PXL TECH

Professionele Bachelor Agro- en Biotechnologie

Biotechnologie – Cel- en Gentechnologie

CLONING OF RING-HYDROXYLATING OXYGENASE GENES AND SCREENING OF BACTERIA FOR PHYTOREMEDIATION

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Promotors:

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Acknowledgments

Me, as a student Cell- and gene technology, took this internship because it fits within the objectives that are expected from me. This internship was carried out at the Centre of Environmental Sciences on behalf of Hogeschool PXL at Diepenbeek.

Under supervision of Dr. Sofie Thijs and Dr. Panos Gkorezis, a tool for the enumeration of ring-hydroxylating oxygenase genes was generated and optimized through a Quantitative Polymerase Chain Reaction. Also, polluted soil and leaf samples were screened for the presence of aromatic degrading bacteria. Last, a genome annotation was performed for the bacterium *Acinetobacter calcoaceticus* strain GK2.

First of all, I want to thank Sofie Thijs and Panos Gkorezis. In this internship, I had the opportunity to learn how to work with a Quantitative Polymerase Chain reaction and to interpret the outcoming results. Further, the different steps to isolate and screen bacteria out of polluted soil for aromatic degrading genes, were very useful to sharpen my laboratory skills and will come to hand later in my career and come in handy when I start to work. Similarly, I learned new software programs and techniques for whole bacterial genome annotation. Their help was greatly appreciated and was invaluable for the progression of the thesis and final written report.

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Cloning of ring-hydroxylating oxygenase genes and screening of bacteria for phytoremediation

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Abstract

For phyto- and phyllo-remediation, it is important to know if there is degradation of the pollutant present in the soil or in the air. To solve this problem, a goal of this study is to optimize a Quantitative Polymerase Chain Reaction (qPCR). Through this molecular method, it would be easy to tell if there are aromatic pollutant degrading microorganisms present or not in the soil or phylloplane. By measuring the abundance of these ring-hydroxylating oxygenase genes, a correlation between the presence of these genes and the concentration of pollutants in the environment can be made. This allows to estimate the degree of phytoremediation taking place at the polluted site. The tested genes here were alkane monooxygenase, phenol hydroxylase and naphthalene dioxygenase. Also, the total population of bacteria and fungi was determined. The genes were Polymerase Chain Reaction (PCR) - amplified and cloned from bacteria. These cloned genes were used to set up the standard curves for the qPCR. Second, bacteria isolated out of polluted soil, were screened on aromatic ring-hydroxylating oxygenase (RHO) genes including alkane monooxygenase, phenol hydroxylase, naphthalene dioxygenase, catechol dioxygenase, xylene monooxygenase, toluene dioxygenase and biphenyl dioxygenase. From this, the positive bacteria can be further used in phytoremediation. A PCR amplifies those genes, so that the presence in the bacteria can be determined. Lastly, the new bacterium *Acinetobacter calcoaceticus* strain Gkorezis (GK) 2, was screened for his degradative and plant growth promoting potential to be used in phytoremediation by means of genome annotation. The tests and optimizations revealed successful new insights. Naphthalene dioxygenase-, phenol hydroxylase- and alkane monooxygenase genes were successfully cloned and the qPCR was optimized. There are significant correlations found between pollutant concentrations and the presence of degradative genes. Also, new aromatic-eating bacteria were isolated and interesting degradation pathways in strain GK2 were found. Possible soil and air pollutant remediation solutions must be further investigated, because of the positive effects these types of plant-microbe interactions have on the environment.

Keywords

Ring-hydroxylating monooxygenase, naphthalene dioxygenase, qPCR, hydrocarbon pollution, soil phytoremediation, air phylloremediation

Introduction

The problem of environmental pollution

Environmental pollution is a serious problem. Air, soil and water pollution kills over twelve million people per year worldwide. This is equivalent with the Belgian population and makes about a quarter of the 55,6 million deaths a year. More and more people also live and work in environments that are contaminated with different kinds of pollutions. This leads to premature dead caused by heart diseases, cancer or respiratory diseases (De Roy 2016).

Soil and air pollution in Belgium

Of all the pollutions in Europe and Belgium, the pollution of soil with oil, heating oil or kerosene is one of the most common and contributes to

over 60 % of the polluted sites (Panagos *et al.*, 2013). Volatile organic compounds (VOCs), caused by the heavy traffic and associated incomplete combustion of fossil fuels, are one of the most important causes of air pollution in Belgium. The presence of these VOCs is very important because this is one of the greatest causes of premature death. It is proven that the telomeres in our cells become shorter because of air pollution, what leads towards chronical health issues (Martens and Nawrot 2016).

In Belgium and Europe, legislation and actions are taken to prevent new causes of pollution and to clean up polluted soil, air and water (Ahluwalia 2015). The government takes preventive measures in order to reduce the chance of future spills and takes remedial actions to limit the impact of the pollution on humans and the environment. To do this, they need to monitor the pollutant concentrations to understand the nature of the pollution, the source of origin and which remedial actions can be taken for this kind of pollution.

Preventive actions are first taken to control the pollution. For soil pollution, Europe has not a specific legalization on soil protection (European Commission 2016). Belgium has a soil decrete, but the preventive actions depend on the use of the land (EMIS VITO 2006). There are a few well studied preventive actions for air pollution such as particulate matter filters in cars, busses and tunnels, installation of low emission zones, speed limits and by industrial emission directives (Dani 2014).

Remediation of polluted sites is necessary to return the environment in a healthy state. A promising green technology is bioremediation (Dani 2014). Hereby, the polluted site is treated by biological processes using plants and (micro-)organisms.

Using plants and bacteria as remediating agents

To clean up large and diffuse contaminated sites, the use of conventional remediation strategies is not always economical and practical feasible. For this reason, the interest to use bioremediation, more specifically phyto- and phyllo-remediation, has grown. Phytoremediation is the degradation of pollutants in soils, while phylloremediation is the degradation of pollutants in the air. The mean agents used in both phyto- as phyllo-remediation are plants and bacteria. The symbiosis between them is harnessed to take-up, transport and degrade the pollutants. The bacteria, that can degrade the toxic aromatic compounds in the air and soil, are not easy to detect and to study in normal conditions because of the low abundance of these bacteria (Seo et al 2009).

Aromatic ring-hydroxylating oxygenase genes

Bacteria can help in the destruction of oil in the soil and VOCs in the air. These organic compounds can even be important food sources for some bacteria. The bacteria, that can digest oil and VOCs, use often a combination of the following 7 important pathways: the alkane monooxygenase system, naphthalene dioxygenase system, phenol hydroxylase system, catechol dioxygenase system, xylene monooxygenase system, toluene dioxygenase system and biphenyl dioxygenase system. These are briefly discussed in the following paragraphs.

The alkane monooxygenase system oxidizes short-chain alkanes, with the result that those alkanes are easily destructed (Cappelletti et al 2011). This system is regulated by the alkBFGHJKL-operon. In brief, when alkanes are present, they are transformed into an alcohol by the addition of an hydroxyl group by the AlkBGTF-gene complex. After this, an H^+-ion is removed from the hydroxyl group by the enzymes, produced by AlkJ and the obtained molecule is further transformed by the gene products of AlkH to a carboxylic acid. The carboxylic acid is activated by the enzyme produced by AlkK, which adds an acetyl-Co-enzyme A (CoA), prior to entry of the molecule in the β-oxidation cycle upon which the

compound is mineralized to carbon dioxide (CO_2) and water. Summarized, the present of alkanes induced this operon to degrade the alkanes in CO2 and water. This operon is found in several soil bacteria (FASEB 1990).

