Analyses of microbial community in nutrient solution with biofertilizer and risk assessment of establishment of pathogens

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Abstract

The societal interest for a more sustainable and circular food production is increasing. Hydroponic farming is an alternative way of growing vegetables that can bring farming into urban environments, reducing long transports of food. In parallel, the focus in waste treatment is being more directed toward resource recovery. Food waste is used as a resource for biogas production, producing a liquid residue that is rich in the nutrients needed for cultivation of crops that can be certified for use as a biofertilizer. The application of this biofertilizer in hydroponic cultivation systems could pave way for a circular urban food production. Striving for this, the project *"Food waste to new food in an urban context - production, risk assessment and consumer acceptance",* that is a collaboration between SLU (Swedish University of Agricultural Sciences) in Alnarp and Ulltuna (Uppsala) and Lund University, led by SLU. The project´s overall scope is to investigate the possibilities of hydroponic cultivation of different vegetables such as pak choi with a biofertilizer produced from anaerobically digested food waste (a residue from biogas production) as a nutrient source, replacing the customary inorganic fertilizer. Since the biofertilizer in hydroponic setups is in direct contact with the crops, ensuring microbiological safety of the biofertilizer in a food safety perspective is paramount. This master thesis project aims to perform an in-depth microbiological risk assessment of the biofertilizer, utilizing 16S rRNA gene amplicon sequencing of samples collected from greenhouse experiments of hydroponic setups with the biofertilizer. Additional investigations of the microbial community was performed utilizing MALDI-TOF MS and calorimetry. As a simulation of a contamination, challenge tests of the biofertilizer with the food-borne pathogens *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes* was performed to investigate their establishment in the biofertilizer. The 16S rRNA gene amplicon sequencing showed that *Mycobacterium* is the most abundant genus of bacteria in the biofertilizer used in the greenhouse experiments. The challenge testing experiments revealed that low levels of *Bacillus cereus* (~10 CFU/ml) is naturally present in the biofertilizer. The inoculation of 10^5 CFU/ml of the three food-borne pathogens resulted in *S. enterica* and *L. monocytogenes* no longer being detectable in the biofertilizer with selective plating after 48 hours of incubation, and four $\log_{10}$ reductions of *B. cereus* within 24 hours of incubation. Additionally, results from the investigation of the biofertilizer using calorimetry indicate that the biofertilizer does not seem to support microbial proliferation without the addition of substrates containing a carbon source.

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Preface

This master's thesis is part of the project "Food waste to new food in an urban context - production, risk assessment and consumer acceptance" funded by FORMAS. The thesis has been carried out at the Division of Applied Microbiology, Department of Chemistry at Lund University. It is registered as the course "KMBM05" and is the last step towards acquiring an MSc in Engineering, Biotechnology, from the Faculty of Engineering (LTH), Lund University. This project has been a deep-search within a broad subject in the field of biotechnology, fulfilling the goals required for the MSc, as well as performing research that strive towards the United Nations' sustainable development goals to pave way for a more sustainable future.

I would like to give a special thanks to all the students and employees at the Department of Applied Microbiology for all the support and help you have provided during this project, especially to Linda Jansson and Johannes Hedman for all the help regarding polymerase chain reactions and DNA extractions, to Kristjan Pullerits for the help and advice regarding filtration and sequencing, and to Christer Larsson for always assisting, both with all your knowledge and with an extra pair of hands in the lab. I would like to thank my examiner Peter Rådström for your valuable inputs, and I would also like to give a special thanks to my main supervisor Jenny Schelin, for always inspiring and supporting in all the crazy situations this spring has entailed; with your guidance this project reached new heights.

Lastly I would also like to give a special thanks to Malin Hultberg and the rest of the team at SLU who have made this master thesis project possible due to all the previous work you have done.

Popular science summary

Mikrobiologisk analys och riskbedömning av biogödsel som näringskälla i hydroponisk odling

Jordens ökande befolkning kräver också en ökande produktion av livsmedel. Ett hållbart och innovativt sätt för ökad livsmedelsproduktion är att använda sig av hydroponisk odlingsteknik, där grödans rötter placeras direkt i en flytande näringslösning istället för jord, vilket kräver mindre yta och möjliggör urban odling. Parallellt med detta så ökar intresset för resursutnyttjning och återvinning. Biogödsel framställt ur matavfall kan fungera som en näringskälla för hydroponisk odling, men frågan återstår: är det säkert att använda denna återvunna produkt för livsmedelsproduktion? För att besvara detta har den mikrobiologiska floran av biogödsel undersökts.

För att tillgodose behovet av livsmedel krävs nya och effektiva typer av jordbruk. Hydroponisk odlingsteknik har många fördelar i att det kräver avsevärt mindre vattenförbrukning än traditionellt jordbruk, det är mer platseffektivt och kan utnyttja urbana ytor, och långa transportsträckor av maten från jordbrukare in till städerna kan undvikas. Användningen av en återvunnen näringskälla är intressant ur ett hållbarhetsperspektiv, men gällande livsmedelsproduktion är den mikrobiologiska säkerheten avgörande. För att undersöka detta har den mikrobiologiska floran av ett biogödsel undersökts, både genom kartläggning av den naturliga mikrofloran i biogödseln under en hydroponisk odling, och även genom att iscensätta en kontaminering av biogödseln genom att tillsätta tre olika patogener; de sjukdomsalstrande bakterierna *Bacillus cereus, Salmonella enterica* och *Listeria monocytogenes* (vilkas förekomst i livsmedel kan orsaka magsjuka), för att undersöka hur de överlever och etablerar sig i biogödseln.

Det visade sig att ingen av de tillsatta bakterierna *B. cereus, S. enterica* och *L. monocytogenes* kunde varken etablera sig eller överleva i biogödseln i skadliga nivåer, vilket bådar gott för biogödselns användning för livsmedelsproduktion. I en förstudie av biogödselns naturliga mikroflora visade det sig att den innehöll en stor mängd och rik variation av mikroorganismer, men att dessa överraskande nog behövde ytterligare näring för att kunna vara aktiva och växa. Detta kan vara en förklaring till att patogenerna inte kunde etablera sig och överleva i biogödseln.

Den naturliga mikrobiologiska floran i biogödseln undersöktes bland annat med 16S-rRNA sekvensering, som är ett sätt att kartlägga den bakteriella kompositionen i ett prov. Det visade sig med denna metod att det mest förekommande släktet bakterier i biogödseln som använts för hydroponisk odling är *Mycobacterium.* Fastän de flesta bakterier inom detta släkte är relativt ofarliga bakterier som finns i jord och vatten, så ingår två bakterier som orsakar tuberkulos och spetälska i detta släkte. För att säkerställa säkerheten av att använda denna biogödsel för livsmedelsproduktion skulle

det vara önskvärt att vidare undersöka vilka arter av *Mycobacterium* som finns i biogödseln.

Genom resultatet att det krävs en ytterligare extern näringskälla för att tillåta mikrobiologisk tillväxt i biogödseln verkar det säkert att bruka den för livsmedelsproduktion. Icke desto mindre vore en ytterligare undersökning av precis vilka faktorer i biogödseln som saknas eller förhindrar mikrobiologisk tillväxt av intresse för att ytterligare kunna försäkra dess säkerhet, exempelvis skulle det också vara intressant att undersöka om tillsatsen av grödans rötter förändrar näringstillgängligheten för mikroorganismer.

Detta examensarbete har varit det första av sitt slag som inkluderat hela kedjan av biogödsel för hydroponisk odling, från tillverkning till tillämpning, i sin analys av den mikrobiologiska naturliga floran, och har därmed genererat många värdefulla insikter som kan ligga till stöd för att motivera dess användning till hydroponisk livsmedelsproduktion.

1. Introduction

The societal interest for a more sustainable and circular food production is increasing, in particular for small scale so called urban farming that would reduce the need for long transportation of foodstuffs from farms to grocery stores. For an effective farming in cities, where possibilities of cultivations in soil is scarce, farming with a lower area requirement is needed. One of the solutions to such a problem is to turn to hydroponic farming, where the crop is placed directly in a nutrient solution, and minimizes the space needed for cultivation. It enables the possibility of farming in varying systems that might be horizontal, vertical, in several layers and can be conducted in basements, on rooftops and in containers. In the current situation, conventional inorganic fertilizer is the main nutrient source in hydroponic setups.

This master thesis project is a part of the project "*Food waste to new food in an urban context - production, risk assessment and consumer acceptance*" that is funded by Formas 2019-2021 within the National research programme for food. The project team consists of researchers from the Swedish agricultural university (SLU) in Alnarp and in Ulltuna (Uppsala) and Lund university and this collaborative project is led by SLU. The overall scope is to investigate the possibilities of hydroponic farming of vegetables with a biofertilizer made from anaerobically digested food waste (originating from a residue of biogas production) as a source of nutrients, instead of the customary inorganic fertilizer. The use of a recycled, biobased fertilizer constitutes another advantage from an environmental perspective considering resource utilization and to advance the development of a circular and biobased economy.

Since the fertilizer in hydroponic setups is in direct contact with the crop, establishing whether this biofertilizer is microbially safe is paramount. This master thesis project aims to perform an in-depth microbial risk assessment of the biofertilizer for the final purpose of consumer use for hydroponic growth of vegetables. To investigate this, samples from a hydroponic greenhouse experiment were analysed with 16S-rRNA sequencing. To simulate the event of a contamination of the biofertilizer, challenge testing of the biofertilizer with major food-borne pathogens *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes* was carried out. In a parallel study at Ulltuna the presence of residual chemicals has been investigated and these results will also be presented and discussed in this report.

1.1. Scope

The scope of this master thesis project is to perform a risk assessment of the use of a biofertilizer for hydroponic growth of vegetables, through analysing the naturally present microbial flora of non-nitrified and nitrified biofertilizer with 16S-rRNA sequencing, and through challenge testing of the nitrified biofertilizer with the food-borne pathogens *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes.*

1.2. Aim

The goals of this project are to

- Design a procedure to successfully extract bacterial DNA to enable mapping of the natural microbial community in the biofertilizer using 16S-rRNA sequencing.
- Investigate microbial survival, growth and behaviour in nitrified biofertilizer using the food-borne pathogens *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes.*

2. Background

For the cultivation of crops on farmland, in greenhouse or hydroponic setups, the addition of a nutrient source is necessary. This section provides information of what is required of a fertilizer with a focus on the application of hydroponic farming, and the current use of hydroponic farming. The manufacturing process of the biofertilizer utilized and investigated in this project is explained, together with requirements in regards of microbiological food safety and the methods utilized in this project for the microbiological risk assessment of the biofertilizer.

2.1. Plant fertilizers

In the current situation, hydroponic setups are mainly based on conventional inorganic fertilizer, while in this project an organic fertilizer produced from digested food waste is investigated for the purpose of hydroponic farming. In a biobased, circular system of utilizing a biofertilizer made from food waste to grow new food, there are several more variable factors in comparison to the production and usage of an inorganic fertilizer. This section aims to present the important factors to consider regarding the use of a biofertilizer produced from digested food waste.

2.1.1. Prerequisites of a plant fertilizer

The main purpose of a plant fertilizer is to provide the essential nutrients that is needed for plant growth, which cannot be retrieved from the air or synthesized by the plant itself. Essential components are mineral nutrients macronutrients nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulphur (S) and magnesium (Mg), along with micronutrients (trace elements) iron (Fe), boron (B), chlorine (Cl), manganese (Mn), zinc (Zn), copper (Cu), molybdenum (Mo) and nickel (Ni) (Barker, 2006). In this study fertilizers in hydroponic setups are investigated, and apart from the essential nutrients the nutrient solution needs to constitute an appropriate pH range (5.5-7.0 for most plants (Perry, 2003)) and a suitable level of electrical conductivity (i.e. salinity). The level of electrical conductivity is important for the plants uptake of both water and nutrients (Asp et al., 2020). An inorganic fertilizer is manufactured to provide an optimal composition of nutrients for the crop, while in the biobased fertilizer the exact composition of nutrients will vary depending on the composition of the substrate (i.e. food waste) that is digested, and might therefore be more difficult to control. It is also required that the biofertilizer is within acceptable range of undesired compounds of heavy metals and micropollutants, also that it is treated so that no food-borne pathogens are transferred from biofertilizer to plant. A previous analysis on the composition of macro and micro nutrients etc. of the biofertilizer to be used in this project compared to that of an inorganic fertilizer can be observed in Table 1.

Table 1. *pH, electrical conductivity, nitrogen compounds, macro- and micronutrients of the nitrified biofertilizer (5-6% v/v) to be used in this project compared to the same values of an inorganic fertilizer. Table collected from the report previously made in the project "Food waste to new food in an urban context - production, risk assessment and consumer acceptance", of investigations on the nitrification process of the biofertilizer by Asp, Bergstrand and Hultberg (Asp et al., 2020).*

	Nitrified biofertilizer	Inorganic fertilizer
pH	5.0-5.5	5.8
EC (mS cm^{-2})	1.8-4.0	1.8-2.0
NH4-N	14-150	17-61
NO3-N	90-180	175-210
NO2-N	0-80	-
P	8-41	38-39
K	240-250	198-200
Mg	9-21	23-34
S	27-36	46-48
Ca	76-99	152-190
Mn	0.1-0.7	0.5-0.6
B	0.1-0.2	0.2-0.3
Cu	0.04-0.1	0.03-0.1
Fe	3.3-11	0.3-1.6
Zn	0.1-0.2	0.2-0.7
Mo	0.01-0.02	0.03-0.04
Cl	70-270	28-29
Na	20-120	8-16

2.1.2. Environmental aspects

Considering the environmental aspects when comparing a synthesized inorganic fertilizer with a biobased organic fertilizer, it is not clear whether the use of a biobased fertilizer is a better alternative in regards of nitrogen leaching and pollution. However, studies have shown that the use of a biobased organic fertilizer does improve soil microbial activity, plant nutrient synthetization, and yield (Lin et al., 2019, Ibrahim et al., 2013).

Nevertheless, the utilization of an organic fertilizer produced from food waste provides a circular source of nutrition. In Sweden, approximately 1.7 million tonnes of biofertilizer was produced at 20 biogas/waste treatment plants, of which 99% where used as a fertilizer on farmland (Avfall Sverige, 2017).

2.1.3. Manufacturing of the biofertilizer utilized in this project

The biofertilizer investigated in this project is a residual product from the production of biogas produced at Karpalund biogas plant located in north-eastern Skåne, Sweden. This is one of the bigger biogas plants in Sweden, treating around 85,000 tonnes of organic waste per year (CTCN). An image showing the biogas plant with its digestion chambers can be observed in Figure 1.

Figure 1. *Karpalund biogas plant. Image downloaded from Wikipedia: Biogasanläggningen Karpalund https://sv.m.wikipedia.org/wiki/Karpalund.*

The biogas is produced from a mixture of household food waste (~40%), manure (~25%), slaughterhouse waste (~20%), and residues from food production industry. The ingoing ingredients are mixed into a slurry with a dry weight of 8-10% The slurry then undergoes a so called hygienization, where it is heated to >70°C during 1 hour. The hygienized material is then anaerobically digested during approximately 25 days, during which the temperature can be somewhere between 37-46°C, and the pH is 7 or higher. Thereafter it undergoes a second digestion during approximately 10 days. After digestion the residue (that is the basis for the biofertilizer) is kept in post-digestion storage at room-temperature during a couple of days before being transported to end-consumer (Stuhre, 2020, oral communication). This product has to be approved and certified as a biofertilizer according to SPCR 120 (Avfall Sverige, 2020). A schematic illustration of the production of the biofertilizer can be observed in Figure 2.

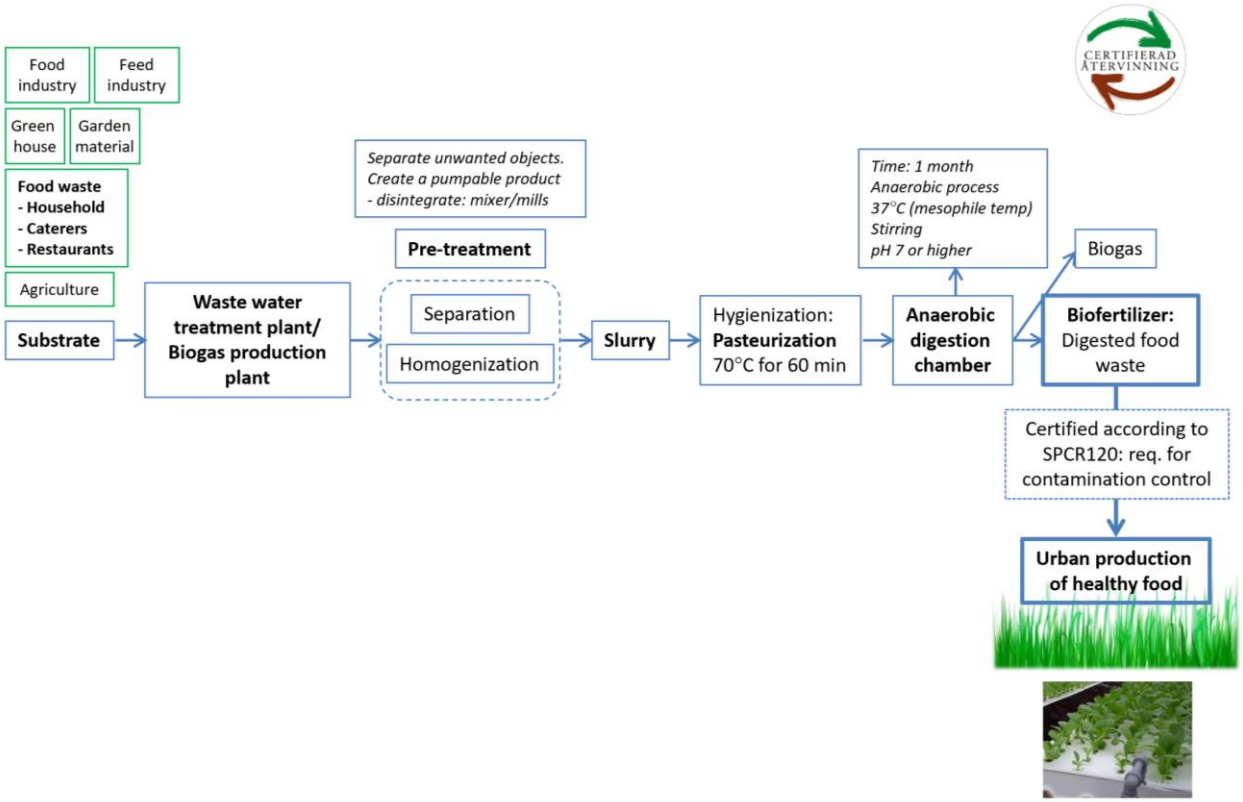


Figure 2. *A schematic illustration of the production of the SPCR 120 certified biofertilizer utilized in this project.*

In the project *Food Waste to New Food*, this residue is then diluted with tap water to a concentration of 6,5%. While it contains high amounts of nitrogen which is needed for plant growth, most of it is present in the form of ammonium, which is toxic to plants in high concentrations. Therefore, the biofertilizer undergoes a nitrification process, where nitrifying bacteria converts the ammonium to nitrate (Asp et al., 2020). After nitrification, the biofertilizer is ready to use for cultivation.

In the current situation, a lot of hydroponic cultivations are performed with inorganic fertilizer. As a control throughout the experiments in this project conventional inorganic fertilizer for soilless production (1+1 g/L, respectively, of Kristalon™ Indigo and Calcinit™; Yara, Oslo, Norway) was used.

2.2. Hydroponic farming

In a world where the population is expected to reach 9 billion by 2050 (Tilman et al., 2002), the food production will also have to reach greater magnitudes. This coupled with the decrease of culturable land due to urbanization and pollution (Chen, 2007), and water shortages caused by drastic climate change predicted to leave 1.8 billion people in countries or regions with absolute water scarcity by 2025 (FAO), there are reasons to look for more integrated and smart solutions for farming.

Hydroponic farming is a soilless cultivation method where crops are cultivated in a water and nutrient solution (Gericke, 1945). This method has received increased attention and interest mainly due to its environmental benefits. The fact that there is no need for large areas of soil

enables the farming to take place in different forms, such as on roof-tops, in basements, and in layers in containers (so called vertical hydroponics), which allows for urban production of food. Positive effects of this could be shorter transport distances from cultivation sites to consumers, a more effective usage of unconventional areas for farming, and a more optimized resource utilization with recirculating systems. Some companies are already using this technology; Swedish companies Grönska and Ikea use vertical hydroponic farming for cultivation of herbs and leafy greens (Obminska, 2020, Obminska, 2018) with success. Examples of what vertical hydroponic farming setups can look like can be observed in Figure 3 and 4 below.

Figure 3. *Example of a vertical hydroponic farming setup. Belgian company Urban Crops's vertical hydroponic farming in container. Image downloaded from Dispatch Weekly, October 2016, "The Future of Vertical Farming in 5 Inspiring Examples" https://dispatchweekly.com/2016/10/future-vertical-farming-5-inspiring-examples/.*

Figure 4. *Example of a vertical hydroponic farming setup. Swedish company Grönska's vertical hydroponic farming, with LED-lights adjusted to mathc the plants' favourite wavelength. Downloaded from Nyteknik, February 2020, "Framtidens odlingsteknik: Så grönskar det inomhus" https://www.nyteknik.se/story/framtidens-odlingsteknik-sa-gronskar-det-inomhus-698734.*

Considering the environmental benefits, theoretical studies comparing the resources needed for growing lettuce with hydroponic farming versus with conventional farming shows that hydroponic farming had a greater yield (41 ± 6.1 kg/m2/y compared to 3.9 ± 0.21 kg/m2/y with conventional farming), required less water (20 ± 3.8 L/kg/y compared to 250 ± 25 L/kg/y with conventional farming). However, hydroponic farming requires a higher energy consumption (90,000 ± 11,000 kJ/kg/y compared to 1100 ± 75 kJ/kg/y with conventional farming) (Barbosa et al., 2015). If the energy consumption is optimized, for example by designing the lighting so accordingly to the needs of the different cultivars of plants in the way Swedish company Grönska does by only utilizing the most energy-consuming blue light for plants that truly need it (as can be observed in Figure 4) (Obminska, 2020), the environmental benefits of hydroponic farming are very promising.

In regards of taste and consumer acceptance, studies performed on sensory evaluation of lettuce cv. Brunella cultivated with hydroponics versus conventional cultivation have been performed at Universidade Federal de São Carlos in Brazil, and concluded that no significant difference of preference could be perceived between the lettuces from the two different ways of cultivation (Fontana et al., 2018). A Canadian study also performed sensory evaluation on different cultivars of tomato, cucumber, rocket lettuce and bibb lettuce that had been grown either hydroponically or with conventional cultivation, and found that the differences between different cultivars of the vegetables had a greater impact on the taste than the choice of cultivation had (Nassar et al., 2015). However, consumer acceptance of hydroponically grown vegetables with a biofertilizer made from food waste remains to be investigated as another part in the project *Food Waste to New Food.*

2.2.1. The hydroponic greenhouse experiments

In the project *Food Waste to New Food*, greenhouse experiments have been performed in which the biofertilizer as described above is utilized to cultivate pak choi (a type of Chinese cabbage) in a hydroponic setup. The plants were grown in a greenhouse at ~20°C with a constant circulation of nutrient solution (Asp et al., 2020). For the sake of comparison, a hydroponic cultivation with conventional inorganic fertilizer was also set up. An illustration of the hydroponic channel with recirculating nitrified biofertilizer reaching the root system of the pak choi can be observed in Figure 5 below.

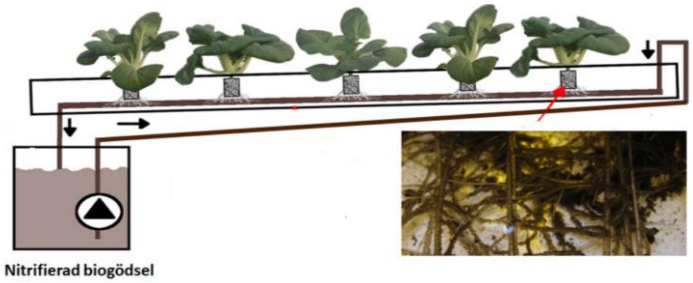


Figure 5. *An illustration of the hydroponic channel with recirculating nitrified biofertilizer reaching the root system of the pak choi. Figure collected from the report previously made in the project "Food waste to new food in an urban context - production, risk assessment and consumer acceptance", of investigations on the nitrification process of the biofertilizer by Asp, Bergstrand and Hultberg (Asp et al., 2020).*

The duration of these experiments was 21 days, the time from start of cultivation to harvesting. Samples from this cultivation lie as a foundation for the microbiological community analysis of the biofertilizer in this master thesis project. An overview of the sampling points from the cultivation experiments using both nitrified biofertilizer and inorganic fertilizer can be observed in Table 2. A sample of non-nitrified biofertilizer was also included for the microbiological community analysis.

