Developing methods for on-site DNA sequencing

Master thesis in biotechnology

Peter Rendbæk

Center for microbial communities

Supervisors

Mads Albertsen

Per Halkjær Nielsen

Rasmus H. Kirkegård

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Author:

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Peter Rendbæk

Supervisor:
Mads Albersen,
Per H. Nilsen.
Rasmus H. Kirkegaard

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AALBORG UNIVERSITY
DENMARK

Department of Chemistry and Bioscience
Fredrik Bajers Vej 7H
DK - 9220 Aalborg Øst
www.ses.aau.dk

Preface

This project has been completed by Peter Rendbæk and submitted as a 9th and 10th semester Master's thesis at Aalborg University.

The objective of the project was to develop a method for infield and on-site DNA extraction to be used together with the MinION DNA sequencer from Oxford Nanopore.

Several persons have contributed academically, practically and with other types of support to this Master's thesis. I would therefore first like to thank my supervisors Mads Albertsen, Per H. Nielsen and Rasmus H. Kirkegaard for their time, valuable input and support throughout the entire master period.

Correspondingly, I would like to thank Jannie Munk Kristensen, Jakob Brandt and Martin Hjorth Andersen for they guidance and supervision in the laboratory. Furthermore, I would like to thank the Environmental Biotechnology(EB) group and Albertsen Lab at Aalborg University for lending a helping hand throughout my project, for guidance, a lot fun and great times throughout the production of this Master's thesis.

To finish, I would like to thank you, the reader, because if you are reading this line, you have at least read one page of my thesis.

Thank You.

Peter Rendbæk

Abstract

The efficiency of wastewater treatment plants is largely determined by their microbial composition. Therefore, identification of the microbial community is an important part of running a particular wastewater treatment plant and understanding how it functions. Currently, this is done in highly specialized laboratories, but this limits the method to being only able to look back at changes, and it is not being used to guide operational decisions. However, ongoing advancement of sequencing technology (Oxford Nanopore MinION) and automated sample preparation makes it theoretically possible to move sequencing out of the laboratory. However, to make this a reality, there is a need for a fast, cheap, reliable and highly mobile DNA extraction that works on par with state-of-the-art extraction methods.

In this thesis, an easy to use, fast and highly mobile DNA extraction method is developed. The method is based on a power tool with a 3D printed adapter for bead-beating based lysis of cells, and DNA is isolated using solid phase reversible immobilization beads. The method was compared to the state-of-the-art and recommended DNA extraction method for the field of activated sludge: the MiDAS field guide. The comparison of the methods was made on several levels including the amount of extracted DNA, purity and fragmentation. Furthermore, 16S rRNA amplicon sequencing was used to evaluate any potential extraction bias in the observed microbial community.

It was shown that the proposed DNA extraction method did not introduce a bias in microbial community composition and performed just as good on yield and purity. Correspondingly, it cut the total time for DNA extraction down to roughly 10 minutes compared to the 1-hour standard protocol.

However, further optimization is needed to make sure the method fulfills the high purity and long DNA fragment length requirement for the MinION. Overall, the developed approach provides a foundation for moving the DNA extraction and sequencing out of the laboratory and into the field.

Synopsis (Danish)

Effektiviteten af spildevandsrensningsanlæg er i høj grad bestemt af deres mikrobielle sammensætning. Derfor er identifikation af det mikrobielle samfund en vigtig del af at køre et bestemt spildevandsrensningsanlæg og forstå, hvordan det fungerer. I øjeblikket gøres dette i højt specialiserede laboratorier, men det begrænser metoden til at se tilbage ved ændringer og bruges ikke til at styre operationelle beslutninger. Imidlertid gøres der løbende fremskridt med sekventeringsteknologi (Oxford Nanopore Minion) og automatiseret prøveforberedelse gør det teoretisk muligt at flytte sekventering ud af laboratoriet. Men for at gøre dette til en realitet er der et behov for en hurtig, billig, pålidelig og meget mobil DNA-ekstraktion, der fungerer i lighed med State-of-the-art ekstraktionsmetoder.

I denne afhandling er der udviklet en brugervenlig, hurtig og meget mobil DNA-ekstraktionsmetode. Metoden er baseret på et kraftværktøj med en 3D-trykt adapter til bead beating baseret lysering af celler, og DNA isoleres ved anvendelse af fastfase-reversible immobiliseringsbeads
. Metoden blev sammenlignet med den nyeste og anbefalede DNA-ekstraktionsmetode for feltet af aktiveret slam-MiDAS-feltguide. Sammenligningen af fremgangsmåderne blev lavet på flere niveauer, inklusive mængden af ekstraheret DNA, renhed og fragmentering. Desuden blev 16S rRNA amplicon-sekventering brugt til at evaluere enhver potentiel ekstraktionsforstyrrelse i det observerede mikrobielle samfund.

Det blev vist, at den foreslåede DNA-ekstraktionsmetode ikke introducerede en bias i mikrobielle samfunds sammensætning og præsterede lige så godt på udbytte og renhed. Tilsvarende reduceres den samlede tid til DNA-extraction ned til ca. 10 minutter sammenlignet med 1-timers standard protokollen.

Der er dog behov for yderligere optimering for at sikre, at metoden opfylder kravet om høj renhed og lang DNA-fragmentlængde for MinION. Samlet set giver den udviklede tilgang et fundament for at flytte DNA-ekstraktionen og sekventeringen ud af laboratoriet og ind i feltet.

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Abbreviations

WWTP:	Waste Water Treatment Plant
MiDAS:	Microbial Database for Activated Sludge
OTU:	Operational Taxonomic Units
PCR:	Polymerase Chan Reaction
ONT:	Oxford Nanopore Technology
SPRI beads:	Solid Phase Reversible Immobilisation beads
EBPR:	Enhanced Biological Phosphorus Removal
PAOs:	Polyphosphate-Accumulating Organisms
VFAs:	Volatile Fatty Acids
PHAs:	PolyHydroxyAlkanoates
Df:	Degrees of Freedom
SEM:	Standard Error of the Mean
PCA:	Principal Component Analysis

1. INTRODUCTION

An important criterion for human civilization is wastewater management (Daims, Taylor and Wagner, 2006). In the management of wastewater, microbes are superior to human-made technology; they have an unmatched ability to degrade organic substance and cycle elements as nitrogen, phosphorus, and carbon (Daims, Taylor and Wagner, 2006). The natural development is to make an exploitation of microbes to clean wastewater, which is achieved in the modern biological wastewater treatment plant (WWTP).

1.1 Wastewater treatment plants

This exploration of the natural processes of microbes can be observed in a modern WWTP. This treatment can be divided three steps, a pretreatment, primary treatment and secondary treatment of wastewater, which is known as activated sludge. An overview of the WWTP and system that leads to the used of activated sludge can be seen in figure 1,

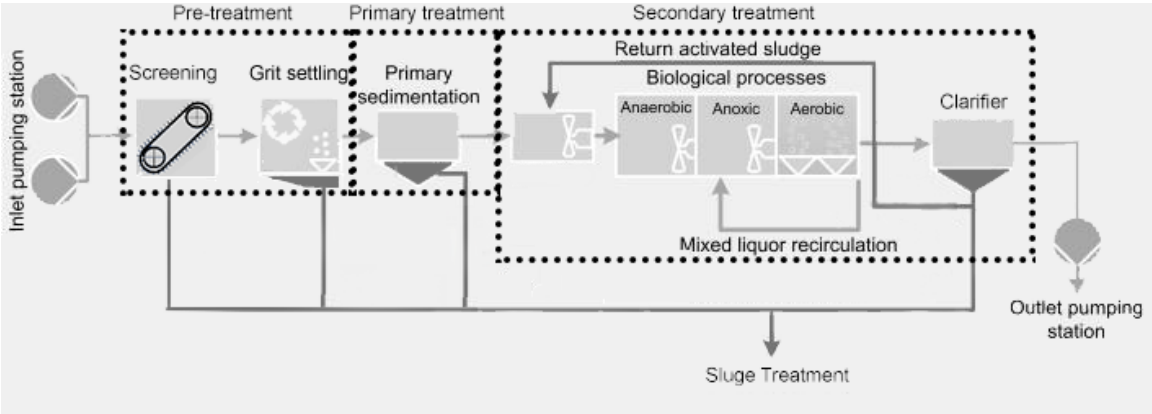


Figure 1: Schematic of a WWTP with activated sludge treatment. Picture modified from (Sulzer, 2017). The configuration shown in the illustration is a setup of the advanced system and is a series of reactors with an anaerobic, anoxic, and aerobic configuration, which has an aerobic mixed liquor recirculation to the anoxic reactor and a return sludge recycle to the anaerobic reactor (Michigan Water Environment Association, 2012).

In the WWTP before the wastewater can be treated by activated sludge, it needs to go through a pre-treatment and primary treatment of wastewater. The first two steps are pretreatment of wastewater. The screening removes objects such as rags, paper, plastics, and metals. The grit settling removes the organic, biodegradable solids heavier than a solid material such as sand (U.S. EPA, 2000). This is done to prevent damage and clogging of downstream equipment piping, and appurtenances (U.S. EPA, 2000).

This leads to the primary treatment in the form of sedimentation, where the settleable solids and floating solids such as grease, oils, plastics, and soap are removed (Water Environment Federation, 2008). Afterward, the secondary treatment of wastewater with activated sludge is applied. The basic activated sludge system uses a mix of microbes under aerobic conditions to remove organic compounds of carbon and nitrifying ammonia (Daims, Taylor and Wagner, 2006; Nielsen *et al.*, 2010; Saunders *et al.*, 2015). The basis system has in principle three main components. A bioreactor is containing an anaerobic and anoxic part, a clarifier to separate activated sludge from solids and the treated wastewater(IWApublishing, 2017). Lastly, it contains a return sludge recycle system that transfers the separated solids from the clarifier to the entrance of the aeration tank hence the name activated sludge (IWApublishing, 2017).

This system can be made more advanced with the addition of an enhanced biological phosphorus removal (EBPR) to remove phosphate using polyphosphate-accumulating organisms(PAOs) and can remove nitrate through denitrification (Saunders et al. 2015; Nielsen et al. 2010; Daims et al. 2006). EBPR works using the PAOs that can form storage compounds called polyphosphates (polyP) in excess of normal cellular requirements under aerobic conditions by utilizing volatile fatty acids (VFAs), such as acetate or propionate and store them as intracellular polymers polyhydroxyalkanoates (PHAs) (Urdalen, 2013). Under anoxic or aerobic conditions these PHAs are used for the formation of polyphosphate and growth of new cells (Sathasivan, 2009). Nitrifying of ammonia occurs in the aerobic reactor and then it is recycled to the anoxic reactor to be denitrified.The function and efficiency of the activated sludge and the wastewater treatment plant are determined by the structure and function of the microbial community (Nielsen *et al.*, 2010). The activated sludge and WWTP is an ideal system to model and understand the dynamic of a complex microbial community (Daims, Taylor and Wagner, 2006).

1.1.2 Microbial Database for Activated Sludge

To optimize the plant design and processes it is important to better understand the microbial community in WWTP (Nielsen *et al.*, 2012). This understanding is achieved through a survey of the core microbes in activated sludge that determines its function and composition of the microbial community (McIlroy *et al.*, 2015; Saunders *et al.*, 2015).

Surveys and the characterization of microbes have been gathered in the Microbial Database for Activated Sludge (MiDAS) which is a database that links the identity of process critical microbes with their function in activated sludge. MiDAS is a powerful tool to get a better understanding of the microbial community and its function. The overview of the MiDAS and its information can be seen in figure 2.

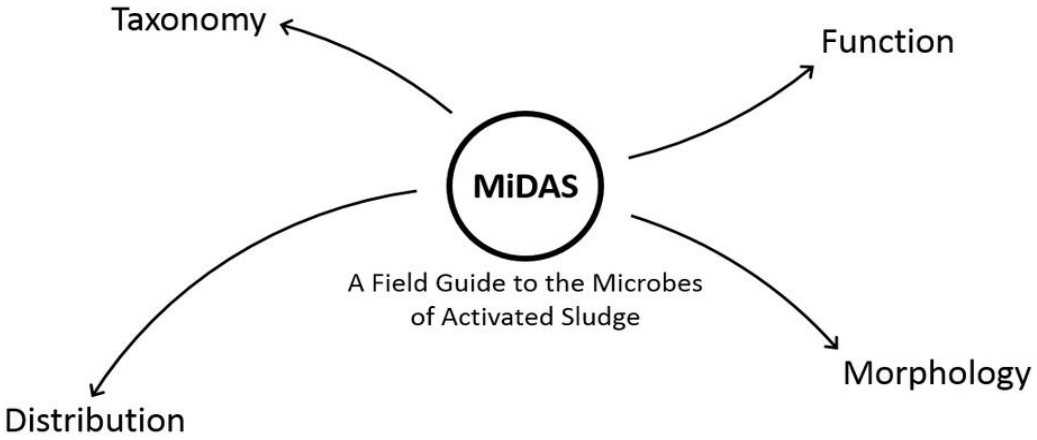


Figure 2: An overview of the MiDAS database.

The database gives more information on the ecology of activated sludge and the role of process critical bacteria in the microbial community, and this, in turn, will provide better design and more optimal running parameters. With the advancement of sequencing and molecular biology technology, it is possible to do a high throughput of samples and thereby determine to identify more bacteria (McIlroy *et al.*, 2015). One of the methods used to identify microbes today is the 16S rRNA gene amplification method, which is also employed in the MiDAS field guide to link identity of microbes with their function.

1.2 Identification of microbes and its community by use of 16S rRNA gene amplification and sequencing

Identifying microbes is an important part of understanding the dynamics in the microbial community, and through advanced sequencing technology microbes in microbial communities can be determined (Goodwin, McPherson and McCombie, 2016). The 16S rRNA gene amplification makes use of a phylogenetic marker in the form of the 16S rRNA gene. A phylogenetic marker is a genetic marker that is present in all organisms. The function of the marker should be highly conserved in functionality, and it must contain highly conserved and variable regions (Patwardhan, Ray and Roy, 2014; Karst *et al.*, 2016). This is fulfilled by the 16S rRNA gene, and it contains nine variable regions, called v1-9, with conserved regions in between (Ashelford *et al.*, 2005). Using this method, it is possible to make an estimation of the composition of the microbial

community and an identification of its constituents. A basic overview of the workflow can be seen in figure 3.

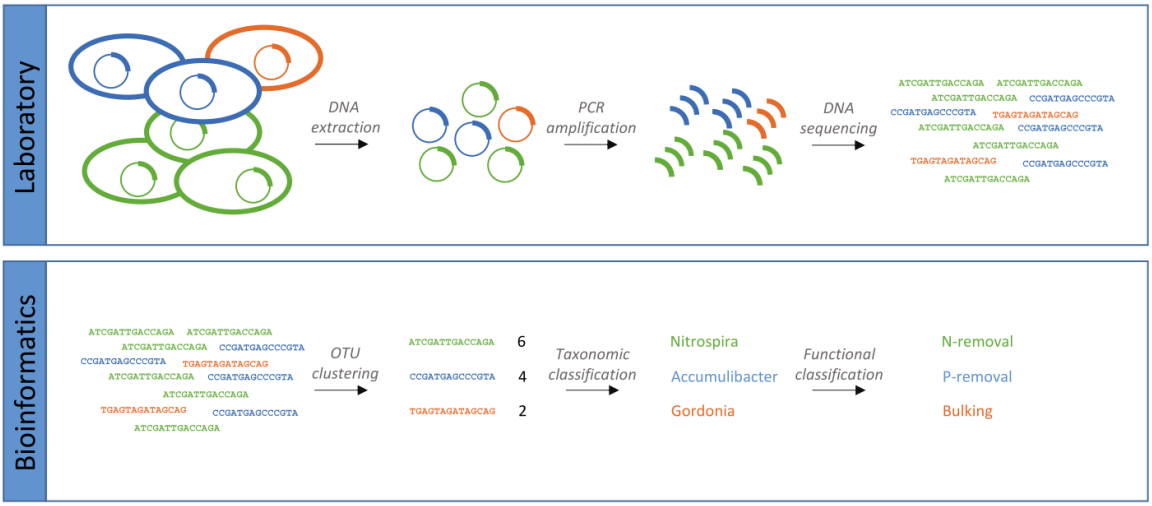


Figure 3: Overview of 16srRNA gene amplicon sequencing, The figure is from (Karst *et al.*, 2016)

The method can be divided into a laboratory and bioinformatics part. In the laboratory, the genomic DNA from the microbial community is extracted, isolated and purified. Afterward, this 16S rRNA gene in the genomic DNA is amplified, using polymerase chain reaction(PCR). The primer used is designed to bind to conserved parts of the 16S rRNA gene; this primer also enables the sequencing of the 16S rRNA amplicons. It is enabled by use of an adapter, which attaches the amplicons and adaptor to the sequencing machine, the adaptor is similarly used as a barcode for multiplexing (Illumina, 2017).