The naphthalene dioxygenase system transforms naphthalene into CO_2 and water using different enzymes (Umar Maigari and Umar Maigari 2015). The first enzyme in the pathway is naphthalene dioxygenase, which incorporates two oxygen molecules into the rings of naphthalene. Second naphthalene dehydrogenase ensures that the remaining oxygen molecules are cleaved and third, downstream genes turns the products into CO_2 and water.

Next, the phenol hydroxylase system transforms phenol into pyruvate and acetyl-CoA. This system is regulated by the dmpKLMNOPQBCDEFGHI–operon, and phenol induces the operon. The first gene in the pathway is phenol hydroxylase which hydrolyzes phenol in catechol (Shuaixin 2013a). This gene is activated when the parts KLMNOP of the operon are induced. Other genes of the catechol degradation pathway including catechol dioxygenase transforms catechol further until pyruvate and acetyl-CoA (Winslow et al 2016).

The transformation of toluene into acetyl-CoA is regulated by the TodDEFGHI–operon. The presence of toluene induces its transcription (Copeland et al 2017, Leicester 2015) and the first gene in the pathway is toluene dioxygenase, which hydrolyze toluene. This operon has already been found in many *Pseudomonas* species (Baldwin et al., 2004).

The xylene monooxygenase system transforms xylene into CO_2 and water, regulated by the XylMABCXYZLEFHIJK–operon (Shuaixin 2013b) (Yano et al 2010). The presence of xylene induces the operon and the first gene in the pathway, which hydrolyzes xylene, is xylene monooxygenase. This gene is activated when part XylMA of the operon is induced.

Highly similar to the previously discussed pathways is the biphenyl dioxygenase-pathway. This system transforms biphenyl in CO_2 and water. The first gene in this system is the biphenyl dioxygenase gene (BpHAEFG). This gene exist of small, large, ferredoxin and ferredoxin reductase subunits and is induced when biphenyl is present as carbon source for the bacteria (Porter et al 2016).

In all, the discussed pathways uses the aromatic ring-hydroxylating group of the produced enzymes. The genes can be used as target to detect aromatic compound degrading bacteria which can subsequently be used and tracked for phyto- and phylloremediation. Also, the degree of gene expression by microbial communities in soil can be measured.

Acinetobacter calcoaceticus strain GK2

Recently the bacterium *Acinetobacter calcoaceticus* strain GK2 was isolated from a heavily polluted oil contaminated soil in Beverlo (Belgium). The strain was full genome sequenced by Ion Torrent technology but not yet been fully annotated. The functional annotation and characterization of ring-hydroxylating enzymes in the bacterial genome is important to enhance our understanding how this strain degrades oil in soil and to determine whether additional inoculations are needed to speed-up the oil remediation process.

Knowledge gaps

Although phytoremediation is used since the early 1970s, there is not yet a complete understanding of all the processes and interactions that take place between plant, microbes and the pollutants in soil. This lack of information often hinders large-scale implementation of phytoremediation and draws concerns about the predictability, efficiency, progression and completeness of the phytoremediation experiment. Easy to implement tools to monitor the presence of

degradation genes in a certain environment and to timely take action when certain genes are missing are needed to reach a higher efficiency of phytoremediation. One approach would be to enhance the cultivability of aromatic pollutant degrading bacteria, however this is not straight forward. Further difficulties for current monitoring are the lack of information regarding which bacteria (genes) are present and which are not. However, these bacteria are necessary for the use of phytoremediation. There is thus a need for rapid screening and detection of the presence of degradative organisms (genes) at a contaminated site to evaluate and monitor the phytoremediation process and to improve it if certain conditions are sub-optimal. So all those boundaries are needed to be taken away.

Aims of this study

At this moment, there are no optimized and easy screening methods to determine whether aromatic compound degrading genes are present in polluted sites. Also, it is not clear if enriching microbes in the soil is successful, *i.e.* if the aromatic compound degrading bacteria effectively are enriched and active at the site of interest. Hereby, a qPCR will be tested to measure the abundance of the ring-hydroxylating oxygenase genes. A correlation between the presence of these genes and the concentration of pollutants in the environment can be made to determine the degree of phytoremediation.

Next to soil pollution, there is also the problem of air pollution. Here, hornbeam- (*Carpinus betulus*) and ivy (*Hedera helix*) plants were screened for the presence of aromatic degrading bacteria using the optimized qPCR protocol and primers. These bacteria could be helpful for the removal of pollutants in the air.

In addition to monitoring the abundance of degradation genes in total microbial communities, a functional genome annotation of *Acinetobacter calcoaceticus* strain GK2 was performed. The bacterium was used for the inoculation of an oil polluted soil in Beverlo, Belgium. We searched for the pathways that could be involved in the degradation of hydrocarbons as well as stimulation of plant growth.

Lastly, we isolated aromatic compound degrading bacteria from contaminated environments by plating soil samples and isolating of bacteria, grown on these plates, on media with pollutants as sole carbon source. When the bacteria could grow on these pollutants, they were isolated and purified. Hereafter, a PCR was carried out to know which aromatics were degraded by these bacteria. The identified bacteria that are positive for the aromatic degrading genes can be used for phytoremediation in later experiments. The sequences of the target genes can be found in annex '1. Sequences from the used genes'.

Materials and methods

Isolation source of the used samples

For the isolation of aromatic degrading bacteria, we started from soil samples originating from an oil polluted site in Beverlo (Belgium) and a crude oil polluted forest in Bobrka (Poland). Further, this study used bacteria that were previously isolated from the leaves of hornbeam trees growing in Bobrka and Warsaw (Poland) (L. Kowalkowski, personal communication).

The positive control and aromatic compound degrading bacteria used in this study included, *Acinetobacter O9, Rhodoccocus D3, Pseudomonas veronii VI4T1 and Pseudomonas sp.* VI4.1 isolated previously from polluted environments (V. Imperato, personal communication).

For the detection and enumeration of aromatic degrading bacteria, we started from DNA-samples of the leaves of ivy growing in the city center of Hasselt and in the nature reserve De Maten, both situated in Belgium. Also DNA-samples were used growing on the polluted site Bobrka, of the nature reserve Bialowiesa and of the city center of Warsaw, all situated in Poland (L. Kowalkowski and V. Imperato performed

the sampling and DNA-extraction). There was also soil DNA-samples from the site in Borbka and Bialowiesa used. Finally, there was DNA extracted from soil originating from an oil polluted site in Beverlo, Belgium.

Cultivation of bacteria

In this project, there were three ways used of cultivating bacteria. The first approach was to isolate them from soil. For this, 5 g of soil was dissolved in 45 mL of liquid 869-media (Mergeay *et al.*, 1985) in a falcon tube (see annex '2.1. Weighing' for measuring the soil and '2.2. 869 Rich-medium' for making the media). Samples were serially diluted till 10^{-3} and the bacterial suspensions were plated onto 1/10 869-media with indole (Mutnuri *et al.*, 2009) (see annex '2.3. Spread plate technique' and '2.4 Isolation of bacteria from soil' for more details).