Table 2. *Sampling points from the greenhouse experiment at SLU Alnarp where nitrified biofertilizer was utilized. Hydroponic growth using inorganic fertilizer was used as a reference. At each sampling point, a defined volume of the circulating biofertilizer was collected from the cultivation channels.*

Sampling point	**Type of fertilizer**		
	Non-nitrified	Nitrified	Inorganic
Start of experiment	X	X	X
10 days		X	X
17 days		X	X
21 days		X	X

2.3. Microbiological risk assessment of the biofertilizer in regards of food safety

In order to assess the practical feasibility of utilizing a biobased fertilizer as a nutrient source in hydroponic farming, a hazard analysis is essential; a process recognizing the physical, chemical and biological hazards that might arise and be significant in regards of food safety. Regarding the risk assessment and hazard analysis of utilizing the biofertilizer in this project, the physical hazards are monitored by the biogas plant as they control the inlet of substrate for biogas (and thus biofertilizer) production. The chemical risk assessment of the biofertilizer has been monitored in the analysis performed as another sub-project of the project *Food Waste to New Food*, conducted by SLU (Swedish University of Agricultural Sciences) in Ulltuna (Uppsala), including analysis of presence of pharmaceuticals, personal care products, and pesticides. For concluding results from this chemical analysis, see Appendix 1. In this master thesis project, a biological risk assessment has been performed to determine the hazards of the biofertilizer and its suitability for hydroponic farming. In this section, the microbiological requirements and previous microbiological investigation of this biofertilizer are presented, as well as the means of determining the microbial safety in this master thesis project.

2.3.1. Microbiological requirements of a certified biofertilizer and additional controls

To be certified for use as a biofertilizer, the liquid residue from biogas production plant must fulfil certain requirementsaccording to SPCR 120 (Avfall Sverige, 2020). After the substrate for the biogas production has been mixed into a slurry, it undergoes a hygienization where it is subjected to a temperature of at least 70°C for at least 60 consecutive minutes. During a hygienization control, microbiological samples are taken before (sample point 1) and after (sample point 2) hygienization, as well as after digestion (sample point 3) when the biofertilizer is ready to be delivered to end user. At sample point 1 and 2 the presence and reduction of *E. coli* and *Enterococcus* are monitored (reduction by at least 4 $\log_{10}$ units), and at sample point 3

both of the previous as well as an absence of *Salmonella* is monitored (not detectable in 25 g of biofertilizer).

In addition to the requirements in the certification, some additional controls were conducted under the project *Food Waste to New Food* at three biogas/waste treatment plants to obtain a further understanding of the microbiological nature of the biofertilizer. The results from these controls revealed that the biofertilizer in fact had unsanitary levels (EFSA, 2005) of food-borne pathogen *Bacillus cereus* at all plants that had been sampled, as can be observed in Table 3.

Table 3. *Previous results from microbiological sampling of biofertilizer at three different biogas plants in the project* Food Waste to New Food. *In addition to the analyses required by the SPCR 120 certification, additional sampling including C. perfringens, B. cereus, ESBL-CARBA (Extended-spectrum beta-lactamases-Carbapenemases resistant intestinal bacteria such as Enterobacteriaceae, Escherichia coli and Klebsiella) and MRSA (methicillin resistant Staphylococcus aureus) was performed. Two biological replicates were analysed per microorganism, sampling point and biogas plant. Dates indicate when samples were collected at the respective biogas plant. After hyg: samples collected immediately after the hygienization step of the biodegradable waste). Final product: samples collected after anaerobic digestion completed. n.d.: not detected. Results highlighted in pale orange indicate increased concentrations and results highlighted in orange (B. cereus) indicate concentration above recommended levels.*

	2019-06-24		**2019-07-11**		**2019-07-11**	
	Karpalund		Uppsala		Västerås	
	After hyg	Final product	After hyg	Final product	After hyg	Final product
E. coli NMKL 125, 4th ed, 2005	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g
	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g
Enterokocker NMKL 68, 5th ed, 2011	< 2.0 log cfu/g	< 2.0 log cfu/g	< 2.0 log cfu/g	< 2.0 log cfu/g	3.0 log cfu/g	4.4 log cfu/g
	< 2.0 log cfu/g	< 2.0 log cfu/g	< 2.0 log cfu/g	< 2.0 log cfu/g	3.5 log cfu/g	< 2.0 log cfu/g
Salmonella NMKL 71, 5th ed, 1999	n.d. in 25 g	n.d. in 25 g	n.d. in 25 g	n.d. in 25 g	n.d. in 25 g	n.d. in 25 g
	n.d. in 25 g	n.d. in 25 g	n.d. in 25 g	n.d. in 25 g	n.d. in 25 g	n.d. in 25 g
C. perfringens NMKL 95, 5th ed, 2009	2.7 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g
	< 1.0 log cfu/g	2.8 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g	< 1.0 log cfu/g
B. cereus NMKL 67, 6th ed, 2010	4.6 log cfu/g	4.3 log cfu/g	4.0 log cfu/g	3.3 log cfu/g	4.8 log cfu/g	4.7 log cfu/g
	4.7 log cfu/g	4.1 log cfu/g	3.8 log cfu/g	3.6 log cfu/g	4.0 log cfu/g	4.7 log cfu/g
ESBL-CARBA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MRSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

2.3.2. Microbiological challenge testing of the biofertilizer

Microbiological challenge testing is an important tool in regards of food safety to investigate what will happen to a product during its processing or handling by following an inoculated microorganism in the product (Notermans et al., 1993). It might be used for determination of shelf life and investigation of microbiological spoilage of products, while in this master thesis project it was used to investigate the potential for growth in the event of a contamination of a food-borne pathogen in the biofertilizer. As the biofertilizer is recirculated in a hydroponic

setup, it will be in continuous contact with the crop during its entire growth cycle, and it is thus of interest to evaluate the hazard of such an event.

To investigate growth potential of food-borne pathogens in the biofertilizer during the process of a plants growth cycle in a hydroponic setup, these conditions were mimicked by incubating flasks of biofertilizer at room temperature and with a stirring of 100 rpm (as to resemble the aeration that is created from the continuous flow of the nutrient solution in the hydroponic channels), and inoculating them with different food-borne pathogens; *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes.* The flasks were then kept incubated like this during the time course of a plant cycle, 21 days, and the growth and establishment of the pathogens in the biofertilizer was monitored by sampling at a number of antecedently determined sampling points. The samples of the three different pathogens were then quantitively monitored for the respective pathogen according to of NMKL (Nordic Committee on Food Analysis) agar plating methods.

2.3.3. Microbial community analysis using 16S rRNA gene amplicon sequencing

As an in-depth microbiological analysis of the biofertilizer, samples were taken from a hydroponic setup with the biofertilizer during different time points of a plant growth cycle to map its microbiological community in detail during such a process. 16S-rRNA sequencing for bacterial identification is possible due to that the 16S-rRNA gene that exists in all bacteria contains regions that are highly conserved among all bacterial species, and in between these conserved regions have 9 hypervariable regions that differ more the more distant the phylogenetic relationship between the genera are. Since the 16S-rRNA gene sequence has been determined for a lot of strains, there is a massive library to which the sequenced sample of interest can be matched to, and thus identified (Clarridge, 2004).

The sequencing itself is essentially a PCR reaction where a primer set is chosen depending on which part of the 16S-rRNA gene that is desired to be sequenced. Choosing primer region is a disputed subject, and there is currently no consensus on which one that provides the most advantageous result. (Pollock et al., 2018). However, it has been shown that the primer choice and design will have an effect on the phylogenetic resolution of the sequencing (Ghyselinck et al., 2013). The primer set chosen in this experiment was 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGACTACNVGGGTWTCTAAT-3') that sequences the V4 region of the 16S-rRNA gene (Apprill et al., 2015). This region was chosen to be sequenced both based on recommendation from the company that performed the sequencing as they had had satisfactory results with this primer set, and also based on other studies where this primer set had been used for similar purposes as in this experiment, for example in the Earth Microbiome Project (Thompson et al., 2017).

The bioinformatic analysis of the 16S rRNA gene amplicon sequencing is performed through clustering the reads from the sequencing at some level of similarity into so called OTUs (Operational Taxonomic Unit), which correspond in a manner to the bacterial genera or species. The sequencing data clustered into OTUs then provide relative abundance of the different OTUs detected in a sample. The bacterial taxa can be assigned the generated OTUs through similarity searching of the reads in public databases (Takayasu et al., 2019).

3. Materials and methods

In this section the materials and methods used in this project are presented. The SPCR 120 certified biofertilizer (digestate from biodegradable waste) analysed throughout the project was collected at the biogas production plant Karpalund in Kristianstad, Sweden. The nitrification of the biofertilizer was performed in SLU, Alnarp prior to the green house experiments.

3.1. Investigation of the microbial community of the biofertilizer

3.1.1. Pre-study of fertilizers by cultivation

With the purpose of being aware of the microbial background before starting the challenge tests, and also to further map the microbial community, a three-week experimental setup to study growth over time was performed on the non-nitrified biofertilizer, the nitrified biofertilizer and the inorganic fertilizer (1+1 g/L, respectively, of Kristalon™ Indigo and Calcinit™; Yara, Oslo, Norway)**.** One hundred (100) ml of the three different fertilizers were incubated in 1000 ml baffled shake flasks (previously sterilized through autoclavation) at 20°C and 100 rpm. Samples were collected at the following time points during incubation: 0 hrs, 4 hrs, 8 hrs, 24 hrs, 48 hrs, 10 days, 17 days and 21 days and analysed through traditional cultivation-based plating on Brain Heart Infusion (BHI) agar (BD Difco™, USA). Samples were diluted by stepwise 10x dilution series to appropriate dilutions with sterile 0.9% NaCl (Merck KGaA, Darmstadt, Germany) and two succeeding dilutions were plated on two BHI agar plates (100 µl/plate). After 24 h incubation at 37°C, the number of colonies on the plates were counted and mean value were used to calculate the logarithmic colony forming units (CFU) per ml, with respect to the dilution factor.

3.1.2. Biotyping using MALDI-TOF mass spectrometry

MALDI-TOF (Matrix Assisted Laser Desorption Ionization – Time of Flight) was used to identify some of the microorganisms found in the biofertilizer in the pre-study. The analysis was performed using equipment at Skåne University Hospital in Lund thanks to the collaboration of Bo Nilson and Lisa Wasserstrom working at the department of Clinical Microbiology at Skåne University Hospital.

The method was performed through first lysing selected colonies grown on BHI agar plates, and then placing the sample together with formic acid that fixes the sample on a small analysis plate, as can be observed in Figure 6. The plate was then placed in the The MALDI Biotyper® (Bruker) instrument (as can be observed in Figure 7) which ionizes the proteins fixed on the plate. When a current is placed over the column, the ionized proteins are transported through it, and are separated by their mass and size, which creates a specific mass-spectrum for the sample in the order of which the ionized reach a detector at the end of the column. The spectrum of each sample was then compared to a library which matched it against a reference library of spectrums specific for different microorganisms, where the matching would obtain a score of 0 to 3. If the score is >2.0, it is considered to be a reliable match, if <2.0 and >1.7 it is a low-confidence match, and if it is <1.7 it is unlikely to be a correct match (Nilson, 2020, Skåne University Hospital Lund, oral communication).

Figure 6. *Target plate for MALDI-TOF Biotyping. Extract of a lysed colony is placed in a sample position (circle) on the plate, and the sample is fixed on the tablet with formic acid. The plate is then ready to be subjected to analysis in the MALDI Biotyper® (Bruker) instrument.*

Figure 7. *The MALDI Biotyper® (Bruker) utilized in this project (image downloaded from Bruker's pamphlet on the use of the MALDI Biotyper® https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/Literature/Brochures/1866135_MALDI_Biotyper_RUO_brochure_01-2019_eBook.pdf)*

3.1.3. Isothermal calorimetery as a tool to investigate microbial activity

The isotherm calorimeter used in this project was a prototype of a Flex/Ultra-instrument (Calmetrix Inc, USA). This instrument contains eight independent calorimeters which all measure at the same constant temperature, which can be set to be between 5-90°C. At temperatures up to 50°C the samples are placed in 20 ml polypropylene vials closed with screw

caps. Each calorimeter has a reference which heat capacity is adjusted to minimize external disturbances. Baselines are measured with samples of inert material; calibrations has been performed with electric heaters in calibration vials. Heat development was registered using PicoLog Recorder software (Picotech) (Wadsö L, 2020, Div. of Building Materials, Dep. of Building and Environmental Technology, Faculty of Engineering, LTH, oral communication).

3.1.3.1. Pre-study of the nitrified biofertilizer

For initial investigation of the microbial community of the biofertilizer, calorimetry was used to investigate microbial activity. 10 ml of nitrified was placed in the 20 ml polypropylene vials described above, in replicates of two. The vials were incubated in the calorimeter at 20°C. After 5 days of incubation, one ml BHI broth ((BD Difco™, USA) was added. After another 3 days, one ml of BHI broth was added once again. After another 24 hours, one ml of glucose (125 g/l) was added.

3.1.3.2. Challenge testing of the biofertilizer

In parallel with the cultivation-based plating of the biofertilizer inoculated with the three food-borne pathogens *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes*, 10 ml of samples of nitrified biofertilizer inoculated with the pathogens to a starting concentration of 10^3 CFU/ml of the pathogens in the biofertilizer were also incubated in the calorimeter to monitor the microbial activity. Replicates of two of each type of inoculation (two samples with *B. cereus* inoculation, two with *S. enterica* inoculation, and two with *L. monocytogenes* inoculation, all samples being) were incubated at 20°C in the calorimeter, and left for 21 days.

3.1.4. Filtration of biofertilizer nutrient samples collected in greenhouse experiment

Filtration and prefiltration of nutrient samples including nitrified biofertilizer collected during two independent hydroponic cultivations in green house at SLU, were performed according to a filtration protocol designed and optimized by Pullerits et al (unpublished, 2020). This filtration was accomplished prior to DNA extraction and 16S rRNA gene amplicon sequencing. The filtration setup consisted of a previously sterilized (autoclaved) sidearm flask connected to a tube sucking out air, creating a vacuum, together with a filter holder and a funnel. The filter holder and the funnel were held together by a clamp. For this experiment, filter holders and funnels in both stainless steel and glass were used ("Stainless steel funnel, filter holder and clam, 47 mm", Merck (Darmstadt, Germany) and "Classic glass filter holders, 47 mm", Merck (Darmstadt, Germany)). Before each use, the funnel and filter holder in stainless steel were sterilized with a Bunsen burner, and the ones in glass were sterilized through autoclaving. The filtration setup can be observed in Figure 8.

Figure 8. *Filtration setup. Stainless steel funnel and filter holder held together with a clamp, inserted into a sidearm flask connected to a tube creating a vacuum. (Photograph by J. Södergren, 2020)*

The nutrient samples obtained from the hydroponic cultivation (greenhouse) experiments were received frozen, and prior to filtration the samples were thawed slowly in a refrigerator (5-8°C). The samples that were going to be filtered had a volume of 100 ml. Given the particle density of the biofertilizer, even with a step of prefiltration, to be able to filtrate the liquid it needed to be divided into smaller volumes. The sample of non-nitrified biofertilizer was divided into 3 parts of 33 ml each as it had a greater particle density, and samples of nitrified biofertilizer and the inorganic fertilizer was divided in two with 50 ml per filtration. The samples of 33 or 50 ml were poured into the funnel with the filter holder that held the prefilter ("Glass Fiber Filter with binder", product number AP2004700, Merck (Darmstadt, Germany)), and the liquid was sucked down into the sidearm flask below as a result of the vacuum created in the sidearm flask. The funnel, filter holder and clamp were then moved to a new sidearm flask that was connected to a tube with suction, and a 0.22 µm filter ("Isopore Membrane Filter" pore size 0.22 µm, product number GTTP04700, Merck (Darmstadt, Germany)) was placed on the filter holder. The

content in the first sidearm flask after prefiltration was then poured into the funnel for the final filtration onto the 0.22 µm filter. The 0.22 µm filters holding the bacterial cells of the filtered samples were placed in sterile petri dishes and stored in a -20°C freezer until DNA extraction would take place. An example of the filters after filtration can be observed in Figure 9 below.

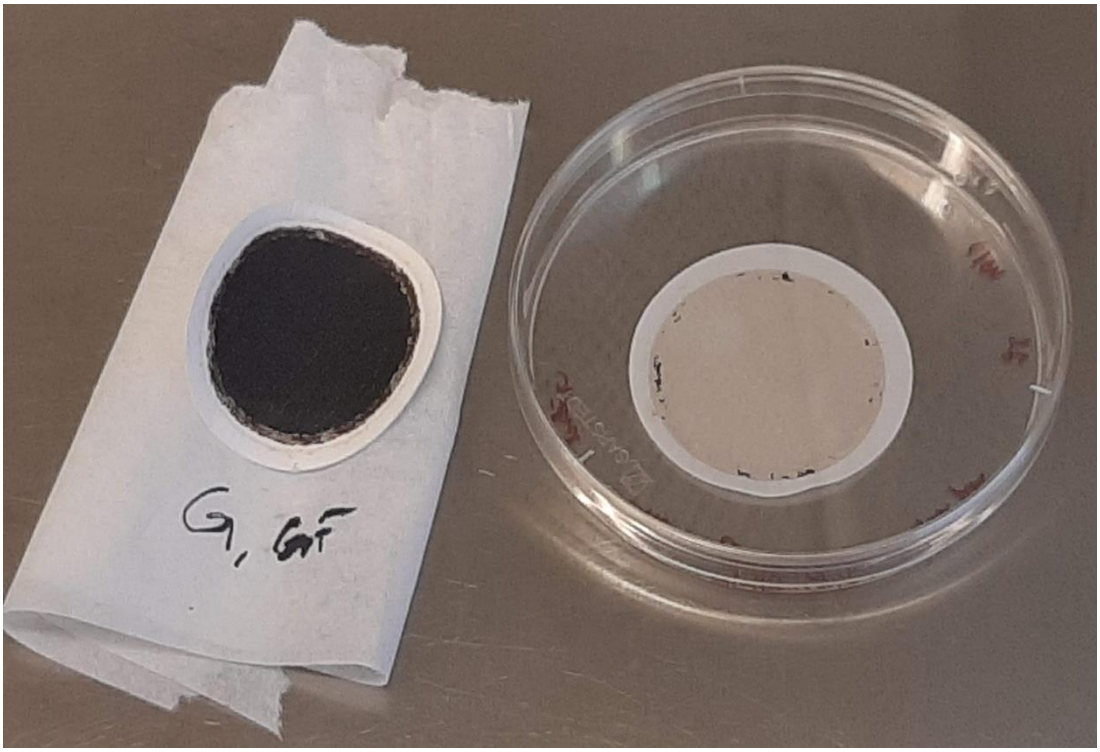


Figure 9. *Example of the filters after filtration. To the left prefilter, to the right 0.22 µm filter after filtration of the non-nitrified biofertilizer. (Photograph by J. Södergren, 2020)*

3.1.5. DNA extraction

3.1.5.1. NUCLISENS® MINIMAG®

The first method of DNA extraction to be tested was the NUCLISENS® MINIMAG®. Samples of the biofertilizer (unfiltered) were poured in volumes of 50 ml into 50 ml Falcon tubes (Sarstedt Inc.), and were then centrifuged at 3200 × g at 4°C in 30 minutes in Centrifuge 5424 (Eppendorf, Hamburg, Germany). The supernatant was discarded, and the pellets were dissolved in 0.9% NaCl (Merck). The reagents from the NUCLISENS® MINIMAG® kit were room-tempered prior to use. Lysing buffer and wash buffer were pre-heated to 37°C. Up to 1000 µl of the samples were added to the lysing buffer tubes and vortexed with Vortex-Genie 2 (Scientific Industries, Inc., New York, USA). The tubes were incubated at room-temperature for 10 minutes, whereupon the silica solution was also vortexed and added in volume of 50 µl to each lysing tube, and the lysing tubes were vortexed immediately. Samples were incubated for 10 minutes at room-temperature, and then centrifuged using Microcentrifuge MiniStar silverline (VWR, Radnor, USA) for 2 minutes at 1500 × g. The supernatant was discarded, and 1.5 ml Eppendorf (Eppendorf, Hamburg, Germany) tubes were placed in the MiniMAG instrument with lids open. The pellets of the samples were dissolved in 400 µl wash buffer, and transferred to the 1.5 ml tubes in the MiniMAG instrument. The magnetic rack was placed

upright, and samples were washed with wash buffer according to the five wash steps, being careful to not disturb the silica particles gathered around the magnet. After washing, all of the washing buffer was removed, samples removed from the MiniMAG instrument and elution buffer was added. Samples were placed in heat block for 5 minutes 60°C at 900 rpm. Samples were then again placed in the MiniMAG instrument, and the extracted DNA was transferred to new microtubes being careful not to transfer magnetic particles. Some of the extracts were further purified utilizing ZYMO spin columns (ssDNA/RNA Clean & Concentrator, ZYMO RESEARCH, USA).

This method of extraction was not used in the final analysis of the microbial community.

3.1.5.2. FastDNA™ SPIN Kit for Soil

Ultimately DNA extraction was performed with FastDNA™ SPIN Kit for Soil, MP Biomedicals. Since the samples to be analysed in this study are of liquid origin, they were prefiltered and then filtered onto a 0.22 µm filter (see section 3.1.4.) Thereby, instead of inserting a soil sample as is suggested by the manual, the 0.22 µm filters containing the bacterial cells of the samples were cut into thin shreds with a sterilized knife, and inserted into the lysing tubes (one filter per tube). Following the manual of the FastDNA™ SPIN Kit for Soil, the samples were then homogenized with a FastPrep instrument (MP Biomedicals) that homogenizes the samples. Due to the presence of the beads in the lysing tube the bacterial cells were mechanically lysed as well. The samples were then centrifuged to pellet debris (for 15 minutes instead of 5-10 as suggested by the manual), and the supernatants were transferred to new 2 ml Eppendorf tubes (Eppendorf, Hamburg, Germany). A protein precipitation solution was added, and the samples were once again centrifuged to pellet the precipitated proteins. The supernatants were transferred to 15 ml Falcon tubes, and a DNA binding matrix was added. The samples were then placed on a rocking table to allow for binding of the DNA to the matrix. The samples were then transferred to SPIN™ Filter Tubes and was centrifuged. The SPIN™ Filters were transferred to new catch tubes, and the pellet containing DNA was resuspended using 100 µl of DNase/Pyrogen-Free Water. The samples were placed on a heat block at 55°C for 5 minutes, and were then centrifuged. The eluted DNA was then pooled together (as the samples had been divided into parts of two or three in the filtration.

3.1.6. Quantitative PCR

Quality control of all DNA extractions were performed using quantitative PCR was performed using LightCycler® Nano (Roche Diagnostics, Basel, Switzerland) and CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA). Two different protocols were used; one for a *Salmonella* assay and one for a General Bacteria assay. The reagents for the two different protocols are presented in Table 4 and 5. The same PCR program was used for both protocols, and can be observed in Table 6.

Table 4. *Salmonella assay. PCR reagents for the amplification of the invA gene present in* Salmonella. *All the concentrations of the reagents (in parenthesis) are stock concentrations.*

Reagent	**µl/well**
SuperQ water	8.5
ImmoBuffer (10X) (Roche Diagnostics, Basel, Switzerland)	2
dNTP (2 mM) (Roche Diagnostics, Basel, Switzerland)	2
$MgCl_2$ (25 mg/µl) (Roche Diagnostics, Basel, Switzerland)	3.2
Primer invA F (10 µM) (Roche Diagnostics, Basel, Switzerland)	0.6
Primer invA R (10 µM) (Roche Diagnostics, Basel, Switzerland)	0.6
Probe invA FAM (10 µM) (Roche Diagnostics, Basel, Switzerland)	0.4
BSA 20 mg/ml (Roche Diagnostics, Basel, Switzerland)	0.5
IMMOLASE™ DNA Polymerase (5 U/µL) (Roche Diagnostics, Basel, Switzerland)	0.2
Sample	2

Table 5. *General Bacteria assay. PCR reagents with general primers for the amplification of bacteria. All the concentrations of the reagents (in parenthesis) are stock concentrations.*

Reagent	**µl/well**
SuperQ water	9.1
ImmoBuffer (10X) (Roche Diagnostics, Basel, Switzerland)	2
dNTP (2 mM) (Roche Diagnostics, Basel, Switzerland)	2
$MgCl_2$ (25 mg/µl) (Roche Diagnostics, Basel, Switzerland)	3.
Primer bact F (10 µM) (Roche Diagnostics, Basel, Switzerland)	0.6
Primer bact R (10 µM) (Roche Diagnostics, Basel, Switzerland)	0.6
EVAGREEN 20X (Roche Diagnostics, Basel, Switzerland)	1
BSA 20 mg/ml (Roche Diagnostics, Basel, Switzerland)	0.5
IMMOLASE™ DNA Polymerase (5 U/µL) (Roche Diagnostics, Basel, Switzerland)	0.2
Sample	2

Table 6. *PCR program*

Step	Initial denaturation	Denaturation	Annealing	Extension
Temp	95°C	95°C	60°C	72°C
Time	10 min	10 sec	6 min	30 sec
		45 cycles		

3.1.7. 16S rRNA gene amplicon sequencing

The Danish company DNASense (https://dnasense.com/) was contacted to perform and analyse the 16S-rRNA gene amplicon sequencing, see Appendix 2. Primers chosen for the sequencing of V4 variable region were the primer pair 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGACTACNVGGGTWTCTAAT-3').

3.2. Challenge testing of the biofertilizer

3.2.1. Pre-cultures for inoculation into the biofertilizer

Three food-borne pathogens were used for the challenge testing of the biofertilizer: *Bacillus cereus* (F2085) (gift from SVA, Swedish National Veterinary Institute) (Fricker et al., 2011),

Salmonella enterica serovar Typhimurium (CCUG-98112-08) and *Listeria monocytogenes* LM052 (a model strain from EU reference laboratories) (Guiller, 2013). The three organisms were collected from glycerol stocks kept in a -80°C freezer, and spread on agar plates, Brain Heart Infusion agar (BD Difco™, USA) for *B. cereus* and *L. monocytogenes* and Luria Bertani agar (BD Difco™, USA) for *Salmonella.* The inverted plates were incubated overnight for approximately 15 hours; *L. monocytogenes* and *Salmonella* at 37°C and *B. cereus* at 30°C. One discrete colony was picked with a sterile plastic loop (VWR International) and placed in a Falcon tube (Sarstedt Inc) in 50 ml of nutrient broth (Brain Heart Infusion broth (BD Difco™, USA) for *B. cereus* and *L. monocytogenes* and Luria-Bertani broth (BD Difco™, USA) for *Salmonella*), and this same procedure was performed with three different Falcon tubes for the three different microorganisms. The tubes were placed on a rocking table and incubated overnight for approximately 15 hours; *L. monocytogenes* and *Salmonella* at 37°C and *B. cereus* at 30°C. The concentration of the cultures after incubation were measured using flow cytometry (BD Accuri™ C6, BD Biosciences, San Jose CA, USA) and optical density at 620 nm (Ultrospec 2100, Amersham Biosciences Corp., USA).

3.2.2. Inoculation, incubation and monitoring of the pathogens in the nitrified biofertilizer

The experiment was performed in three independent biological replicates as to account for potential biological variance within the sample, and provide a more reliable result. The initial aim was to inoculate 10^3 CFU/ml of the cultured microorganisms into the nitrified biofertilizer, but due to the results of the challenge testing from the first experiment the inoculation dosage was increased to 10^5 CFU/ml for the following two experiments. Hence, in the first biological replicate three 1000 ml baffled (previously autoclaved) shake flasks containing 200 ml of nitrified biofertilizer was inoculated with one food-borne pathogen each (a visualization of the inoculation can be observed in Figure 10) to obtain a starting inoculation concentration of 10^3 CFU/ml. In the two following biological replicates, three 1000 ml baffled shake flasks containing 200 ml of nitrified biofertilizer was inoculated with one food-borne pathogen each to obtain a starting inoculation concentration of 10^5 CFU/ml.

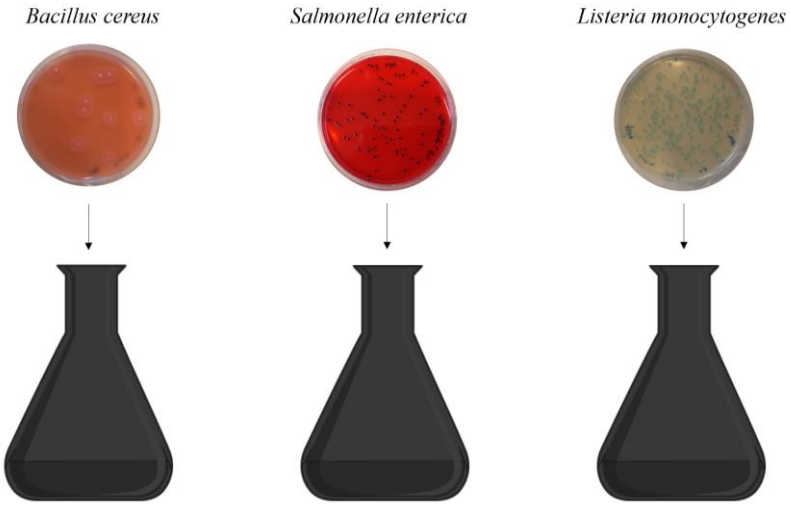


Figure 10. *A visual clarification on how the inoculation was performed. Three different food-borne pathogens were inoculated in three different baffled shake flasks containing nitrified biofertilizer.*

After inoculation, the flasks were incubated under aerobic conditions at 20°C and 100 rpm. One flask containing only the nitrified biofertilizer (no inoculation) was also incubated along with the three other flasks as control/reference. The survival and establishment of the pathogens in the nitrified biofertilizer was monitored through plating on selective agar. This procedure was performed according to instructions from NMKL (Nordic Committee on Food Analysis). For *B. cereus* MYP (Mannitol egg Yolk Polymyxin) agar (Merck KGaA, Darmstadt, Germany) was used, for *Salmonella* XLD (Xylose Lysine Deoxycholate) agar (Merck KGaA, Darmstadt, Germany) and for *L. monocytogenes* ALOA (Agar Listeria according to Ottaviani and Agosti) (Merck KGaA, Darmstadt, Germany) was used. Samples were collected at the following time points during incubation: 0 hrs, 4 hrs, 8 hrs, 24 hrs, 48 hrs, 10 days, 17 days and 21 days. Samples were diluted by stepwise 10x dilution series to appropriate dilutions with sterile 0.9% NaCl (Merck KGaA, Darmstadt, Germany) and two succeeding dilutions were plated on three agar plates (100 µl/plate). After 24 h incubation at 37°C, the number of colonies on the plates were counted and mean value were used to calculate the logarithmic colony forming units (CFU) per ml, with respect to the dilution factor.

3.2.2.1. Standard plate count

A protocol on how to enumerate colonies was established based on FDA BAM Chapter 3: Aerobic Plate Count https://www.fda.gov/food/laboratory-methods-food/bam-chapter-3-aerobic-plate-count. Based on guidelines from this manual the following criteria were established to ensure consistency in the enumeration: Plates containing between 25-250 colony forming units were counted. Plates of the lowest dilution possible that had <25 CFU per plate were counted anyway, and reported as an estimated count. Plate counts were reported as the mean of the three replicates of the dilution counted and converted into logarithmic values for easier comparison. Estimated counts was marked with an asterisk. ANOVA statistical analysis was performed with MS excel to investigate if there was a statistical difference between the growth curves within each biological replicate.

4. Results

In this project different sets of samples have been analysed in order to understand microbial presence and composition, survival, growth and behavior in an organic biofertilizer. The biofertilizer is composed of anaerobically digested food waste which is the residual produced during biogas production. The main set of samples to be analysed was the "greenhouse samples" that were collected from hydroponic growth experiments using the biofertilizer as the source of nutrients. In addition pre-study experiments and challenge tests were performed directly in the biofertilizer generating a set of samples that were further analyzed. Traditional cultivation-based plating was used to investigate presence, survival and growth of microorganisms present in the biofertilizer along with complementary methods such as isothermal calorimetry, MALDI-TOF biotyping and microbial community analysis using DNA sequencing of the 16S rRNA gene.

4.1. Investigation of the microbial community naturally present in the biofertilizer

While the main purpose of this master thesis project was to microbiologically investigate the nitrified biofertilizer, as it is the one to be used for hydroponic farming, it is also compared to the non-nitrified biofertilizer, to investigate what changes the nitrification of the biofertilizer entails. The two biofertilizers were also compared to a conventional inorganic fertilizer which is the type of fertilizer conventionally used in hydroponic farming. The three fertilizers used in this experiment can be observed in Figure 11 below.

Figure 11. *The three types of fertilizer investigated in this master thesis project. From left to right: Non-nitrified biofertilizer, nitrified biofertilizer, and inorganic fertilizer. (Photograph by J. Södergren, 2020)*

4.1.1. Pre-study of fertilizers by cultivation

A pre-study was performed initially in order to further get to know the matrix that the biofertilizer constitutes in regards to microbial flora. Shake flasks with nitrified biofertilizer were incubated during 21 days at 20°C and 100 rpm (a slow mixing to resemble the conditions from the greenhouse experiment where the nutrient solution slowly circulated in the growth channels. Samples were collected at the following time points during incubation: 0 hrs, 4 hrs, 8 hrs, 24 hrs, 48 hrs, 10 days, 17 days and 21 days and analysed through traditional cultivation-based plating on a rich general (non-selective) substrate. Considering that both the greenhouse samples to be sequenced and the challenge testing experiments were performed over a course of 21 days following the growth cycle of the plants in the greenhouse experiments, also this pre-study was performed for 21 days and samples were collected at the same time points as in the other experiments

4.1.1.1. Selection of rich general substrate and conditions of cultivation

To investigate which rich general substrate that would best support growth of the microflora naturally present in the biofertilizer, an experiment plating the incubated biofertilizer on both LB agar and BHI agar was performed. The biofertilizer was spread on the surface of the agar with spread plate technique and incubated at 37°C. This experiment showed that the plates with BHI agar both had a greater diversity of colonies and also a greater number of colonies, and therefore the BHI substrate was chosen to be used for further investigations of the microbial community of the fertilizers.

After BHI agar had been chosen as substrate to be used for the pre-study investigation of the microflora naturally present in the three types of fertilizer, 100 ml of each type in 1000 ml baffled shake flasks were incubated under previously stated conditions. The samples collected over said time points from all three different fertilizers were appropriately diluted and plated on BHI agar with spread plate technique (on the surface of the agar) and incubated in inverted position at 37°C. This most likely selects for only a part of the microflora of the fertilizers. However, these conditions of cultivation were chosen on the basis that the purpose of the cultivation was to be aware of the background flora that might appear on the plates in the challenge testing experiments (and also, to see if there was any microbial flora present at all after the hygienization of the biofertilizer). Therefore, while this cultivation only provides an inkling of the microbial flora of the biofertilizers, exactly that was the intended purpose.

4.1.1.2. Growth of the three fertilizers during 21 days

The plating of samples taken from the three fertilizers over the course of 21 days revealed a variety of microorganisms in all three types of fertilizer. Both the nitrified and non-nitrified biofertilizer showed a rich and varying microflora during the full length of the experiment. An overview of the results are presented in Table 7. The relative colony density is relative that of the first measurement to illustrate its alteration over time, and a brief description of the morphology and appearance of the colonies in each sampling point is provided. An example of the rich microflora on BHI agar can be observed in Figure 12 below. Growth from the samples taken during the first 48 hours of incubation showed quite similar colony appearances for both the nitrified and non-nitrified biofertilizer, however, there is a notable difference between the two biofertilizers in that in the non-nitrified, small colonies appeared that were completely absent in the samples from the nitrified, as can be observed in Figure 13.

Table 7. *Description of colony density and appearance of the three fertilizers incubated during 21 days. The relative colony density is relative in comparison to the first plating (0 hr). "-" means less density of colonies, "+" means a higher density of colonies, and "=" means an equal colony density.*

	Non-nitrified		Nitrified		Inorganic	
	Relative colony density	Colony morphology	Relative colony density	Colony morphology	Relative colony density	Colony morphology
0 hr		Irregular, umbonate, mucoid Circular, convex, shiny		Irregular, umbonate, mucoid Circular, convex, shiny	No colonies	-
4 hr	=	Irregular, umbonate, mucoid Circular, convex, shiny	=	Irregular, umbonate, mucoid Circular, convex, shiny	No colonies	-
8 hr	=	Irregular, umbonate, mucoid Circular, convex, shiny	+	Irregular, umbonate, mucoid Circular, convex, shiny	No colonies	-
24 hr	=	Filamentous (swarming) Irregular, umbonate, mucoid Circular, convex, shiny	+	Filamentous (swarming) Irregular, round, shiny, shiny, dull, moist	No colonies	-
48 hr	-	Filamentous (swarming) Irregular, umbonate, mucoid Circular, convex, shiny	=	Filamentous (swarming) Irregular, umbonate, mucoid Circular, convex, shiny	+	Round, shiny
10 days	-	Filamentous (swarming) Irregular, umbonate, mucoid Circular, convex, shiny	-	Filamentous (swarming) Irregular, umbonate, mucoid Circular, convex, shiny	=	Round, shiny
17 days	-	Filamentous (swarming) Irregular, umbonate, mucoid Circular, convex, shiny	-	Filamentous (swarming) Irregular, umbonate, mucoid Circular, convex, shiny, white	+	Round, shiny
21 days	-	Filamentous (swarming) Irregular, umbonate, mucoid Circular, convex, shiny	-	Filamentous (swarming) Irregular, umbonate, mucoid Circular, convex, shiny	=	Round, shiny

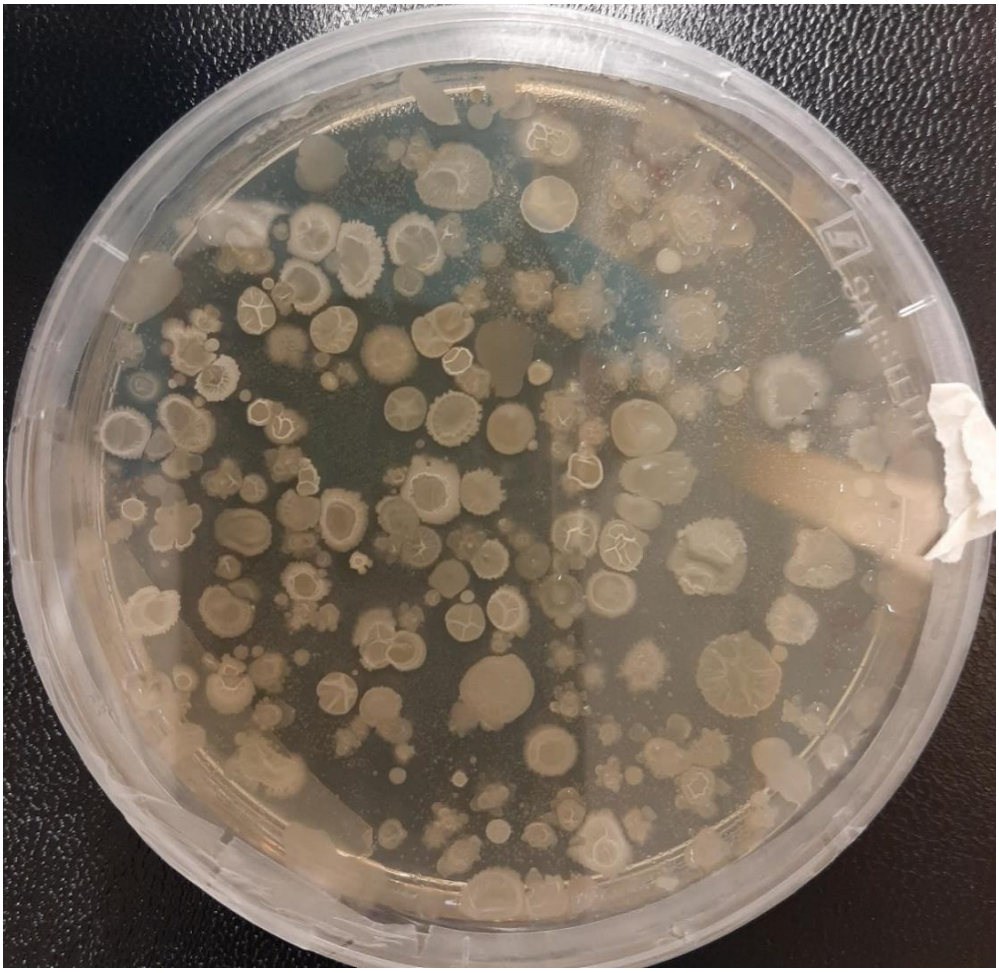


Figure 12. *A rich and varying microflora can be observed when plating the biofertilizer on BHI agar. In this image the variety in microflora from the non-nitrified biofertilizer can be observed.*

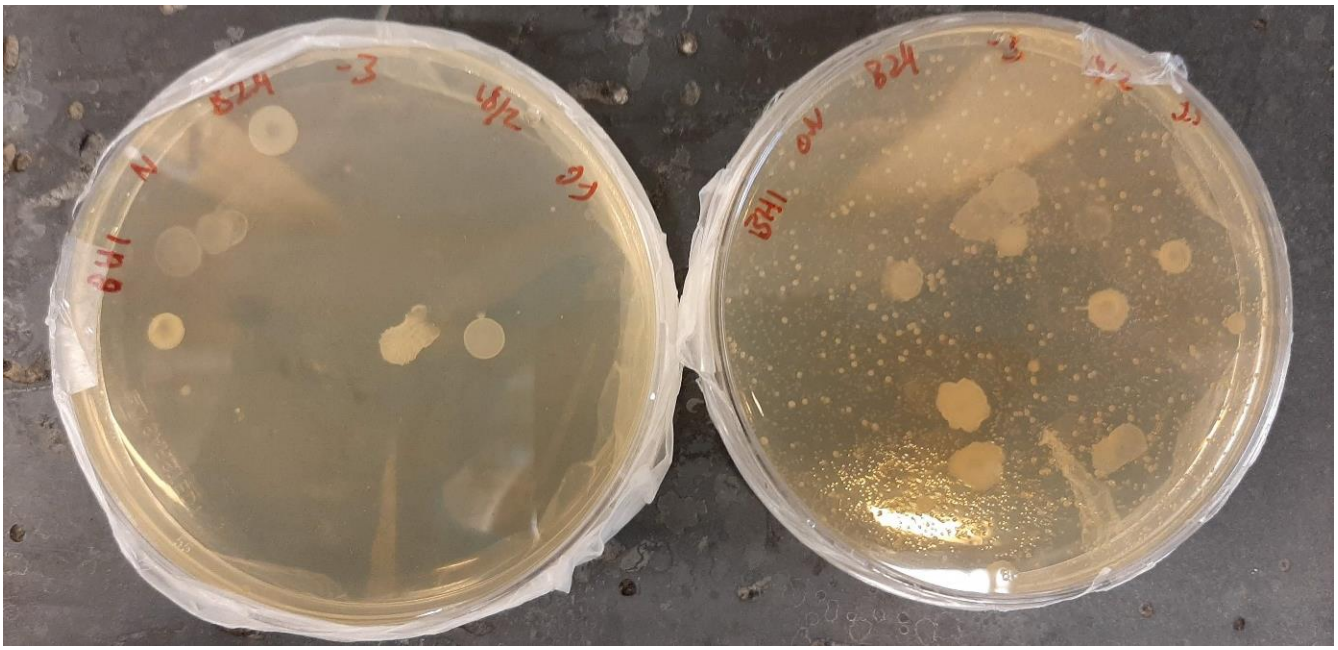


Figure 13. *A clear difference in microbial load and variety could be observed between the two biofertilizers when plating aerobically on BHI agar. To the left, nitrified biofertilizer after 24 hours of incubation plated on BHI agar (dilution 1:1000) can be observed, and to the right, non-nitrified biofertilizer after 24 hours of incubation plated on BHI agar (dilution 1:1000) can be observed.*

During the course of the experiment the microflora varies indeed, but it is difficult to determine any pattern in particular. When comparing the plates from all of the time points, it seems that the occurrence of swarming colonies (in both the nitrified and non-nitrified biofertilizer, example of a swarming colony in Figure 14 below) started after 24 hours of incubation and at the time points thereafter. However, the same colony appearance as during the first eight hours also persisted. For example, in Figure 15 below can be observed plates from the samples taken after 10 days of incubation, with both swarming and non-swarming colonies.

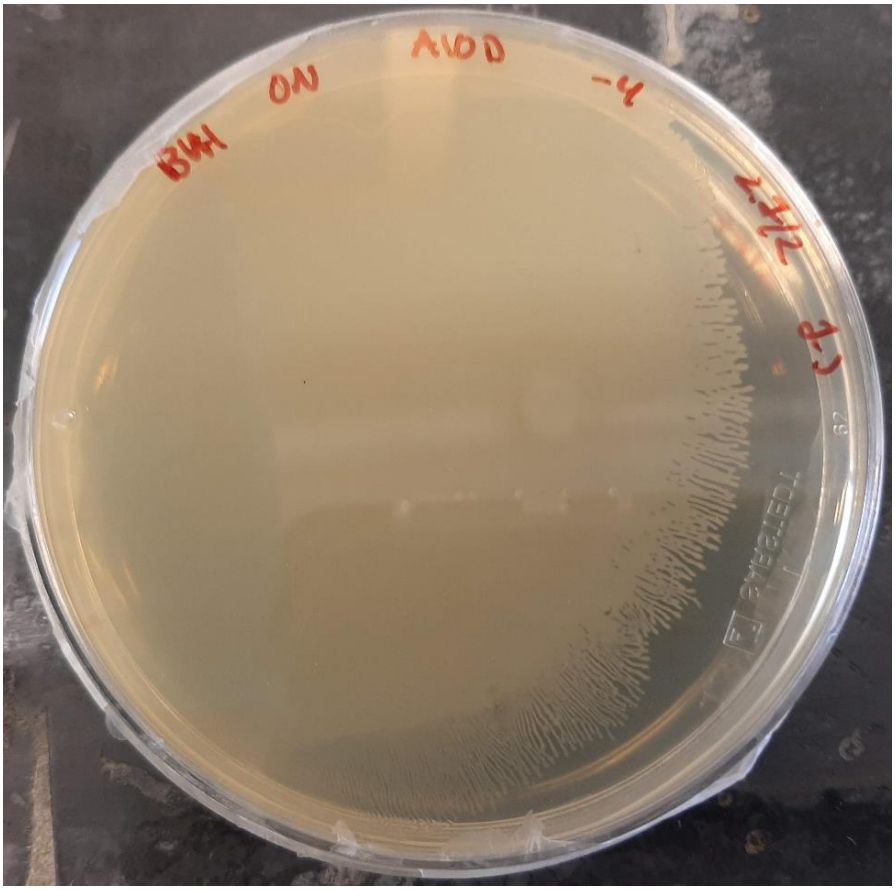


Figure 14. *Swarming colonies appeared on samples taken from incubation after 24 hours and at the time points thereafter. In this image a swarming colony can be observed in the 10 days after incubation sample of non-nitrified biofertilizer plated on BHI agar (dilution 1:10000).*

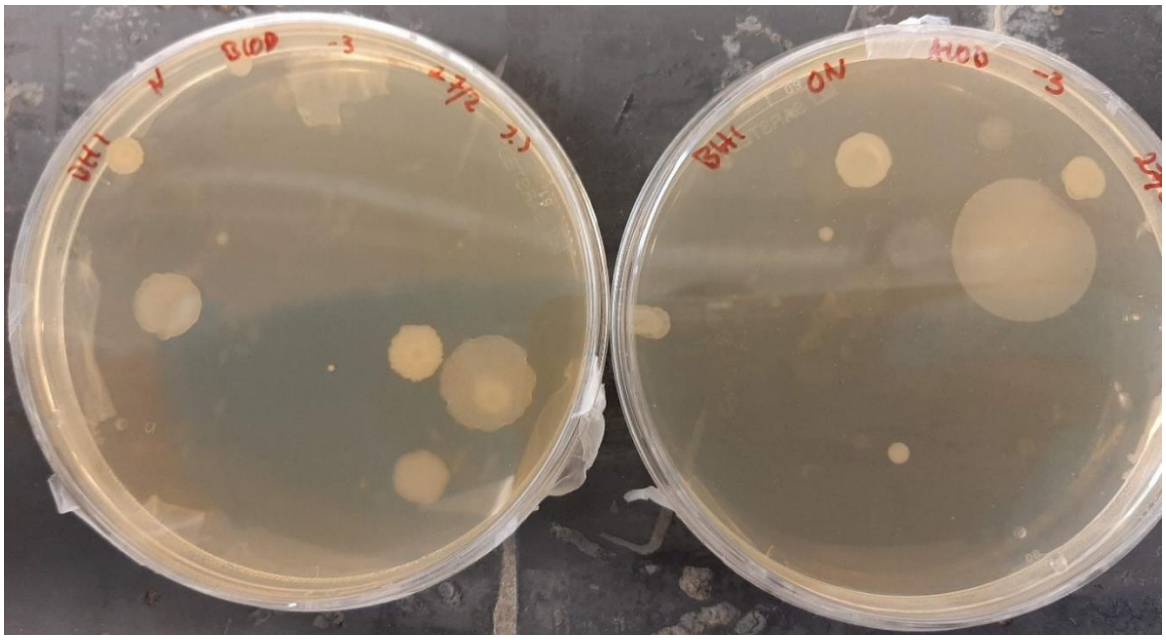


Figure 15. *In the plates without swarming colonies, colony appearances similar to those in the samples taken during the first 24 hours of incubation can be observed in the seen in both the nitrified biofertilizer (to the left, dilution 1:1000) and the non-nitrified biofertilizer (to the right, dilution 1:1000) after 10 days of incubation. Note that the "small colonies" that could be clearly seen after 24 hours of incubation in the non-nitrified biofertilizer are much less pronounced at this time point.*

Initially, no growth was observed in the inorganic fertilizer, but in the sample plated after 48 hours of incubation a uniform appearance of colonies could be observed (Figure 16 below), and this colony appearance could then be observed throughout the rest of the experiment as well.

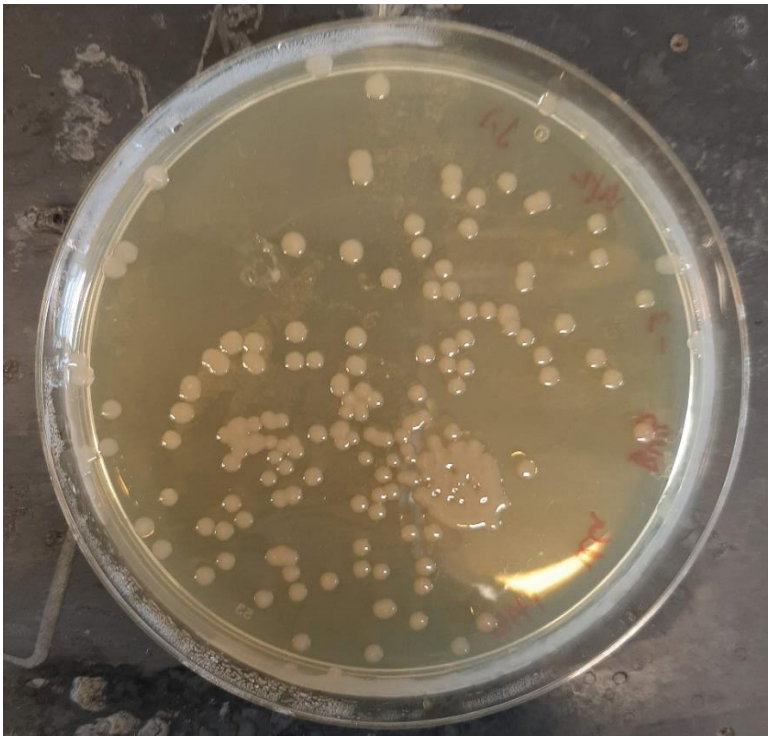


Figure 16. *Initially no growth could be observed in the inorganic fertilizer, but after 48 hours and during the rest of the experiment a uniform colony appearance could be observed when plating on BHI agar. In this image a sample of inorganic fertilizer incubated for 48 hour plated on BHI agar (dilution 1:1000) can be observed.*

4.1.1.3. Calorimetry as a tool to investigate microbial activity

As microorganisms live and metabolize, heat is developed. Therefore, a way of measuring metabolic activity can be through the use of isothermal calorimetry. The calorimeter is isothermal and keeps the incubated samples at a certain temperature. The changes in heat that microbial activity of the samples produce is detected as an electric signal, and connected to a software this signal is displayed as a graph presenting the heat development over time.