The final product after the PCR is called a 16S rRNA gene amplicon sequencing library, which can be sequencing on an Illumina modern sequencing machine (Karst et al., 2016). The data is generated by sequencing and is made into high quality through a bioinformatic process which filters out low-quality reads from the data (Karst et al., 2016). The high-quality reads are clustered into operational taxonomic units(OTU) in a process called OTU clustering (Karst et al., 2016). The clustering is achieved with a sequence identity of 97% (Karst et al., 2016). Reads mapping to each OTU are counted, and a representative read is selected. This is used in the following taxonomic classification; the representative reads are then compared against a reference database of 16S rRNA gene sequences, in this case, the MiDAS database (Karst et al., 2016). The result from this process is an OTU table where each row is a different OTU with its taxonomic classification, and the column represents each sample, and, each cell has the count of reads (Karst et al., 2016). This table can then be used for data analyses of the microbial community.

This method is not without drawbacks. It introduces bias into the analyses; which will affect the final observed microbial community. Two major biases in this method are the 16S rRNA gene copy number, where a microbe can contain up to 15 gene copies (Větrovský and Baldrian, 2013). This affects the count of an OTU and microbes with more than one 16S rRNA genes would be artificially inflated and seem to have a higher abundance. The next major bias is the primer affinity since not all primers have the same affinity for the microbial genes in the sample. Therefore, a species in one sample can get under or over-represented (Albertsen *et al.*, 2015; Brooks *et al.*, 2015). These are biases introduced in the method, but there are also more general biases, not specific for this approach introduced in the DNA extraction. If a different DNA extraction kit is implored, it can introduce bias based on the kits used which will affect the final microbial community (Guo and Zhang, 2013; Albertsen *et al.*, 2015). Even with its drawbacks, it is still a powerful tool to identify microbes in a community

However, as described in MiDAS, the bacterial metabolism determines the effectiveness of the biological treatment. However, the understanding of the relationship between the operational parameter of the WWTP and the microbial community is still limited (Cydzik-Kwiatkowska and Zieliinska, 2016). This limited understanding combines with the need for information on the microbial community to increase the effectiveness of decision-making and WWTP. This information can be obtained with use of 16S rRNA amplicon method, but it is not a method that is usable to do this kind of on-site survey. The main problem is that the method is a time-consuming method where it can take days to weeks to get the needed information for operational decisions making, and it requires specialized laboratories. Similarly, the transportation of samples from the WWTP to the laboratory if not done correctly, can have an effect on the overall microbial community which can influence the conclusion of a weekly timeframe (Albertsen *et al.*, 2015).
The solution needs to be faster and more mobile.

1.3 Need for on-site identification of microbial community.

To archived this, a development of fast and on-site microbial identification is necessary. With the advancement in the sequencing technology, this on-site and real-time sequencing is becoming a reality, and in the future, it could become as easy as PH measurement at the WWTP (Goodwin, McPherson and McCombie, 2016). However, the current technology (Illumina MiSeq) used for the 16S rRNA gene amplification relies on batch runs to obtain a larger amount of DNA sequence data (Illumina, 2017; Mitsuhashi *et al.*, 2017). The benefits of this method are the low price per sample and high throughput. But the main drawback and the reason why the Illumina MiSeq is not optimal for an on-site microbial identification solution is it is large and heavy size (Illumina,

2017). However, a new technology that has a small enough footprint to be quite mobile and has a possibility to do real time sequencing is the Oxford Nanopore sequencing.

1.3.1 Oxford Nanopore sequencing

Many companies have developed nanopore sequencing technology, and many methods have been developed, but only the MinION from the Oxford Nanopore Technology (ONT) has been successfully employed by independent genomic laboratories(Jain *et al.*, 2016). The MinION is a 90-g portable USB powered, nanopore-based DNA sequencing platform. The MinION is an array of protein nanopores placed in an impermeable polymer membrane which results in an ionic current through the pore (Jain *et al.*, 2016; Johnson *et al.*, 2017; Smith *et al.*, 2017).

By measuring the ionic current changes of DNA passing through this nanopore in single nucleotide steps regulated by a processive enzyme, this generates a characteristic current change which enables the nucleotides to be sequencing. This gives a possibility to produce a massively parallel continuous read length of over >100 kb (Mikheyev and Tin, 2014; Jain *et al.*, 2016; Johnson *et al.*, 2017). A picture of the MinION and overview of how the MinION works can be seen in figure 4.

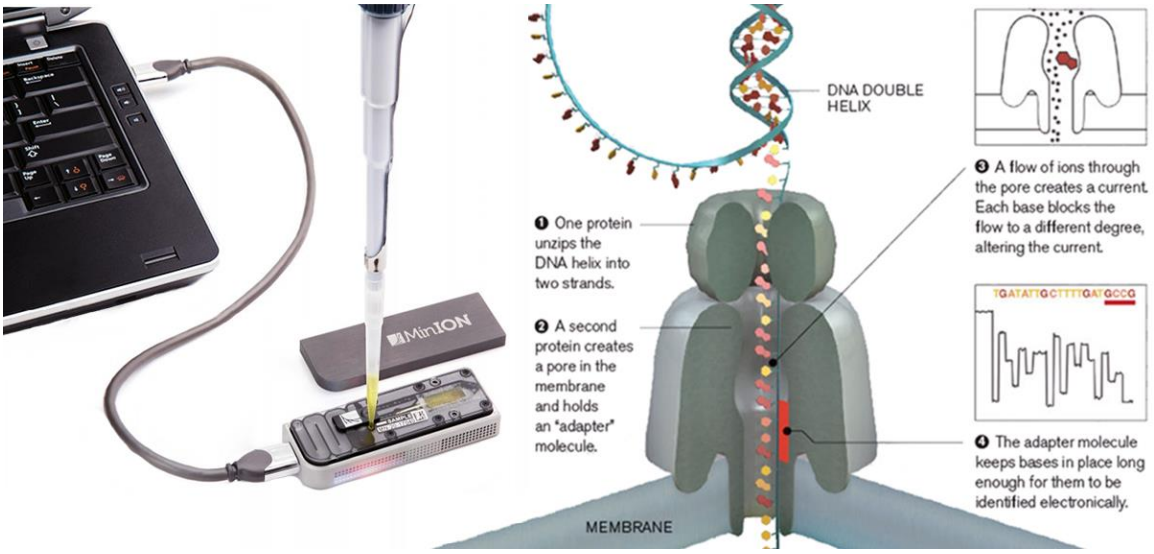


Figure 4: The figure shows a MinION. **Left**: a MinION is getting connected to a computer and is getting loaded with a sample. The figure is from (Regalado, 2016) **Right**: a schematic on how the Minion works. DNA can be sequenced by threading it through a microscopic pore in a membrane where the bases are identified by the way they affections flowing through the pore from one side of the membrane to the other. The figure and text are from (SCHAFFER, 2012)

One of the vast possibilities of this technology is to enable the real-time sequencing and identification of a microbial community. This means that there is no fixed run time with the MinION, and the sequencing can run until sufficient data is garnered (Oxford Nanopore Technologies, 2017b). Another possibility is to circumvent the uses of PCR and allowed reading full-length 16S-gene or smaller fragmentation directly from the genomic DNA or reading by 16S rRNA directly from the microbial community (Rosselli *et al.*, 2016; Smith *et al.*, 2017).

One of the main problems in achieving this is the lack of high-quality and full-length reference sequences being deposited into reference databases since the main method used today is the shorter fragment, high-throughput methods such as Illumina (Schloss *et al.*, 2015). The real-time sequencing lowers the time as it has been demonstrated that only 5 min of sequencing time is needed to detect all bacteria in a mock community (Mitsuhashi *et al.*, 2017).

Combining this with the 10 min rapid library preparation kit from ONT, it is possible to archive rapid sequencing and identification of the microbial community using only a MinION and laptop computer in small laboratory settings (Edwards *et al.*, 2016; Mitsuhashi *et al.*, 2017).

1.3.2 The use of the MinION sequencing mobility

The use of the MinIONs portability, real-time and flexibility have been shown in several fields. Two of the fields, which have shown significant improvement, are the genomic epidemiology and metagenomics.

Two examples of the genomic epidemiology are the genome sequencing of the West African Ebola virus outbreak in 2014-2015 and the ZiBRA project. In the first case, they made a genome surveillance system that was portable and included three MinION instruments, four laptops, a thermocycler, a heat block, pipettes and sufficient reagents and consumables to make genome sequencing of the Ebola virus (Quick *et al.*, 2016). All this was packed into an <50kg of standard airline travel luggage (Quick *et al.*, 2016). With this system, it was possible to achieve real-time genomic surveillance in resource-limited settings that is easily transported and can be established rapidly to monitor outbreaks (Quick *et al.*, 2016).

The next example is the ZiBRA project which, inspired by the success of the Ebola virus genomic surveillance, mapped and monitored the Zika virus outbreak in Brazil in the field (Faria *et al.*, 2016). In this project, a mobile transportable genome sequencing laboratory is constructed in a caravan to be towed after a car (Faria *et al.*, 2016).

Another field benefitting from the MinION is the field of metagenomics, where the technology is used to make infield metagenomic DNA sequencing. The metagenomic field has the problem of transfer of microbial samples to a laboratory to perform a characterization of microbial diversity since there are many logistical challenges in this. A solution to this is the portability of the MinION that is reported in (Edwards *et al.*, 2016) where they used it to characterize the microbiota of high arctic glacier in Svalbard. The laboratory used was contained in 2X23 kg deployment bags that still required mains electricity and internet access. Afterward, they optimized the field laboratory to achieve off-grid metagenomics.

The optimization was a reduction of weight to 12 kg 45-liter rucksacks that contain the means for DNA extraction, quality control, and DNA sequencing. The laptop used to contain the bioinformatic package capable of conducting metagenomics worked without internet connectivity and electricity. The initial testing of the rucksacks was a success, but they were limited by short read lengths and low yield of DNA (Edwards *et al.*, 2016). They experienced two problems for the on-site DNA sequencing. The first problems is sample preparation and the second issue is the DNA extraction.

1.3.3 VolTRAX: Automated sample and library preparation.

Besides preparing and loading the sample on the MinION, the most difficult practical part of the sequencing is the sample preparation. Thus to simplify, ONT developed the VolTRAX; an automated sample and library preparation device (Oxford Nanopore Technologies, 2017d). It uses a disposable array of pixels to apply a charge to move micro liquids; it also contains a heating zone for heating up samples if PCR is needed.
By combining the microfluidic control of VolTRAX with the 1D rapid library prep, it is possible to simplify the sample and library preparation and make the transition to move sample preparation out of the lab easier. However, this technology is still in its early stage, it needs to be improved to be used optimally and will be dockable with the MinION, a picture and overview of working can be viewed in figure 5 (Oxford Nanopore Technologies, 2017d).

Figure 5: Left: The VoltTRAX with its disposal workcell **right:** overview of how the VolTRAX works by adding a sample and using a charge to move them. The pictures are from (Oxford Nanopore Technologies, 2017d)

1.3.4 A need for a mobile solution for DNA extraction,

The norm is still to use a DNA extraction kit that is tailored to be employed in a laboratory setting and not designed to follow the mobility and flexibility the Minion offers. Therefore, the development of a DNA extraction method that can follow suit is needed. It is also important that the DNA extraction method developed be adapted to be used on environment samples such as activated sludge and will give sufficient DNA yield and fragment length. As mentioned earlier, a DNA extraction kit can introduce bias. Hence it is necessary to show that the DNA extraction kit used does not introduce major bias (Guo and Zhang, 2013; Albertsen *et al.*, 2015). It is important to test the DNA extraction kit thoroughly; this is achieved by comparing it against a standard DNA extraction method. The method developed is compared to the MiDAS protocol, which is a golden standard DNA extraction method that is optimized to be used on activated sludge. Thus, there is a need and a requirement to develop and evaluate a DNA extraction

method which is portable, fast, easy to use and suited to be used together with the MinION to move sequencing out of the laboratory and into the field.

2. AIM

The objective of this project is to develop a mobile, fast, robust and easy to use DNA extraction protocol to enable close to real-time and on-site identification of microbes in activated sludge and compare it to the current state-of-the-art methods.

Specific objectives to address:

- Design and evaluate a cheap mobile bead beating tool
- Develop and evaluate a fast DNA extraction protocol.
- Compare all results to the golden standard within the field.

3. MATERIALS AND METHODS

3.1 Strategy on evaluation the mobile method tested in this thesis.

In this project 16S rRNA, gene amplification is used to evaluate a mobile DNA extraction method and compare it to a standard of DNA extraction from activated sludge and general recommendation by the MiDAS field guide. The comparison is achieved by using a standard sample that is handled and stored at -20^{o}C identically. The standard sample used in this thesis is activated sludge from Aalborg West WWTP.

The DNA was then extracted with the variations of the mobile method proposed in this thesis and the standard DNA extraction recommendation by the MiDAS with three replicas of each. The extraction was compared to the quantity and quality of the extracted DNA. The DNA quantity was determined by use of the quantitative fluorometric method QuBit.

The DNA quantity is stated as total DNA amount in the samples since the same sample input amount, and elution volume is used across all methods. The DNA purity is examined by use of the Nanodrop to determine the $A_{260/280}$ and the $A_{260/230}$ ratio. $A_{260/280}$ determines the purity of DNA in the sample and should lie around 1.8, and $A_{260/230}$ is a secondary measurement to determine purity and should be over 2.0 (ThermoScientific, 2011).

All the replicas and measures are shown as the mean of the three replicas and the standard error of the mean (SEM). The quality is analyzed by viewing the DNA length; this is archived using agarose gel on an Agilent 2200 Tapestation and Genomic DNA screentapes (Agilent, USA). After the DNA quantity and quality were determined, the microbial communities are studied, this is achieved by using 16S rRNA gene amplification of the region V1-3, and the sequencing was done on an Illumina MiSeq. The data was put through the bioinformatic workflow as described in material and methods. The sequencing reads are clustered into operational taxonomic units (OTUs) that are assigned taxonomy using the MiDAS database for each sample. Samples with less than 3000 reads and low abundance OTU less than 10 reads are removed from further analysis. Afterward, the methods are compared with the use of heat map comparing the top most abundant OTUs in the samples. The total microbial community is then analyzed by use of principal community analysis(PCA). An overview of the determined pipeline can be seen in figure 6.