The second way is to purify bacteria by spreading them on a plate following the protocol in annex '2.3. Spread plate technique'. Single colonies were picked up from the plate, and quadrant streaked onto fresh 869-media (Mergeay *et al.*, 1985) (annex '2.7. Purification using quadrant streaking').

The third method used to cultivate bacteria involved to grow bacteria in a masterblock with 1 mL liquid 869-medium (Mergeay *et al.*, 1985). Each well of the masterblock contained one bacterium which was picked up from a plate and previously purified following the protocol in annex '2.3. Spread plate technique'. The method how the masterblock was inoculated, is explained in the protocol in annex '2.5. Cultivation of bacteria in a masterblock'.

Finally, for long-term storage, all bacteria pellets were suspended in 1 mL of 15% glycerol and 0,75 % NaCl solution and stored at -45°C. The glycerol was made following the protocol in annex '2.6. 15% Glycerol stock'.

DNA-extraction

DNA-extraction was carried out with four different kits depending on the type of starting material used (cells or soil).

Total DNA was extracted from 1,5 mL bacterial suspensions using the Qiagen Blood and Tissue kit (Qiagen Inc., Valencia, CA, USA), the E.Z.N.A. Bacterial DNA kit (Omega bio-tek, Norcross, GA, USA), or the Promega Wizard DNA purification kit (Promega, Madison, WI, USA) according to manufacturer's instructions. (see annex '2.8. DNA-extraction Qiagen Blood and Tissue kit', '2.9 DNA-extraction E.Z.N.A. Bacterial DNA kit' and '2.10 DNA-extraction Promega Wizard Purification kit'). With the Qiagen kit, there was DNA extracted from *Acinetobacter O9, Rhodoccocus D3, Pseudomonas veronii V4T1 and Pseudomonas sp. V4.1*. For the EZNA kit, the incubation is extended after addition of the lysozyme to 30 min. instead of 10 min. to improve the lysis. For the Promega kit, a custom lysis buffer was used as described in annex '2.10. DNA-extraction Promega Wizard DNA Purification kit'.

Total DNA was extracted from 2 g soil using the MOBIO soil DNA-extraction kit (MOBIO, Carlsbad, CA, USA) with modifications, which followed annex '2.11. MOBIO soil DNA extraction kit'. Products of the MOBIO-kit were custom made and the capturing of the DNA was performed with magnetic beads. The used washing solutions were those prescribed by the Promega Wizard Purification kit.

The DNA-concentration and purity were measured by the Qubit dsDNA High Sensitive assay (ThermoFisher Scientific, Waltham, MA, USA) or the NanoDrop. The protocols of how these parameters are measured, can be found in annex '2.12. Qubit dsDNA HS Assay' and '2.13. NanoDrop'.

Primer design and in silico-analysis of the primers for qPCR

All of the primers used for a qPCR of aromatic ring-hydroxylating genes, were collected from the literature (Baldwin et al 2003, Jurelevicius et al 2013, Schulz et al 2012) and synthetized using the high purity method at Biolegio (Biolegio, Nijmegen, Nederland) (Table 1). The in xilico-analysis of the primers was performed by the program 'Snapgene', where a virtual PCR

was carried out. Strains, which gave positive amplicons of the correct insert and annotation, were used for wet lab PCRs.

PCR-optimization

The primers were first tested using a gradient-PCR to determine the optimal annealingtemperature (Ta) for subsequent PCRs or qPCRs. Table 1 shows the optimal Ta determined for all the primer pairs. The PCR reactions were set-up in 25 µL composed of 1 x DreamTaq buffer, 0,2 mM dNTPs, 1,5 mM $MgSO_4$ and 1U of Dream Taq polymerase. 1 µL of DNA template was used. The run conditions were 1 cycle of 180s at 95°C; 30 cycles of 20s at 95°C, the different Ta's and 20s at 72°C and 1 cycle of 420s at 72°C in the Bio-Rad C-1000 Touch (Bio-Rad , Hercules, CA, USA), with a Ta from 60°C to 50,0°C in steps of 1,2 °C decrease (see annex '2.14 Cloning reaction'). The size and intensity of the PCR-products was checked on a 1,5% agarose gel. (see the protocol in annex '2.15. Agarose gel-electrophoresis). The second gradient-PCR for AlkB has the same PCR-program, only with a Ta from 58°C to 48°C in steps of 1,2°C decrease.

The PCR-products were purified by using the Qiagen QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA), following annex '2.16. PCR purification' and manufacturer's instructions. Purified PCR products were quantified using fluorescent dyes and the Qubit as described above.

Cloning of the PCR products

To ligate the PCR-products in the vector, 2 µL of the PCR-products were added to the PGEM-T vector (Promega, Madison, WI, USA) following the instructions. Competent *E. coli*-cells were used to take up and clone the vector. Screening of the competent cells was carried out by a blue-white screening using x-gal according to standard procedures (see annex '2.3. Spread plate technique' and '2.14. Cloning reaction').

The plasmid-extraction was carried out with the GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, Waltham, MA, USA) and the concentration of the plasmid DNA was determined using NanoDrop (see annex '2.13. NanoDrop' and annex '2.17. Plasmid-extraction'). To verify the insert, plasmid DNA was cut with restriction enzyme EcoR1 using 10 µL of the plasmid DNA to 1 x restriction buffer (BioLab, Ipswich, MA, USA) (see annex '2.14. Cloning Reaction'). 5 µL of the restriction-products were tested on a 1,5% agarose gel (see annex '2.15 Agarose gel-electrophoresis') and the correct ligated, competent cells were sent to sequencing by Macrogen (Netherlands) with the universal primer M13F-pUC.

qPCR

The standard curves were made with the bacteria sent to sequencing and with the correct sequence (see annex '1. Sequences from the used genes'). For the 16S- and ITS-gene, the standard curves were already available from a previous project (S. Thijs, personal communication). For the phenol hydroxylase gene, the DNA plasmid templates were $1/10^{th}$ diluted in a series of 10^8 till 10^2 copies per µL. For the AlkB-gene and the naphthalene dioxygenase gene, the plasmid DNA was $1/5^{th}$ diluted in a series of dilution from 10^9 till $6{,}4^4$. The protocol for these standard curves was explained in annex '2.18. Quantitative Polymerase Chain Reaction'. The obtained standard curves are given in annex '3. Standard curves'.

