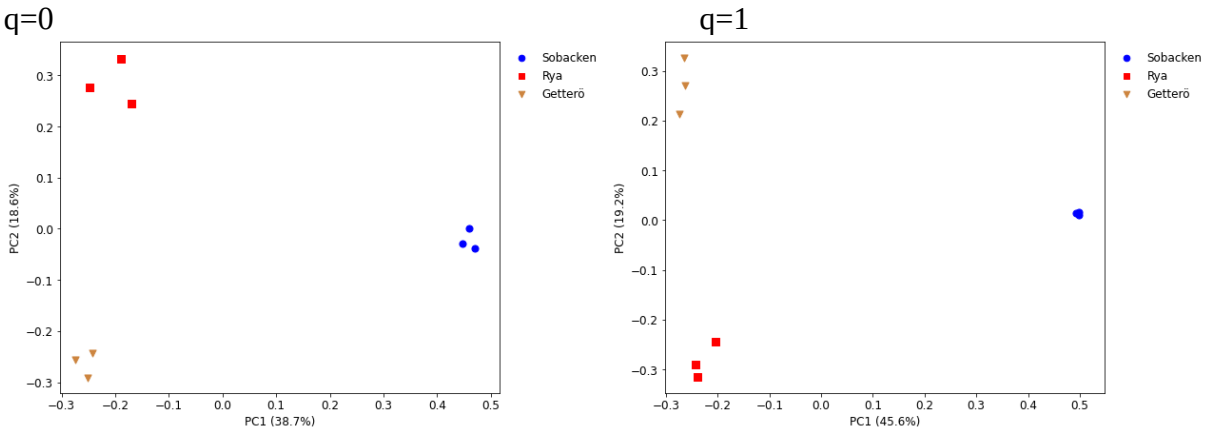

Figure H.24. PCoA plot for SingleM protein 10 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

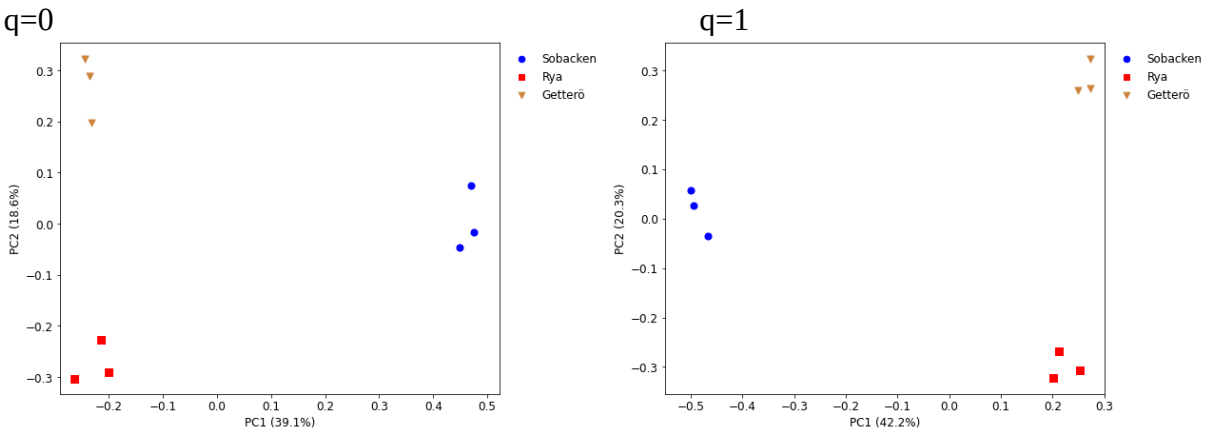

Figure H.25. PCoA plot for SingleM protein 11 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