In parallel with the initial cultivation-based characterization of the three fertilizers, measurements of microbial activity was also performed using a calorimeter. The basis of this investigation is that any metabolic activity within the samples will generate heat that is detected by the calorimeter. This is a way of detecting activity of microorganisms that may not be viable when plating on agar substrate.

Samples of nitrified biofertilizer in duplicates were incubated in the calorimeter. During the first 5 days of incubation no heat development could be observed, and thus no metabolic activity. After 5 days of incubation one ml of BHI broth was added to the samples to investigate if the metabolic activity was affected. Approximately three hours after the addition a peak of heat development was generated as can be observed in Figure 17. After 8 days of incubation another addition (one ml) of BHI was made, with the same result as can be observed in the figure (the second peak, generated shortly after addition of BHI). As this was an indicator that an addition of nutrients was necessary to allow growth of present microorganisms, one ml of glucose solution (125 g/l) was added 24 hours after the latest addition of substrate, to investigate whether a source of carbon would be able to promote growth and thus might be a potential factor that was missing in the biofertilizer. After this addition, another peak of heat development could be seen shortly after the addition of glucose. Once the metabolic activity had ceased (after 2 more days), the samples were temporarily removed from the calorimeter with the purpose to aerate the sample.The lid of each vial was carefully opened, the vial was swirled and then re-closed and replaced in the calorimeter to investigate whether aeration of the samples had an effect on the growth or not. After opening the samples, no new peaks of heat development could be seen.

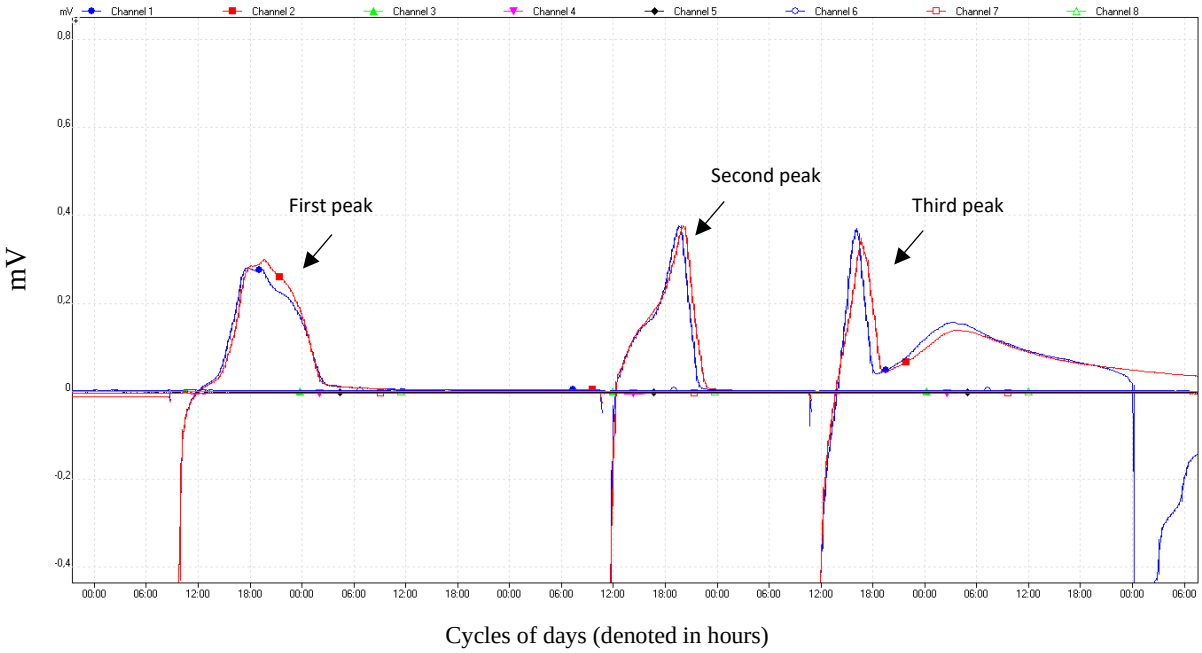


Figure 17. *Heat development of samples of nitrified biofertilizer in the calorimeter after addition of sources of nutrition. The first two peaks in the diagram appeared after the addition of BHI broth, and the third peak appeared after the addition of glucose.*

4.1.1.4. Biotyping using MALDI-TOF mass spectrometry

Matrix-Assisted Laser Desorption/ Ionization – Time Of Flight (MALDI-TOF) is a method that can be utilized for the identification of microorganisms. By inserting lysed, fresh (i.e. recently cultivated) colony samples in a MALDI-TOF instrument where they are ionized, the current applied in the instrument causes growth proteins of the bacterial cells to be transported across the column to a detector. This causes a specific spectrum for each individual species and strains of microorganisms, and the spectrum generated for each sample is matched to a digital reference library, which enables identification of the microorganism. In this project, MALDI-TOF biotyping was used to identify some microorganisms of interest from the pre-study experiment of the three fertilizers.

The microorganisms chosen to be analysed were the ones deemed the most interesting from the plating-over-time experiments, and the microorganism chosen and the reasoning behind the choosing can be observed in Table 8 below.

Table 8. *Presentation of the colonies chosen for idenfication with MALDI-TOF MS.*

Type of fertilizer	Time point	Reason	Name in MALDI-TOF report	Type of microorganism
Nitrified biofertilizer	24 hrs	Appeared frequently in only nitrified biofertilizer	NGUL	Unidentified
Nitrified biofertilizer	17 days	Appeared predominantly in nitrified biofertilizer in 17 and 21 days' measurement	NVIT	*Candida palmioleophila*
Non-nitrified biofertilizer	24 hrs	Appeared only in non-nitrified biofertilizer	ON1	Unidentified
Non-nitrified biofertilizer	24 hrs	Appeared only in non-nitrified biofertilizer	ON2	Unidentified
Non-nitrified biofertilizer	24 hrs	Appeared in both nitrified and non-nitrified biofertilizer	ON3	*Bacillus amyloliquefaciens*
Inorganic fertilizer	48 hrs	First observed type of colony in inorganic fertilizer	KGLITEN	(low score) *Pseudomonas sp*
Nitrified biofertilizer	-	This colony was taken from an experiment made outside of the growth experiment, where agar plates were made with nitrified biofertilizer and glucose as substrate	GODSEL	*Candida palmioleophila*

The full results from the MALDI-TOF run of the selected microorganisms can be viewed in Appendix 3. Some of the microorganisms remained unidentified also after the MALDI-TOF analysis due to not having a match in the library of biotyped microorganisms. However, two of the microorganisms could be identified with a high certainty (score value greater than 2). One of the two was *Bacillus amyloliquefaciens ssp plantarum*, a root-colonizing, plant growth-promoting bacteria (Niazi et al., 2014), which can be seen in Figure 18 below. This bacteria could be found in both the non-nitrified and nitrified biofertilizer.

Figure 18. Bacillus amyloliquefaciens *on BHI agar*

Another identified microorganism was *Candida palmioleophila*, a yeast pathogen (Jensen and Arendrup, 2011), which can be observed in Figure 19 below. It was found to be the predominant microorganism in the nitrified biofertilizer after day 17 of the experiment (see Figure 20).

Figure 19. Candida palmioleophila *on BHI agar*.

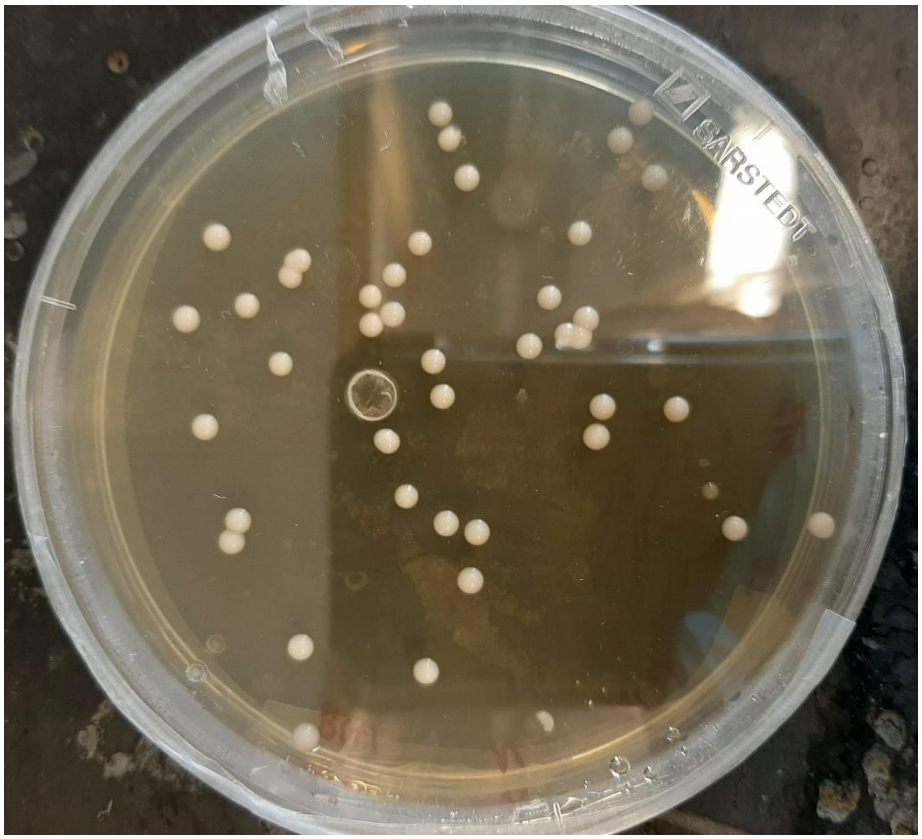


Figure 20. Candida palmioleophila *etc on BHI agar in nitrified biofertilizer, day 17 after incubation.*

Another microorganism which could be identified, albeit not with the same reliability (with a score of 1.72) was *Pseudomonas sp* which could be observed when plating a sample from the inorganic fertilizer 48 hours after incubation as can be observed in Figure 21 below.

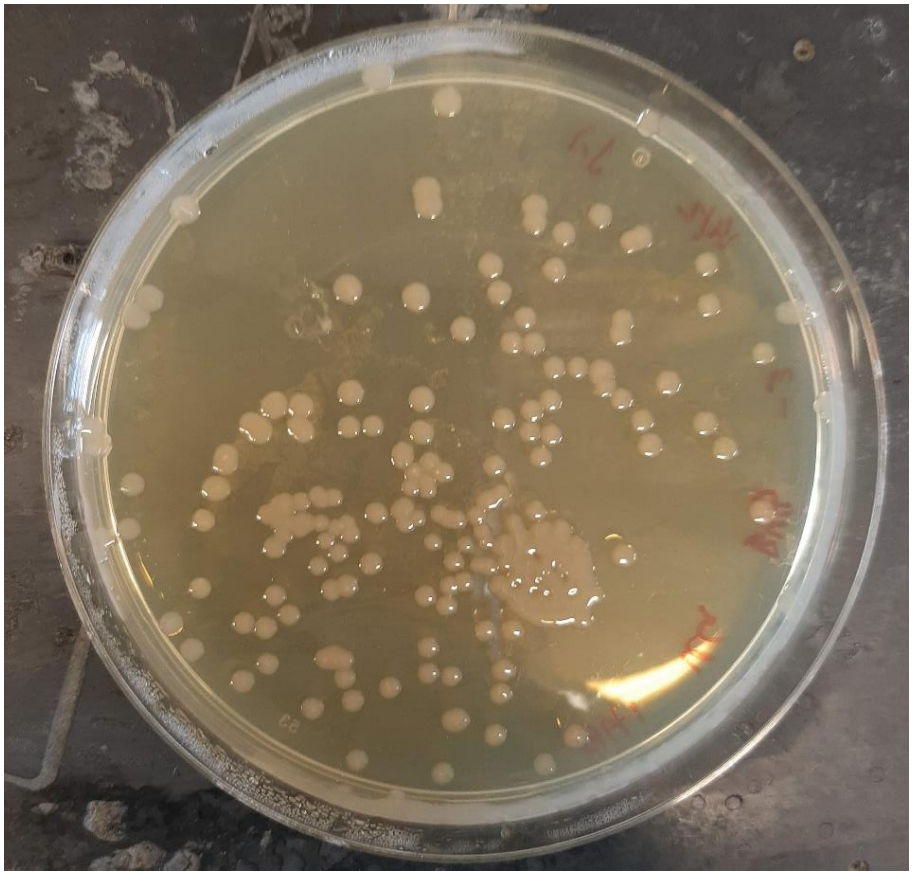


Figure 21. *Presumably* Pseudomonas *on BHI agar*.

4.1.2. Microbial community analysis

After the initial pre-study experiments were complete, investigation of the microbial community was performed using 16S rRNA gene amplicon sequencing. The purpose of this was to obtain a more complete picture of the microbial community within the nitrified biofertilizer, the non-nitrified biofertilizer and the inorganic fertilizer during a plant's growth cycle in a hydroponic setup.

Due to the physical nature of the biofertilizer (liquid, and rich in impurities such as humic substances that might inhibit subsequent PCR reactions to be performed in the sequencing), the biofertilizer needed to be treated filtered to gather the bacterial cells, and treated in some way to remove inhibitory substances before it could undergo DNA extraction and sequencing. For this purpose, it was ultimately decided that a prefiltration would be necessary in order to successfully extract DNA and perform a satisfactory sequencing.

Both the filtration and the DNA extraction were optimized to keep as much of the bacterial cells

as possible while removing as much impurities as possible to provide as truthful a result from the 16S-rRNA sequencing and avoid as much inhibition as possible.

4.1.2.1. Filtration

The final goal of the filtration was to be able to gather the microorganisms present in the biofertilizer on a 0,22 µm filter, from which their DNA could then be extracted. As stated, as not to introduce bias of the 16S-rRNA sequencing by the loss of bacterial cells in pre-filtration, a pre-filter was chosen on the basis of allowing as much of the sample through as possible while still removing so much of the impurities that the 0,22 µm filter would not be clogged. After some experimentation with equipment and filters at hand at the department, and after consultation about filtration of the liquid with technical support at Merck and Matts Ramstorp, a filtration expert, it was decided that a glass-fibre prefilter with a pore size of 2,0 µm would be suitable.

4.1.2.2. DNA extraction

Due to the great particle-density of the biofertilizer and the risk for inhibition of PCR that the impurities in it might constitute, the method of extraction of DNA was optimized to extract as much as possible with as little inhibition as possible. Two different methods, NUCLISENS® MINIMAG® and FastDNA™ SPIN Kit for Soil, were tried out to see which one fulfilled this purpose the best.

4.1.2.2.1. NUCLISENS® MINIMAG®

The NUCLISENS® MINIMAG® was chosen to be one of the DNA extraction methods to be evaluated in this project, partly due to its claim to remove inhibitors but also because it removed the need for filtration; as mentioned in section 3.1.5.1 the material to be subjected for extraction is in this method collected through centrifugation. This was considered interesting as it seemed that this kind of treatment of the sample would introduce less bias (in the case of filtration, some cells could be filtered away in the pre-filtration, giving a skewed representation of the microflora of the sample in the following 16S-rRNA sequencing).

In order to evaluate the effectivity of this type of DNA extraction method, samples of nitrified biofertilizer were spiked with *Salmonella.* As inhibitory compounds were believed to be present in the biofertilizer, the samples were subjected to another purification step (DNA Clean and Concentrator ZYMO) which consisted of placing the extracted DNA in a spin column that removes salts and purifies the DNA extract. The effectivity of both the method of DNA extraction and the additional purification step with ZYMO was then controlled with quantitative, real time PCR (qPCR, LightCycler® Nano, Roche Diagnostics), with a *Salmonella* assay (primers targeted for *Salmonella*), and the results represented in the form of the cycle of quantification are shown in Table 9 below. The negative control consists only of the master mix for the PCR.

Table 9. *Results from qPCR of DNA samples extracted with NUCLISENS® MINIMAG®.*

Sample number	Sample content	C_q
1	1 ml nitrified biofertilizer (50 ml) spiked with Salmonella O/N culture (200 µl)	23.87
2	0.5 ml nitrified biofertilizer (50 ml) spiked with Salmonella O/N culture (200 µl)	23.53
3	0.5 ml nitrified biofertilizer	-
4	0.154 ml Salmonella O/N culture (equal to the amount of Salmonella spiked in sample 1 and 2)	19.11
5	Identical as sample 1, subjected to ZYMO	22.85
6	Identical as sample 2, subjected to ZYMO	22.71
7	Identical as sample 3, subjected to ZYMO	-
8	Identical as sample 4, subjected to ZYMO	18.69
9	Negative control	-

4.1.2.2.2. FastDNA™ SPIN Kit for Soil

The FastDNA™ SPIN Kit for Soil was chosen to be one of the DNA extraction methods to be evaluated in this project because of its recommended use for soil and compost samples, which the biofertilizer resembles to a high degree, and because of its claim to effectively remove humic acids and other PCR inhibitors. Since this method included a bead-beating step, it provided the possibility to insert a filter with the bacterial cells of the sample collected on it (instead of inserting a soil sample), which allowed for a greater concentration of bacterial cells to be lysed and be subjected to DNA extraction. This method was thus only tested on filtered samples.

The effectivity of the DNA extraction was controlled with quantitative, real time PCR (qPCR, LightCycler® Nano, Roche Diagnostics), with a *Salmonella* assay (primers targeted for *Salmonella*) in the same way as described above, and the results represented in the form of the cycle of quantification are shown in Table 10 below. The negative control consists only of the master mix for the PCR.

Table 10. *Results from qPCR of DNA samples extracted with FastDNA™ SPIN Kit for Soil.*

Sample number	Sample content	C_q
1	Filtered nitrified biofertilizer (50 ml) spiked with Salmonella O/N culture (200 µl)	17.98
2	Identical to sample 1, subjected to ZYMO	17.91
3	Nitrified biofertilizer (50 ml)	-
4	0.154 ml Salmonella O/N culture in 50 ml NaCl 0.9 % (equal to the amount of Salmonella spiked in sample 1 and 2)	19.72
5	Negative control	-

Since this method provided lower C_q-values (and thus the samples contained a larger quantity of DNA after extraction) it was selected to be the one to use for the samples to be sequenced.

4.1.2.3. 16S rRNA gene amplicon sequencing

The purpose of performing a 16S rRNA gene amplicon sequencing was to investigate the composition of the microflora of the biofertilizer from different perspectives. The samples that had undergone sequencing were taken from the greenhouse experiments (see Table 2). In the bioinformatic analysis from the sequencing, the non-nitrified biofertilizer was denominated ON, the nitrified fertilizer was denominated G and the inorganic fertilizer was denominated NPK. The different vantage points of this investigation was to:

- Investigate how the microflora of the biofertilizer changes after it has undergone a nitrification process.
- Investigate how the microflora of the nitrified biofertilizer changes over time during the growth cycle of the plant.
- Investigate the variance of microflora of the nitrified biofertilizer of samples taken at the same time points but from different channels.
- Compare the microflora of the nitrified biofertilizer with the microflora of the conventional inorganic fertilizer during the course of the growth cycle of the plant.

The samples from the greenhouse experiment were taken at the time points seen in table x, upon which they were frozen and kept frozen until the time of filtration and extraction. Before filtration, the samples were thawed slowly in a refrigerator (5-8°C). The thawed samples were filtered with the filtrate gathered on the 0.22 µm filter, which was placed in a sterile petri dish for each filter and kept in a -20°C freezer until they were subjected to DNA extraction.

Before sending the extracted DNA samples to be sequenced to DNASense, a qPCR (Bio-Rad) was performed as a quality control to confirm that the samples indeed contained DNA. The qPCR was performed with a General Bacteria Assay. Standard curves were generated from samples with known DNA concentrations from *Listeria monocytogenes* to be able to determine the amount of DNA in the samples to be sequenced. A graph of the amplification results generated from the qPCR (intensity of signal vs number of cycles) can be observed in Figure

22. The full table with the yields from the DNA-extraction can be observed in Appendix 4. The DNA concentration of the non-nitrified biofertilizer was measured to be 18.572 ng/µl, and the nitrified biofertilizer at Day 0 was measured to be 0.042 ng/µl. The DNA concentrations of the rest of the samples of nitrified biofertilizer ranged between 3.43×10^{-5}-5.52×10^{-4} ng/µl (day 10), 1.91×10^{-6}-7.32×10^{-6} ng/µl (day 17) and 2.28×10^{-4}-5.47×10^{-6} ng/µl (day 21). The samples of inorganic fertilizer was below 1.27×10^{-5} ng/µl at all time points.

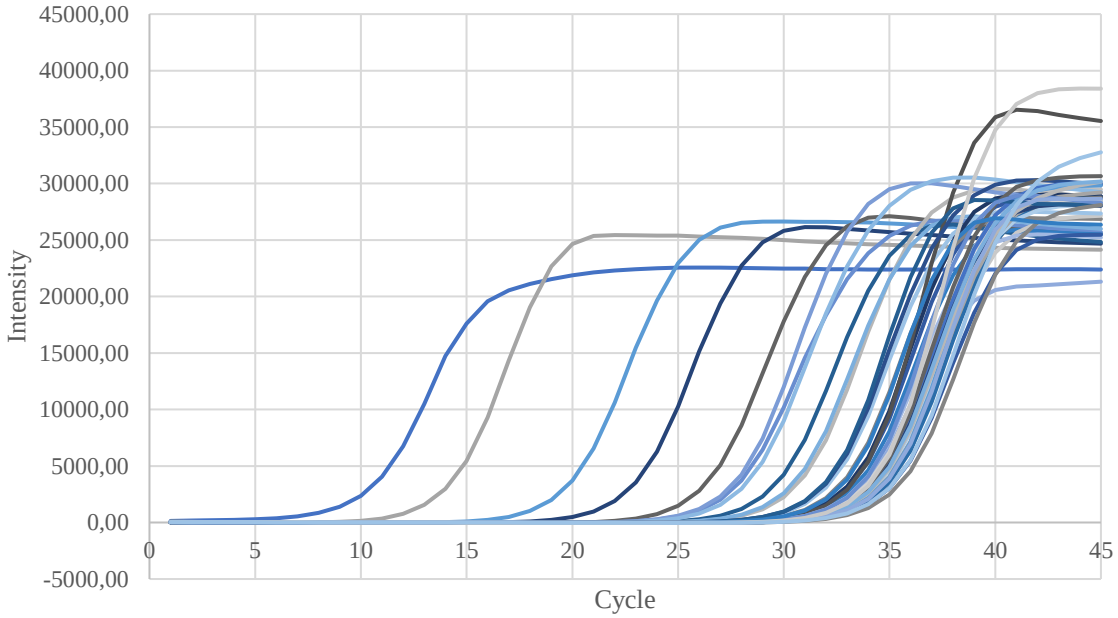


Figure 22. *Results of the quality control qPCR run on the DNA extracts of the samples from the greenhouse experiments. Note that the first five curves to the left are the* Listeria *standard curves.*

The library preparation of the 16S rRNA gene amplicon sequencing yielded quite low amounts of DNA (<1 ng/µl) for all samples except for the samples of non-nitrified biofertilizer (>10 ng/µl) and the starting sample of nitrified biofertilizer (before addition to the furrrows) (>10 ng/µl). A negative control was included as well in the library preparation. The 25 most abundant genera across all samples can be observed in Figure 23 below. If no genus level classification could be obtained, the lowest assigned taxonomic classification was given. In addition, the phylum level classification was given (Proteobacteria at class level). The microbial communities of the samples were also analysed with multivariate statistical analysis (principal component analysis), demonstrating the similarity in microbial community between the samples, as can be observed in Figure 24. The dots represent the microbial communities of the different samples, and the closer they are together, the more similar are the microbial communities.