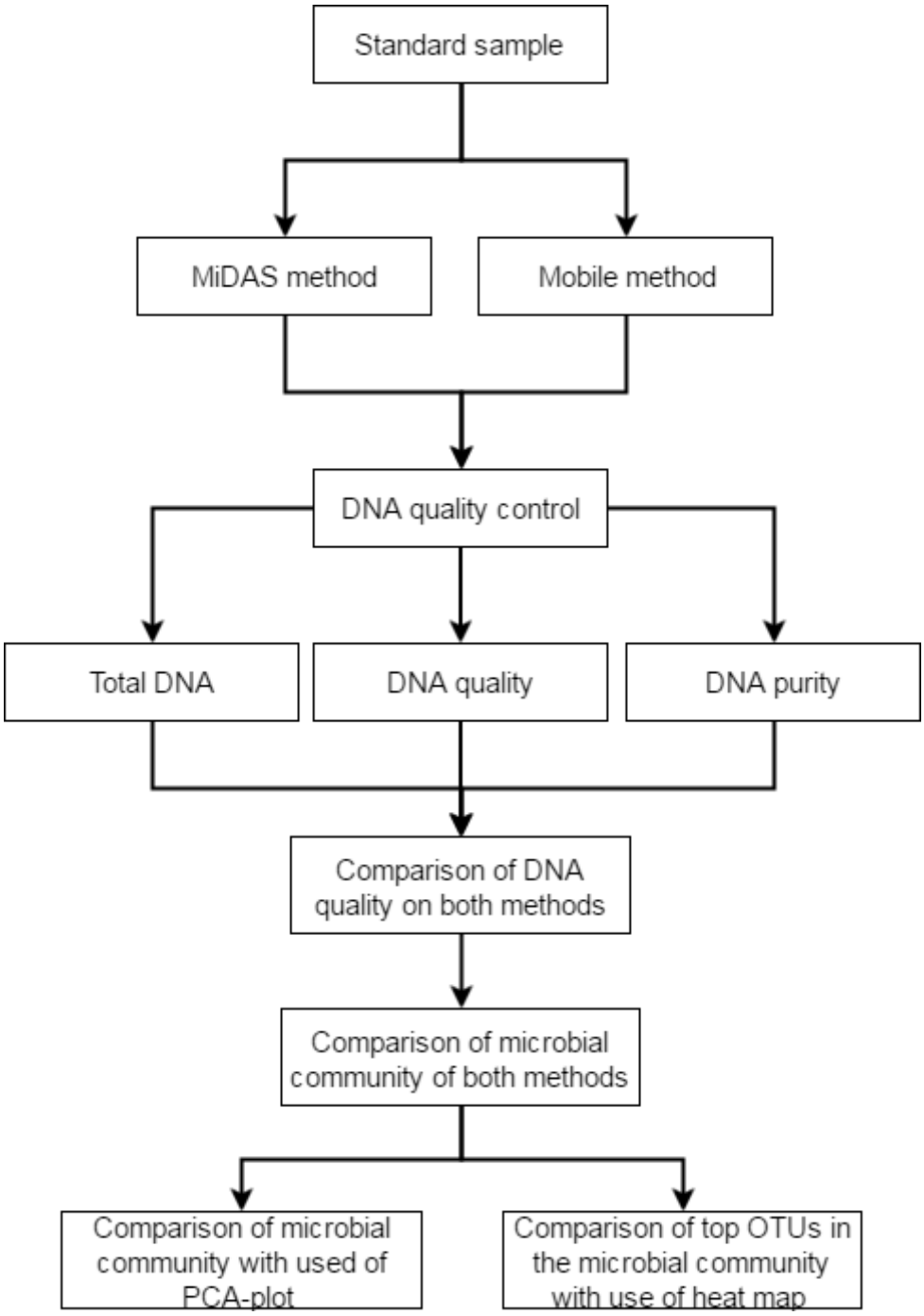


Figure 6: Overwiew of the eksarimental test done in this thesis, the new method repersent variastion of the new mobile method tested in this thesis

3.2 Sample collection

The samples were collected by the method described in the MiDAS protocol (Aalborg University, 2017b), four liters were collected the 20/9-2016, homogenized and distributed into two mL sample tubes, after that the sample were stored at -20°C until the DNA extraction.

3.3 DNA extraction method

The DNA extraction and isolation were performed with two methods. The MiDAS extraction protocol and Mobile method with variations.

3.4.1 MiDAS protocol

The Midas method is described at the MiDAS database homepage (Aalborg University, 2017b). The extraction used the Fast-spin kit for soil from MP Biomedical as a basis, just with modification, mainly streamlining and longer bead beating time. After DNA extraction and before the sequencing libraries preparation, the sample is stored at -20°C.

3.4.2 Design and assembly of the Mobile Bead-beater.

The Mobile Bead-beater is a power-tool with an MBB adaptor.
The power-tool is for the mobile bead-beater is an Akku multi cutter model nr: DM8503+71348. The MBB adaptor is designed in SolidWorks 3D computer assisted design 2017 and is uploaded onto the web page Thingiverse.com(Rendbæk, 2017) for easy access. The model from Thingiverse.com and final assembly can be seen figure 7.

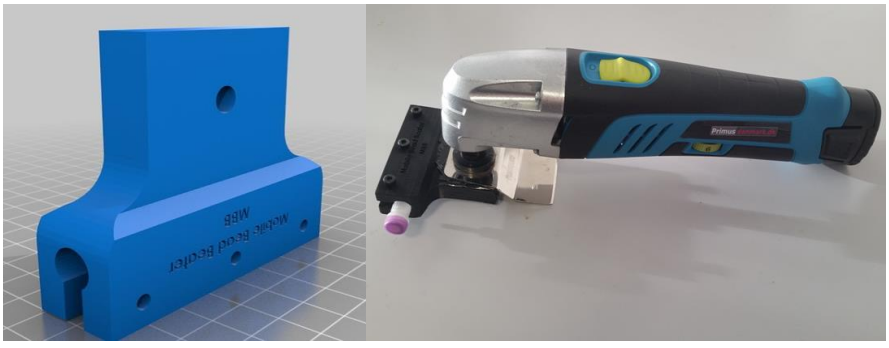


Figure 7: Left is the model of the MBB adaptor as seen on thing verse (Rendbæk 2017). Right is an picture of an final assemble mobile bead beader.

The design was printed in an CreatBot DX with PLA plastic. The setting of the Mobile bead beater was an infill density of 60% and a shell thickness of 1mm, and with support.

Rest of the setting is the core setting for PLA plastic on the CreatBot DX as seen on (Henan Suwei Electronics Technology co., 2017).
The mobile was adding with screws to add integrity and the hole that the holds the beating tubes were grinded to grab the beating types firmly. The adaptor is mounted on the power tool as seen in figure 7.

3.4.3 Mobile method

The sample was prepared as described in MiDAS protocol, were 500 µL sample were transfer to the Lysing Matrix E-type with 480µL PBS buffer and 120 µL MT Buffer from FastDNA® Spin kit for soil (Aalborg University, 2017b). The bead-beating were performed with the mobile bead-beater, the beating time was carried out with two variations of time with can be seen in Table 1. The sample was spun down with a centrifuge; this was performed with 3 variations and can be seen in Table 1.
Subsequently, the 850uL supernatant was transferred to a tube containing diluted SPRI beads in the form of Agencourt AMpure XP(Beckman Coulter, 2016). The beads were diluted in SPRI beads dilution buffer with a variation ratio of 1:5(170uL SPRI beads volume) and 1:10(80uL SPRI beads volume) this can be seen in Table 1.

The beads were incubated 5 min and placed on a magnet stand for 2.5 min to magnetic separated the beads from the supernatant. The supernatant was removed, and the beads were washed with 1mL 70%ethanol two times. The tube contains the beads were centrifuged short(5sec) to dry the beads. The tube was removed from the magnetic stand, and the beads were resuspended in 63 µL nuclease-free water to elute the purified DNA on the beads. The tube is placed on the magnet stand to separate the beads from the purified DNA. 60 µL from the tube is transferred to a new container. The variation of the method can be seen in Table 1.

Table 1: The variation of the mobile method tested in this survey.

Name	Bead beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	10min 14000xg	170
Mobile 2	4 x 40 sec.	1min 14000xg	170
Mobile 3	4 x 40 sec.	1min 2000xg	170
Mobile 4	60 sec.	1min 2000xg	170
Mobile 5	60 sec.	1min 2000xg	80

3.4.4 SPRI beads dilution buffer

8g of PEG-8000 is diluted into 20mL 5M NaCl; this is then heated to dissolve the PEG-8000. Afterward, the solution is diluted to 40 mL with DNA free water, giving a final solution of 20 w/w% PEG-8000 and 2.5 M NaCl.

3.4.5 Quality control of DNA extractions

After DNA extraction, the purity of the DNA was evaluated spectrophotometrically from Nanodrop1000 and using A260/230nm and A260/280nm (Thermo Fisher Scientific, USA) (Albertsen *et al.*, 2015). Also, the length and quality of the DNA were estimated by use of agarose gel electrophoresis, using the Tapestation 2200 and Genomic DNA screentapes (Agilent, USA) (Albertsen *et al.*, 2015). The DNA concentration was measured fluorometrically by use of Quant-iT DNA BR DNA assay (ThermoFisher Scientific, 2016), with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA).

3.5 Sample preparation and sequencing of 16S rRNA gene libraries

Samples from the extraction method were prepared for sequencing of the V1-3 variable region of the 16S rRNA gene were done as described by (Karst *et al.*, 2016). The sample were prepared for PCR by using 2µL DNA from the extraction as temple, reaction also contained, Platinum®Taq DNA polymerase High Fidelity(1U), 1Xplatinum High Fidelity buffer, dNTPs (400 nM of each), MgSO4(1.5mM) and last barcode library adaptors(400 nM) with a total volume of 25µL. The library was made with duplicates. The PCR thermocycler setting can be seen in Table 2 where the cycle repeated 30 times.

Table 2: PRC setting used in 16s rRNA gene amplification. Table modified form (Karst *et al.*, 2016)

Step	Temperature	Time	
Denaturation	95 °C	2 min	
	40 cycles		
Denaturation	95 °C	20 s	
Annealing	56 °C	30 s	X 30
Amplification	72 °C	60 s	
Amplification	72 °C	5 min	
Storage	4 °C	Forever	

A negative control contains nuclease-free water, and the positive contain extracted digester DNA, this was were included in all PCR steps and sequencing. The Amplicon libraries duplicates from the PCR were pooled, and the PCR were purified using AMpure XP bead (Beckman Coulter, 2016).

The AMpure XP beads were added to the sample with a 40uL to the 50uL library and were eluted into 23 uL nuclease-free water, 20 uL were transferred to a new container. The library concentration was measured with Quant-it HS DNA assay and visualized on tape station 2200 with D1K ScreenTapes. The sample was equimolar pool concentration on to a type. After the library pools had been done, the sequence was carried out on an Illumina MiSeq. The protocol and chemical can be view at the MiDAS database homepage (Aalborg University, 2017a).

3.5 Bioinformatics

The raw data in from of paired-end reads from the Miseq were trimmed using Trimmomatic (v.0.32)(Bolger, Lohse and Usadel, 2014) and after that, the trimmed reads were merged by FLASH(v.1.2.10) (Magoč and Salzberg, 2011). After the merge, potential phix contamination was removed by use of USEARCH(9.2) (Edgar, 2010), was phix is a virus genome that is used as a positive control in the sequencing. After that, the reads from the run were clustered into OTU using USEARCH, with a sequence identity threshold of 97%.

After this taxonomic classification was done by using the Ribosomal Database Project classifier(v.2.2) (Wang *et al.*, 2007)with the MiDAS database (v.2.1.2) (McIlroy *et al.*, 2015). The sequencing data were analyzed in R (3.4.0) (R Core Team, 2017) with Rstudio (1.0.143) (RStudio Team, 2017) using amvis package (Albertsen *et al.*, 2015) for visualization of the sequestering data.

4. RESULTS AND DISCUSSION

4.1 Improving and simplifying DNA extraction

DNA extraction can be a time-consuming progress but is an essential step in microbial community analyses. The DNA extraction is divided into cell lysis, cell debris removal, and DNA isolation. DNA extraction has been optimized and developed by the following criteria.

4.1.1 Criteria for improvement

Safety: The requirements to the method developed are that it must be safe to use and using dangerous chemicals must be avoided; this is crucial to make on-site identification of microbes. DNA extraction must be done without the need of a protection suit or special training to handle chemicals or use the equipment.

Mobility: The equipment utilized for the method must be easy to transport, and chemicals used must be able to be stored at room temperature for a day or two. The equipment and chemicals used for the method must be light in weight so it can be carried by hand or in a backpack.

Compatibility with Oxford Nanopore Technology: The DNA extraction from the method must fulfill the requirements for being used in with the MinION sequencer. Oxford Nanopore has made demands that for optimal usage of the rapid sequencing kit. DNA purity measured using Nanodrop must have an OD 260/280 of 1.8 and OD 260/230 over 2.0. Average fragment size of >30kb and input mass, measured by Qubit, 200 ng and no detergents or surfactants in the buffer.

Reproducibility: It is necessary that the method can reproduce the same results with small variations in the microbial community.

The following sections will describe the results leading to the final mobile DNA extraction method. This method will be referred to as the mobile method and in figures just as mobile. The mobile method is described in three major steps in DNA extraction; cell lysis, cell debris removal, and DNA isolation. The mobile method is continually compared to the method recommended by MiDAS field guide (Aalborg University, 2017b). This approach is referred to as the MiDAS method in the following sections.

4.1.2 Description of the mobile method and improvement of cell lysis, cell debris removal, DNA isolation and compared to MiDAS

The first step of DNA extraction is cell lysis. The mobile and MiDAS method both utilize a combination of mechanical and chemical lysis. Both approaches use the bead-beating tubes and lysis buffer from Fast-spin kit for soil from MP Biomedical. The difference between the MiDAS method and the mobile method is the tool used. The MiDAS method uses bead-beater that is specially made for the kit from MP Biomedical, whereas the mobile method uses a converted power-tool. The difference between the two approaches can be seen in Table 3.

Table 3: Table with picture and data for the bead-beater used in the two methods. Picture and data for the FastPrep are from (MP Biomedicals, 2017)

Method	MiDAS	Mobile method
Comparison	Standard bead-beater: FastPrep®-24	Mobile bead-beater
Weight	20 kg	1 kg
Sample nr.	24	2
Price	62366 dkk.	500 ddk.
Picture		

The mobile bead-beater used in the mobile method is a modified multi cutter that is typically used for removing paint and cutting in lighter materials. The modification from the standard multi cutter is a 3D printed adapter, which is made to hold two samples. This adaptor was printed with PLS plastic with a 60% infill density, but there is a need for the adaptor to be printed in a more durable material if a longer operation is necessary. By comparing the two bead-beaters, the first major difference is the weight. The FastPrep weighs 20 kg, and the mobile bead-beater weighs one kg. The weight reduction made it handier to transport the mobile bead-beater, but at the cost of the number of samples. The mobile bead-beater can handle two samples whereas FastPrep can handle 22. It is possible to increase the number of samples by changing the MBB adaptor, but increasing weight is a limiting factor since it would put a strain on the power tool. Another large

difference between the two tools is the price since the Mobile bead-beater has a price reduction of 99% compared to the FastPrep.

The seconded step of DNA extraction is cell debris removal. The mobile and MiDAS method both utilize centrifugation to remove cell debris from cell lysis. In the MiDAS method, a centrifugation of 10 min at 14000xg is used. This centrifuge time and speed are quite excessive and a reduction of centrifuge time and speed are tested for the mobile method. Two faster approaches made and tested, centrifuged at 14000xg and 2000Xg for 1 min.

The third and last step of DNA extraction is DNA isolation. The MiDAS method uses a binding matrix to bind DNA, and a spin column is used to isolate the sample. This is a time-consuming process, so in the mobile method DNA isolation was done by using Solid Phase Reversible Immobilisation (SPRI) beads, frequently used to purify DNA after PCR. The protocol is the same as for purification of DNA after PCR, but with some modification to make the protocol more suitable to handle the large quantities of genomic DNA. The volume of beads is also reduced since a 1:1 ratio of sample/beads solution is not needed. Instead, the beads solutions are diluted in buffer contain PEG-8000, 2.5 M NaCl.

4.1.3 Comparison of the mobile method with MiDAS and commercially available DNA extraction kit, in price and extraction time.