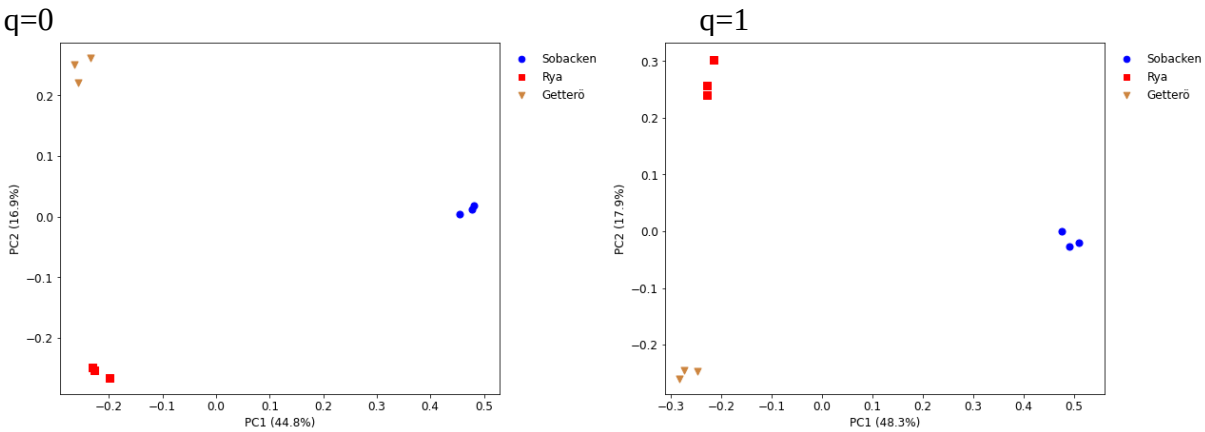

Figure H.26. PCoA plot for SingleM protein 12 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

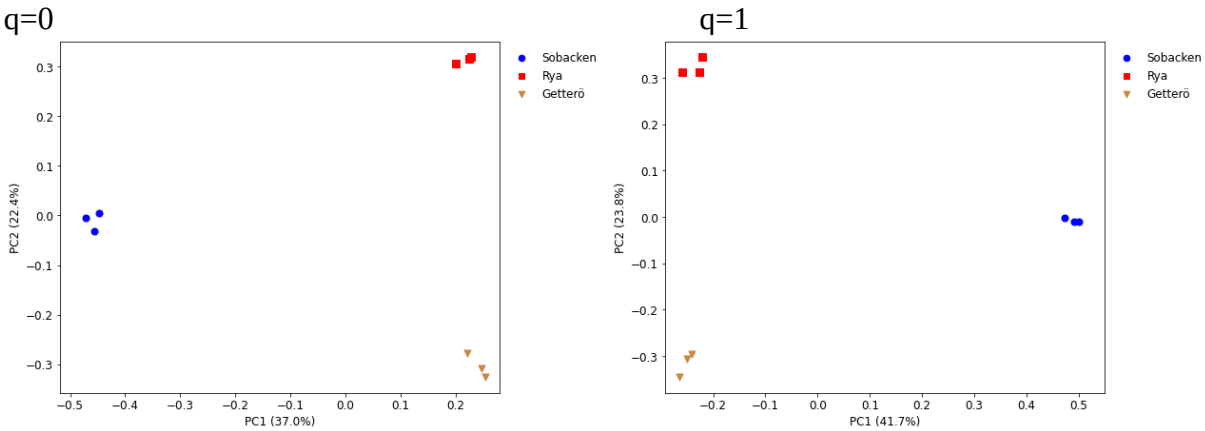

Figure H.27. PCoA plot for SingleM protein 13 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

q=0 q=1

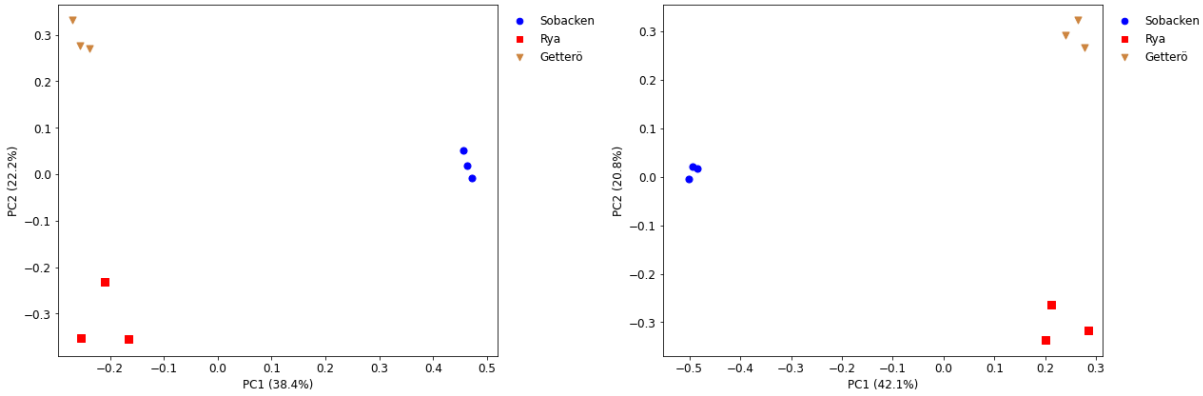

Figure H.28. PCoA plot for SingleM protein 14 displaying the samples from Sobacken, Rya and Getterö WWTP. The plot is based on a beta diversity dissimilarity matrices of diversity order *q*=0 to the left and *q*=1 to the right.

DEPARTMENT OF ARCHITECHTURE AND CIVIL ENGINEERING

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