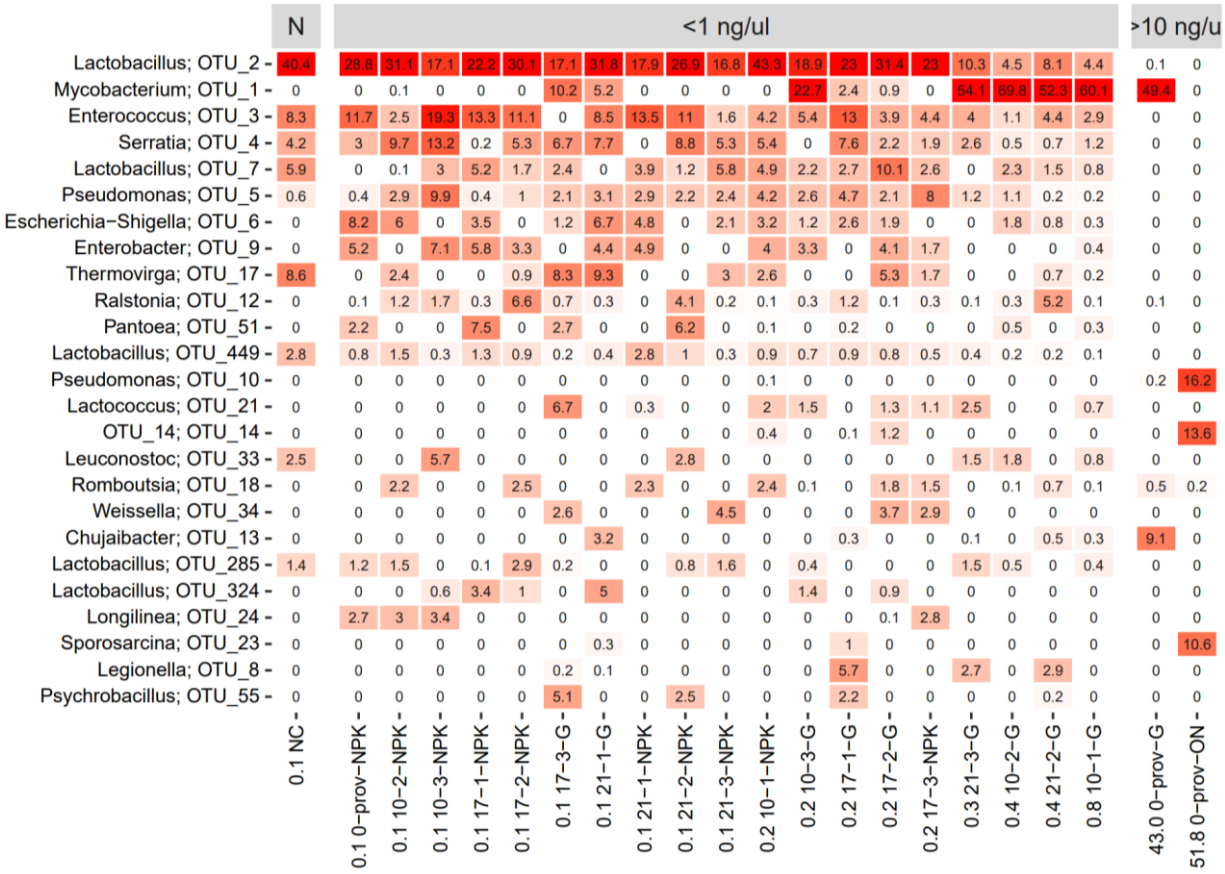


Figure 23. *Heatmap of the 25 most abundant genera across the samples. The intensity of red represents the relative abundance, with a darker red being a higher relative abundance. Furthest to the left is the negative control (containing sequenced background from the PCR reagents), with the samples containing <1 ng/µl of DNA in the middle, and samples containing >10 ng/µl of DNA furthest to the right.*

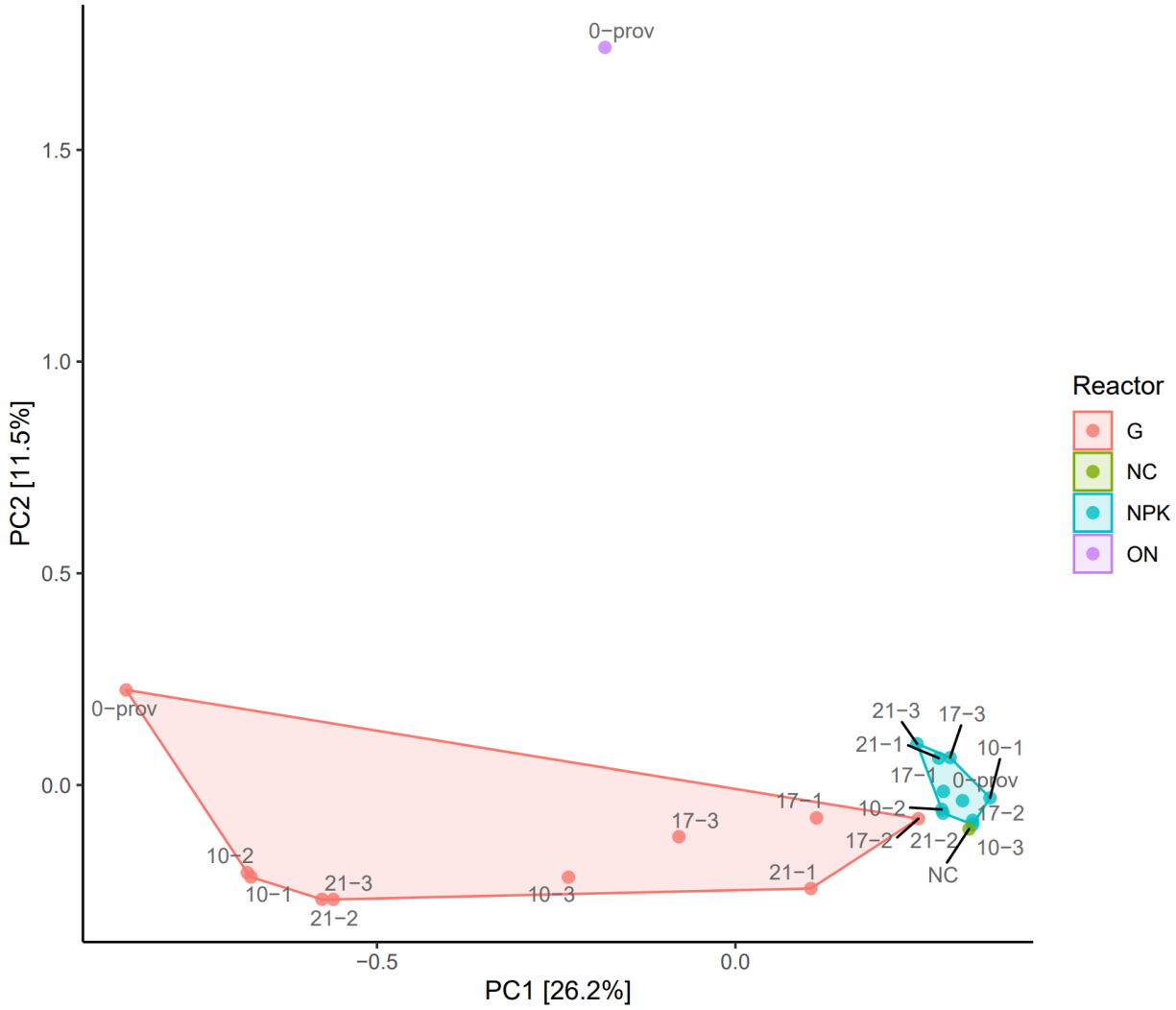


Figure 24. *Principal component analysis of the samples. The phylogenetic relationship is illustrated through this statistical analysis, showing that the non-nitrified biofertilizer (ON 0-prov) has a distinctly different microbial community than the other samples. The PCA illustrates how the microbial community of the nitrified biofertilizer (G) changes between the different timepoints of the hydroponic growth experiments.*

From these results it is clear that the nitrification of the biofertilizer indeed changes the microbial community greatly. From figure x it can be observed that the genera *Mycobacterium* is not present in the non-nitrified biofertilizer (nor in the inorganic fertilizer), but is abundant in the nitrified biofertilizer. The microflora of the nitrified biofertilizer indeed changes over time, as can be observed in the principal component analysis. The microflora of the nitrified biofertilizer also varies between the samples taken at the same time point but from different channels, as a comparison the samples of the inorganic fertilizer as much more clustered and vary less in between samples. However, it is important to note that the negative control (NC) showed a high abundance of Lactobacillus, which was also found in all samples with amplicon concentrations below 1 ng/µl but is less abundant in the samples with concentrations close to 1 ng/µl. This indicates that this OTU could be sequenced background which is common for samples with very low DNA concentration, which also should be taken into consideration when drawing conclusions about the relationships of the different samples.

4.2. Challenge testing of the nitrified biofertilizer with *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes*

A possible scenario of contamination of the biofertilizer is problematic in a growth setup such as the one in the greenhouse experiments, as the biofertilizer is recirculated and the plant is exposed to it during its entire growth cycle, especially since some bacteria, e.g. *Salmonella*, can internalize into plant cells and thus will not be ridden by washing of the plant (Murphy et al., 2016). Hence, it is of interest to investigate whether an establishment of pathogens in the biofertilizer would occur upon the event of a contamination.

To simulate the event of a contamination of the biofertilizer, challenge testing of the nitrified biofertilizer was performed through the addition of the following three food-borne pathogens: *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes* in three different shake flasks with biofertilizer (i.e. one species inoculated in one flask). Together with the three flasks was also a control flask that contained biofertilizer with no inoculation. The flasks were then incubated for 21 days at 20 °C to at 100 rpm to resemble the conditions in the greenhouse experiments. Samples were collected at the following time points after incubation: 0 hrs, 4 hrs, 8 hrs, 24 hrs, 48 hrs, 10 days, 17 days and 21 days.

4.2.1. Establishment of the pathogens in the nitrified biofertilizer

The challenge testing was followed through traditional plate count on selective agar for respective pathogen. The samples collected from the flask inoculated with *B. cereus* was plated on MYP agar (typical colony appearance can be observed in Figure 25 below). The samples collected from the flask inoculated with *Salmonella* was plated on XLD agar (typical colony appearance in Figure 26). The samples collected from the flask inoculated with *Listeria* was plated on ALOA agar (typical colony appearance in Figure 27). The control flask with non-inoculated biofertilizer was also plated on all three types of agar at each sampling point. The samples were plated in replicates of three using the appropriate dilutions.

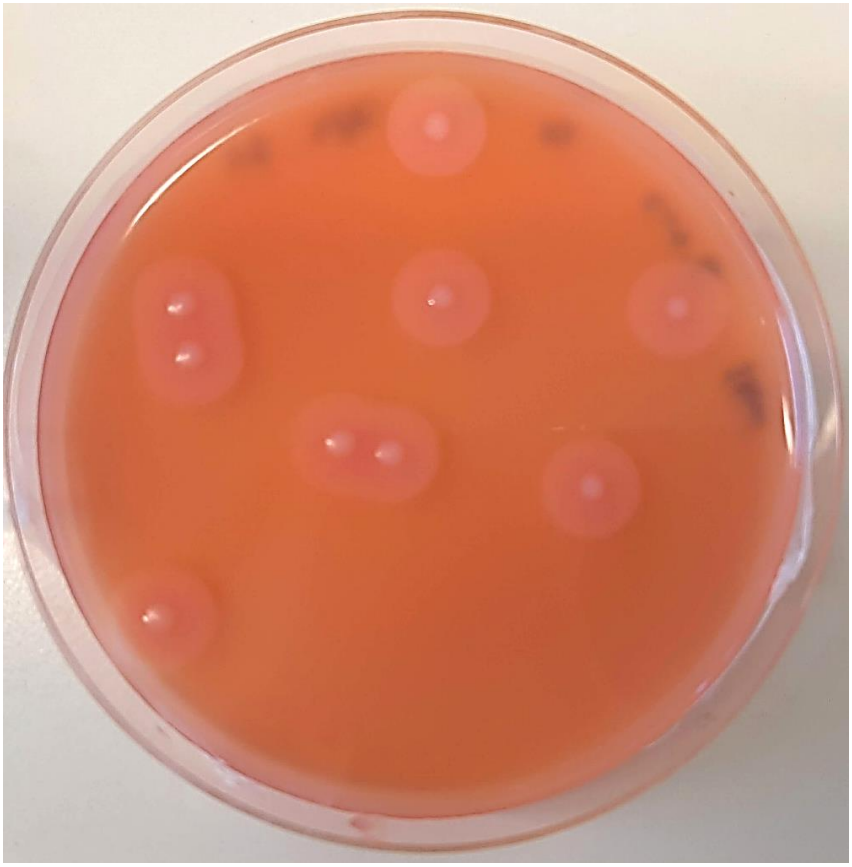


Figure 25. *Colony morphology and appearance of* Bacillus cereus *on MYP agar*

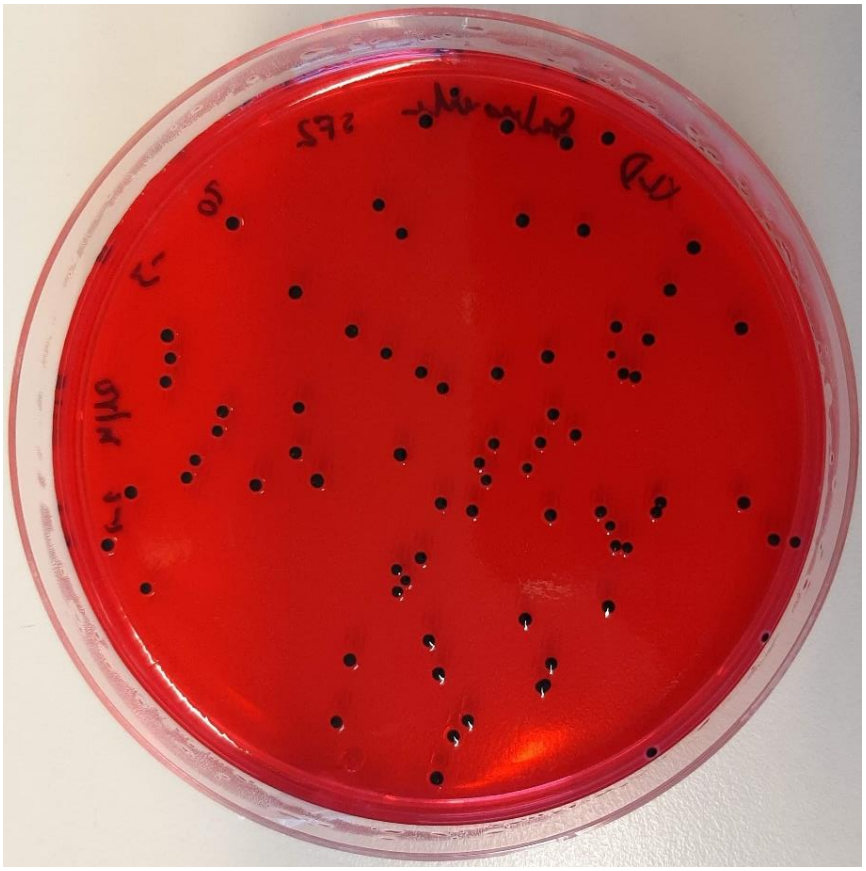


Figure 26. *Colony morphology and appearance of* Salmonella enterica serovar typhimurium *on XLD agar.*

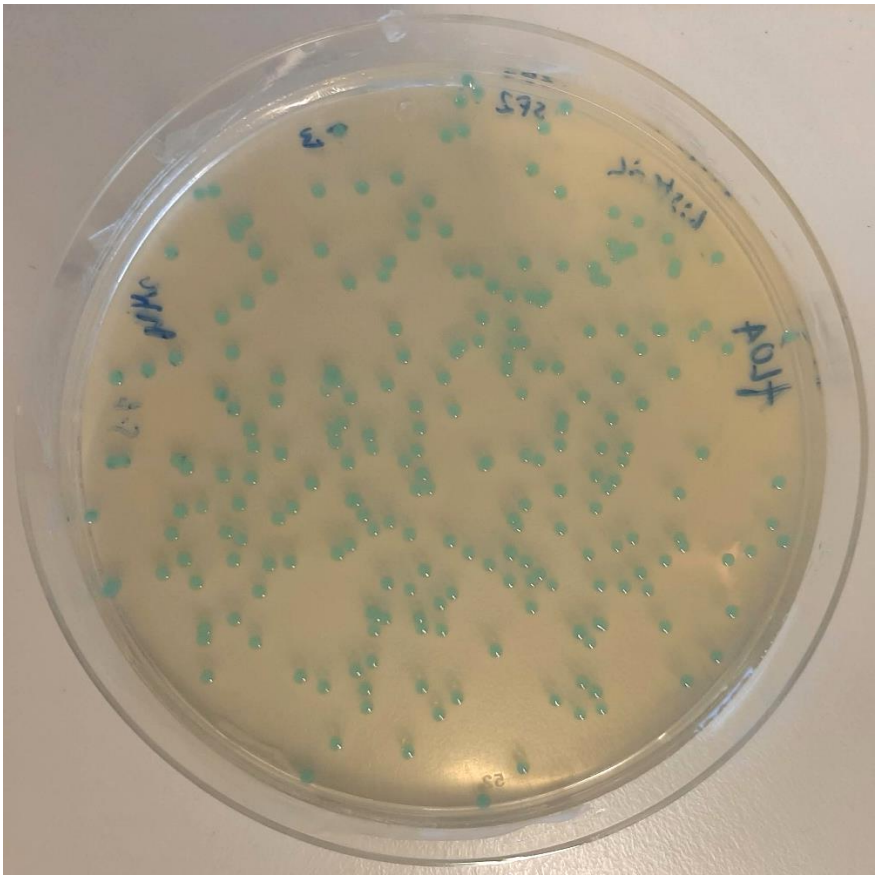


Figure 27. *Colony morphology and appearance of* Listeria monocytogenes *on ALOA agar.*

The concentrations of the respective pre-cultures were measured prior to inoculation of nitrified biofertilizer in order to obtain a starting concentration of the experimental cultures of 10^3 CFU/ml. As it was discovered after the first biological replicate of the challenge testing experiments that the bacteria decreased in concentration faster than expected, and very quickly entered the unaccepted range regarding enumeration, the starting concentration of the experimental cultures was altered to 10^5 CFU/ml. It was also observed that FC estimated a somewhat higher value of cells/ml than what was later shown when plated immediately after inoculation, and therefore the concentration of cells of the pre-cultures were also measured with optical density (OD) of the pre-cultures, and the values for the three different bacteria where then compared to values found in literature (Kaptan Ölmez and Aran, 2005, Islam et al., 2004, Francois et al., 2005).

The three different flasks of nitrified biofertilizer was inoculated with the three different pathogens to obtain a starting concentration of 10^3 CFU/ml of the pathogen in the biofertilizer in the first biological replicate and 10^5 CFU/ml of the pathogen in the biofertilizer in the second and third biological replicate. The growth curve for the microorganisms, are presented in Figures 28, 29 and 30 below. Estimated counts are marked with an asterisk. The non-inoculated biofertilizer that was plated on all three selective agars at each sampling point showed growth of *Bacillus cereus* on MYP agar throughout all three biological replicates. This was expected as previous samplings at the biogas plant had shown a presence of *Bacillus cereus.*

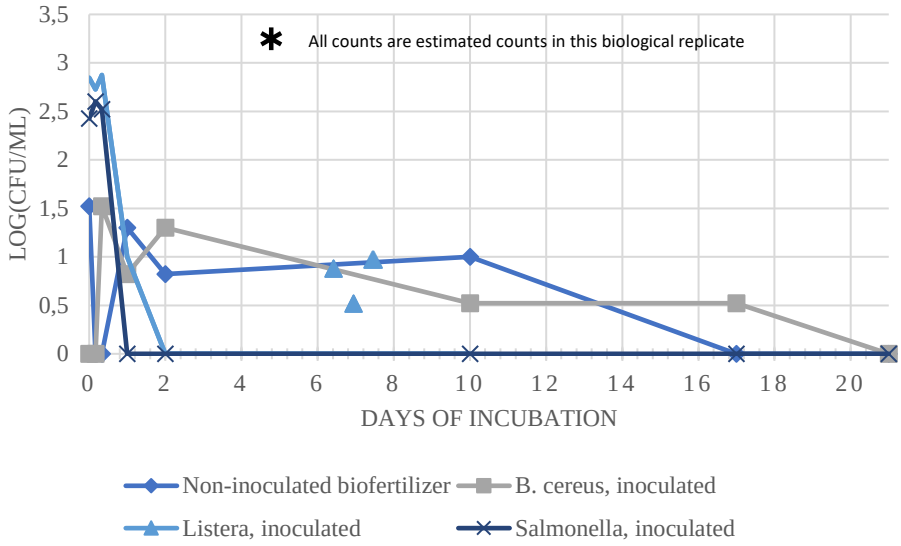


Figure 28. *Plate count of* Bacillus cereus, Listeria monocytogenes *and* Salmonella enterica *in the nitrified biofertilizer in the first biological replicate of the challenge testing. The non-inoculated biofertilizer showed growth of* B. cereus *colonies on MYP agar, which are the ones counted and represented in this graph. Logarithm of mean values of three plates (technical replicates) are presented. Estimated counts (<25 CFU/plate) are marked with an asterisk.*

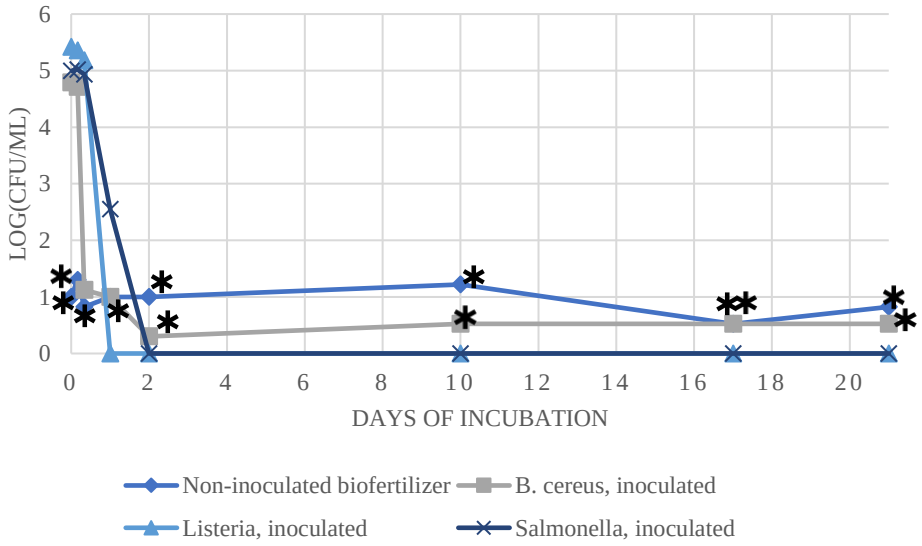


Figure 28. *Plate count of* Bacillus cereus, Listeria monocytogenes *and* Salmonella enterica *in the nitrified biofertilizer in the second biological replicate of the challenge testing. The non-inoculated biofertilizer showed growth of* B. cereus *colonies on MYP agar, which are the ones counted and represented in this graph. Logarithm of mean values of three plates (technical replicates) are presented. Estimated counts (<25 CFU/plate) are marked with an asterisk.*

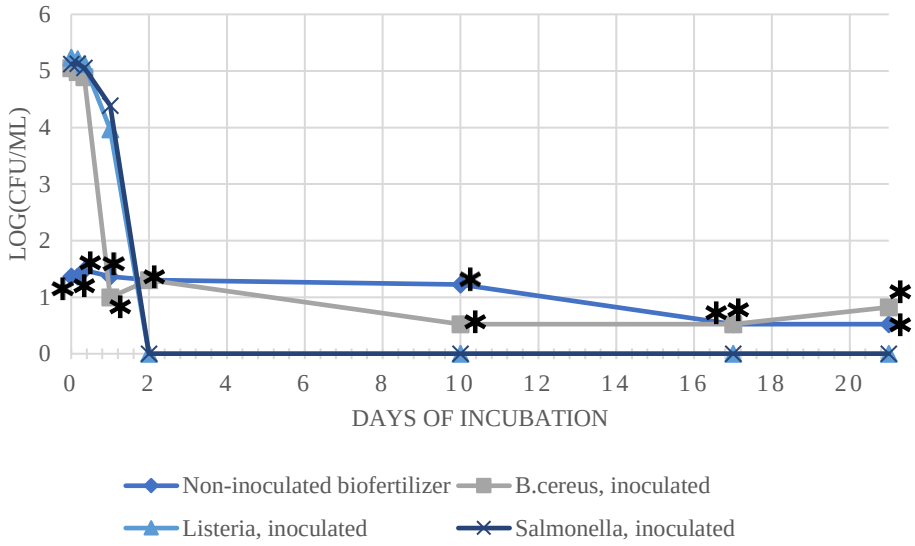


Figure 29. *Plate count of* Bacillus cereus, Listeria monocytogenes *and* Salmonella enterica *in the nitrified biofertilizer in the third biological replicate of the challenge testing. The non-inoculated biofertilizer showed growth of* B. cereus *colonies on MYP agar, which are the ones counted and represented in this graph. Logarithm of mean values of three plates (technical replicates) are presented. Estimated counts (<25 CFU/plate) are marked with an asterisk.*

4.2.2. Calorimetry as a tool to investigate microbial activity in the challenge tests

Although the results from the pre-study indicated that the biofertilizer alone was not enough to support growth (or even allow metabolic activity to befall), as it was unknown how the pathogens would behave in this matrix, samples with biofertilizer inoculated with the pathogens in replicates of two (in total six samples) were placed in the calorimeter. This was performed in parallel with the challenge test experiments.

No heat development could be observed during the timeframe of the experiment, and thus no metabolic activity of microorganisms was detected. This correlated with the results obtained in the cultivation-based analyses on selective agar in the challenge tests presented above.

5. Discussion

5.1. Microbial community analysis

Considering the analysis of the microbial community in an unknown matrix, traditionally cultivation-based techniques have been used for such assessments. However, choice of substrate and conditions of cultivation inevitably selects for a part of the microflora, and previous studies performed on soil claim that only 0.1% of the total flora is detectable with culture-dependent techniques (Hill et al., 2000). Today, when analysing microbial communities the use of methods utilizing culture-independent techniques based on nucleic acid analysis are commonplace, with high throughput sequencing technology being the most frequently used method of investigation microbiological diversity and community structure within a sample (Meng et al., 2019). In several recent studies concerning mapping of a previously unknown microflora of a soil or compost sample (such as the sample of investigation in this study resembles), 16S rRNA sequencing has been utilized with amplification of variable region V4 or V3 and V4 (McPherson et al., 2018, Antunes et al., 2016).