The qPCR reactions were set-up in 10 µL reaction volume with Qiagen Quantinova SYBR green PCR kit (Qiagen Inc., Valencia, CA, USA). The primers that were used are listed in Table 1. The qPCR reaction mixture was composed of 5 µL 1 x buffer, 50 µM ROX, 300 nM primer for naphthalene dioxygenase and phenol-hydroxylase, or 400 nM for alkane-monooxygenase, 16S and ITS, dH_2O to make 8 µL and then 2 µL of 1/20 diluted DNA template. The reactions were set-up in 96-well plates (MLP-9601 Multiplate High-Profile 96-Well Unskirted PCR Plates, Bio-Rad laboratories, Netherlands). The program run was the following: 1 cycle of 90s at 95°C; 40 cycles of 15s at 94°C, 30s at 54°C and 30s at 72°C and 1 cycle of 15s at 95°C, 60s at 60°C, 15s at 95°C and

Table 1:Primerdata used for performing a gradient-PCR, conventional PCR and qPCR

Gene/region	Name	Sequence (5' – 3')	Size (bp)	Ta (literature)	Strain used for cloning and in silico PCR	Literature reference
AlkB	Alkb-1f-Schulz2013 Alkb-1r-Schulz2013 alk-H1F-Chen2003 alk-H3R-Chen2003	AAYACNGCNCAYGARCTNGGNCAYAA GCITGITGITCISWRTGICGYTG CIGIICACGAIITIGGIC ACAAGAAGG IGCITGITGATCIIIGTGICGCTGIAG	548	54°C (multiplex)	*Rhodococcus sp.* BCP1 (HM771646)	(Jia Chen et al 1994, Schulz et al 2012)
Naphthalene dioxygenase	NAH-F NAH-R	CAAAARCACCTGATTYATGG AYRCGRGSGACTTCTTTCAA	377	47°C	*Pseudomonas veronii* strain VI4T1 (MULN00000000)	(Baldwin et al 2003)
Phenol monooxygenase	PHE-F PHE-R	GTGCTGACSAAYCTGYTGTTC CGCCAGAACCAYTTRTC	202	49°C	*Pseudomonas sp.* strain VI4.1 (MULM00000000)	(Baldwin et al 2003)
Xylene monooxygenase	TOL-F TOL-R	TGAGGCTGAAACTTTACGTAGA CTCACCTGGAGTTGCGTAC	475	55°C	*Pseudomonas putida* F1 (CP000712)	(Baldwin et al 2003)
Toluene dioxygenase	TOD-F TOD-R	ACCGATGARGAYCTGTACC CTTCGGTCMAGTAGCTGGTG	754	53°C	*Pseudomonas putida* HS1 (AB434906)	(Baldwin et al 2003)
Catechol dioxygenase	CATA-F CATA-R	ACVCCVCGHACCATYGAAGG CGSGTNGCAWANGCAAAGT	470	54°C	*Pseudomonas putida* F1 (CP000712)	(Jureleviciu s et al 2013)
Biphenyl dioxygenase	BPH2-F BPH2-R	GACGCCCGCCCCTATATGGA AGCCGACGTTGCCAGGAAAAT	724	65°C	/	(Baldwin et al 2003)
16S	27F 1492R Eub 338 Eub 518	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	1465 180	54°C 54°C	/ /	/ /
ITS	EUK 345F EUK 499R	AAGGAAGGCAGCAGGCG CACCAGACTTGCCCTCYAAT	154	54°C	/	/

60s at 60°C in the 7500 Fast Real-Time PCR-system (Applied Biosystems, Foster City, CA, USA) (annex '2.18. Quantitative Polymerase Chain Reaction'). Excel was used to generate the standard curves and to determine the efficiency, R^2 and rico (Figure S4a,b,c,d,e). The specificity of the qPCR is tested with the melting curves of the amplicons.

Screening of bacteria for RHOs

The bacteria, cultivated in the masterblocks (Greiner Bio-One, Kremsmünster, Austria) and some on plates, were screened for the presence of RHO-genes. 80 µL of bacterial suspension in the masterblocks was transferred to test tubes with 5 mL of 284-media (Schlegel *et al.*, 1991) (see annex '2.19. 284 Selective-medium' for making the media), and 10 µL of an aromatic compound, either toluene, n-xylene, ethylbenzene, benzene or diesel. After two weeks, the tubes were screened for a pellet. The tubes with a pellet were spread out on plates, composed of 1/10 869-media with indole (see annex 2.20. Cultivation of bacteria on aromatic compounds). Colonies were purified, DNA was extracted and cells were stored at -45°C in a 15 % glycerol solution.

The purified DNA was used for PCR amplifications using the RHO primers (Table 1, marked blue). The PCR reaction mixture was composed of 1 x buffer, 200 nM dNTP's (Promega, Madison, WI, USA), 200 nM primer, 0,25 U of Dreamtaq polymerase (ThermoFisher Scientific, Waltham, MA, USA), dH2O to make 24 µL and then 1 µL of DNA template. The reactions were set-up in 0,2 mL microtubes.

The program run was the following: 1 cycle of 120s at 95°C; 35 cycles of 60s at 95°C, 30s at 54°C and 120s at 72°C and 1 cycle of 300s at 72°C in the Bio-Rad C-1000 Touch (Bio-Rad , Hercules, CA, USA) (annex '2.21. Polymerase Chain Reaction'). 5 µL of the PCR-products were tested on a 1,5% agarose gel (see annex '2.15 Agarose gel-electrophoresis').

10 µL of the PCR-products, from the positive bacteria, were run on a 1,5% agarose gel (see annex '2.15 Agarose gel-electrophoresis') and the ones with again a positive result, were cut out of the gel, extracted using the QIAquick Gel extraction kit (Qiagen Inc., Valencia, CA, USA) followed annex '2.22. QIAquick Gel Extraction kit'. DNA from the positive bacteria was send to sequencing at Macrogen Netherlands, by the primers used in the PCR.

Genome annotation of *Acinetobacter calcoaceticus* strain GK2

For the annotation of *Acinetobacter calcoaceticus* strain GK2, the Genome Workbench (NCBI, MA, USA) and custom blast pipelines were used (see annex '2.23. Custom Blast').

Statistical analysis

The results of qPCR were checked for equal variance and normal distribution. If the criteria passed, a one-factorial ANOVA for multiple groups was carried out using the software SigmaPlot 11.0 (Systat software Inc., San Jose, CA, USA).

Results

Cloning reaction

Gradient-PCR

To test which optimum annealing temperature for the primers, a gradient-PCR was carried out. On the first gel, the AlkB-, PHE- and NAH-primers are tested. The AlkB-primers gave a result by 50,7°C at the height of 550 bp. By the PHE-primers there is, by a temperature of 50,0°C and 51,9°C, a band at the height of 200 bp. For the NAH-primers, by the temperatures 50,0°C; 51,9°C; 58,6°C; 59,2°C and 60,0°C and a height of 400 bp, a result was detected. The AlkB-primers were tested again with different temperatures. On the gel (annex '4.1. Gradient-PCR'), there was a band by each temperature at the height of 550 bp, except for 57,2°C and the two blanks. The lower the temperature, the less bright the band. By the AlkB-primers tested at *Pseudomonas sp.* 4T1, a second band was present at the height of 1000 bp.

Restriction-analysis

To see if the genes were correctly ligated in the vector, a restriction analysis was performed. The expected height was 800 bp for alkane monooxygenase, 250 bp for phenol hydroxylase and 450 bp for naphthalene dioxygenase. Lane 1 until 17 on the gel (annex '4.2. Restriction-analysis'), represent the results of the restriction-analysis. There was an outcome for each of the samples at the height of 1500 bp. A second band was present for all the samples. From lane 1 until 5, this was at the height of 800 bp. For the samples in lane 6, 8, 9 and 10, the second band has a height of 250 bp. Lane 7 has a second band at the height of 400 bp and from lane 11 until 16 this was at the height of 450 bp.

qPCR

qPCR for RHOs genes in epiphytes of Bialowiesa, Bobrka and Warsaw samples

With the qPCR, the expression of RHOs genes in epiphytes on a control (Bialowiesa) and two polluted sites (Bobrka and Warsaw) was determined. This was done to see if the pollution has an effect on the expression of the RHO genes. The raw data can be found in annex '5.1. Raw data of phyllosphere bacteria on epiphytes in Bialowiesa, Bobrka and Warsaw'.