A comparison of the method is made in the following category: price, time, the maximum number of the samples and additional required equipment. An estimation of the price per sample for the mobile method is in Appendix A. The cost of the MiDAS method is the price of the Fast spin DNA kit for soil. The following comparison is made from the data found in the rapport: *Technical Evaluation of Sample-processing, Collection, and Preservation method* done for the US. Army Chemical Biological Defense Department (Betters *et al.*, 2014). A selection of the 6 most prominent commercial DNA extraction kits in the form of cost per sample and processing time have been selected and can be seen in Table 4.

Table 4: An overview of extraction method costs per sample, time, the maximum number of the samples and an additional required equipment. Data and table are from (Betters *et al*., 2014), and the price for the MP FastDNA Spin Kit for Soil are from MP BIOMEDICALS

Method	Cost/sample (DKK)	Processing time(min)	Maximum No. Samples	Additional Required Equipment
Mobile Method	30	11:30	2	Magnet stand Mobil beater
MP FastDNA Spin kit for Soil (MiDAS)	58	50	22	Fastprep instrument / FastPrep®-24
AutoGen QuickGene-Mini80	2	60-90	8	QuickGene instrument, AutoGen reagents
Bio-Rad InstaGene Matrix	5	30-45	30-45	N/A
Claremont PureLyse	52	5	1	N/A
MoBio PowerSoil	34	40	22	Vortex adaptor
Akonni TruTip	36	15-60	8	Rainin pipette, thermomixer

The first comparison is the cost per sample, the cheapest method is the QuickGene-Mini80 DNA purification system, these costs only 2 DKK per sample, but the method requires that QuickGene BioRad's Instrument is bought which costs 12.600 DKK and AutoGen reagents (Betters *et al.*, 2014). The method following this in price is the Bio-Rad Instagene Matrix the cost of which is 5 DKK per sample without additional equipment. The third cheapest method is a mobile method that costs around 30 DKK per sample, but the mobile method requires the mobile bead-beater with a price of 500 DKK and a magnet stand for SPRI beads. MoBio PowerSoil kit has a cost of 34 DKK but requires a Vortex adaptor. Finally, we have the MiDAS with the MP FastDNA Spin Kit for Soil at the price of 58 DKK, but this also requires for optimal use that a Fastprep instrument is bought, as seen in Table 3 this tool costs 62366 DKK (Betters *et al.*, 2014).

The next comparison is the processing time from sample to extracted DNA. This criterion is where multiple methods are sorted off since the only method that beats the mobile method was the Claremont Purelyse gDNA extraction kit. The Purelyse method

could do sample preparation in 5 min, whereas the mobile method could do it in 11.30 min.

The Purelyse gDNA extraction kits were tested in early stages in this thesis but were not further used since it could not handle the active sludge flocks. The MiDAS Method is at the higher end of the processing time, but the MiDAS method has a high throughput in the number of the samples it can process at the same time which is fitting for laboratory settings.

However, this shows that the mobile method is one of the cheaper alternatives and the second fastest method compared to the outer commercially available DNA extraction kit in this comparison. The price and processing time of a method is unimported if the method cannot estimate the proper microbial community of a sample.

4.2 Evaluation of the effect of variation of different steps in the mobile method and it performs in comparison to MiDAS method

DNA extraction, using different kits has been shown to introduce potentially significant bias and can give a difference in the determined microbial community (Brooks *et al.*, 2015). Consequently, it is important to analyze the mobile method and determine that it does not pose significant bias and has a microbial community that is related to the standard extraction method recommended by MiDAS field guide.

The difference between these two approaches is analyzed in the following chapters. The mobile method is varied in the three steps: bead-beating time, centrifuge time and the amount of SPRI beads used for DNA isolation. This is done to find the best-suited method and correspondingly a method that fits the criteria for usage of the MinION technology. This variation is then compared to each other and the MiDAS method. The strategy to evaluate the different and how the data is presented can be seen in section (3.1) in material and method.

4.2.1 The effect of bead-beating time and performance of the mobile bead beater

The first variation step of the mobile method tested is the bead beating time. The variation was tested to determine if its possible to get the same microbial community and feasible DNA with shorter beating time and evaluated the performance of the 3d printed mobile beadbeater. Beating time and speed have been shown to have a positive effect on microbial lysis of gram positive (de Boer *et al.*, 2010; Albertsen *et al.*, 2015). Therefore, the mobile method is varied; the first is **mobile3** with 160sec divide into 40 sec with 2 min cooling. The second is **mobile4** with the 60-sec beating. The **MiDAS** is a 160sec beating divide into 40 sec with 2 min cooling on ice. These two methods were tested and compared to each other and MiDAS.

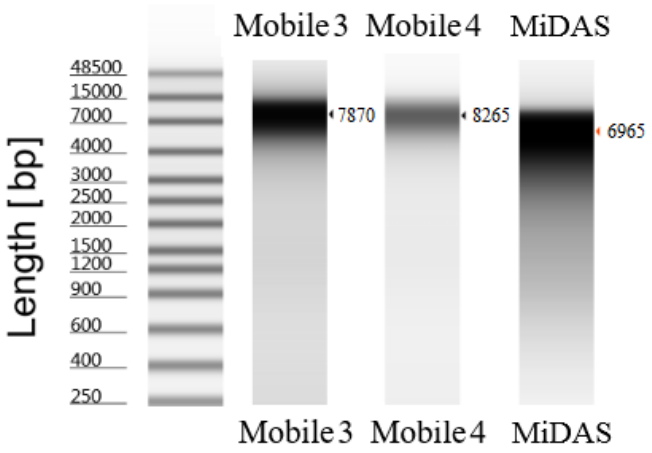
4.2.1.1 Effect of bead beating time on DNA quality and amount

The DNA quantity and quality of the three methods are examined. The data is obtained as described in material and method in section (3.1). The results of the total DNA measured fluorometric, the $A_{260/A280}$ and $A_{260/230}$ ratio and the DNA length from agarose gel can be viewed in Figure 8.

A

Name	Total DNA [µg]]	A_{260}/A_{280}	A_{260}/A_{230}
Mobile 3	7.62 ±0.24	1.71 ±0.025	1.12 ±0.014
Mobile 4	7.17 ±1.08	1.84 ±0.037	1.49 ±0.025
MiDAS	11.46 ±1.48	1.88 ±0.012	0.34 ±0.014

B



C

Name	Beat beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	10min 14000xg	170
Mobile 2	4 x 40 sec.	1min 14000xg	170
Mobile 3	**4 x 40 sec.**	1min 2000xg	170
Mobile 4	**60 sec.**	1min 2000xg	170
Mobile 5	60 sec.	1min 2000xg	80

Figure 8: The figure displays the results from the qualitative and quantitative measurement of the variation beating time**. A**: Table with the results from the qualitative and purity measurement of the DNA, total DNA of each method, the $A_{260/280}$ ratio and $A_{260/230}$ ratio. The data show a mean of three replicas with the ± SEM. **B**: Agarose gel of the three-method tested with a ladder, Number to the right of the agarose gel is the average fragment length **C**: The table shows an overview of all variations tested, the parts marked in the table are the variations seen in figure A and B.

The total DNA has small decreases with reductions of beating time from the mobile 3 to Mobile 4. However, this difference is quite small, and the SEM of the sample are overlapping. It cannot be determined if there is a difference between the total DNA of both methods. Comparing Mobile 3 and 4 with the MiDAS method, the MiDAS gives an increase of 52% in total DNA from mobile3. When comparing with literature, it corresponds with the results; the literature shows that an increase of beating time and speed would lead to a larger amount and higher yield and therefore a higher total DNA (Bürgmann *et al.*, 2001). The next comparison is the purity of the sample; all methods lie close to 1.8 marks, with the $A_{260/A280}$ ratio. The Mobile 4 has the best ratio following MiDAS and Mobile 3. The next ratio is the $A_{260/230}$ where no method fulfills the

requirement of a ratio of over 2.0; this indicates contamination that absorbs at A_{230} and is present in all methods, but it can be observed that Mobile 4 has the highest ratio following Mobile 3 and lastly MiDAS. The contamination is likely; humic acid which can affect subsequent molecular studies (McIlroy *et al.*, 2009). The absorption ratio is better with a lowering of beating time, which could indicate that a lower beating time would give higher ratio and thereby a smaller contamination. More information is needed before making conclusions.

Comparing all the methods, the MiDAS method had a low $A_{260/A230}$, ratio. An explanation of this can be found in the form of the FAST SPIN DNA kit for soil used. One of the steps in the Fast Spin DNA kit for soil used a solution that contains guanidine thiocyanate which likely is not adequately removed (MP BIOMEDICALS, 2016). Guanidine thiocyanate has a high absorption at A_{230} and would highly influence the measurement (Desjardins and Conklin, 2010; ThermoScientific, 2011).

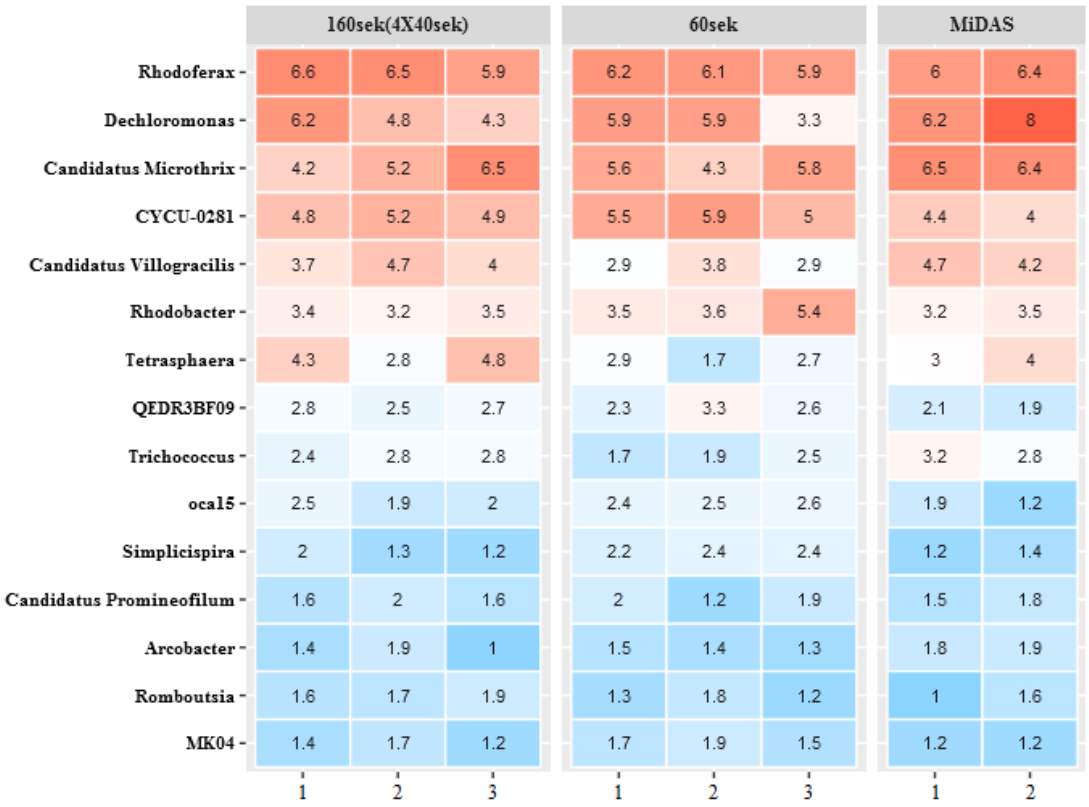
The effect of bead beating on DNA integrity can be observed in figure 8A. This figure shows a decrease of bead beating time resulted in less fragmented DNA and higher average fragment size. The method with the least fragmentation was the Mobile4, following Mobile 3 and last MiDAS. When compared with literature and according to the results, an increase of beating time and speed would increase the DNA shredding in the sample and thereby increase the DNA fragmentation (Bürgmann *et al.*, 2001; Albertsen *et al.*, 2015).

From this, the mobile bead-beater can also be evaluated, since beating time and speed influence DNA shredding amount and yield. The mobile bead-beater gave less total DNA and a smaller amount of DNA shredding, this indicated that the mobile bead-beater have lower beating speed than the FastPrep®-24 used in the MiDAS method. However, how well the mobile bead beater does compare to the FastPrep®-24 cannot determine from this data since there are used two different DNA isolation methods. However, it can be determent that the mobile bead beater has sufficient speed and power to performed bead-beating.

4.2.1.2 Effect of bead beating on microbial composition

An evaluation of the effect of bead-beating on the microbial community is made. The data is obtained as defined in material and method in section (3.1), with 16S rRNA-gen amplification of reagionV1-3. All samples from the mobile method had over 3000 reads except one replica from the MiDAS which had less than 3000 reads. A visualization of the sequencing data is displayed in figure 9.

A



B

Name	Beat beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	10min 14000xg	170
Mobile 2	4 x 40 sec.	1min 14000xg	170
Mobile 3	**4 x 40 sec.**	1min 2000xg	170
Mobile 4	**60 sec.**	1min 2000xg	170
Mobile 5	60 sec.	1min 2000xg	80

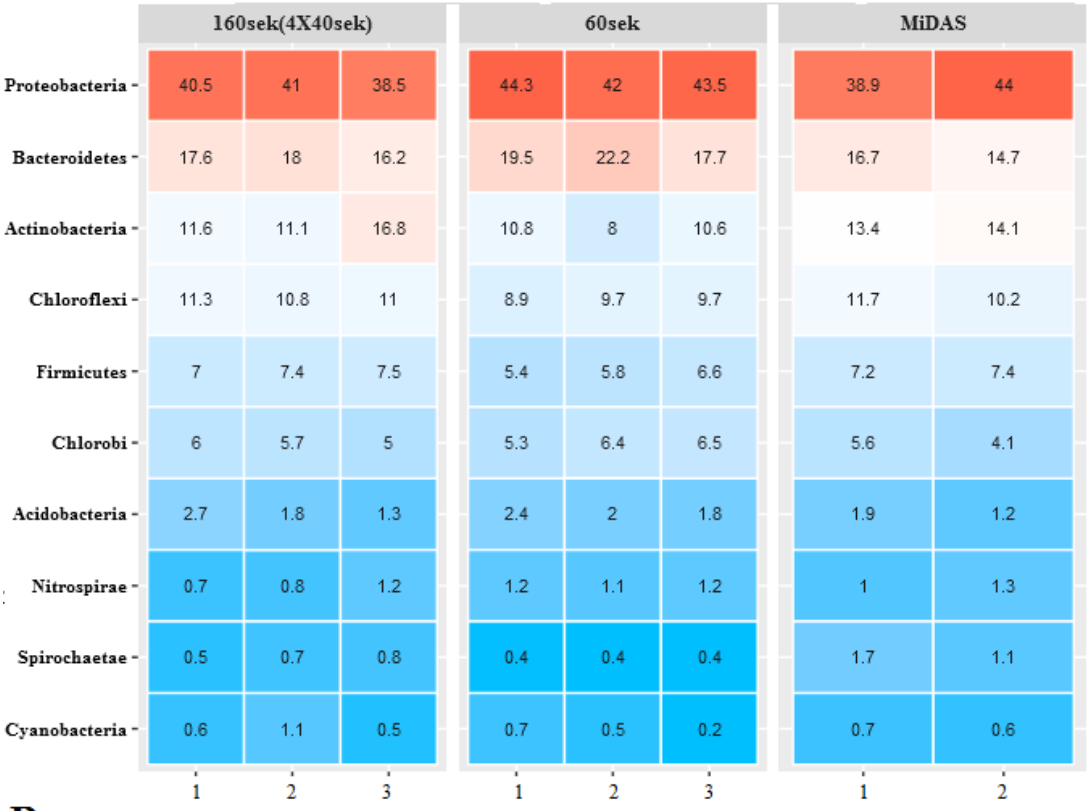
Figure 9 **A**: Heatmap of the variable beating time. The Y-axis ranks the 15 most abundant OTUs over all samples. The OTUs are assigned to its genus classification or the closest possible taxonomic rank by using the MiDAS database. The x- axis represents samples grouped into the bead-beating time, where each 1,2,3 represents a replication. Each number on the figure represented the relative read abundance of the OTUs **B**: An overview of all methods and variations tested in this report, the marked parts are the variations that are shown on the heat map.