5.1.1. Pre-study of fertilizers by cultivation

The purpose of the pre-study of the fertilizers through cultivation was to provide more insight in the microbial community of the fertilizers. The reason behind the choice of both the method of investigation and the culture medium was to enable as objective, broad and non-biased representation of the microflora as possible. One important reason to have knowledge about this natural background was in relation to the challenge testing experiments to be performed in a later stage of the project. Challenge testing experiments require full control of the microbial status of the matrix in order to be able to elucidate what results originate from the deliberate inoculation, and what is background microflora of the matrix. While it is understood that utilizing a cultivation-based method most likely selects for only a part of the total microflora, the conditions of cultivation was deemed adequate for this pre-study, both considering that it would indeed provide and inkling of the microflora of the biofertilizer (in which the microbial community has not been investigated up until this point), and also considering that the challenge testing would be performed under the same conditions of cultivation.

5.1.2. Calorimetry as a tool to investigate microbial activity

One of the most surprising results in this project was the discoveries obtained when utilizing the calorimeter, to detect growth or microbial activity, as a complement to the traditional cultivation-based methods. The initial purpose and motivation of using this technique was to detect and monitor any viable but non-culturable cells, that existed in the biofertilizer but was unable to be cultured on agar plates. Isothermal calorimetry has previously been used in all kinds of purposes where microbial activity is to be studied, and have many applications in for example food science, such as investigations of assessing properties of bacterial cultures in fermented foods, or the influence of thermal treatments on the shelf life of products (Wadsö and Gómez Galindo, 2009). It also has been shown to be useful in medical applications, for example for differentiation of methicillin-susceptible *Staphylococcus aureus* from methicillin-resistant *S. aureus* within five hours, also while being able to detect the microbial activity from as few as 10,000-100,000 cells (Braissant et al., 2010).

In the current study however, while observing colonies on the BHI agar plates when the biofertilizer was plated (and thus indicating that it indeed contained microorganisms that were able to form CFU), no signs of metabolic activity could be observed in the calorimeter. Since

the presence of nutrients in the agar substrate obviously allowed present microorganism to be able to form visible and countable CFU on the agar plates, the same was tried in the calorimeter with the addition of BHI broth to the samples. As presumed, microbial activity was then observed. This lead to the suspicion that perhaps the biofertilizer was missing a carbon source needed to support microbial growth. This hypothesis was tried by the addition of solely glucose (125 g/l), and microbial activity could once again be observed. The fact that the biofertilizer might have a scarcity of adequate carbon sources to support microbial growth might not come as such a surprise considering that it has been anaerobically digested by microorganisms for over a month, but was not something that had been reflected upon at the beginning of this project. Interestingly, other studies performed on comparable samples (soil and compost) does not seem to encounter this problem in the literature investigated. In a study performed on soil comparing counting on agar plates versus utilizing calorimetry to measure microbial growth, they were able to observe heat development even in their negative control sample (consisting of 100.0 g soil and 20% water) (Critter et al., 2002). In another study that evaluated microbial activity in compost using microcalorimetry, compost (which the biofertilizer presumably resembles in many ways) without addition of any other substrate also develop heat when placed in the calorimeter (Laor et al., 2004). Possibly, the long period of digestion of the biofertilizer (over one month) renders the biofertilizer depleted of key factors for microbial proliferation.

Considering this insight, a potential microbial contamination of the biofertilizer (such as investigated in the challenge testing in this project) might not be an issue at all if the pathogen is not able to establish itself due to the lack of key factors to support growth. Even if it might be so that biofertilizer lacks key factors for growth the question then emerges whether the roots of the plant in a hydroponic setup might alter the matrix that the biofertilizer constitutes in such a way that it might convey a carbon source. Further investigation using calorimetry for the investigation of the support of microbial growth of the microorganism is recommended to determine exactly what factors are deficient in the biofertilizer. Moreover, even though it might not show support of growth in this type of experiment, further investigation on the influence that the insertion of the plant roots into the biofertilizer might have on the are recommended before being able to fully state that the biofertilizer is a microbiologically safe substrate in hydroponic setups (because of its potential deficiency of essential factors for microbial establishment and growth).

5.1.3. Biotyping using MALDI-TOF mass spectrometry

Using MALDI-TOF mass spectrometry as a tool for bacterial identification has its advantages in it being a fast, high accuracy method (Hou et al., 2019). Indications that it might provide a better resolution at species level than 16S rRNA gene amplicon sequencing (Shin et al., 2015) suggests that it has potential in performing microbial community analyses on unknown samples. However, it is still cultivation dependent. Today, MALDI-TOF MS is mainly used for clinical purposes, and therefore the existing commercial reference databases of microorganisms consist mainly of species related to infection and disease (Sampedro et al., 2018), being limited in the detection of microorganisms not as clinically interesting. A study encountering this problem when analysing microbial isolates from spacecrafts constructed their own MALDI-TOF MS reference database as a complement to the commercially existing ones (Seuylemezian et al., 2018).

In this project, the biotyping using MALDI-TOF mass spectrometry resulted in three out of the seven colonies being identified. Not having a majority of the colonies identified was a

somewhat expected result as the reference database that the hospital uses for identification mainly consists of human pathogens (although it also had some species and strains more relevant for veterinary medicine and food industry). Since the biofertilizer has been hygienized and anaerobically digested for over one month, these types of bacteria were not expected to be present. Naturally, these kinds of pathogens are the ones most relevant to be able to identify rapidly in the case of infections, and therefore references for environmental microorganisms are not desired in the same extent. However, in the case of having access of a reference library that also contains environmental microorganisms, the mass spectrums from the unidentified microorganisms in this experiment could perhaps find a match.

The software performing the matching of the mass spectrum detected against the database lists the species and strains of these species that match the mass spectrum the most accurately. The mass spectrum generated from a colony collected from the inorganic fertilizer was matched to being a *Pseudomonas spp*. The matching obtained a score of 1.72, which indicates a low confidence match. However, the rest of the microorganisms in the list are also *Pseudomonas*, which provides a higher certainty of the reliability of the result, and suggests that it might indeed be a *Pseudomonas* but perhaps of a different strain that does not exist in the database. Interestingly, *Pseudomonas* was also identified in the all the samples of inorganic fertilizer from the greenhouse experiments in the 16S rRNA gene amplicon sequencing. Since it is a bacteria wide-spread in the environment (Mercado-Blanco and Bakker, 2007), the occurrence of it in the inorganic fertilizer might very well be a contamination. However, *Pseudomonas* has also been shown to be a very hardy bacteria enduring extreme environments such as extreme salinity (Elabed et al., 2019), and therefore might possess the ability to endure in the inorganic fertilizer.

The lists of the best matches of the remaining (three) unidentified colonies were composed of a great variety of different species of microorganisms. An explanation to this might be either that it truly is an unknown species that does not have a match in the database, or that the colony might be heterogeneous, i.e. containing more than one single species. Since the biofertilizer contains a variety of microorganism, picking and plating one single colony might be difficult (colonies of different microorganism might be merged). A future prospect of performing biotyping using MALDI-TOF mass spectrometry on samples that have a rich microflora, would be to isolate the colonies of interest and streak them separately to obtain discrete colony forming units. In some cases the procedure of re-streaking on fresh agar plates would have to be repeated several times to ensure plates with pure colonies.

5.1.4. Filtration and DNA extraction

A challenge that comes along with purification of a sample that is to be analysed for its microbial community is the introduction of bias. A concern regarding the filtration of the samples was that it might distort the analysis of the microflora in some way; larger microorganisms might adhere to the prefilter in a larger extent, and provide an askew presentation on the microbial composition of the sample, or some microorganisms might be adhered to the particles that are filtered out in the prefilter. The same reasoning regarding bias applies in the extraction of the DNA of the samples; with every step in the extraction process some DNA is lost. Considering that the result of the DNA extraction from the samples collected from the greenhouse experiments yielded low DNA concentrations, potential bias becomes even more prominent. All the same, while it is known that with each step of purification of a sample DNA is lost and that this will influence the final analysis of the microbiological

community, a purification of the samples to be sequenced was necessary to be able to perform the sequencing successfully.

The low yield of DNA from the DNA extraction could be explained on one hand by losses during the purification of the sample, and on the other hand by the storage of the samples. The samples collected from the greenhouse experiment was stored in 100 ml plastic vials and frozen until the time of extraction. This might not have been the optimal way of treating samples from which microorganisms were to be extracted, as the ice formation upon freezing might have disrupted the cell membranes of the bacterial cells and lysed them. In a future prospect, samples collected for this kind of analysis should be centrifuged, and the pellet from the centrifugation should be kept for analysis.

5.1.5. Microbial community analysis through 16S rRNA gene amplicon sequencing

The goal of the sequencing was to provide a truthful representation of the microbial community in the samples collected from the greenhouse experiment. For this purpose, the variable region V4 was the one chosen to be sequenced. The selection of which variable region/regions to sequence for is an equilibrium of including an objective representation of the microbial community within the sample and providing a good resolution of it (i.e. displaying the variance within the sample at an adequate level). While the sequencing of this region had been shown to give successful results for this kind of sample in previous studies (Thompson et al., 2017), it also naturally entails a selection of a certain microflora (the kind of flora expected to be present in this kind of sample).

The 16S rRNA gene amplicon sequencing revealed that the most abundant genus within all the samples of nitrified biofertilizer was *Mycobacterium*. This genus could not be detected at all in the samples of non-nitrified biofertilizer or the inorganic fertilizer. Since it is present also in the Day 0 sample (i.e. before addition to the hydroponic channels), a transfer or contamination from the plant roots to the biofertilizer can be ruled out. Seeing that the microbial communities of the nitrified and non-nitrified biofertilizer changes so drastically in the microbial community composition, there is reason to believe that the nitrification process, including lowering of the pH from 8 to 5.5, aeration, and changes in the composition of nitrogen compounds, might be affecting the microflora present. Perhaps *Mycobacterium* can handle this process/change in environment due to its very thick cell walls (Percival and Williams, 2014). However, since it is not detected in the non-nitrified biofertilizer it indicates that it has been transferred to the biofertilizer in some step after nitrification. Notwithstanding, a count of 0 in relative abundance is not straightforward, as it might mean not that the genus is absent, but that it is below the limit of detection (Hugerth and Andersson, 2017). Regardless, finding *Mycobacterium* in a sample such as the biofertilizer would not at all be unreasonable since they are soil- and water-bound saprophytes which feed on decomposing organic material (Murty Ds, 2014). Since some *Mycobacterium* (e.g. *Mycobacterium tuberculosis* and *Mycobacterium leprae*) are human pathogens, further investigation on exactly what species of *Mycobacterium* is present in the nitrified biofertilizer is recommended to ensure the safety of using it for food production.

In the non-nitrified biofertilizer the most abundant genera from the16S rRNA gene amplicon sequencing were *Pseudomonas, Leuconostoc* and *Sporosarcina*. None of these are surprising to find as both *Pseudomonas* and *Sporosarcina* are naturally found in soil (Mercado-Blanco and Bakker, 2007, Pregerson, 1973), and *Leuconostoc* normally found wide-spread in the

environment, in fermented foods and in plant matter (Holland and Liu, 2011). An interesting observation is that some species of *Sporosarcina (S. ureae),* produce ammonium in the presence of urea, and as mentioned in section x, ammonium are present in high levels in the non-nitrified biofertilizer. *S. ureae* also reportedly requires a high concentration of ammonium and high alkalinity in order to grow (Mörsdorf and Kaltwasser, 1989).

In the inorganic fertilizer, the most abundant genera was *Lactobacillus, Enterococcus, Serratia,* and *Pseudomonas,* the last also being identified in the inorganic fertilizer with MALDI-TOF MS. The high relative abundance of OTUs detected in the negative control in the samples is a result of the low DNA yield of the samples (mainly in the samples from hydroponic channels with the inorganic fertilizer), rendering the sequenced background more prominent. After some consultation with the company performing the sequencing and analysis it is therefore concluded that it is not probable that the OTUs detected in the negative control are really present in the samples. The origin of the sequenced background is that the ingredients of the PCR reaction of the sequencing may contain bacterial DNA, which is of common occurrence. (Bech Lukassen, DNASense, 2020, oral communication). It was furthermore not expected to have high yields of DNA in the inorganic fertilizer. In the inorganic fertilizer the most abundant genera were *Lactobacillus,* although this was also the most abundant in the negative control and is believed to be sequenced background, *Enterococcus,* although as well present in negative control in but in generally lower abundance, *Serratia,* also present in negative control, and *Pseudomonas* present in negative control but in very low relative abundance. *Pseudomonas* was however also identified in the inorganic fertilizer with MALDI-TOF biotyping in the pre-study experiment of the fertilizers, and thus is presumably indeed present. It is uncertain however how it has been introduced into the inorganic fertilizer, which might very well be possible due to it being wide-spread in the environment (Mercado-Blanco and Bakker, 2007).

5.2. Challenge testing of the nitrified biofertilizer with *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes*

Simulating the event of a contamination of the biofertilizer with the food-borne pathogens *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes* yielded promising results in regards of food safety. Both *Salmonella* and *Listeria* were non-detectable on their respective selective agar plates within 48 hours after incubation, in all three replicates of the experiment. Regarding *B. cereus,* the interpretation of the results is a bit more complex due to the microorganism already occurring in the biofertilizer, as was found in the previous microbiological controls that had been performed on the biofertilizer at the biogas/waste treatments plants (see Table 3). Throughout the three biological replicates it was steadily present in the control flask of non-inoculated nitrified fertilizer in around 10 CFU/ml (estimated count). The flask with nitrified biofertilizer inoculated with *B. cereus* dropped to these levels within 24 hours after incubation. A hypothesis regarding the ceaseless presence of *B. cereus* concerns its spore-forming trait (Bottone, 2010). The bacteria's ability to form spores provides an explanation as to how it can be present in such high amounts after hygienization and digestion of the biofertilizer, and also to how it can be steadily present in the biofertilizer in the challenge testing experiments despite a large amount of the inoculation dying off after a very short time after incubation.

The fact that all the bacteria do not establish themselves, even seemingly dying off within 48 hours after incubation in the biofertilizer, implies that the biofertilizer constitutes a highly inhospitable environment for these bacteria. The pH of the biofertilizer is measured at the beginning of all biological replicates, and lies between 5.8-6.1, and thus the pH of the biofertilizer should not be hindering the establishment of the bacteria. Considering the chemical analysis that has previously been performed at Ulltuna (see Appendix x), the analysis of PPCP's (Pharmaceuticals and Personal Care Products), the samples collected from Karpalund (from where this biofertilizer is collected) shows considerable levels (150 ng/g) of fenbendazole (a compound used to treat roundworm in animals (Zamanian et al., 2018)), however this compound is not reported to have an antimicrobial activity (EMEA, 2004). Antimicrobial agent sulfaclozine (used for treatment of various poultry diseases (Sentepe and Eraslan, 2010)) was found in levels of 60 ng/g in samples collected from Karpalund, which might very well have an impact on the establishment of the pathogens. Theobromine, antimicrobial bitter compound (Lakshmi et al., 2019) found in cocoa was detected in levels of 2000 ng/g, and caffeine (which also possesses an antimicrobial activity (Pruthviraj et al., 2011)) found in levels of ~5000 ng/g which also very well might have an effect on the establishment of the pathogens. Fungicides propiconazole, fludioxonil (Twizeyimana et al., 2013)and imazalil (Altieri et al., 2013) were detected in considerable amounts (~90 ng/g, ~80 ng/g and 850 ng/g respectively). Possibly the presence of all of these compounds makes the biofertilizer an inhospitable environment for the pathogens to establish themselves. Another possibility is the apparent lack of nutrients (seemingly a carbon source) that the results from the calorimetry implies. As well as in the pre-study, the samples with nitrified biofertilizer inoculated with the pathogens placed in the calorimeter show no signs of microbial activity. Possibly, the lack of key factors for growth in the biofertilizer does not support the survival of these pathogenic bacteria, or the combination of lack of nutrients and presence of harmful compounds render them viable but not countable, leaving them unable to form colony forming units when plating on their respective selective medium.

5.3. Societal relevance

The investigations in this project are of importance for urban food security, and in turn for the development of a circular food production. It directly addresses and strives towards some of the United Nations' (UN) sustainability developmental goals:

- Goal 2: Zero hunger, more specifically the target 2.4: Sustainable food production and resilient agricultural practices.

  The utilization of recycled resources paired with a setup that can be placed essentially anywhere (e.g. in urban environments) is a promising alternative to tackle the changes that agricultural practices face as a result of climate change.

- Goal 8: Decent Work and Economic Growth, more specifically the targets: 8.2 Diversify, Innovate and Upgrade for Economic Productivity; 8.4 Improve Resource Efficiency in Consumption and Production.

  The use of this biofertilizer as a circular resource utilization of produced food waste.

- Goal 12: Responsible Consumption and Production, more specifically targets: 12.2 Sustainable Management and Use of Natural Resources; 12.4 Responsible Management of Chemicals and Waste; 12.5 Substantially Reduce Waste Generation.

These targets are endeavored through taking part in valorization of food waste and allow urban production of healthy, safe and attractive produces.

6. Conclusions

As a step towards a more circular, biobased society and food production systems, the project *"Food waste to new food"* investigates the possibilities of utilizing an urban-friendly hydroponic cultivation system for production of pak choi, based on having an organic biofertilizer produced from food waste as a nutrient source. Ensuring microbial safety is crucial in such a short nutrient loop, advocating for a microbiological risk assessment to assure that the biofertilizer is suitable for food production.

This project has collected insights of the microbial community of a biofertilizer produced from food waste. The microbiological community of the biofertilizer changes distinctly through a necessary initial nitrification process, and the nitrified biofertilizer does not provide a favourable environment for the food-borne pathogens *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes* to neither grow nor establish. The following conclusions can be made based on the results obtained in this master thesis project:

- The most abundant genus of bacteria in the nitrified biofertilizer is *Mycobacterium.*
- Low levels of *Bacillus cereus* (~10 CFU/ml) is naturally present in the nitrified biofertilizer.
- Four $\log_{10}$ reductions of *B. cereus* could be observed after its inoculation in the nitrified biofertilizer within 24 hours at 20°C.
- An inoculation of 10^5 CFU/ml of *S. enterica* and *L. monocytogenes* in the nitrified biofertilizer was no longer detectable with selective plating after 48 hours of incubation at 20°C.
- The nitrified biofertilizer does not seem to support microbial proliferation without the addition of substrates containing a carbon source.

7. Future perspectives

In this master thesis project experiments have been performed to assess the microbiological safety of a nitrified biofertilizer in the application of hydroponic farming. However, discoveries from this risk assessment would need further investigation to be fully aware of the microbiological risks that would arise in the utilization of this biofertilizer for hydroponic farming. A further analysis on what species of *Mycobacterium* that is present in the biofertilizer to exclude pathogenic species that might constitute a food safety concern is required. A possible method of investigation could be to use MALDI-TOF MS for species level identification of *Mycobacterium* present in the nitrified biofertilizer.

It might also be of interest to further optimize the method used for microbial community analyses of the biofertilizer. Since the DNA yield was quite low in this study, ways of increasing it such as storing the samples collected from the hydroponic channels in a more appropriate way, for example the bacterial content could be collected though centrifugation of the sample, discarding the liquid and freezing only the solid content for later DNA-extraction. Another way of increasing the DNA yield might be to collect samples of larger volume. More thorough and systematic recovery studies to see how much is actually lost through the sample preparation (filtration and DNA-extraction) may also be of relevance.

The challenge tests in this study was performed in solely the nitrified biofertilizer, however it might be relevant to investigate whether the addition of the plant's roots alters the nutrient solution in some way so that an establishment of a pathogen would be possible. It is also of interest to perform a more extensive analysis on what factors that are hindering the establishment of the food-borne pathogens *Bacillus cereus, Salmonella enterica* and *Listeria monocytogenes* (or other microbiological contaminants), be it a deficiency of a key factor for microbiological growth or an interaction caused by the PPCP's discovered in the chemical analysis.

8. References

ALTIERI, G., DI RENZO, G. C., GENOVESE, F., CALANDRA, M. & STRANO, M. C. 2013. A new method for the postharvest application of imazalil fungicide to citrus fruit. *Biosystems Engineering,* 115**,** 434-443.

ANTUNES, L. P., MARTINS, L. F., PEREIRA, R. V., THOMAS, A. M., BARBOSA, D., LEMOS, L. N., SILVA, G. M. M., MOURA, L. M. S., EPAMINO, G. W. C., DIGIAMPIETRI, L. A., LOMBARDI, K. C., RAMOS, P. L., QUAGGIO, R. B., DE OLIVEIRA, J. C. F., PASCON, R. C., CRUZ, J. B. D., DA SILVA, A. M. & SETUBAL, J. C. 2016. Microbial community structure and dynamics in thermophilic composting viewed through metagenomics and metatranscriptomics. *Scientific reports,* 6**,** 38915-38915.

APPRILL, A., MCNALLY, S., PARSONS, R. & WEBER, L. 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology,* 75.

ASP, H., BERGSTRAND, K.-J. & HULTBERG, M. 2020. Biogödsel som näringskälla vid hydroponisk odling - nitrifiering och pH. *LTV-fakultetens faktablad (BIB14970973).*

AVFALL SVERIGE. 2017. *Basfakta om biogödsel* [Online]. www.biogodsel.se. [Accessed 2020-06-01].

AVFALL SVERIGE 2020. SPCR 120: Certification rules for digestate from biodegradable waste by the quality assurance system of Avfall Sverige – The Swedish Waste Management Association www.avfallsverige.se.

BARBOSA, G. L., GADELHA, F. D. A., KUBLIK, N., PROCTOR, A., REICHELM, L., WEISSINGER, E., WOHLLEB, G. M. & HALDEN, R. U. 2015. Comparison of Land, Water, and Energy Requirements of Lettuce Grown Using Hydroponic vs. Conventional Agricultural Methods. *International journal of environmental research and public health,* 12**,** 6879-6891.

BARKER, A. V., PILBEAM, DAVID J. 2006. Handbook of Plant Nutrition. CRC Press.

BOTTONE, E. J. 2010. Bacillus cereus, a volatile human pathogen. *Clinical microbiology reviews,* 23**,** 382-398.

BRAISSANT, O., WIRZ, D., GÖPFERT, B. & DANIELS, A. U. 2010. Use of isothermal microcalorimetry to monitor microbial activities. *FEMS Microbiology Letters,* 303**,** 1-8.

CHEN, J. 2007. Rapid urbanization in China: A real challenge to soil protection and food security. *CATENA,* 69**,** 1-15.

CLARRIDGE, J. E., 3RD 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical microbiology reviews,* 17**,** 840-862.

CRITTER, S. A. M., FREITAS, S. S. & AIROLDI, C. 2002. Comparison between microorganism counting and a calorimetric method applied to tropical soils. *Thermochimica Acta,* 394**,** 133-144.

CTCN, C. T. C. N. *Karpalund Biogas Plant, Kristianstad* [Online]. Available: https://www.ctc-n.org/products/karpalund-biogas-plant-kristianstad [Accessed].

EFSA 2005. Opinion of the Scientific Panel on Biological Hazards on Bacillus cereus and other Bacillus spp in foodstuffs. 1. *The EFSA Journal (2005),* 175.

ELABED, H., GONZÁLEZ-TORTUERO, E., IBACACHE-QUIROGA, C., BAKHROUF, A., JOHNSTON, P., GADDOUR, K., BLÁZQUEZ, J. & RODRÍGUEZ-ROJAS, A. 2019. Seawater salt-trapped Pseudomonas aeruginosa survives for years and gets primed for salinity tolerance. *BMC microbiology,* 19**,** 142-142.

EMEA 2004. COMMITTEE FOR MEDICINAL PRODUCTS FOR VETERINARY USE, FENBENDAZOLE (Extrapolation to all ruminants) European Medicines Agency, Veterinary Medicines and Inspections.

FAO Climate change, water and food security. *In:* NATIONS, F. A. A. O. O. T. U. (ed.). unwater.org.

FONTANA, L., ROSSI, C., HUBINGER, S., FERREIRA, M., SPOTO, M., SALA, F. & VERRUMA-BERNARDI, M. 2018. Physicochemical characterization and sensory evaluation of lettuce cultivated in three growing systems. *Horticultura Brasileira,* 36**,** 20-26.

FRANCOIS, K., DEVLIEGHERE, F., STANDAERT, A. R., GEERAERD, A. H., COOLS, I., VAN IMPE, J. F. & DEBEVERE, J. 2005. Environmental factors influencing the relationship between optical density and cell count for Listeria monocytogenes. *Journal of Applied Microbiology,* 99**,** 1503-1515.

FRICKER, M., ÅGREN, J., SEGERMAN, B., KNUTSSON, R. & EHLING-SCHULZ, M. 2011. Evaluation of Bacillus strains as model systems for the work on Bacillus anthracis spores. *Int J Food Microbiol,* 145 Suppl 1**,** S129-36.

GERICKE, W. F. 1945. THE MEANING OF HYDROPONICS. *Science,* 101**,** 142.

GHYSELINCK, J., PFEIFFER, S., HEYLEN, K., SESSITSCH, A. & DE VOS, P. 2013. The Effect of Primer Choice and Short Read Sequences on the Outcome of 16S rRNA Gene Based Diversity Studies. *PLOS ONE,* 8**,** e71360.