The AlkB-gene was detected in Bialowiesa (no-polluted forest), Bobrka (oil polluted forest) as well as Warsaw samples (trees next to street) (Figure 1). There were more copies of this gene present in Warsaw, compared to Bobrka and Bialowiesa. There was no significant difference between Warsaw, Bobrka and Bialowiesa. The normality-test failed (see annex '5.2.1. Alkane monooxygenase gene').

Phenol hydroxylase was detected in all of the sites (Figure 1). The most copies of this gene, were found in Bialowiesa, followed by Bobrka and last Warsaw. There was not a significant difference for this gene (p=0,057). The performed ANOVA can be found in annex '5.2.2. Phenol hydroxylase gene'.

There was no naphthalene dioxygenase gene detected in all of the sites (Figure 1).

qPCR for RHOs genes in phyllosphere bacteria, on ivy of Hasselt and De Maten samples

The expression of RHO genes in phyllosphere bacteria on the leaves of ivy were determined with a qPCR. This for both a control site (De Maten) and a polluted site (Hasselt). Out of this, the effect of the pollution could be determined. The raw data can be found in annex '5.3. Raw data of phyllosphere bacteria on ivy in Hasselt and De Maten'.

Alkane monooxygenase, phenol hydroxylase and naphthalene dioxygenase were detected in De Maten (no-polluted forest) as well as Hasselt samples (trees next to street) (Figure 2a). There were more copies of all the genes in De Maten, then in Hasselt. The normality-test failed for both alkane monooxygenase as phenol hydroxylase (see annex '5.4.1. Alkane monooxygenase gene' and '5.4.2. Phenol hydroxylase gene'). The equal variance-test failed for naphthalene dioxygenase (see annex '5.4.3. Naphthalene dioxygenase gene').

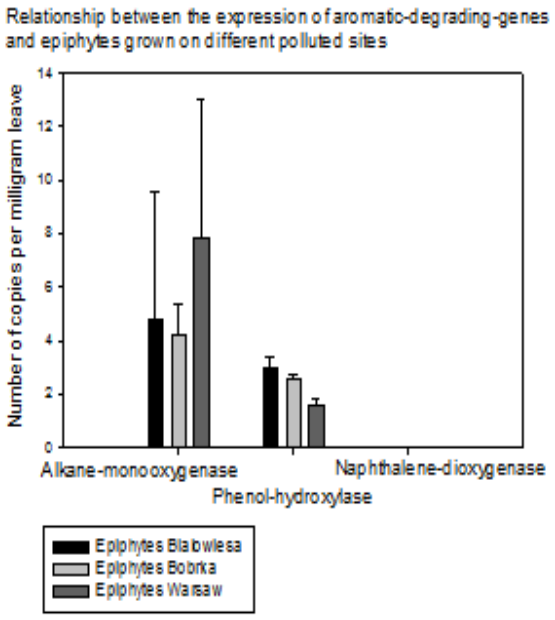

*Figure 1: Log copy number of RHOs genes per site with the relationship between the expression of aromatic degrading genes and epiphytes grown on different polluted sites. Statistically significant effects are indicated with an * (p < 0,005). Mean values with standard error bars are indicated of n=1;7;7 experiments for the different site for AlkB and n=6;2;5 for phenol hydroxylase.*

The absolute abundance of bacteria was also estimated based on the 16 rRNA gene copies. There are more copies of 16S rRNA gene in De Maten than in Hasselt (Figure 2b). The normality-test before the ANOVA failed (see annex '5.4.4. Total population of bacteria').

The absolute abundance of fungi was estimated based on the ITS-gene copies. There were more copies of ITS-gene in De Maten than in Hasselt (Figure 2b). The normality-test before the ANOVA has failed (see annex '5.4.5. Total population of fungi').

qPCR for RHOs genes in soil-bacteria of Bialowiesa and Bobrka samples

To determine if the expression of RHOs genes in soil-bacteria is different from a polluted site (Bobrka) relative to a control site (Bialowiesa), a qPCR was carried out. The raw data can be found in annex '5.6. Raw data of soil-bacteria in Bialowiesa and Bobrka'.

The AlkB-gene and phenol hydroxylase gene were detected in Bialowiesa (no-polluted forest) as well as Bobrka samples (polluted oil site) (Figure 2c). There were more copies of both genes present in Bobrka, compared Bialowiesa. There was not a statistically significant difference between both site for alkane monooxygenase. The ANOVA calculated a p-value of 0,060. For phenol hydroxylase, the p-value is 0,014. This means that there was a significant difference between both places for this gene. The results of the ANOVAs can be found in annex '5.7.1. Alkane monooxygenase gene' and '5.7.2. Phenol hydroxylase gene.

The naphthalene dioxygenase gene was only detected in Bobrka (Figure 2c). An ANOVA could not be performed.

There were more copies of 16S rRNA gene and ITS-gene in Bobrka than in Bialowiesa (Figure 2d). There was a statistically significant difference between both sites for these genes. The p-value for the 16S rRNA gene was 0,047 and for the ITS-gene 0,004. The performed ANOVAs can be found in annex '5.7.3. Total population of bacteria' and '5.7.4. Total population of fungi'.

qPCR for RHOs genes in soil-bacteria on Beverlo samples before and after inoculation

The inoculation with aromatic degrading bacteria could have an effect on the expression of RHOs genes in soil-bacteria. With the qPCR, the rate of expression of these genes was determined before and after inoculation. The raw data can be found in annex '5.8. Raw data of soil-bacteria in Beverlo before and after inoculation'.

Alkane monooxygenase and phenol hydroxylase were detected in Beverlo (polluted site) before inoculation with degrading bacteria, as well after inoculation (Figure 2e). There were more copies of this genes present before inoculation than after inoculation. Alkane monooxygenase was significant different between both sites (p=0,027). The equal variance-test failed for phenol hydroxylase. The performed ANOVAs can be found in annex '5.9.1. Alkane monooxygenase gene' and '5.9.2. Phenol hydroxylase gene'.

The naphthalene dioxygenase gene was only detected after inoculation (Figure 2e). An ANOVA could not be performed.

There were slightly more copies of 16S rRNA gene after inoculation than before inoculation (Figure 2f). There was not a significant difference between the inoculation and the gene (p=0,690). The ANOVA can be found in annex '5.9.3. Total population of bacteria'.

There were more copies of ITS-gene after inoculation than before inoculation (Figure 2f). A significant difference between the inoculation and the gene was not statistically confirmed (p=0,568). The result of this can be found in annex '5.9.4. Total population of fungi'.

Screening of bacteria

Genotypic identification of the bacteria by 16S rRNA gene amplification and sequencing

To identify the different isolated bacteria, a 16S rRNA gene amplification was carried out. The sequencing results were present in annex '6.2. Sequencing of bacteria'. Out of the sequencing results, a phylogenetic tree of all of the sequenced bacteria was drafted (Figure 3). Some of the bacteria were closely related, like AS_02 and AS_20. Others are very diverted from each other, like AS_01 and AS_30.