On figure 9 all top 15 OTUs are expected as genus typically seen in active sludge on the MiDAS database. All OTUs under the top 8 OTUs have a variation of between 0.5-1% abundance across all replicas in the methods. The OTU under the top 8 of the mobile method had a slight deviation of relative abundant OTUs compared to the MiDAS. It's only in the Top 8 OTUs where the difference between the methods is more noticeable. One of the genus where there is a higher variation in and between the method is *Dechloromonas*. This gives a problem in filtrating one of the MiDAS replicas off is showing since between replica one and two there is a difference of 2%. Therefore, it was difficult to indicate which relative abundant of the two replicas are the correct one.

However, it appearances there is a small reduction of the abundance of the gram-positive *Tetrasphaera* genus with lower beating time. Literature shows that gram-positive bacteria are resistant to mechanical disruption because of their thick cell wall and therefore are hard to lysis(Guo and Zhang, 2013; Albertsen *et al.*, 2015). A higher beating and longer beating time would give a better estimation of gram-positive bacteria (Guo and Zhang, 2013; Albertsen *et al.*, 2015).

To better visualize the effect of beating time on the gram-positive bacteria, the taxonomical level of the heat map is changed from genus to phylum. The visualization of the phylum heat map can be seen in figure 10.

A



B

Name	Beat beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	10min 14000xg	170
Mobile 2	4 x 40 sec.	1min 14000xg	170
Mobile 3	**4 x 40 sec.**	1min 2000xg	170
Mobile 4	**60 sec.**	1min 2000xg	170
Mobile 5	60 sec.	1min 2000xg	80

Figure 10: **A**: Heatmap of the variable beating time. The Y-axis ranks the 15 most abundant OTUs over all samples. The OTUs are assigned to its phylum classification or the closest possible taxonomic rank by using the MiDAS database. The x- axis represents samples grouped into the bead-beating time, where each 1,2,3 represents a replication. Each number on the figure represented the relative read abundance of the OTUs **B**: An overview of all methods and variations tested in this report, the marked parts are the variations that are showed on the heat map.

Observing the microbial community change at the phylum level, a general increase of abundance of the gram-positive phylum Actinobacteria is showed with an increase of bead beating time. The same effect can also be seen for the gram-positive Firmicutes. However, this also shows that even with a lower beating time, the mobile method still shows the same overall composition as the MiDAS method. Even with a decrease of 100 sec. of beating time from Mobile 3 to 4, it only resulted in a reduction of relative read

abounds of 2% for the Actinobacteria and Firmicutes. It also affirmative that the mobile bead beater has sufficient speed and power to performed bead-beating to lysis hard to lysis bakteria. The top OTUs of the sample still does not estimate how the whole microbial community is compared to each other. To estimate how the microbial communities are compared to each other a Principal Component Analysis (PCA)-plot is made. This plot is visualized in Figure 11. On this plot, each dot represents a replica, and the closer the dots are, the more similar the bacterial composition.

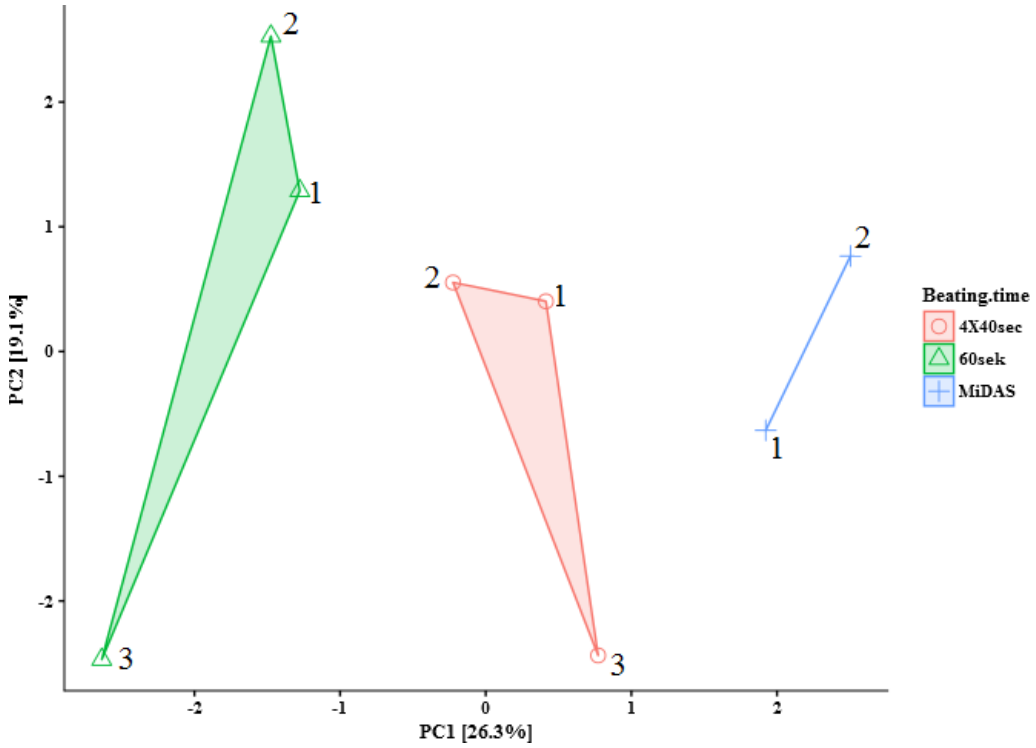


Figure 11: PCA plot comparing the effect of different beating time. Red represents mobile 3 with 160sec beating time; green represents mobile 4 with 60 sec beating time; Blue represents MiDAS. Each point with a number accounts for a replicate of the method. PC1 and PC2 explain 26.3% and 19.1 per cent of the total variation in the microbial community.

In general, each method clusters together, but there is a general trend that replica 3 of each mobile method is more different then replica 1 and 2. An explanation of this can be replica 1 and 2 are beaten together, and replica 3 is beaten alone. The replica of 1 and 2 of the mobile method is clustered the same as the MiDAS protocol. However, the replica 3 microbial compensation is more different then replica 1 and 2. This indicates that only sample beating together can be optimal compared. If this is true the 3d printed adapter and power tool may need to be more optimized to become more reducible, but more data is a needed to determine this. Overall, the PCA plot shows that when the bead-beading time increases, the replicas cluster more closely together, and the Mobile resembles the MiDAS more in the microbial community.

4.2.2 The effect of centrifuge time

The next step tested is the effect of different centrifuge speed and time. Centrifugation is used both in the MiDAS and mobile method to remove cell debris that could influence DNA isolation step. In **MiDAS** a 10min 14000xg setting is used to remove cell debris. By reducing the time and speed of centrifugation, a size reduction of the requirement equipment in the mobile method can be made. Therefore a decrease of both centrifuge time and speed is tested. An overview of the centrifuge speed and time-tested can be seen in Table 5.

Table 5: Table of the centrifuge time that is tested in this project, the marked parts are the variations tested.

Name	Beat beating time	Centrifuge time	SRPI. [uL]
Mobile 1	160sek(4x40sek)	**10minX14000g**	170
Mobile 2	160sek(4x40sek)	**1minX14000g**	170
Mobile 3	160sek (4x40sek)	**1minX2000g**	170

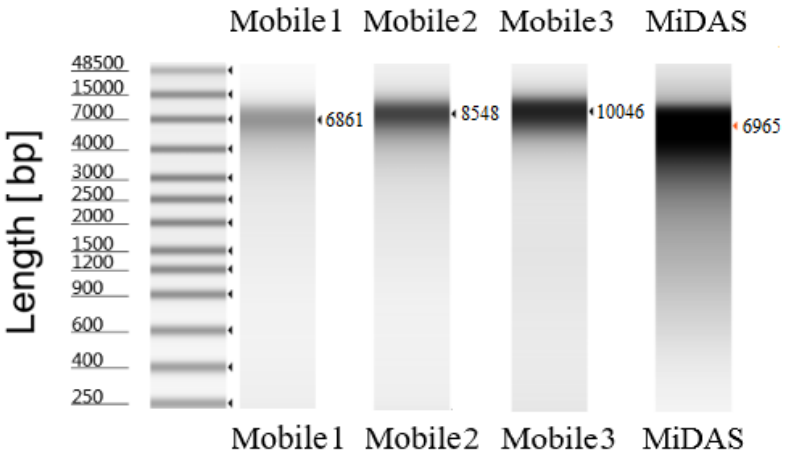
4.2.2.1 Effect of centrifuge time on DNA quality and amount

The DNA quantity and quality of the four methods is examined. The data is obtained as described in material and method in section (3.1). The results of the Total DNA measured fluorometrically, the $A_{260/A280}$ and $A_{260/230}$ ratio and the DNA length from agarose gel can be viewed in figure 12.

A

Name	Total DNA [µg]]	A_{260}/A_{280}	A_{260}/A_{230}
Mobile 1	8.74 ±0.0048	1.93 ±0	1.88 ± 0.073
Mobile 2	6.96 ±0.24	1.91 ±0.0033	1.86 ± 0.088
Mobile 3	7.62 ±1.17	1.71 ±0.025	1.12 ± 0.14
MiDAS	11.46 ±1.48	1.88 ±0.012	0.34 ± 0.014

B



C

Name	Beat beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	**10min 14000xg**	170
Mobile 2	4 x 40 sec.	**1min 14000xg**	170
Mobile 3	4 x 40 sec.	**1min 2000xg**	170
Mobile 4	60 sec.	1min 2000xg	170
Mobile 5	60 sec.	1min 2000xg	80

Figure 12: Figure displaying the results from the qualitative and quantitative measurement of the variation in beating time. **A**: Table with the results from the qualitative and purity measurement of the DNA, total DNA of each method, the $A_{260/280}$ ratio and $A_{260/230}$ ratio. Data shown is a mean of three replicas with the ± SEM. **B**: Agarose gel of the three-method tested with a ladder. The number to the right of the agarose gel is the average fragment length **C**: The table shows an overview of all variations tested, the parts marked in the table are the variations, see figure A and B

In figure 12 a decrease of centrifugation time resulted in a reduction of the total DNA, but when observing the SEM of both Mobile 2 and 3, they are booth overlapping, therefore it hard to determine if they different. However, the MiDAS method gets still 31% more total DNA compared to the Mobile 1.

When comparing the purity of the $A_{260/280,}$ it decreases with a reduction in centrifuge time in the mobile method. The same can be seen for the $A_{260/230}$ ratio, but still, no method fulfills the requirements of the MinION, this low $A_{260/230}$ is contamination that absorbs at A230. The difference between the Mobile 1 and 2 is about 1% in both $A_{260/280,}$ and $A_{260/230}$ which can indicate the 10-min centrifugation is a redundancy since the 1min centrifugation almost gives the same purity. However, the purity in the $A_{260/230}$ ratio drops with 0.7 with 1 min 2000xg.

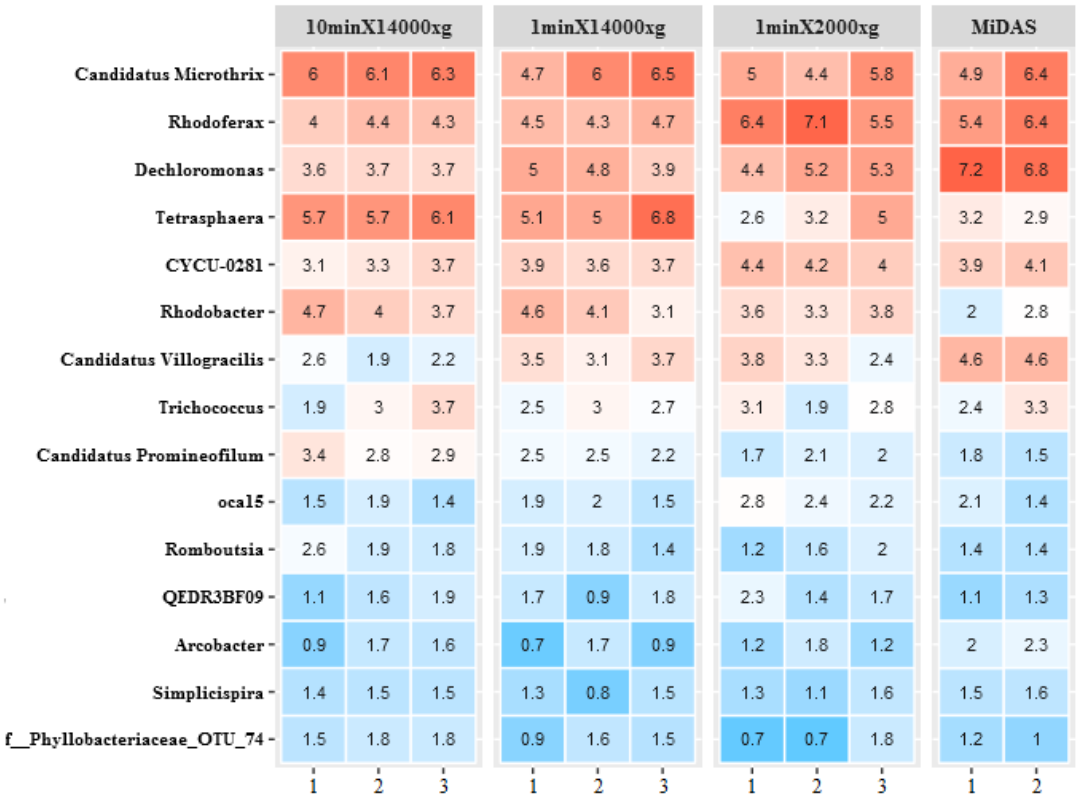
When viewing the DNA fragment length in figure 12B, it shows a general trend that a decrease of centrifuge time gives an increase of DNA fragment length. A possible explanation of this can be that the gravitational force has an effect on the separation of a particle in a solution depends on the particle size and density, particles of higher density or larger size are typically separated faster than their smaller counterpart(Sharpe, 2012). This can explain why an increase of fragment size is observed since a longer fragment of DNA is kept in the supernatant of the solution with reduction of centrifugation time.

To test if this explanation is true a statistically significant difference in fragment length must be observed between the three methods. To determine this, a one-way ANOVA test was conducted ($F(2,6) = 1.86$, $p = 0.23$) at the $p<0.05$ level on all three replicas and three mobile methods. It revealed there was no statistically significant difference between the three Mobile methods and their fragment length. However, there are chances of getting a false negative since the statistical test has a small sample size and more data is needed to make a conclusion. The average fragment length, mean and ANOVA can be seen in Appendix D2.

4.2.2.2 Effect of centrifuge time on microbial composition

An evaluation of the effect of centrifuge time on the microbial community is made. The data is obtained as in material and method in section (3.1), where the 16S rRNA-gene amplification of reagionV1-3 is used. All samples from the mobile method had over 3000 reads except one replica from the MiDAS which had less than 3000 reads. A visualization of the sequencing data is displayed in Figure 13.

A



B

Name	Beat beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	**10min 14000xg**	170
Mobile 2	4 x 40 sec.	**1min 14000xg**	170
Mobile 3	4 x 40 sec.	**1min 2000xg**	170
Mobile 4	60 sec.	1min 2000xg	170
Mobile 5	60 sec.	1min 2000xg	80

Figure13: Heatmap of the variable centrifuge time. The Y-axis ranks the 15 most abundant OTUs over all samples. The OTUs are assigned to their genus classification or the closest possible taxonomic rank by using the MiDAS database. The x- axis represents samples grouped according to the bead-beating time, were each 1,2,3 represent a replication. Each number on the figure represents the relative read abundance of the OTUs **B**: An overview of all methods and variations tested in this report, the marked parts are the variations that are shown on the heat map.