GUILLER, E. A. 2013. Developement of a set of Listeria monocytogenes strains for conducting challenge tests. *ANSES-Food Safety Laboratory, Maisons-Alfort*.

HILL, G. T., MITKOWSKI, N. A., ALDRICH-WOLFE, L., EMELE, L. R., JURKONIE, D. D., FICKE, A., MALDONADO-RAMÍREZ, S., LYNCH, S. T. & NELSON, E. B. 2000. Methods for assessing the composition and diversity of soil microbial communities. *Applied Soil Ecology,* 15**,** 25-36.

HOLLAND, R. & LIU, S. Q. 2011. Lactic Acid Bacteria | Leuconostoc spp. *In:* FUQUAY, J. W. (ed.) *Encyclopedia of Dairy Sciences (Second Edition).* San Diego: Academic Press.

HOU, T.-Y., CHIANG-NI, C. & TENG, S.-H. 2019. Current status of MALDI-TOF mass spectrometry in clinical microbiology. *Journal of Food and Drug Analysis,* 27**,** 404-414.

HUGERTH, L. W. & ANDERSSON, A. F. 2017. Analysing Microbial Community Composition through Amplicon Sequencing: From Sampling to Hypothesis Testing. *Frontiers in microbiology,* 8**,** 1561-1561.

IBRAHIM, M. H., JAAFAR, H. Z. E., KARIMI, E. & GHASEMZADEH, A. 2013. Impact of organic and inorganic fertilizers application on the phytochemical and antioxidant activity of Kacip Fatimah (Labisia pumila Benth). *Molecules (Basel, Switzerland),* 18**,** 10973-10988.

ISLAM, M., MORGAN, J., DOYLE, M. P., PHATAK, S. C., MILLNER, P. & JIANG, X. 2004. Fate of Salmonella enterica serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Appl Environ Microbiol,* 70**,** 2497-502.

JENSEN, R. H. & ARENDRUP, M. C. 2011. Characterization of a Previously Overlooked Pathogen and Its Unique Susceptibility Profile in Comparison with Five Related Species. *Journal of Clinical Microbiology,* 49**,** 549.

KAPTAN ÖLMEZ, H. & ARAN, N. 2005. Modeling the growth kinetics of Bacillus cereus as a function of temperature, pH, sodium lactate and sodium chloride concentrations. *International Journal of Food Microbiology,* 98**,** 135-143.

LAKSHMI, A., VISHNUREKHA, C. & BAGHKOMEH, P. N. 2019. Effect of theobromine in antimicrobial activity: An in vitro study. *Dental research journal,* 16**,** 76-80.
LAOR, Y., RAVIV, M. & BORISOVER, M. 2004. Evaluating microbial activity in composts using microcalorimetry. *Thermochimica Acta,* 420**,** 119-125.
LIN, W., LIN, M., ZHOU, H., WU, H., LI, Z. & LIN, W. 2019. The effects of chemical and organic fertilizer usage on rhizosphere soil in tea orchards. *PloS one,* 14**,** e0217018-e0217018.
MCPHERSON, M. R., WANG, P., MARSH, E. L., MITCHELL, R. B. & SCHACHTMAN, D. P. 2018. Isolation and Analysis of Microbial Communities in Soil, Rhizosphere, and Roots in Perennial Grass Experiments. *Journal of visualized experiments : JoVE***,** 57932.
MENG, Q., YANG, W., MEN, M., BELLO, A., XU, X., XU, B., DENG, L., JIANG, X., SHENG, S., WU, X., HAN, Y. & ZHU, H. 2019. Microbial Community Succession and Response to Environmental Variables During Cow Manure and Corn Straw Composting. *Frontiers in Microbiology,* 10.
MERCADO-BLANCO, J. & BAKKER, P. 2007. Interactions between plants and beneficial Pseudomonas spp.: Exploiting bacterial traits for crop protection. *Antonie van Leeuwenhoek,* 92**,** 367-89.
MURPHY, S., GAFFNEY, M. T., FANNING, S. & BURGESS, C. M. 2016. Potential for transfer of Escherichia coli O157:H7, Listeria monocytogenes and Salmonella Senftenberg from contaminated food waste derived compost and anaerobic digestate liquid to lettuce plants. *Food Microbiology,* 59**,** 7-13.
MURTY DS, P. K. 2014. A Brief review on Ecology and Evolution of Mycobacteria. *Mycobacterial Diseases,* 04.
MÖRSDORF, G. & KALTWASSER, H. 1989. Ammonium assimilation in Proteus vulgaris, Bacillus pasteurii, and Sporosarcina ureae. *Archives of Microbiology,* 152**,** 125-131.
NASSAR, A. M. K., KUBOW, S. & DONNELLY, D. J. 2015. High-Throughput Screening of Sensory and Nutritional Characteristics for Cultivar Selection in Commercial Hydroponic Greenhouse Crop Production. *International Journal of Agronomy,* 2015**,** 376417.
NIAZI, A., MANZOOR, S., ASARI, S., BEJAI, S., MEIJER, J. & BONGCAM-RUDLOFF, E. 2014. Genome analysis of Bacillus amyloliquefaciens Subsp. plantarum UCMB5113: a rhizobacterium that improves plant growth and stress management. *PloS one,* 9**,** e104651-e104651.
NOTERMANS, S., IN'T VELD, P., WIJTZES, T. & MEAD, G. C. 1993. A user's guide to microbial challenge testing for ensuring the safety and stability of food products. *Food Microbiology,* 10**,** 145-157.
OBMINSKA, A. 2018. Containerodling ska göra Ikea självförsörjande på sallad. *NyTeknik,* 2018-12-13.
OBMINSKA, A. 2020. Framtidens odlingsteknik: Så grönskar det inomhus. *NyTeknik,* 2020-02-13.
PERCIVAL, S. L. & WILLIAMS, D. W. 2014. Chapter Nine - Mycobacterium. *In:* PERCIVAL, S. L., YATES, M. V., WILLIAMS, D. W., CHALMERS, R. M. & GRAY, N. F. (eds.) *Microbiology of Waterborne Diseases (Second Edition).* London: Academic Press.
PERRY, L. 2003. pH for the Garden. *University of Vermont Extension Department of Plant and Soil Science* [Online]. Available: http://pss.uvm.edu/ppp/pubs/oh34.htm.
POLLOCK, J., GLENDINNING, L., WISEDCHANWET, T. & WATSON, M. 2018. The Madness of Microbiome: Attempting To Find Consensus "Best Practice" for 16S Microbiome Studies. *Appl Environ Microbiol,* 84.

PREGERSON, B. S. 1973. *The Distribution and Physiology of Sporosarcina Ureae*, California State University, Northridge.
PRUTHVIRAJ, P., SUCHITA, B., SHITAL, K. & SHILPA, K. 2011. Evaluation of antibacterial activity of caffeine. *International Journal of Research in Ayurveda and Pharmacy,* 2**,** 1354-1357.
SAMPEDRO, A., CEBALLOS MENDIOLA, J. & ALIAGA MARTÍNEZ, L. 2018. Chapter Three - MALDI-TOF Commercial Platforms for Bacterial Identification. *In:* COBO, F. (ed.) *The Use of Mass Spectrometry Technology (MALDI-TOF) in Clinical Microbiology*. Academic Press.
SENTEPE, I. & ERASLAN, G. 2010. Pharmacokinetic of sulfaclozine in broiler chickens. *Food Chem Toxicol,* 48**,** 448-51.
SEUYLEMEZIAN, A., ARONSON, H. S., TAN, J., LIN, M., SCHUBERT, W. & VAISHAMPAYAN, P. 2018. Development of a Custom MALDI-TOF MS Database for Species-Level Identification of Bacterial Isolates Collected From Spacecraft and Associated Surfaces. *Frontiers in microbiology,* 9**,** 780-780.
SHIN, H. B., YOON, J., LEE, Y., KIM, M. S. & LEE, K. 2015. Comparison of MALDI-TOF MS, housekeeping gene sequencing, and 16S rRNA gene sequencing for identification of Aeromonas clinical isolates. *Yonsei Med J,* 56**,** 550-5.
TAKAYASU, L., SUDA, W. & HATTORI, M. 2019. Mapping the Environmental Microbiome. *In:* RANGANATHAN, S., GRIBSKOV, M., NAKAI, K. & SCHÖNBACH, C. (eds.) *Encyclopedia of Bioinformatics and Computational Biology*. Oxford: Academic Press.
THOMPSON, L. R., SANDERS, J. G., MCDONALD, D., AMIR, A., LADAU, J., LOCEY, K. J., PRILL, R. J., TRIPATHI, A., GIBBONS, S. M., ACKERMANN, G., NAVAS-MOLINA, J. A., JANSSEN, S., KOPYLOVA, E., VÁZQUEZ-BAEZA, Y., GONZÁLEZ, A., MORTON, J. T., MIRARAB, S., ZECH XU, Z., JIANG, L., HAROON, M. F., KANBAR, J., ZHU, Q., JIN SONG, S., KOSCIOLEK, T., BOKULICH, N. A., LEFLER, J., BRISLAWN, C. J., HUMPHREY, G., OWENS, S. M., HAMPTON-MARCELL, J., BERG-LYONS, D., MCKENZIE, V., FIERER, N., FUHRMAN, J. A., CLAUSET, A., STEVENS, R. L., SHADE, A., POLLARD, K. S., GOODWIN, K. D., JANSSON, J. K., GILBERT, J. A., KNIGHT, R., RIVERA, J. L. A., AL-MOOSAWI, L., ALVERDY, J., AMATO, K. R., ANDRAS, J., ANGENENT, L. T., ANTONOPOULOS, D. A., APPRILL, A., ARMITAGE, D., BALLANTINE, K., BÁRTA, J. Í., BAUM, J. K., BERRY, A., BHATNAGAR, A., BHATNAGAR, M., BIDDLE, J. F., BITTNER, L., BOLDGIV, B., BOTTOS, E., BOYER, D. M., BRAUN, J., BRAZELTON, W., BREARLEY, F. Q., CAMPBELL, A. H., CAPORASO, J. G., CARDONA, C., CARROLL, J., CARY, S. C., CASPER, B. B., CHARLES, T. C., CHU, H., CLAAR, D. C., CLARK, R. G., CLAYTON, J. B., CLEMENTE, J. C., COCHRAN, A., COLEMAN, M. L., COLLINS, G., COLWELL, R. R., CONTRERAS, M., CRARY, B. B., CREER, S., CRISTOL, D. A., CRUMP, B. C., CUI, D., DALY, S. E., DAVALOS, L., DAWSON, R. D., DEFAZIO, J., DELSUC, F., DIONISI, H. M., DOMINGUEZ-BELLO, M. G., DOWELL, R., DUBINSKY, E. A., DUNN, P. O., ERCOLINI, D., ESPINOZA, R. E., EZENWA, V., et al. 2017. A communal catalogue reveals Earth's multiscale microbial diversity. *Nature,* 551**,** 457-463.
TILMAN, D., CASSMAN, K. G., MATSON, P. A., NAYLOR, R. & POLASKY, S. 2002. Agricultural sustainability and intensive production practices. *Nature,* 418**,** 671-677.
TWIZEYIMANA, M., MCDONALD, V., MAYORQUIN, J. S., WANG, D. H., NA, F., AKGÜL, D. S. & ESKALEN, A. 2013. Effect of Fungicide Application on the

Management of Avocado Branch Canker (Formerly Dothiorella Canker) in California. *Plant Dis,* 97**,** 897-902.
WADSÖ, L. & GÓMEZ GALINDO, F. 2009. Isothermal calorimetry for biological applications in food science and technology. *Food Control,* 20**,** 956-961.
ZAMANIAN, M., COOK, D. E., ZDRALJEVIC, S., BRADY, S. C., LEE, D., LEE, J. & ANDERSEN, E. C. 2018. Discovery of genomic intervals that underlie nematode responses to benzimidazoles. *PLoS Negl Trop Dis,* 12**,** e0006368.

Image references

Figure 1. Wikipedia: Karpalund. *Karpalund Biogasanläggning* https://sv.m.wikipedia.org/wiki/Karpalund. Downloaded 2020-06-07.

Figure 3. DispatchWeekly: *The Future of Vertical Farming in 5 Inspiring Examples* https://dispatchweekly.com/2016/10/future-vertical-farming-5-inspiring-examples/. Downloaded 2020-06-02.

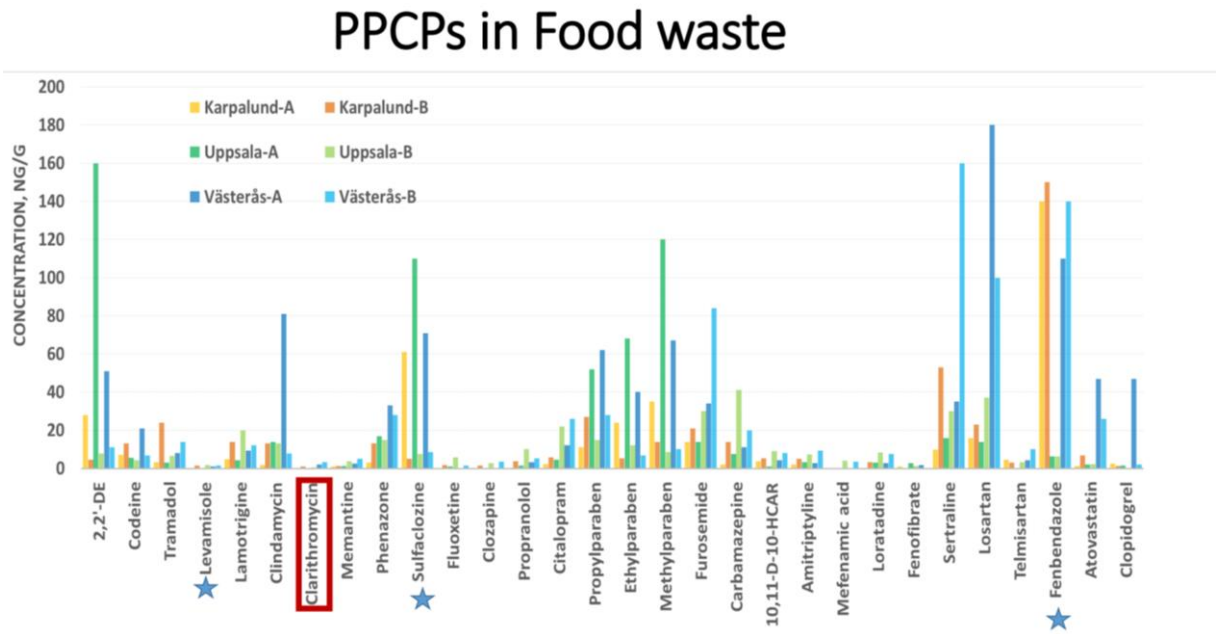
Figure 4. NyTeknik: *Framtidens odlingsteknik: Så grönskar det inomhus.* https://www.nyteknik.se/story/framtidens-odlingsteknik-sa-gronskar-det-inomhus-6987341. Downloaded 2020-06-02.

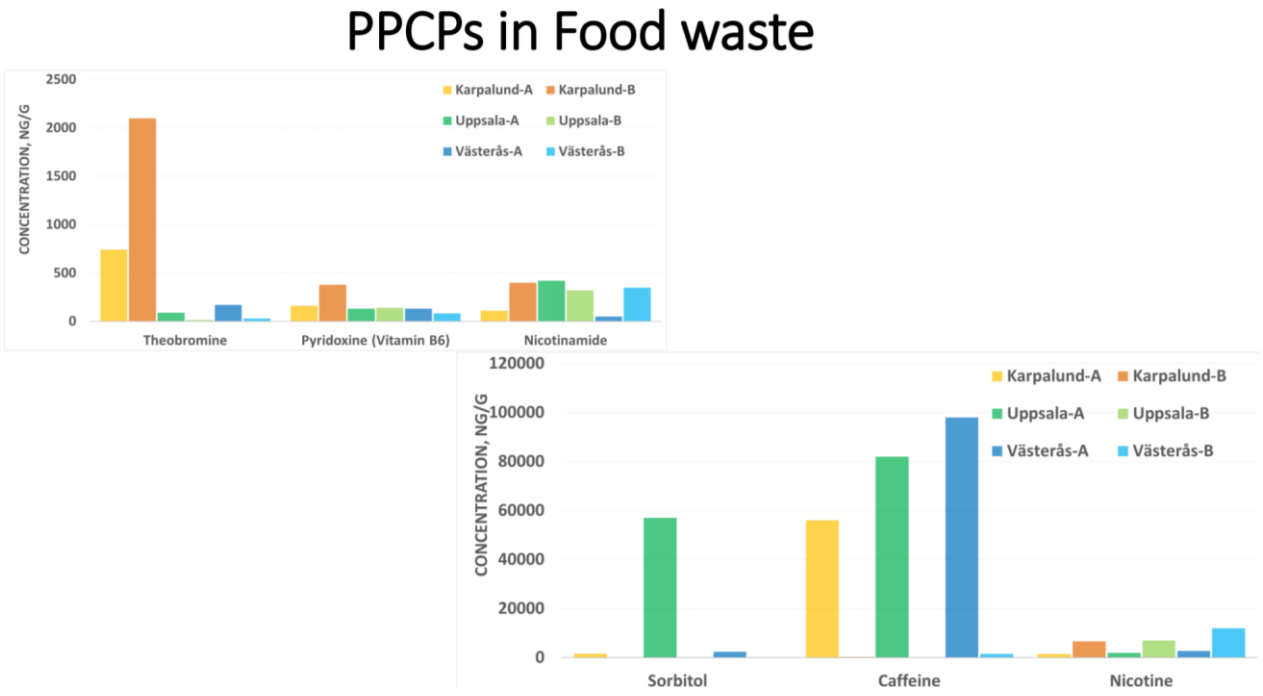
Figure 7. Bruker: The MALDI Biotyper®, brochure.

https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/Literature/Brochures/1866135_MALDI_Biotyper_RUO_brochure_01-2019_eBook.pdf). Downloaded 2020-06-05.

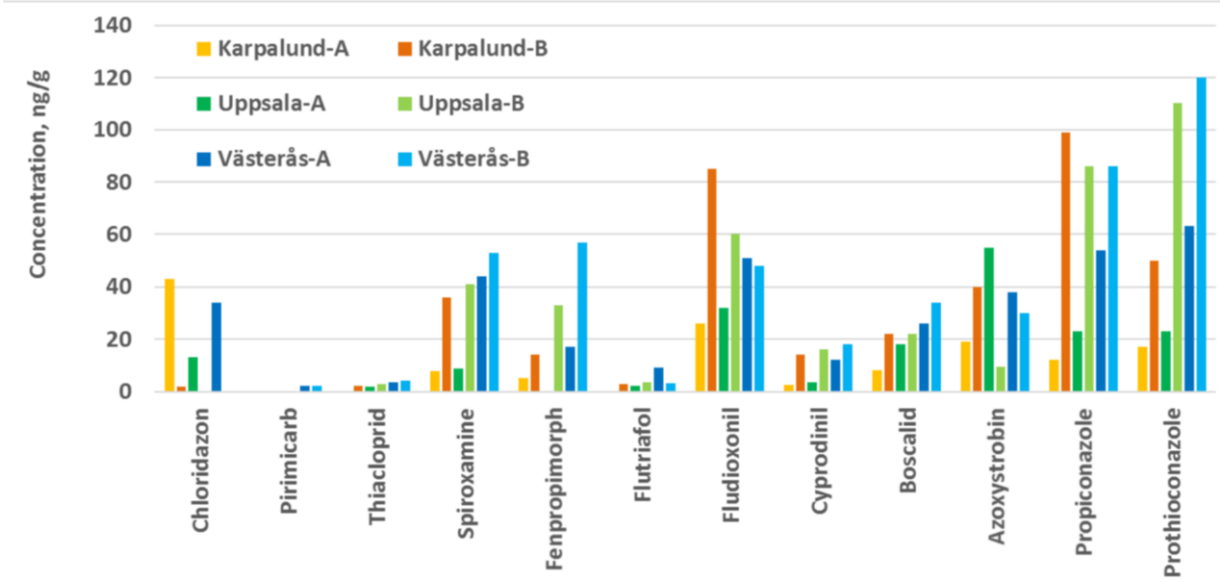
Appendix 1. Results from SLU, Ulltuna's chemical analysis of PPCPs and pesticides in food waste

PPCPs (Pharmaceuticals, Personal Care Products), and Pesticides. The samples marked "Karpalund-B" are the ones collected from the same origin as the biofertilizer investigated in this project.

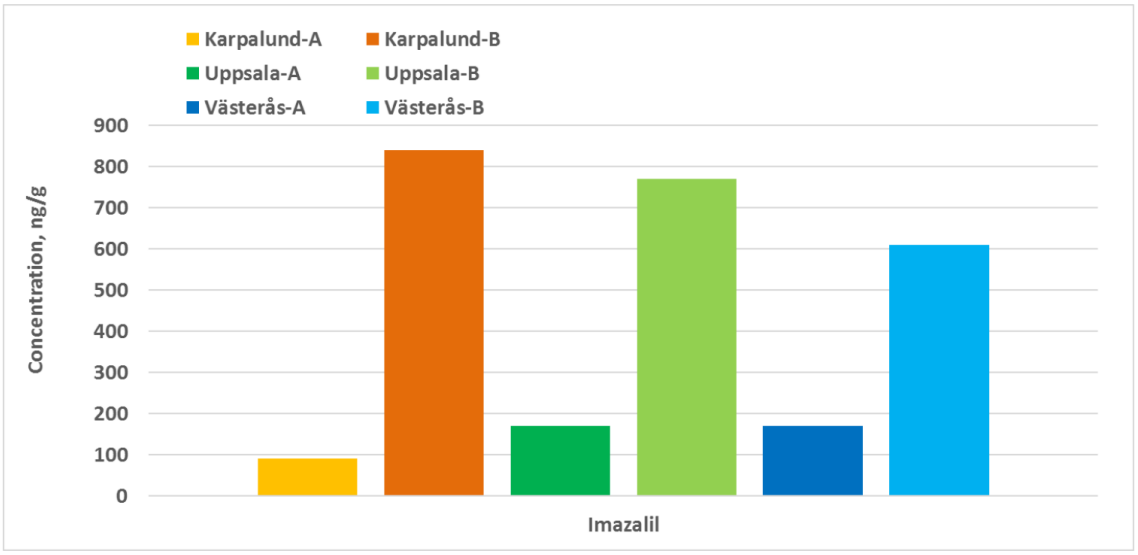




Pesticides in Food waste



Pesticides in Food waste



Imazalil is allowed for some different uses in Sweden: in seed treatment of potatoes and barley and against mildew in cucumbers in greenhouses.

Appendix 2. Materials and methods of the 16s rRNA gene amplicon sequencing and analysis, performed by DNASense

Materials and methods

Archaeal and bacterial community analysis targeting 16S V4 rRNA

Library preparation

Archaea and Bacteria, 16S rRNA gene region V4 sequencing libraries were prepared by a custom protocol based on an Illumina protocol (Illumina, 2015). Up to 10 ng of extracted DNA was used as template for PCR amplification of the Archaea and Bacteria, 16S rRNA gene region V4 amplicons. Each PCR reaction (25 µL) contained (12.5 µL) PCRBIO Ultra mix (PCR Biosystems, USA) and 400 nM of each forward and reverse tailed primer mix. PCR was conducted with the following program: Initial denaturation at 95 °C for 2 min, 30 cycles of amplification (95 °C for 15 s, 55 °C for 15 s, 72 °C for 50 s) and a final elongation at 72 °C for 5 min. Duplicate PCR reactions were performed for each sample and the duplicates were pooled after PCR. The forward and reverse tailed primers were designed according to (Illumina, 2015) and contain primers targeting the Archaea and Bacteria, 16S rRNA gene region V4: [515FB] GTGYCAGCMGCCGCGGTAA and [806RB] GGACTACNVGGGTWTCTAAT (Apprill et al., 2015). The primer tails enable attachment of Illumina Nextera adaptors necessary for sequencing in a subsequent PCR. The resulting amplicon libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter, USA) with a bead to sample ratio of 4:5. DNA was eluted in 25 µL of nuclease free water (Qiagen, Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries.

Sequencing libraries were prepared from the purified amplicon libraries using a second PCR. Each PCR reaction (25 µL) contained PCRBIO HiFi buffer (1x), PCRBIO HiFi Polymerase (1 U/reaction) (PCRBiosystems, UK), adaptor mix (400 nM of each forward and reverse) and up to 10 ng of amplicon library template. PCR was conducted with the following program: Initial denaturation at 95 °C for 2 min, 8 cycles of amplification (95 °C for 20 s, 55 °C for 30 s, 72 °C for 60 s) and a final elongation at 72 °C for 5 min. The resulting sequencing libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter, USA) with a bead to sample ratio of 4:5. DNA was eluted in 25 µL of nuclease free water (Qiagen, Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries.

DNA sequencing

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 2 nM. The samples were paired-end sequenced (2x300 bp) on a MiSeq (Illumina, USA) using a MiSeq Reagent kit v3 (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. >10% PhiX control library was spiked in to overcome low complexity issues often observed with amplicon samples.

Bioinformatic processing

Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger et al., 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN: 225 . The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoč and Salzberg, 2011) with the settings -m 10 -M 250. The trimmed reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090 -usearch_global command with -id 0.97 -maxaccepts 0 -maxrejects 0. Taxonomy was assigned using the RDP classifier (Wang et al., 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso et al., 2010), using –confidence 0.8 and the SILVA database, release 132 (Quast et al., 2013). The results were analysed in R v. 3.5.1 (R Core Team, 2017) through the Rstudio IDE using the ampvis package v.2.5.8 (Albertsen et al., 2015).