PCR screening for RHOs genes

The bacteria were screened on the present of RHO genes to determine if phytoremediation is possible with these bacteria. From the RHOs PCRs, 30 bacteria had a band on the expected height for Alkb-PCR (Figure S8a,b), 7 bacteria were positive for PHE (Figure S9a,b), 17 for CATA (Figure S11a,b), 9 for the TOD-primers (Figure S12), 9 for the xylene monooxygenase gene (Figure S12) and 17 for the BPH-PCR (Figure S13). Some PCRs gave multiple bands of different heights, this specific amplification was unavoidable, but the products of the expected size were gel-purified to reduce the chance of multi-template for sequencing.

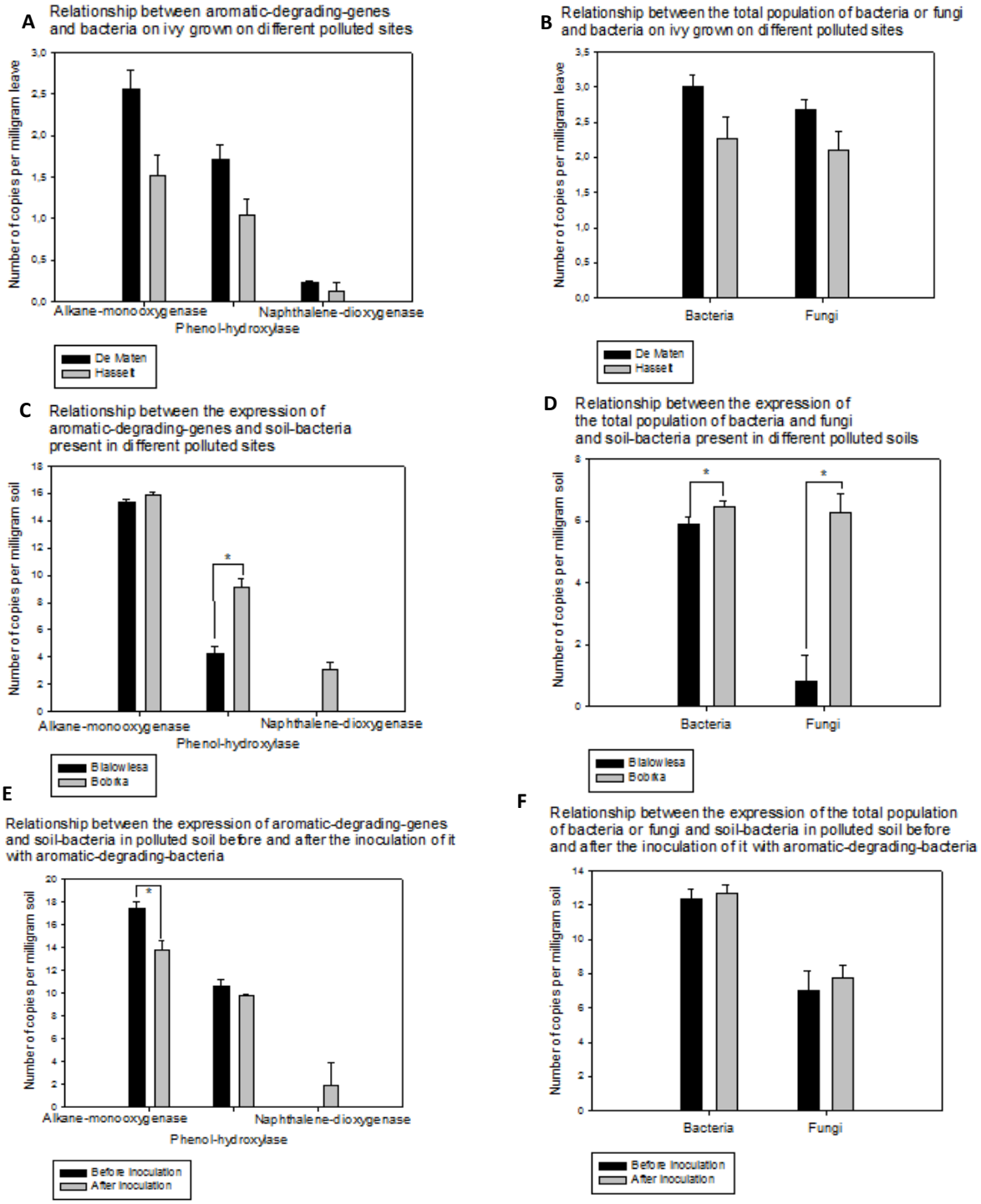

*Figure 2: Log copy number of RHOs genes per site with (a) the relationship between the expression of aromatic degrading genes and bacteria on ivy grown on different polluted sites (b) the relationship between the expression of total population of bacteria or fungi and bacteria on ivy grown on different polluted sites (c) the relationship between the expression of aromatic degrading genes and soil-bacteria in different polluted sites (d) the relationship between the expression of total population of bacteria or fungi and soil-bacteria in different polluted sites (e) the relationship between the expression of aromatic degrading genes and soil bacteria in polluted soil before and after the inoculation of it with aromatic degrading bacteria (f) the relationship between the expression of total population of bacteria or fungi and soil bacteria in polluted soil before and after the inoculation of it with aromatic degrading bacteria . Statistically significant effects are indicated with an * ($p < 0,005$). Mean values with standard error bars are indicated of (a) n=7;5 experiments for the different sites for AlkB, n=13;13 for phenol hydroxylase and n=2;2 for naphthalene dioxygenase (b) n=17;17 for total population of bacteria and fungi (c) n=9;15 for AlkB, n=2;18 for phenol hydroxylase and n=12 for naphthalene dioxygenase (d) n=9;18 for total population of bacteria and n=2;17 for total population of fungi (e) n=3;5 for AlkB, n=4;4 for phenol hydroxylase and n=1 for naphthalene dioxygenase (f) n=4;5 for total population of bacteria and n=4;9 for total population of fungi.*

Second PCR screening for RHO-gene

The positive bacteria from the high-throughput screening were again loaded on a higher resolution gel to better define the positive bacteria. The AlkB-positive bacteria were not screened again.

From the first screening, 6 bacteria were positive for PHE (Figure S14), 12 for CATA (Figure S15a,b), 6 for the TOD-primers (Figure S17a,b), three for the xylene monooxygenase gene (Figure 17a,b) and 7 for the BPH-PCR (Figure S16a,b).

The bacteria with their sample code and the summary of the results, can be found in annex '6.2.2. Results of the 16S-sequencing', '6.3.7. Summary of the positive and negative bacteria for the different aromatic degrading genes after the first screening' and '6.4.5. Summary of the positive and negative bacteria for the different aromatic degrading genes after the second screening'. The red boxes were the bacteria that, in the first screening, were positive but after the second one negative. The yellow boxes were the bacteria that, in the first screening, were negative but after the second one positive.

The positive purified amplicons were identified by sequencing. Only four of these purified amplicons could be sequenced. These were all catechol dioxygenase (see annex '6.5. Sequences of ring-hydroxylating oxygenase genes).