The change of centrifuge time does not have a larger effect on the microbial comparison of the top 15 OTUs. However, there is still variation in the method, as seen in the *Tetrasphera* genus where it has a relative read abundance of 5-6, in the mobile method 1 and 2. When looking at mobile method 3, replica 1 and 2 have a relative read abundance of 2-4%, the same as MiDAS and it seems that the abundance of Mobile 3 resembles MiDAS the most. It appearances the genus *Dechloromonas* is underestimated in all the

mobile method and has variation in and between method as seen in the variation of beat beating time. To determine how the total Microbial composition from each method is compared to each other, a PCA-plot is made. This plot is visualized in figure 14 and on this plot, each dot represents a replica, and the closer the dots, the more similar their bacterial composition.

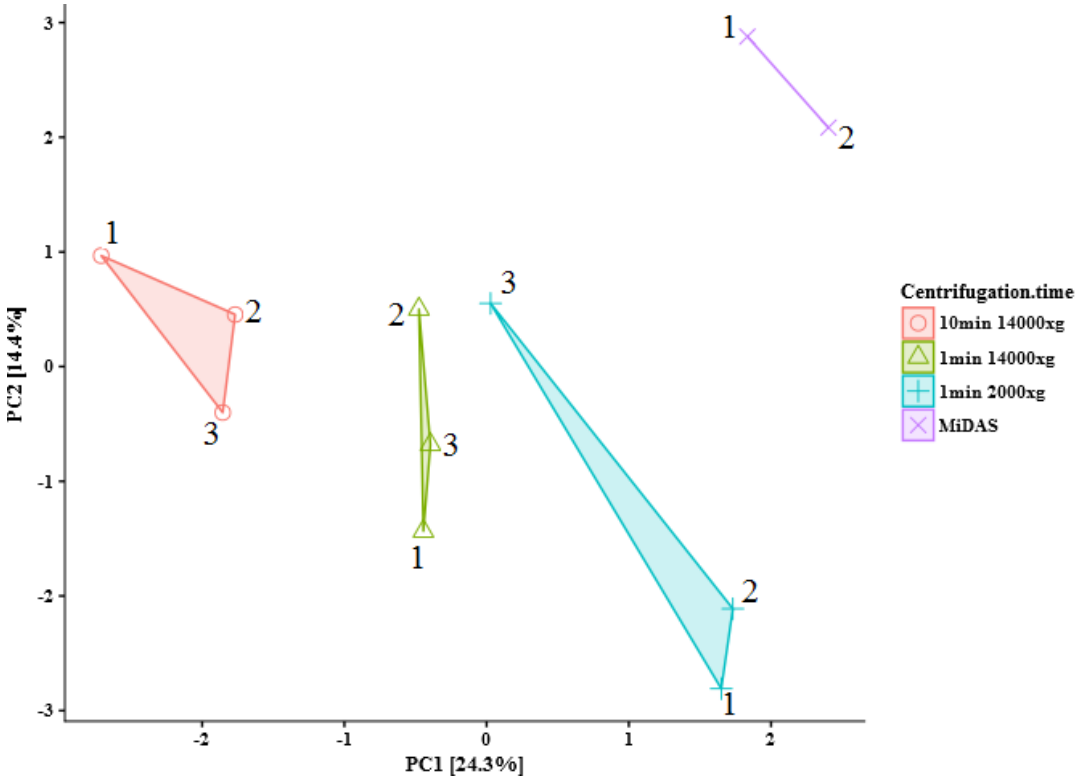


Figure 14: PCA plot comparing the effect of different beating time. Red represents mobile method 1 with 10min 14000xg; green represents mobile method 2 with 1min 14000xg; Blue represents mobile method 3 with 1min 2000xg and purple represents MiDAS. Each point with a number accounts for a replicate of the method. PC1 and PC2 explain 24.3% and 14.4 per cent of the total

It can be observed that mobile method 3 with 1min 2000xg has the same separation of replica 1 and 2 from replica 3 as seen in the variation of beating time. However this is not observed in the outer two mobile methods, and a higher centrifugation in Mobile method makes, the replicas clustered closer together. When comparing the Mobile with MiDAS, it can be noted that the Mobile 3 with 1minX2000xg centrifugation is most closely related with the MiDAS.

4.2.3 The effect of SPRI beads volume

The last step tested is the DNA isolation with the use of SPRI in the form of AMpure XP from Beckman. This variation tested is the volume of SPRI utilized in the DNA isolation step. Since the price of SPRI beads is high, a reduction of the volume used would result in a lower price per sample. The volume tested is 180uL used of the original bead solution called **mobile method 4** and 80uL bead solution call **mobile method 5**. These two beads volumes are then diluted in a cheaper buffer as described in material and methods.

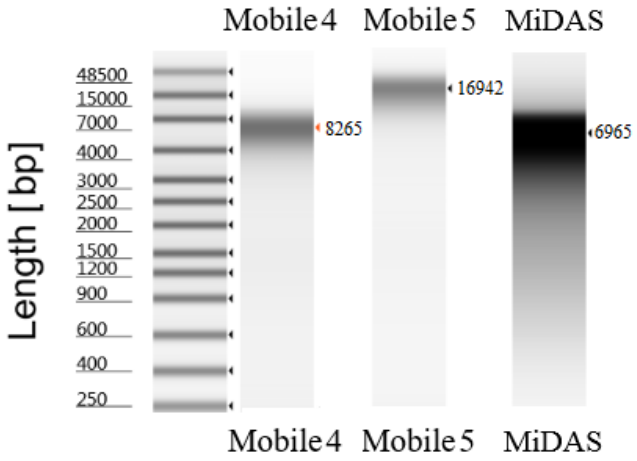
4.2.3.1 Effect of SPRI beads volume on DNA quality and amount

The DNA quantity and quality of the two beads volumes used and MiDAS are examined. The data is obtained as in material and method in section (3.1). The results of the total DNA measured fluorometrically, the $A_{260/A280}$ and $A_{260/230}$ ratio and the DNA length from agarose gel can be viewed in figure 15.

A

Name	Total DNA [µg]]	A_{260}/A_{280}	A_{260}/A_{230}
Mobile 4	7.16 ±1.08	1.84 ± 0.037	1.49 ± 0.025 -2
Mobile 5	2.57 ±0.65	1.79 ± 0.037	1.72 ± 0.012 -2
MiDAS	11.46 ±1.48	1.88 ± 0.012	0.34 ± 0.014 -2

B



C

Name	Beat beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	10min 14000xg	170
Mobile 2	4 x 40 sec.	1min 14000xg	170
Mobile 3	4 x 40 sec.	1min 2000xg	170
Mobile 4	60 sec.	1min 2000xg	**170**
Mobile 5	60 sec.	1min 2000xg	**80**

Figure 15: Figure displaying the results from the qualitative and quantitative measurement for the different SPRI beads volume**. A**: Table with the results from the qualitative and purity mesuerment of the DNA, total DNA of each method, the $A_{260/A280}$ ratio and $A_{260/A230}$ ratio. Data show is a mean of three replicas with the ± SEM. **B**: Agarose gel of the three-method tested with a ladder, Number to the right of the agarose gel is the average fragment length **C**: The table shows an overview of all variations tested, the parts marked in the table are the variations seen in figure A and B.

The total DNA decreases as less SPRI beads are used, but when comparing the purity of the two Mobile, it can be noticed that the $A_{260/280}$ ratio of Mobile4 is 2,79% larger than Mobile5 and the $A_{260/230}$ ratio of Mobile5 is 15,43% higher than Mobile 4.
An explaining of this increase of $A_{260/230}$ ratio is found in the form of the decreased volume of SPRI beads. When the number of beads used is decreased, the surface to volume is larger when the magnetic beads are drawn to the side with a magnet stander.
Therefore, when the beads are washed with ethanol, a larger amount of surface is washed and more impurities are removed.

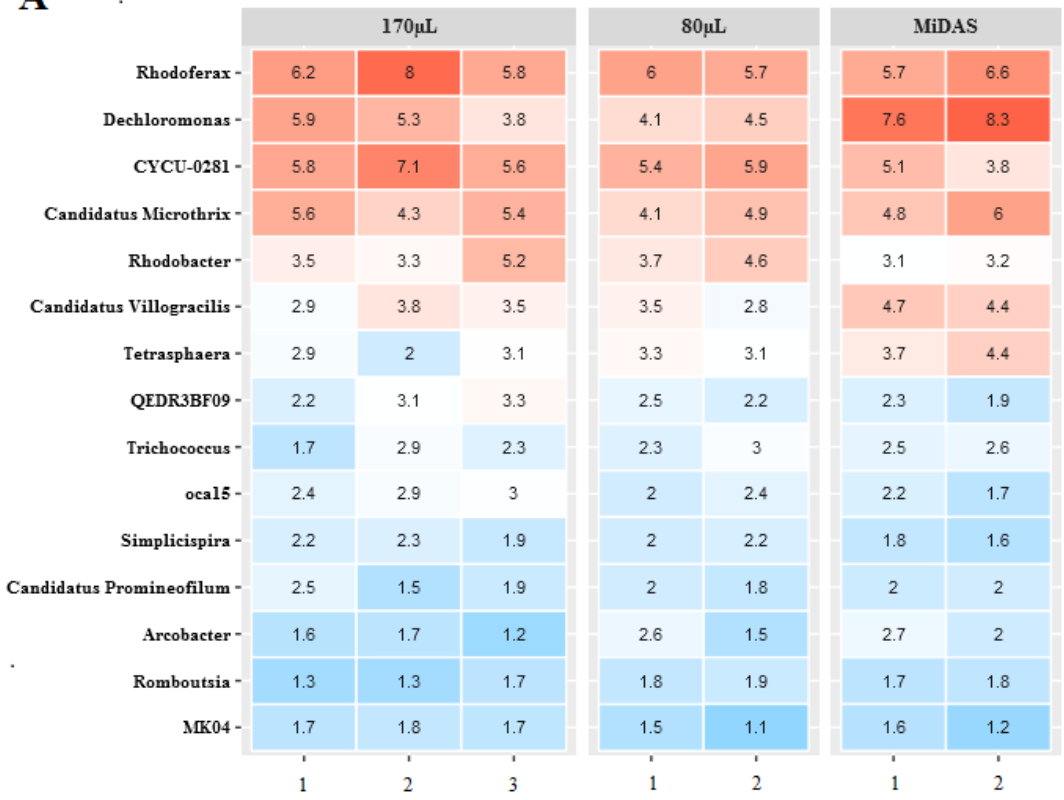
It is seen that a decrease of SPRI beads also resulted in an increase of DNA fragment in figure 15B. The reason for this is that the SPRI beads can make size selection of the fragment size (Lis and Schleif, 1975; Hawkins *et al.*, 1994; Lennon *et al.*, 2010). This size selection happens when the ratio of sample to beads and PEG-8000 are variated. So when the sample/beads ratio and PEG-8000 concentration is lowered it results in a cutoff of smaller fragments (Lis and Schleif, 1975; Hawkins *et al.*, 1994; Lennon *et al.*, 2010).

However, to better determined if mobile method 5 fragment length is a significant difference then mobile method 4 an independent-sample one-tail t-test is used on the three replicas. There was an significant difference between the two fragment length t-test T(2) =[-3.46], p = [0.037]. These suggest that the volume of SPRI influence fragment lengthen and can make cutoff of smaller fragments for smaller fragment lengthen. However, since the test has a small sample size, so it is not fully reliable, and more data is needed to make a concluded. The average fragment length, mean and t-test can be seen in Appendix D1.

4.2.3.1 Effect of SPRI beads volume used in microbial composition

An analysis of the composition and effect of SPRI beads used for the microbial community is undertaken. The data is obtained as defined in material and method in section (3.1), were the 16S rRNA-gen amplification of reagionV1-3 is used. All samples expect one from Mobile 5 and MiDAS had over 3000 reads. A visualization of the sequencing data is displayed in Figure 16.

A



B

Name	Beat beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	10min 14000xg	170
Mobile 2	4 x 40 sec.	1min 14000xg	170
Mobile 3	4 x 40 sec.	1min 2000xg	170
Mobile 4	60 sec.	1min 2000xg	**170**
Mobile 5	60 sec.	1min 2000xg	**80**

Figure16: Heatmap of the variable volume of SPRI beads. The Y-axis ranks the 15 most abundant OTUs over all samples. The OTUs are assigned to their genus classification or the closest possible taxonomic rank by using the MiDAS database. The x- axis represents samples grouped into bead-beating time, where each 1,2,3 represents a replication. Each number in the figure represents the relative read abundance of the OTUs **B**: An overview of all methods and variations tested in this report, the marked parts are the variations that are shown on the heat map.

There is no sizable difference between the composition of the top 15 OTU. There is a difference between the relative abundance of the two mobile methods. This can be observed at the genus *Dechloromonas* which is underestimated in the two mobile methods compared to MiDAS. It cannot be determined if the genus *Cycu-0281* are over- or underestimated since there are a difference in relative abundance of this genus in MiDAS method. To estimate, if there is any change in the total microbial community a PCA plot is made. The plot is visualized in figure 17.

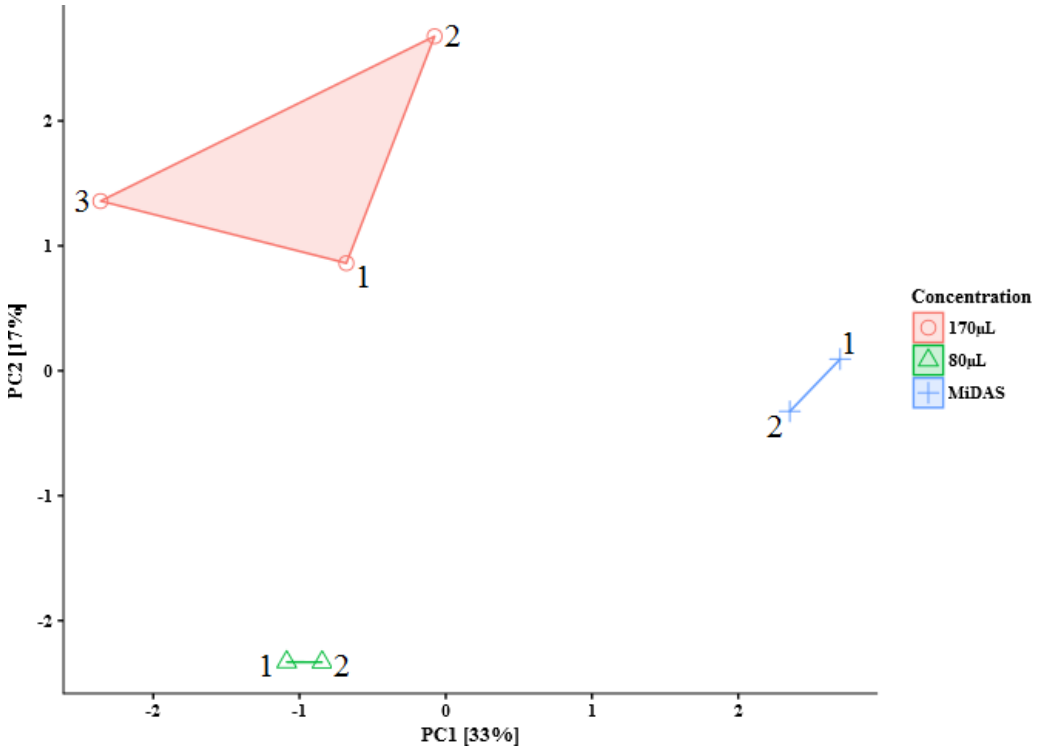


Figure 17: PCA plot comparing the effect of different beating time. Red represents mobile method 4 with 170µL SRPI beads used.; green represents mobile method 5 with 80µL beads used; Blue represents MiDAS. Each point with a number accounts for a replicate of the method. PC1 and PC2 explain 33% and 17% per cent of the total variation in the microbial community.