Appendix 3. Results from MALDI-TOF MS

Run Identifier:
20200313_1351_Projekt_Biogosel_Julia_BoN_2930

Run Creation Date/Time: 2020-03-13T13:32:13.762

Sample 1

Sample Name:	C1
Sample Description:	
Sample ID:	NGUL#1
Sample Creation Date/Time:	2020-03-13T13:32:13.782
Sample Type:	Standard sample
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
ACQ Timestamp:	2020-03-13T13:33:36.194
AutoXecute Method:	MBT_AutoX
Applied MSP Library(ies):	BDAL / contains 8468 MSPs / dee78ccc-c113-4ede-b02e-392d0cde98c1 / 2019-11-05T18:15:45.616, SR Taxonomy 72 MSPs 2018-10-19 (reduced size) / contains 72 MSPs reduced size (do not include Shigella, B anthacis, S typhi and Y pestis strains) / 628751e6-0226-413e-912c-9953513e31e3 / 2018-10-19T09:00:54.238, NIH Fungi Library 364 MSPs 2017-10-05 (one strain deleted) / contains 364 MSPs library according to Lau et al 2013 (one strain deleted AL65) / 154f101f-2800-4507-8c33-7e7cbc41c71f / 2018-10-19T08:56:12.750, Folkhalsomyndigheten 237 MSPs 2017-10-05 / contains 273 MSPs / b8fb573c-cd35-4f0c-9864-bb2a0120abd2 / 2018-10-19T08:52:39.536, Aerococcus SLA 2015 (0/40) / contains 40 MSPs Aerococcus strains from SLA in Denmark / 211923ec-b1be-4a9b-bd71-195b90e8e993 / 2018-10-19T08:43:06.543, Mycobacteria Library (bead method) / contains 952 MSPs / 0734bc24-ffaa-49e2-bc83-5edfbf91c60f / 2019-11-05T17:31:35.484, Filamentous Fungi / contains 577 MSPs / 95680832-cb78-4786-9f45-d67971dd8c1e / 2019-11-05T16:59:37.368, Staph-lib 20MSP(9S argenteus+11 S aureus) 2019-11-08 / Contains 20 MSP, 9 MSP S argenteus , 11 MSP S aureus / 2d6e9496-385f-4d5c-bd52-0187844e789b / 2019-11-08T16:06:30.399

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (-)	Glutamicibacter creatinolyticus DSM 15881T DSM	1.32	1663
2 (-)	Paeniglutamicibacter sulfureus DSM 20167T DSM	1.26	43666
3 (-)	Citrobacter sedlakii DSM 17674T DSM	1.18	67826
4 (-)	Escherichia coli W3350 MMG	1.18	562
5 (-)	Legionella feeleii ATCC 35072T TW VUN	1.18	453
6 (-)	Brevundimonas aurantiaca DSM 4731T HAM	1.15	74316

Result table for sample 1--continued on next page

Result table for sample 1--continued from previous page			
Rank (Quality)	**Matched Pattern**	**Score Value**	**NCBI Identifier**
7 (-)	Raoultella ornithinolytica MB_18887 CHB	1.15	54291
8 (-)	Candida lusitaniae 45 PSB	1.14	36911
9 (-)	Citrobacter freundii 22054_1 CHB	1.13	546
10 (-)	Pseudomonas lundensis DSM 6252T HAM	1.13	86185

Sample 3

Sample Name:	C3
Sample Description:	
Sample ID:	NVIT#1
Sample Creation Date/Time:	2020-03-13T13:32:13.785
Sample Type:	Standard sample
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
ACQ Timestamp:	2020-03-13T13:34:05.935
AutoXecute Method:	MBT_AutoX
Applied MSP Library(ies):	BDAL / contains 8468 MSPs / dee78ccc-c113-4ede-b02e-392d0cde98c1 / 2019-11-05T18:15:45.616, SR Taxonomy 72 MSPs 2018-10-19 (reduced size) / contains 72 MSPs reduced size (do not include Shigella, B anthacis, S typhi and Y pestis strains) / 628751e6-0226-413e-912c-9953513e31e3 / 2018-10-19T09:00:54.238, NIH Fungi Library 364 MSPs 2017-10-05 (one strain deleted) / contains 364 MSPs library according to Lau et al 2013 (one strain deleted AL65) / 154f101f-2800-4507-8c33-7e7cbc41c71f / 2018-10-19T08:56:12.750, Folkhalsomyndigheten 237 MSPs 2017-10-05 / contains 273 MSPs / b8fb573c-cd35-4f0c-9864-bb2a0120abd2 / 2018-10-19T08:52:39.536, Aerococcus SLA 2015 (0/40) / contains 40 MSPs Aerococcus strains from SLA in Denmark / 211923ec-b1be-4a9b-bd71-195b90e8e993 / 2018-10-19T08:43:06.543, Mycobacteria Library (bead method) / contains 952 MSPs / 0734bc24-ffaa-49e2-bc83-5edfbf91c60f / 2019-11-05T17:31:35.484, Filamentous Fungi / contains 577 MSPs / 95680832-cb78-4786-9f45-d67971dd8c1e / 2019-11-05T16:59:37.368, Staph-lib 20MSP(9S argenteus+11 S aureus) 2019-11-08 / Contains 20 MSP, 9 MSP S argenteus , 11 MSP S aureus / 2d6e9496-385f-4d5c-bd52-0187844e789b / 2019-11-08T16:06:30.399

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+++)	Candida palmioleophila MY00850_07 ERL	2.32	45574
2 (+++)	Candida palmioleophila MY938_07 ERL	2.13	45574
3 (+)	Candida palmioleophila 69 PIM	1.92	45574
4 (-)	Candida palmioleophila 108870_2010 IMHM	1.52	45574
5 (-)	Candida lusitaniae MY102_10 ERL	1.35	36911
6 (-)	Candida tropicalis DSM 5991 DSM	1.30	5482

Result table for sample 3--continued on next page

Result table for sample 3--continued from previous page			
Rank (Quality)	**Matched Pattern**	**Score Value**	**NCBI Identifier**
7 (-)	Candida lusitaniae CBS 4870 CBS	1.26	36911
8 (-)	Candida lusitaniae CBS 7270 CBS	1.25	36911
9 (-)	Candida lusitaniae CBS 6936 CBS	1.24	36911
10 (-)	Candida lusitaniae CBS 4414 CBS	1.24	36911

Sample 5

Sample Name:	C5
Sample Description:	
Sample ID:	GOSEL#1
Sample Creation Date/Time:	2020-03-13T13:32:13.787
Sample Type:	Standard sample
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
ACQ Timestamp:	2020-03-13T13:34:31.120
AutoXecute Method:	MBT_AutoX
Applied MSP Library(ies):	BDAL / contains 8468 MSPs / dee78ccc-c113-4ede-b02e-392d0cde98c1 / 2019-11-05T18:15:45.616, SR Taxonomy 72 MSPs 2018-10-19 (reduced size) / contains 72 MSPs reduced size (do not include Shigella, B anthacis, S typhi and Y pestis strains) / 628751e6-0226-413e-912c-9953513e31e3 / 2018-10-19T09:00:54.238, NIH Fungi Library 364 MSPs 2017-10-05 (one strain deleted) / contains 364 MSPs library according to Lau et al 2013 (one strain deleted AL65) / 154f101f-2800-4507-8c33-7e7cbc41c71f / 2018-10-19T08:56:12.750, Folkhalsomyndigheten 237 MSPs 2017-10-05 / contains 273 MSPs / b8fb573c-cd35-4f0c-9864-bb2a0120abd2 / 2018-10-19T08:52:39.536, Aerococcus SLA 2015 (0/40) / contains 40 MSPs Aerococcus strains from SLA in Denmark / 211923ec-b1be-4a9b-bd71-195b90e8e993 / 2018-10-19T08:43:06.543, Mycobacteria Library (bead method) / contains 952 MSPs / 0734bc24-ffaa-49e2-bc83-5edfbf91c60f / 2019-11-05T17:31:35.484, Filamentous Fungi / contains 577 MSPs / 95680832-cb78-4786-9f45-d67971dd8c1e / 2019-11-05T16:59:37.368, Staph-lib 20MSP(9S argenteus+11 S aureus) 2019-11-08 / Contains 20 MSP, 9 MSP S argenteus , 11 MSP S aureus / 2d6e9496-385f-4d5c-bd52-0187844e789b / 2019-11-08T16:06:30.399

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+++)	Candida palmioleophila MY00850_07 ERL	2.21	45574
2 (+++)	Candida palmioleophila MY938_07 ERL	2.04	45574
3 (+)	Candida palmioleophila 69 PIM	1.92	45574
4 (-)	Candida palmioleophila 108870_2010 IMHM	1.59	45574
5 (-)	Lachancea thermotolerans DSM 3434 DSM	1.39	381046
6 (-)	Kytococcus sedentarius IMET 11362T HKJ	1.24	1276

Result table for sample 5--continued on next page

Result table for sample 5--continued from previous page			
Rank (Quality)	**Matched Pattern**	**Score Value**	**NCBI Identifier**
7 (-)	Candida bracarensis CBS 10154T CBS	1.23	273131
8 (-)	Candida mesenterica DSM 70759 DSM	1.22	45568
9 (-)	Lactobacillus kalixensis DSM 16043T DSM	1.20	227944
10 (-)	Lactobacillus kalixensis DSM 16044 DSM	1.19	227944

Sample 9

Sample Name:	C9
Sample Description:	
Sample ID:	ON3#1
Sample Creation Date/Time:	2020-03-13T13:32:13.792
Sample Type:	Standard sample
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
ACQ Timestamp:	2020-03-13T13:35:25.896
AutoXecute Method:	MBT_AutoX
Applied MSP Library(ies):	BDAL / contains 8468 MSPs / dee78ccc-c113-4ede-b02e-392d0cde98c1 / 2019-11-05T18:15:45.616, SR Taxonomy 72 MSPs 2018-10-19 (reduced size) / contains 72 MSPs reduced size (do not include Shigella, B anthacis, S typhi and Y pestis strains) / 628751e6-0226-413e-912c-9953513e31e3 / 2018-10-19T09:00:54.238, NIH Fungi Library 364 MSPs 2017-10-05 (one strain deleted) / contains 364 MSPs library according to Lau et al 2013 (one strain deleted AL65) / 154f101f-2800-4507-8c33-7e7cbc41c71f / 2018-10-19T08:56:12.750, Folkhalsomyndigheten 237 MSPs 2017-10-05 / contains 273 MSPs / b8fb573c-cd35-4f0c-9864-bb2a0120abd2 / 2018-10-19T08:52:39.536, Aerococcus SLA 2015 (0/40) / contains 40 MSPs Aerococcus strains from SLA in Denmark / 211923ec-b1be-4a9b-bd71-195b90e8e993 / 2018-10-19T08:43:06.543, Mycobacteria Library (bead method) / contains 952 MSPs / 0734bc24-ffaa-49e2-bc83-5edfbf91c60f / 2019-11-05T17:31:35.484, Filamentous Fungi / contains 577 MSPs / 95680832-cb78-4786-9f45-d67971dd8c1e / 2019-11-05T16:59:37.368, Staph-lib 20MSP(9S argenteus+11 S aureus) 2019-11-08 / Contains 20 MSP, 9 MSP S argenteus , 11 MSP S aureus / 2d6e9496-385f-4d5c-bd52-0187844e789b / 2019-11-08T16:06:30.399

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+++)	Bacillus amyloliquefaciens_ssp_plantarum CICC 23985_b CICC	2.32	279145
2 (+++)	Bacillus amyloliquefaciens ssp amyloliquefaciens CICC 10075 CICC	2.12	1390
3 (+++)	Bacillus amyloliquefaciens_ssp_plantarum CICC 20037 CICC	2.07	279145
4 (+++)	Bacillus amyloliquefaciens_ssp_plantarum CICC 23981_b CICC	2.04	279145
5 (+++)	Bacillus amyloliquefaciens ssp amyloliquefaciens CICC 10079 CICC	2.03	1390
6 (+)	Bacillus subtilis ssp subtilis CICC 23950 CICC	1.86	135461

Result table for sample 9--continued on next page

Result table for sample 9--continued from previous page

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
7 (+)	Bacillus vallismortis DSM 11031T DSM	1.72	72361
8 (-)	Bacillus amyloliquefaciens CIP 103265T CIP	1.63	1390
9 (-)	Bacillus atrophaeus CICC 23987 CICC	1.62	1452
10 (-)	Bacillus subtilis ssp spizizenii DSM 618 DSM	1.61	96241

Sample 11

Sample Name:	C11
Sample Description:	
Sample ID:	ON1#1
Sample Creation Date/Time:	2020-03-13T13:32:13.795
Sample Type:	Standard sample
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
ACQ Timestamp:	2020-03-13T13:35:57.065
AutoXecute Method:	MBT_AutoX
Applied MSP Library(ies):	BDAL / contains 8468 MSPs / dee78ccc-c113-4ede-b02e-392d0cde98c1 / 2019-11-05T18:15:45.616, SR Taxonomy 72 MSPs 2018-10-19 (reduced size) / contains 72 MSPs reduced size (do not include Shigella, B anthacis, S typhi and Y pestis strains) / 628751e6-0226-413e-912c-9953513e31e3 / 2018-10-19T09:00:54.238, NIH Fungi Library 364 MSPs 2017-10-05 (one strain deleted) / contains 364 MSPs library according to Lau et al 2013 (one strain deleted AL65) / 154f101f-2800-4507-8c33-7e7cbc41c71f / 2018-10-19T08:56:12.750, Folkhalsomyndigheten 237 MSPs 2017-10-05 / contains 273 MSPs / b8fb573c-cd35-4f0c-9864-bb2a0120abd2 / 2018-10-19T08:52:39.536, Aerococcus SLA 2015 (0/40) / contains 40 MSPs Aerococcus strains from SLA in Denmark / 211923ec-b1be-4a9b-bd71-195b90e8e993 / 2018-10-19T08:43:06.543, Mycobacteria Library (bead method) / contains 952 MSPs / 0734bc24-ffaa-49e2-bc83-5edfbf91c60f / 2019-11-05T17:31:35.484, Filamentous Fungi / contains 577 MSPs / 95680832-cb78-4786-9f45-d67971dd8c1e / 2019-11-05T16:59:37.368, Staph-lib 20MSP(9S argenteus+11 S aureus) 2019-11-08 / Contains 20 MSP, 9 MSP S argenteus , 11 MSP S aureus / 2d6e9496-385f-4d5c-bd52-0187844e789b / 2019-11-08T16:06:30.399

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (-)	Acidovorax defluvii DSM 12644T HAM	1.38	86669
2 (-)	Pseudomonas nitroreducens DSM 14399T HAM	1.37	46680
3 (-)	Aeromonas molluscorum 848T DSM	1.30	271417
4 (-)	Lactobacillus mali DSM 20483 DSM	1.28	1618
5 (-)	Lactobacillus amylolyticus DSM 11664T DSM	1.25	83683
6 (-)	Clostridium spiroforme 1047_NCTC 11211T BOG	1.23	29348

Result table for sample 11--continued on next page

Result table for sample 11--continued from previous page			
Rank (Quality)	**Matched Pattern**	**Score Value**	**NCBI Identifier**
7 (-)	Aromatoleum evansii KB740 MPB	1.22	12960
8 (-)	Chryseobacterium joostei LMG 18212T HAM	1.22	112234
9 (-)	Staphylococcus vitulinus DSM 9930 DSM	1.19	71237
10 (-)	Lactobacillus crispatus DSM 20356 DSM	1.19	47770

Sample 13

Sample Name:	D1
Sample Description:	
Sample ID:	ON2#1
Sample Creation Date/Time:	2020-03-13T13:32:13.798
Sample Type:	Standard sample
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
ACQ Timestamp:	2020-03-13T13:36:26.397
AutoXecute Method:	MBT_AutoX
Applied MSP Library(ies):	BDAL / contains 8468 MSPs / dee78ccc-c113-4ede-b02e-392d0cde98c1 / 2019-11-05T18:15:45.616, SR Taxonomy 72 MSPs 2018-10-19 (reduced size) / contains 72 MSPs reduced size (do not include Shigella, B anthacis, S typhi and Y pestis strains) / 628751e6-0226-413e-912c-9953513e31e3 / 2018-10-19T09:00:54.238, NIH Fungi Library 364 MSPs 2017-10-05 (one strain deleted) / contains 364 MSPs library according to Lau et al 2013 (one strain deleted AL65) / 154f101f-2800-4507-8c33-7e7cbc41c71f / 2018-10-19T08:56:12.750, Folkhalsomyndigheten 237 MSPs 2017-10-05 / contains 273 MSPs / b8fb573c-cd35-4f0c-9864-bb2a0120abd2 / 2018-10-19T08:52:39.536, Aerococcus SLA 2015 (0/40) / contains 40 MSPs Aerococcus strains from SLA in Denmark / 211923ec-b1be-4a9b-bd71-195b90e8e993 / 2018-10-19T08:43:06.543, Mycobacteria Library (bead method) / contains 952 MSPs / 0734bc24-ffaa-49e2-bc83-5edfbf91c60f / 2019-11-05T17:31:35.484, Filamentous Fungi / contains 577 MSPs / 95680832-cb78-4786-9f45-d67971dd8c1e / 2019-11-05T16:59:37.368, Staph-lib 20MSP(9S argenteus+11 S aureus) 2019-11-08 / Contains 20 MSP, 9 MSP S argenteus , 11 MSP S aureus / 2d6e9496-385f-4d5c-bd52-0187844e789b / 2019-11-08T16:06:30.399

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (-)	Clostridium clostridioforme 1021_NCTC 11224T BOG	1.33	1531
2 (-)	Acidovorax defluvii DSM 12644T HAM	1.32	86669
3 (-)	Lactobacillus salivarius DSM 20554 DSM	1.31	1624
4 (-)	Pseudomonas nitroreducens DSM 14399T HAM	1.29	46680
5 (-)	Lactobacillus salivarius DSM 20492 DSM	1.29	1624
6 (-)	Paenalcaligenes hominis MCW_10219 MCW	1.29	506

Result table for sample 13--continued on next page

Result table for sample 13--continued from previous page			
Rank (Quality)	**Matched Pattern**	**Score Value**	**NCBI Identifier**
7 (-)	Lactobacillus salivarius DSM 20555T DSM	1.27	1624
8 (-)	Lactobacillus amylolyticus DSM 11664T DSM	1.26	83683
9 (-)	Aeromonas molluscorum 848T DSM	1.26	271417
10 (-)	Chryseobacterium joostei LMG 18212T HAM	1.24	112234

Sample 9

Sample Name:	A9
Sample Description:	
Sample ID:	KGLITEN#1
Sample Creation Date/Time:	2020-03-13T10:52:40.154
Sample Type:	Standard sample
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
ACQ Timestamp:	2020-03-13T10:54:20.435
AutoXecute Method:	MBT_AutoX
Applied MSP Library(ies):	BDAL / contains 8468 MSPs / dee78ccc-c113-4ede-b02e-392d0cde98c1 / 2019-11-05T18:15:45.616, SR Taxonomy 72 MSPs 2018-10-19 (reduced size) / contains 72 MSPs reduced size (do not include Shigella, B anthacis, S typhi and Y pestis strains) / 628751e6-0226-413e-912c-9953513e31e3 / 2018-10-19T09:00:54.238, NIH Fungi Library 364 MSPs 2017-10-05 (one strain deleted) / contains 364 MSPs library according to Lau et al 2013 (one strain deleted AL65) / 154f101f-2800-4507-8c33-7e7cbc41c71f / 2018-10-19T08:56:12.750, Folkhalsomyndigheten 237 MSPs 2017-10-05 / contains 273 MSPs / b8fb573c-cd35-4f0c-9864-bb2a0120abd2 / 2018-10-19T08:52:39.536, Aerococcus SLA 2015 (0/40) / contains 40 MSPs Aerococcus strains from SLA in Denmark / 211923ec-b1be-4a9b-bd71-195b90e8e993 / 2018-10-19T08:43:06.543, Mycobacteria Library (bead method) / contains 952 MSPs / 0734bc24-ffaa-49e2-bc83-5edfbf91c60f / 2019-11-05T17:31:35.484, Filamentous Fungi / contains 577 MSPs / 95680832-cb78-4786-9f45-d67971dd8c1e / 2019-11-05T16:59:37.368, Staph-lib 20MSP(9S argenteus+11 S aureus) 2019-11-08 / Contains 20 MSP, 9 MSP S argenteus , 11 MSP S aureus / 2d6e9496-385f-4d5c-bd52-0187844e789b / 2019-11-08T16:06:30.399

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+)	Pseudomonas sp 01_12605128 MVK	1.72	286
2 (-)	Pseudomonas alcaligenes DSM 50342T HAM	1.53	43263
3 (-)	Pseudomonas aeruginosa 8147_2 CHB	1.31	287
4 (-)	Pseudomonas sp B538 UFL	1.31	286
5 (-)	Pseudomonas jinjuensis LMG 21316T HAM	1.28	198616
6 (-)	Pseudomonas antarctica DSM 15318T HAM	1.27	219572

Result table for sample 9--continued on next page

Result table for sample 9--continued from previous page			
Rank (Quality)	**Matched Pattern**	**Score Value**	**NCBI Identifier**
7 (-)	Pseudomonas pertucinogena LMG 1874T HAM	1.26	86175
8 (-)	Pseudomonas panipatensis DSM 21819T LGL	1.22	428992
9 (-)	Lactobacillus salivarius DSM 20492 DSM	1.21	1624
10 (-)	Pseudomonas nitroreducens LMG 20221T HAM	1.19	46680

Appendix 4. Complete results of quality control qPCR of samples from greenhouse experiment to be sequenced

Sample number	Sample content	C_q	DNA-concentration ng/µl
1	Non-nitrified biofertilizer	1	18,57182217
2	Non-nitrified biofertilizer diluted 1:10	15,34	1,961155244
3	Nitrified biofertilizer Day 0	21,05	0,041858143
4	Nitrified biofertilizer diluted Day 0 1:10	24,12	0,005283998
5	Nitrified biofertilizer, Day 10, channel 1	27,48	0,00055247
6	Nitrified biofertilizer, Day 10, channel 1 diluted 1:10	30,82	5,8144E-05
7	Nitrified biofertilizer, Day 10, channel 2	29,09	0,000186584
8	Nitrified biofertilizer, Day 10, channel 2 diluted 1:10	31,83	2,94026E-05
9	Nitrified biofertilizer, Day 10, channel 3	31,60	3,42779E-05
10	Nitrified biofertilizer, Day 10, channel 3 diluted 1:10	34,37	5,3178E-06
11	Nitrified biofertilizer, Day 17, channel 1	33,90	7,31656E-06
12	Nitrified biofertilizer, Day 17, channel 1 diluted 1:10	34,62	4,4912E-06
13	Nitrified biofertilizer, Day 17, channel 2	35,06	3,34257E-06
14	Nitrified biofertilizer, Day 17, channel 2 diluted 1:10	35,89	1,91351E-06
15	Nitrified biofertilizer, Day 17, channel 3	33,31	1,08854E-05
16	Nitrified biofertilizer, Day 17, channel 3 diluted 1:10	34,24	5,7973E-06
17	Nitrified biofertilizer, Day 21, channel 1	34,33	5,47374E-06
18	Nitrified biofertilizer, Day 21, channel 1 diluted 1:10	35,95	1,83822E-06
19	Nitrified biofertilizer, Day 21, channel 2	28,79	0,000227987
20	Nitrified biofertilizer, Day 21, channel 2 diluted 1:10	35,38	2,68617E-06
21	Nitrified biofertilizer, Day 21, channel 3	29,38	0,000152919
22	Nitrified biofertilizer, Day 21, channel 3 diluted 1:10	33,16	1,20176E-05

21	Inorganic fertilizer	35,90	1,89336E-06
22	Inorganic fertilizer diluted 1:10	36,10	1,66219E-06
23	Inorganic fertilizer Day 10, channel 1	34,86	3,81377E-06
24	Inorganic fertilizer Day 10, channel 1 diluted 1:10	35,13	3,17861E-06
25	Inorganic fertilizer Day 10, channel 2	35,64	2,25784E-06
26	Inorganic fertilizer Day 10, channel 2 diluted 1:10	35,37	2,70805E-06
27	Inorganic fertilizer Day 10, channel 3	35,86	1,94709E-06
28	Inorganic fertilizer Day 10, channel 3 diluted 1:10	35,89	1,90768E-06
29	Inorganic fertilizer Day 17, channel 1	35,45	2,57092E-06
30	Inorganic fertilizer Day 17, channel 1 diluted 1:10	35,26	2,9125E-06
31	Inorganic fertilizer Day 17, channel 2	33,08	1,27154E-05
32	Inorganic fertilizer Day 17, channel 2 diluted 1:10	34,83	3,89477E-06
33	Inorganic fertilizer Day 17, channel 3	35,57	2,36292E-06
34	Inorganic fertilizer Day 17, channel 3 diluted 1:10	35,51	2,46908E-06
35	Inorganic fertilizer Day 21, channel 1	36,34	1,41189E-06
36	Inorganic fertilizer Day 21, channel 1 diluted 1:10	36,66	1,13437E-06
37	Inorganic fertilizer Day 21, channel 2	33,99	6,88214E-06
38	Inorganic fertilizer , Day 21, channel 2 diluted 1:10	35,94	1,8487E-06
39	Inorganic fertilizer Day 21, channel 3	35,17	3,10781E-06
40	Inorganic fertilizer Day 21, channel 3 diluted 1:10	36,31	1,44023E-06