Genome annotation

Genes that encode for proteins involved in the degradation of aromatic compounds were located in the genome of GK2, including anaerobic 4-ethylphenol degradation (12 genes of chnA, 11 genes of eba309, one gene of xccB and 4 genes of xccC), 4-nitrophenol degradation I (14 genes of pdcG, one gene of pnpB, 15 genes of pnpD and 14 genes of pnpE), 4-nitrophenol degradation II (one gene of macA, three genes of npcB, one gene of npcC, two genes of nphA2 and one gene of pcpE), bisphenol A (one gene of EBBID32_42220) and phenylacetate degradation I (one gene of paaA, one gene of paaB, one gene of paaC, three genes of paaE, 11 genes of paaF, 6 genes of paaG, 9 genes of paaJ and one gene of paaK).

Genes that encode for proteins involved in the degradation of alkanes and alcohols were located, including xylene degradation (three genes of xylA, two genes of xylB and 9 genes of xylC), catechol degradation I (two genes of catA, one gene of catB, one gene of catC, one gene of salC, two genes of salD and two genes of pcaD), catechol degradation III (9 genes of

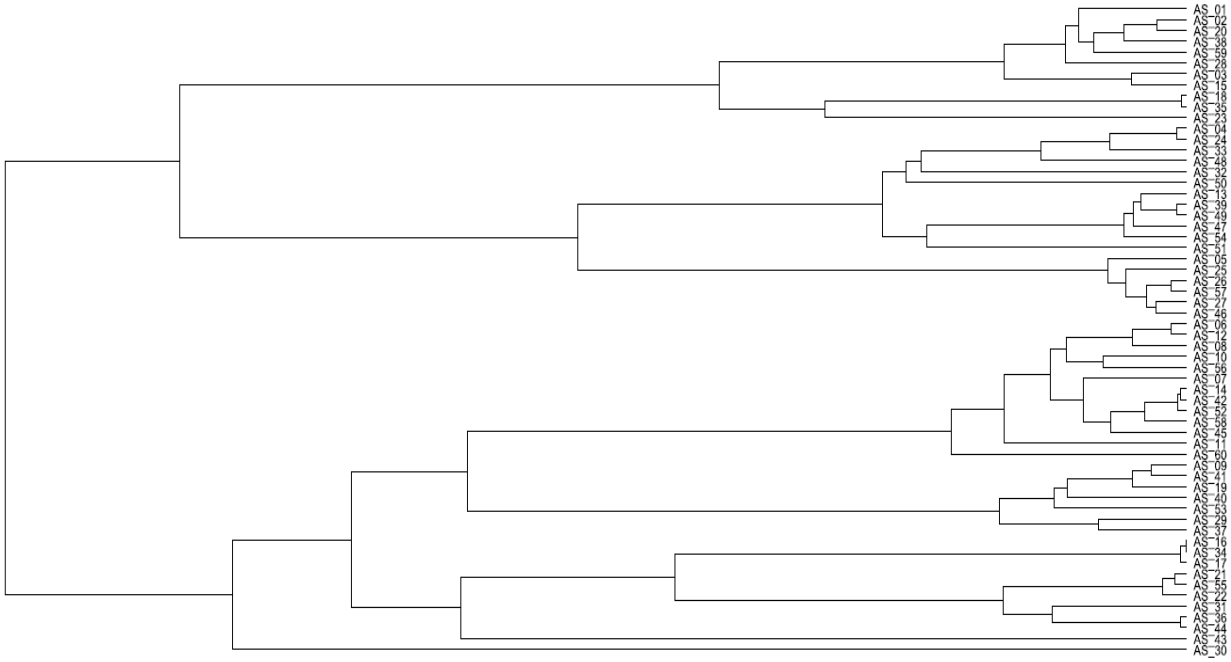

Figure 3: Taxonomy of the different screened bacteria

pcaF, 5 genes of pcaI and 5 genes of pcaJ), naphthalene degradation (three genes of nahB, one gene of nahE, 10 genes of nahF, three genes of ndoA and one gene of ndoB) and octane degradation (one gene of AlkB, one gene of alkF, two genes of alkG, one gene of alkJ, one gene of alkK, four genes of alkT).

The genes encoding for proteins involved in plant growth promotion were located, including indol-3-acetac acid (IAA) II (one gene of AMI1), IAA III (two genes of iaaH and one gene of tms2), L-tryptophan degradation VII (two genes of ipdC) and steroid hormone biosynthesis (one gene of AKR1C3, 5 genes of HSD17B3 and four genes of HSD17B7).

Discussion

Cloning reaction

Gradient-PCR

On the first gel, the results for the AlkB-primers were hardly visible. Because of this, the PCR was carried out again. All the genes had results at the expected height of 545 bp for AlkB, 202 bp for phenol hydroxylase and 377 bp for naphthalene dioxygenase.

The absence of certain bands could be explained by a human mistake. There was no DNA added to the mastermix. Second bands at 1000 bp were aspecific bands, because of the absence at high temperatures and presence at low temperatures. The optimum annealing-temperature for AlkB lays between 51,9°C and 58,0°C; for PHE between 50,0°C and 51,9°C and for NAH 51,9°C and 60,0°C.

A temperature of 54°C was chosen to carry out all of the PCRs. This temperature does not match with the annealingtemperatures out of the literature. A possible reason could be another PCR-application, or other PCR conditions, for example another polymerase or dNTPs (Baldwin et al 2003, Jurelevicius et al 2013, Schulz et al 2012). For the BPH-primers, a temperature of 65°C is chosen.

Restriction-analysis

The bands, present at a height of 1500 bp, were aspecific bands at a second restriction place (Promega 2015). The result of the restriction-analysis had an expected height of 800 bp for the AlkB-samples, 250 bp for the PHE-samples and 400 bp for the NAH-samples when correctly ligated into the vector. All the samples were correct ligated in the vector, except sample PHE 2. This sample was inverse ligated in the vector.

qPCR

The standard curves were reliable. The efficiency must be over 99%, which was the case for every standard curve (Larionov et al 2005).

Phyllosphere bacteria on epiphytes in Bialowiesa, Bobrka and Warsaw

The AlkB-gene was most present in the epiphytes, planted on the polluted site of Warsaw. The copy number was low for the different sites in comparison with the literature, due to a small number of samples or a bad sampling method (Liu et al 2015). The standard errors were high due to a small number of samples. Due to the failed normality-test, the ANOVA could not prove a significant difference between the different sites. This was also because of a small number of samples.

Phenol hydroxylase was most present in the epiphytes, planted in the nature reserve Bialowiesa. The gene had a low copy number for all the sites, compared to the literature, due to the same reason described above (Peng et al 2015). Differences between this gene and his presence in the epiphytes were due to random sampling variability.

The naphthalene dioxygenase gene was not present in the tested epiphytes. The air-quality of the tested sites was not poor enough to cause the appearance of this gene (Ní Chadhain et al 2006).

Phyllosphere bacteria on ivy in Hasselt and De Maten

The AlkB-gene, phenol hydroxylase gene and naphthalene dioxygenase gene were most present on the ivy, planted in nature reserve De Maten. The genes had a low copy number for all the sites, compared to the literature, due to a small number of samples or a bad air-quality (Liu et al 2015, Peng et al 2015). This was also the case if compared to the total population of bacteria, present on the ivy. AlkB and phenol hydroxylase both failed the normality-test. Naphthalene dioxygenase failed the equal-variance-test. Because of this, the ANOVA did not show a significant difference between the different sites. This was due to a low number of samples.