The difference between the two mobile methods is only alone PC1 that explains 17% of the total variation, the Mobile 5 80uL looks more clustered than MiDAS. Comparing the two mobile methods, it appears that the two approaches differ equally from MiDAS, only Mobile 5 80uL has less spread.

4.2.4 Comparison of all the methods microbial composition,

A final comparison of the microbial community is made, across all methods, as seen in figure 18. Where an heatmap of the top 12 OTUs of all method is made, on figure 18A. There can be noted that the overall comparing the composition of the top 12 OTUs on figure 18A of the mobile methods with the MiDAS, the overall compensation is the same, but there is the difference between the relative read abundance as seen with the genus *Dechloromonas*.

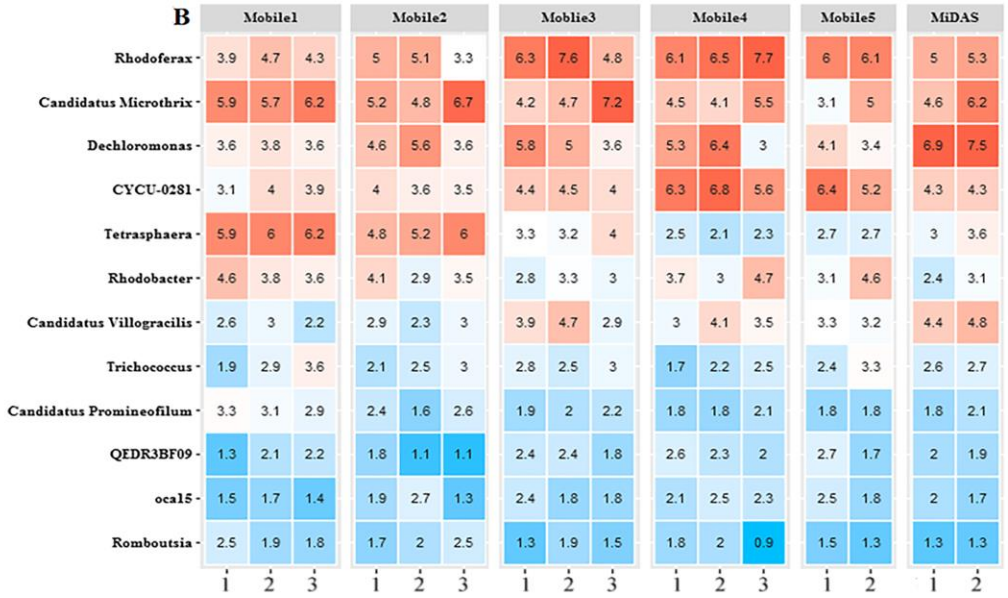
To determine the mobile method that has estimated the microbial community that is closed related to MiDAS a PCA plot with all method is made which can be seen on figure18C.

Here it can be seen that the Mobile method is many separated, alone PC1 and that MiDAS is located with mobile method 3 and 5. But when comparing the PC2 it that MiDAS is separated alone form the Mobile method tested. But the two method that lie closest to the MiDAS sample are mobile3 and mobile5.

Also, the difference of the between replica 1 and 2 compared to replica 3 as observed in figure 11 the PCA plot of bead-beating is evaluated. A possible indication of this can be seen in the figure 18B heat map with Mobile3, were some of the OTU from replica 3 have a higher or lower relative read abundance then replica 1 and 2, but this is an indication since this is not observed in all OTU. Also, this effect can only be obverses on mobile4 and to a small degree mobile3 on figure18C. However, this cannot be determined if this is random or a bias and a larger data size and further investigation and more data are needed to conclude.

A

Name	Beat beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	10min 14000xg	170
Mobile 2	4 x 40 sec.	1min 14000xg	170
Mobile 3	4 x 40 sec.	1min 2000xg	170
Mobile 4	60 sec.	1min 2000xg	170
Mobile 5	60 sec.	1min 2000xg	80



A

Name	Beat beating time	Centrifuge time	SPRI volume [µL]
Mobile 1	4 x 40 sec.	10min 14000xg	170
Mobile 2	4 x 40 sec.	1min 14000xg	170
Mobile 3	4 x 40 sec.	1min 2000xg	170
Mobile 4	60 sec.	1min 2000xg	170
Mobile 5	60 sec.	1min 2000xg	80

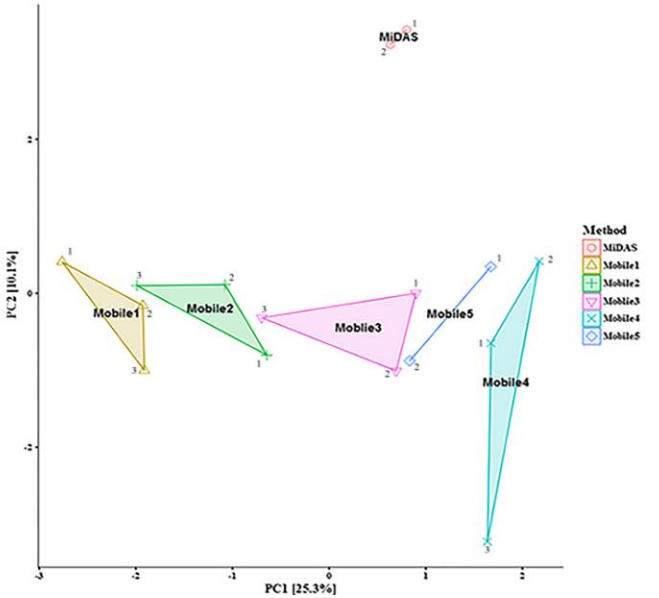


Figure 18: A: An overview of all mobile methods and variations tested in this report. **B**: Heatmap of the all the mobile method in the report. The Y-axis ranks the 12 most abundant OTUs over all samples. The OTUs are assigned to their genus classification or the closest possible taxonomic rank by using the MiDAS database. The x- axis represents samples grouped into DNA extraction, where each 1,2,3 represents a replication. Each number in the figure represents the relative read abundance of the OTUs **C:** PCA plot comparing all mobile method tested in the report. Each point with a number accounts for a replicate of the method. PC1 and PC2 explain 25.3% and 10.1% percent of the total variation in the microbial community.

5. CONCLUSION

In this project, a fast, mobile, cheap, and easy to use DNA extraction protocol was developed and evaluated against state-of-the-art lab procedures. The variation of the method has been tested and analyzed in the three steps. Therefore, an overview of the data is made in three steps, and an optimal method is chosen.

Comparison of Beating time and performers of a 3D print handheld beater

In the variation of beating time, it showed a beating time of 60sec in the mobile method gave the best resulted in the form of quality and purity. However, when comparing the microbial community, it was seen that a higher beating time gave a better representation of hard to lysis gram-positive bacteria. Also, the microbial community determined for the mobile method appeared more like MiDAS with an increase of beating time. Likewise, a spread of the replicas compared to the beating is seen; when samples were beaten together, they showed closer relating microbial community.

The 3D printed handheld mobile beating tool developed in this thesis performed almost as well as the traditional tabletop bead-beater just with lower power output seen in the lower amount of lysis gram-positive bacteria but had sufficient speed to performed bead-beading.

Centrifuge.

A comparison of centrifuge time and speed showed that the centrifuge time had little effect on total DNA. However, it influenced the purity and quality of the DNA. All methods had pure DNA and had still contamination in the in the $A_{260/230}$ ratio. However, it is seen that the 10min 14000xg and 1min 14000xg gave the highest ratio. It can also be noted that there was no significant difference between the two speeds. In DNA quality, an increase of fragment DNA with lower speed could be observed, but there was no statistical significance difference when comparing all average fragment length of all replicas in the mobile method. The microbial community compaction showed that the 1min 2000xg composition appeared most like the MiDAS.

SPRI beads

A comparison of the different volume of SPRI was made and it was demonstrated that an 80uL of beads volume gave a higher DNA purity. A statistical significance higher DNA fragment length was also observed with a decrease of SPRI beads volume used. The microbial community revealed that there was a difference between the two SPRI beads volume used, but this difference is small and the 80uL beads used gave the smallest

spread. The difference between the two SPRI beads volume used and MiDAS was the same.

The optimal mobile method from the resulted.

The optimal method is chosen from a set of criteria established in this thesis. The mobile method fulfilled the requirements of mobility since the tools and chemicals used in the method are easy to transport. The method also fulfilled the requirements of safety since there are no dangerous chemicals is used in the mobile method.
The two last required to be fulfilled the requirement set for being sequenced by the MinION with the Rapid Sequencing Kit, and the performs in this thesis with reproducibility.

In regrades to sequenced by the MinION with the Rapid Sequencing Kit. The first requirement was an input mass of 200 ng, which was fulfilled by all the mobile method tested.
The second is the $A_{260/280}$ of close to 1.8; here all mobile method also fulfilled the requirement. In the $A_{260/230}$ of over 2, no method fulfilled this, but the methods that came nearest was a mobile 1, 2 and 5.
The third criteria were an average fragment size of >30kb, this was not fulfilled by any mobile method, but the highest fragment length was mobile 5.
However, when comparing the microbial community of the methods, the one who had the microbial community that lies closer to the MiDAS is the Mobile method 3 and 5.
The mobile method which had the best performed in the form of quality and purity are mobile5 with a 60sec beating time, 1minx2000g centrifugation and 80uL beads used.

Therefore, for future development of the mobile method, this setup is recommended.
The method stilled require some optimization before the sample processed by the mobile method can be used optimally for the MinION sequencer estimated from requirement for the Rapid Sequencing Kit. There was also an indication of the method may stilled need to optimized in beading tool to be more reproducibility.

However, It was possible to design an cheap mobile bead beating tool that performs almost as well as the traditional tabletop bead beater. Also, the method developed in this thesis was mobile, safe, easy to be used, cheap and can make DNA extraction in about 10 min. This DNA extraction method can form the foundation for moving DNA sequencing out of the lab and closer to application

6. PERSPECTIVE AND FURTHER DEVELOPMENT

The developed mobile DNA extraction method, have been showed to be able to perform as well as state-of-the-art DNA extraction methods. It still needs optimization, but the principle of the method will not change. Therefore, in this section of the thesis, a rough overview of a mobile lab is given where the DNA extraction developed in this thesis is used. This can make onsite DNA sequencing possible and allow potential further development.

6.1 Mobile lab for onsite DNA extraction and future development.

As stated in the introduction, the research in making a portable laboratory that makes use of the MinION portability is already in progress. The closest yet to archive this is described in the paper by Edwards et al. 2017 that have achieved to make a rucksack contain everything to do on-site off-grid metagenomics, everything from DNA extraction to DNA sequencing and analyses. However, as also mentioned in the introduction, they encountered problems with the DNA extraction method used. The problem was the method used gave low yield and DNA integrity which can be an issue for the MinION. This was not optimal, but the DNA extraction method developed in this thesis can be a possible solution. With inspiration from the rucksack devolved in the Edwards et al. 2017 paper, a mobile lab is constructed an overview of which can be seen in figure 19.

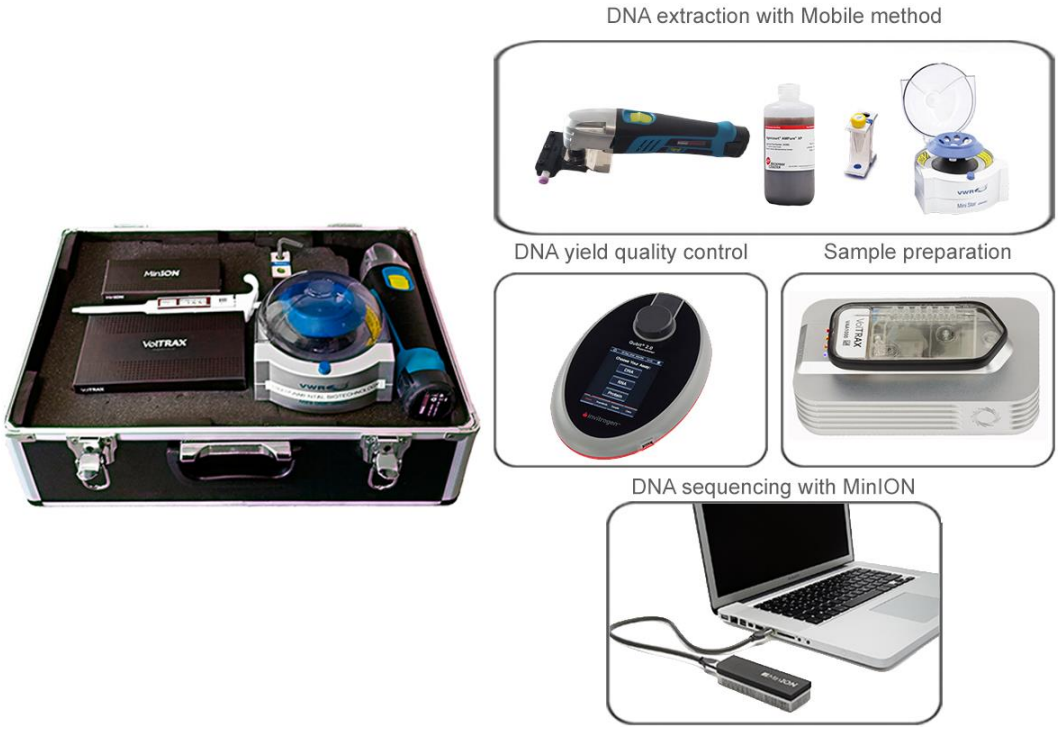


Figure 19: An overview of the mobile lab, the lab contains the mobile method developed in this project, It contains the Qubit 2.0, VolTRAX, a laptop with sufficient hardware to run the minION. It should be noted that the lab also should contain the pippetes, and the chemicals for doing the DNA extraction, DNA quality control, sample Preparation, but these are not shown.

The mobile lab shown in figure 19 makes use of the DNA extraction method developed in this project. It contains a Qubit 2.0 to determine the yield of the DNA extraction and a VolTRAX to do sample preparation and with later development PCR if 16srRNA gene amplification is needed. It also contains a laptop with the required hardware to run the MinION and to make analyses. The mobile lab should make it possible to do the on-site extraction of the sample at a WWTP. As descript in the paper by Edwards et al. 2017 it is possible to use a power bank if mains electricity is not available. However, even with a rough overview of the mobile DNA lab, there is still room for further development of the mobile lab.

6.1.1 Automatization of the mobile lab

The first optimization is the DNA extraction method, which can make use of the automatization given by the VolTRAX. Since it can do bead-based cleanup, it can be utilized to do the SPRI clean-up of genomic DNA in the mobile method developed in this thesis (Oxford Nanopore Technologies, 2017d). ONT stated that the VolTRAX would be

dockable with the MinION and therefore automates the loading process of the samples prepared on the VolTRAX to the MinION (Oxford Nanopore Technologies, 2017d). It gives the possibility to automate a large part of the process. An overview of the automatization can be seen in figure 20.

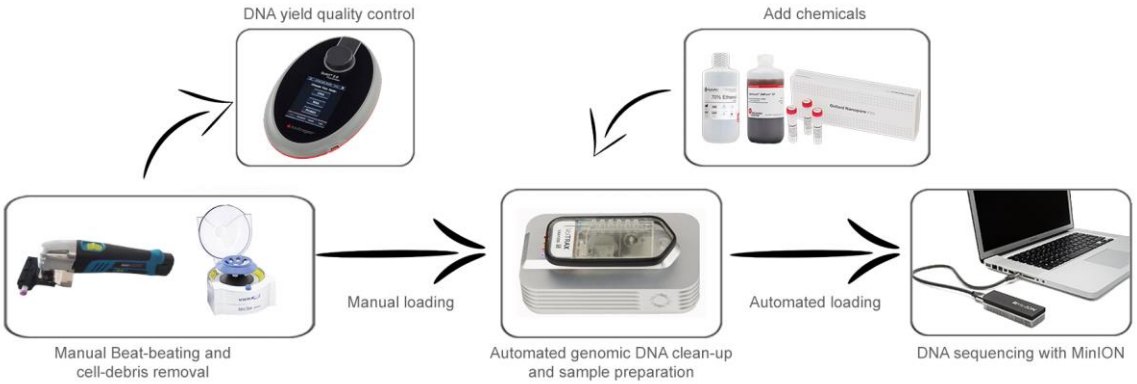


Figure 20: An overview of the automatics of on-site DNA sequencing with a mobile lab.

The only manual part of the process, is the bead-beating, the cell debris removal, the DNA quality control and adding of chemicals to the VolTRAX.
The progress can be set up in three step. First, the sample is bead-beaten and centrifuged to remove cell debris were after the DNA yield is controlled. The second phase is where the sample is loaded onto the VolTRAX with the chemicals needed to do genomic SPRI clean-up and sample preparation for the MinION. The VolTRAX handles the rest of the process and were the last phase is where the sample with DNA ready for sequencing is loads onto the docked MinION. Before this is possible, the VolTRAX still needs further development, but the automatization will bring advantages in the form of increased reproducibility since, in the genomic DNA clean-up and sample preparation, the human influences can be largely removed.

This automation can potential give a steady time reduction since the DNA extraction manual takes about 10 min, so if a significant portion of pipetting is taken out of human hands, it should be faster. Also by using the 10-min rapid library preparation kit from ONT, the time it takes to get from environmental sample to DNA ready for sequencing is about 20 min. Also as mentioned in the introduction, the DNA sequencing is real time. Therefore the objective to detect if a microorganism is in a sample has been shown that this can be achieved after 5 min (Mitsuhashi *et al.*, 2017). With this automation it gives the possibility to go from environmental sample to detection of a microorganism in under 30 mins and if 16S rRNA gene amplification is used in 40 mins (Oxford Nanopore Technologies, 2017a).

6.1.2 Reduction of the DNA sequencing

Since portability is an important part of a mobile lab, the idea is still to make the mobile lab more portable and easy to transport. One possibility is a size reduction of the DNA sequencing; here, ONT also has made further development in the form of the SmidgION; a DNA sequencing device that uses the same core nanopore sequencing technology as the MinION (Oxford Nanopore Technologies, 2017c). This can be seen in figure 21.

Figure 21: Picture of the SmidgION taken from (Oxford Nanopore Technologies, 2017b).

The SmidegION is a DNA sequencing that can be powered and run with a smartphone and makes the sequencing more "mobile" (Oxford Nanopore Technologies, 2017c).

6.1.3 Direct RNA sequencing of 16S ribosomal RNA using nanopore

As described in the introduction the idea to circumvent the traditional bias in the use of PCR amplification used in 16srRNA identification of microbial would be a vast improvement. The problem in using the 16srRNA identification of microbes without PCR is when extracted DNA the 16S rRNA gene is overly diluted into the genomic DNA (Rosselli *et al.*, 2016). However, a principle method to circumvent the PCR amplification has been made by the paper (Smith *et al.*, 2017). The method developed has an enrichment and selective of the 16S rRNA out of the total bacterial RNA, which is achieved by using streptavidin-conjugated magnetic beads and an adapter that hybridized to 16S rRNA and binds to streptavidin-conjugated magnetic beads. The early release direct RNA sequencing kit from ONT can be used, and the native 16S rRNA can be sequencing. This method can be utilized for 16S rRNA in a complex clinical or environmental sample and go from sample to detections within 2 hours.

This approach gives benefits in the full-length 16S rRNA sequencing that will provide an improved taxonomic classification and will remove PCR bias with a removal of the PCR amplification (Smith *et al.*, 2017). This method can also be used to detect antimicrobial epigenetic modifications on 16S rRNA since many antibiotics target prokaryotic ribosomes, where a resistance of microbes can be gained or lost by nucleotide substitutions or base modification (Smith *et al.*, 2017). This base modification is normally hard to detect by use of sequencing by synthesis since this is removed but can be detected by MinION (Smith *et al.*, 2017).

However, in the identification of microbes with direct 16S rRNA sequencing from total RNA, each taxon presence and its relative activity within a community should be proportional to the number of its ribosomes (Rosselli *et al.*, 2016). Also, there is a difference between the 16S rRNA gene amplification and direct 16sRNA sequencing. In 16sRNA gene amplification are influenced by ribosomal operon copy number in the genome, and cell number (Rosselli *et al.*, 2016).

The directed 16sRNA sequencing is centered on the potential protein synthesis activity as a function of the number of ribosomes per single cell and is not necessarily coupled to total cell number nor population growth (Rosselli *et al.*, 2016).
Also gives the possibility to detect bacteria, which are typically overlooked by PCR because of primer mismatch and bias (Rosselli *et al.*, 2016).

However, in this thesis, it has been shown that bead-based purification can be used on genomic DNA.Therefore it can be hypothesized that the mobile method developed in this thesis can be changed to incorporate the used of magnetic bead-based purification of total RNA after that the method can be employed.

6.2 Overview of on-site DNA sequencing of microbe's overview

Overall this gives the potential to have many applications from anti-microbial resistance to environmental research. Since this thesis objective is to develop a DNA extraction to be used for on-site DNA severing, an overview will be given of how the on-site DNA severing to identifying the microbial community on-site WWTP could be achieved. The use of on-site DNA severing can be used to determine if their bacteria can hinder the operation of the WWTP. One of the problems can be bulking and foaming which is mainly associated with filamentous bacteria(Mielczarek *et al.*, 2012).
A rough overview of this pipeline can be seen in figure 21.

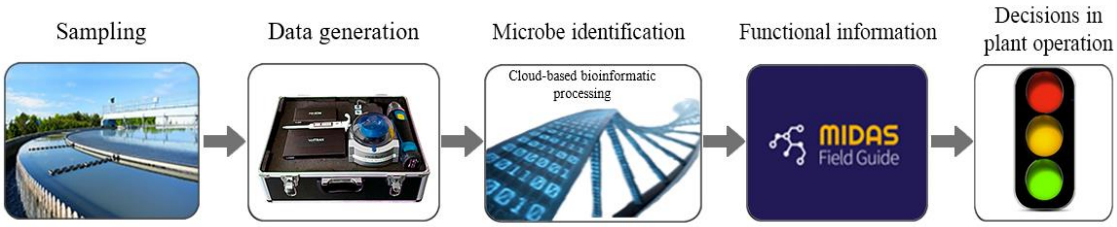


Figure 21: Overview of the Online DNA seqversing. Where an sample is taken at the WWTP, thereafter the data is generated by the used of the mobile lab. Afterward the Microbes are identified by the used of cloud-based bioinformatic processing. Then the MiDAS field guide is used to determine the function of this microbs. Then lastly this information can be used to better decision in the wastewater treatment plants.

Therefore, by sampling on-site and using the mobile lab proposed earlier to generate data. This should take about 40 mins if 16sRNA gene amplification is used. There that the by using the real-time cloud-based bioinformatic in the form of EPI2ME hosted by Metrichor, the microbial identity can be determined. After the microbe is identified the function of the microbial in the community can be determined by using the MiDAS database. This data and function can be can easily be analyzed and visualized by use of online bioinformatic apps. An example of this is the Amplicon-visualizer (Andersen, 2017), an app made of the ampvis (Albertsen *et al.*, 2015) the bioinformatic packed used in this project.

This would give the information on the microbial composition of the WWTP at regular intervals time and thereby obtain a better and cheaper operation. This gives the possibility to the WWTP to optimize and qualify decision-making when to use chemicals used to counter bulking and foaming problems and therefore reduce operation cost.

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8. APPENDIX

APPENDIX A: Price estimation of mobile method

Price estimation for cell lysis.
Since the beating tubes and lysis buffer are from a kit, it makes it hard to estimate the price for one sample for the mobile method. An estimation for the beads tubes and lysis instead the price is estimated from close relate material form outer companies.

Tabel 5: Containing the replacement of the beating tubes and Lysis buffer used in the fast-spin DNA form soil kit

Material	Replacement	Price per sample
Beading tubes	Bashing Bead Lysis (Zymo Research, no date)	19 DKK
Lysis buffer	Lysis buffer (BIO-RAD, 2017).	1 DKK

Price estimation for DNA isolation.

The price for the AMpure XP beads form Beckman is quite expensive. Since there are used two concentrations of AMPure beads, the 170 and 80 ul. The price for 60 mL are 7257 DKK, the price for 170 and 80 uL is 20 and 10 DKK respectably. The price for the buffer that the beads are diluted is not estimated with. The method showed in the rapport is the 80uL volume

Total price for the Mobile DNA extraction method.
Table 6 Table with estimate price if there are use 170uL and 80uL SPRI beads.

Method	Price per sample	Time	Maximum number of sample.	Addition equipment required
mobile 170uL	40	11:30	2	Magnet stand Mobile beater
mobile 80uL	30	11:30	2	Magnet stand Mobil beater

APPENDIX B: Heatmap of all mobile methods: genus, phylum level

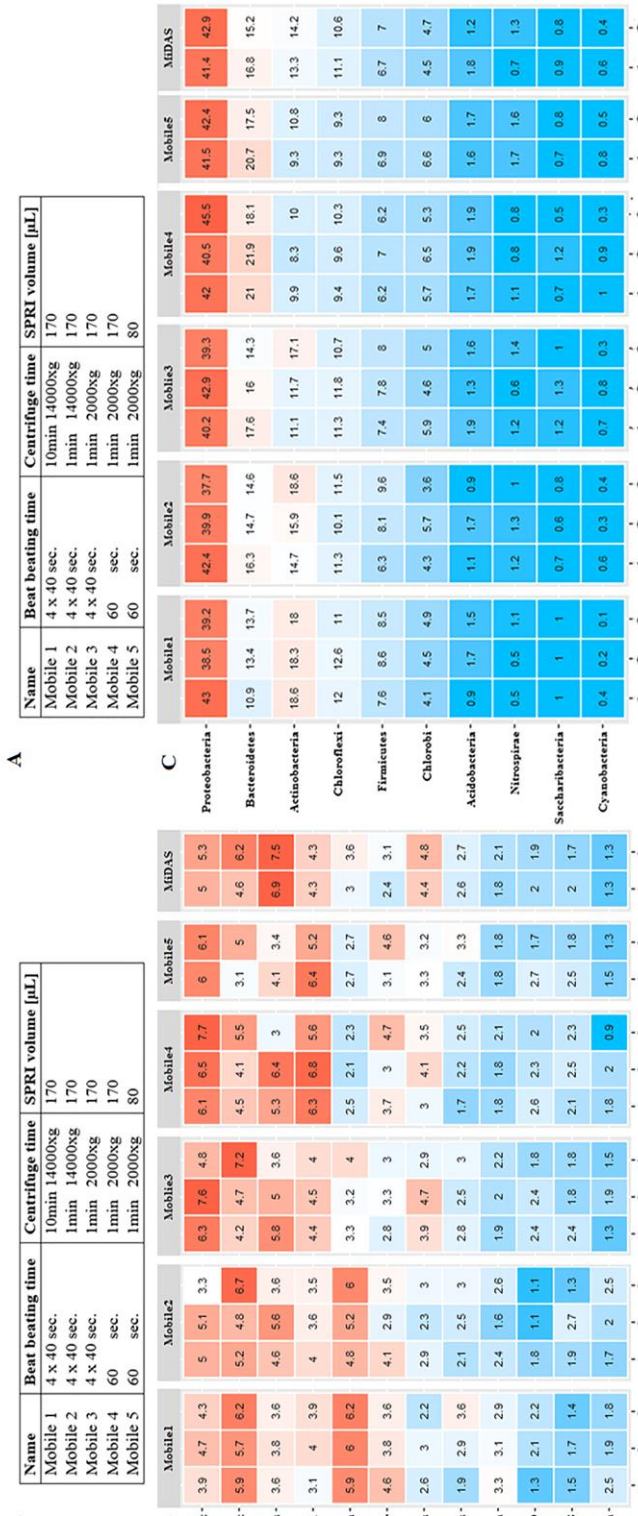

Figure 22: A An overview of all mobile methods and variations tested in this report. **B**: Heatmap of the all the mobile method in the report. The Y-axis ranks the 12 most abundant OTUs over all samples. The OTUs are assigned to their genus classification or the closest possible taxonomic rank by using the MiDAS database. The x- axis represents samples grouped into DNA extraction method, where each 1,2,3 represents a replication. Each number in the figure represents the relative read abundance of the OTUs **C:** Heatmap of the all the mobile method in the report. The Y-axis ranks the 12 most abundant OTUs over all samples. The OTUs are assigned to their phylum classification or the closest possible taxonomic rank by using the MiDAS database. The x- axis represents samples grouped into DNA extraction method, where each 1,2,3 represents a replication. Each number in the figure represents the relative read abundance of the OTUs.

APPENDIX C: Tables with all mobile method data for DNA yield, Total DNA, A260/A280 and A260/A230 ratio.

The DNA yield is messused according to Qubit fluorometry, the Total DNA is calculated from the total volume of the sample and yield. The A260/A280 and A260/A230 ratio is measured form Nanodrop

	DNA yield [ng μL^{-1}]	Total DNA [µg]	$A_{260/280}$	$A_{260/230}$
Mobile 1				
1	149	8.94	1.91	1.85
2	147	8.82	1.91	1.88
3	141	8.46	1.9	1.86
Average	145.67 ± 2.4	8.74 ± 0.0048	1.91 ± 0	1.86 ± 0.073
Mobile 2				
1	116	6.96	1.74	1.19
2	123	7.38	1.73	1.33
3	109	6.54	1.66	0.85
Average	116 ± 4.0	6.96 ± 0.24	1.71 ± 0.0033	1.12 ± 0.088
Mobile 3				
1	110	6.6	1.84	1.47
2	105	6.3	1.9	1.54
3	166	9.96	1.77	1.46
Average	127 ± 19.6	7.62 ± 1.17	1.84 ± 0.025	1.49 ± 0.014
Mobile 4				
1	123	7.38	1.77	1.46
2	149	8.94	1.9	1.54
3	86.4	5.18	1.84	1.47
Average	119.5 ± 18.2	7.17 ± 1.08	1.84 ± 0.037	1.49 ± 0.025
Mobile 5				
1	51.2	3.072	1.85	1.7
2	56.1	3.366	1.76	1.51
3	21.1	1.266	1.75	1.95
Average	42.8 ± 10.9	2.568 ± 0.65	1.79 ± 0.031	1.72 ± 0.012
MiDAS				
1	146	8.76	1.9	0.36
2	196	11.76	1.89	0.31
3	231	13.86	1.86	0.34
Average	191 ± 24.7	11.46 ± 1.48	1.88 ± 0.012	0.34 ± 0.014

APPENDIX D1: One-way ANOVA

One-way ANOVA test of the average fragment length for centrifugation.

Method	Mobile1 [bp]	Mobile2 [bp]	Mobile3 [bp]
1	6861	8548	10046
2	5782	8911	8243
3	6861	6653	6705

One-way Anova

SUMMARY				
Groups	***Count***	***Sum***	***Average***	***Variance***
Mobile1	3	19504	6501,333	388080
Mobile2	3	24112	8037,333	1470226
Mobile3	3	24994	8331,333	2796422

ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	5794632	2	2897316	1,87	0,234
Within Groups	9309458	6	1551576		
Total	15104090	8			

APPENDIX D2: T-test of the average fragment length for the SPRI volume

Method	Mobile4 [bp]	Mobile5 [bp]
1	9279	16942
2	8978	11618
3	9236	15355

T-Test: Two-Sample Assuming Unequal Variances

	Mobile4	*Mobile5*
Mean	9164	14638
Variance	26502	7471452
Observations	3,000	3,000
df	2,000	
t Stat	-3,463	
P(T<=t) one-tail	0,037	
t Critical one-tail	2,920	