The copies from the total population of bacteria or fungi were the most on ivy planted, on De Maten. In comparison to the literature, the number of copies were lower for both sites (Peng et al 2015). This had the same reason as described above. The ANOVA could not prove a significant difference between the different sites, due to a failed normality-test. This also had the same reason as described above.

Soil-bacteria in Bialowiesa and Bobrka

AlkB and phenol hydroxylase were most present in the oil-polluted soil of Bobrka. Compared to the literature, the number of copies were lower for the different places due to a small number of samples or a bad sample method (Liu et al 2015, Peng et al 2015). In comparison to the total population of bacteria present, the number of copies of AlkB was three times higher. Copies of phenol hydroxylase were also higher for Bobrka, and lower for Bialowiesa, in comparison to the bacteria. For AlkB, differences between the sites were due to random sampling variability. For phenol hydroxylase, there was a statistical significant difference for this gene between the different sites.

Naphthalene dioxygenase was only present in Bobrka. The soil pollution concentrations of Bialowiesa were not high enough to cause the appearance of this gene. The copy number of the gene was lower compared to the literature as the total population of bacteria, due to a small number of samples or a bad sample method (Ní Chadhain et al 2006).

The number of copies from the total population of bacteria or fungi, were the most in Bobrka. The genes had a low copy number, compared to the literature, due to a small number of samples or a bad sample method (Peng et al 2015). There was, for both genes, a statistical significant difference.

Soil-bacteria in Beverlo before and after inoculation

The copies of the AlkB-gene and phenol hydroxylase gene were higher before inoculation. The genes had a lower copy number, compared to the literature, due to a small number of samples or a bad sample method (Liu et al 2015, Peng et al 2015). In comparison to the total population of bacteria, AlkB had a higher copy number and phenol hydroxylase had a lower copy number for both sites. This was due to the same reason as described above. For AlkB, there was a statistical significant difference. Due to the failed equal variance-test, the ANOVA could not prove a significant difference between the different sites. This was because of a small number of samples.

Naphthalene dioxygenase was only present after inoculation. The inoculation was necessary to attract bacteria with this gene(Ní Chadhain et al 2006). The standard errors were high due to a small number of samples.

The number of copies from the total population of bacteria or fungi, were higher after inoculation. (Peng et al 2015). Differences between both genes and their presence in the soil were due to random sampling variability.

Screening of bacteria

Sequencing bacteria

The 16S-PCR succeeded for all the bacteria. For most of the bacteria, the sequencing succeeded. Some bacteria could not be

sequenced due to a mixed template or a low DNA-concentration (Janda and Abbott 2007, Macrogen 2017). This was caused by a bad purifying method or a human mistake. The taxonomy of the bacteria were divergent. Some were closely related, others not. The polluted soil, that attracts many organisms, could be an explanation for this.

First screening

The bacteria were screened for the genes AlkB, phenol hydroxylase and naphthalene dioxygenase. They had an expected height of 545 bp, 202 bp and 377 bp. There were aspecific bands, due to secondary binding places of the primers. The bacteria with a band on the expected height, were seen as positive bacteria for this particulate gene.

Second screening

The resolution of the previous gel was too low. A secondary screening with a higher resolution was necessary. There were again aspecific bands due to secondary binding places of the primers. Some of the bacteria that were positive by the first screening, were now negative. Some of them were negative, but were now positive.

Genome annotation

The strain has many genes, but can only cause the degradation of bisphenol A, phenylacetate degradation I, xylene degradation, catechol degradation I and III and octane oxidation. The bacterium has the plant growth promotion factor L-tryptophan degradation VII (S. Thijs, Personal Communication).

Conclusion

The optimum annealingtemperature for a conventional PCR with the AlkB-, PHE-, NAH-CATA-, TOL-, and TOD-primers is 54°C and for the BPH-primers is 65°C.

All of the cloned genes can be used in the standard curves, except for PHE 2.

qPCR can be used as a tool to provide information about the expression of aromatic degrading genes. Alkane monooxygenase is the most expressed by bacteria on the leaves of hornbeam, planted on a polluted site were the air-quality is low. Phenol hydroxylase is the most expressed by bacteria on the leaves of hornbeam, planted on a control site that is not polluted and were the air-quality is good. Naphthalene dioxygenase is not expressed. All of the aromatic degrading genes, the bacteria and the fungi are the most expressed by bacteria on the leaves of ivy, planted on a control soil were that is not polluted and were the air-quality is good. This is also the case for bacteria, found in an oil-polluted soil. Expression of alkane monooxygenase and phenol hydroxylase is the highest before inoculation with aromatic degrading bacteria. Naphthalene dioxygenase, bacteria and fungi is the most expressed after inoculation with aromatic degrading bacteria.

From the screened bacteria, 30 have the alkane monooxygenase gene. 6 have the phenol hydroxylase gene. 12 bacteria have the catechol dioxygenase gene. Three have the xylene monooxygenase gene. Another 6 have the toluene dioxygenase gene and 7 have the biphenyl dioxygenase gene. None of the bacteria have the naphthalene dioxygenase gene.

The bacterium *Acinetobacter calcoaceticus* strain GK2 can degrade bisphenol A, phenylacetate degradation I, xylene degradation, catechol degradation I and III and octane oxidation. The bacteria has the plant growth promotion factor L-tryptophan degradation VII.

To have more significant correlations by the qPCR, more samples need to be tested. Possible soil- and air-pollutant remediation solutions must be further investigated, because of the positive effects these types of bioremediation have on the environment. The newly found aromatic degrading bacteria and strain GK2 could help with this.

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Annexes

1. Sequences from the used genes

1.1. Alkane monooxygenase gene (Cappelletti et al 2011)

AAYACNGCNCAYGARCTNGGNCAYAAACCCAAGCCGCTGGAAGTATTCCTGGCCAAAGTCACTTTGGCGC
CAACGTTCTACGGGCACTTCTATACCGAACATAACCGGGGTCATCACGTCCGTGTCGCCACCCCGGAAGAT
CCGGCCAGCTCGCGGCTGGGGGAAAGTTTCTGGGCTTTCCTGCCGCGCTCGGTGTGGTTCAGCGCACGCTC
GGCGTGGAACCTGGAGCGCGAACGCCTGCGCAAACTCGGCCTGCCGGCCTGGCACTGGAAAAACGCCGT
GCTCAGCGCCTGGCTGTACAGCGTGGTGTTGTGGGGCGTGATGATTGCCTGGTTGGGGGCGGCGGTGATT
CCGTTCCTGATCATCCAGGGCATCTACGGCTTCTCGCTGCTGGAAGTGGTGAACTACGTCGAACACTACGG
CCTTAAACGCCAGAAGTTGCCCAACGGCCGTTATGAGCGTTGCTCGCCGCGACATTCGTGGAACAGTAACC
GGATCGTGACCAATATCTTTCTGTTCCAACTGCARCGNCAYTCNGAYCAYCAYGC

1.2. Naphthalene dioxygenase gene (Thijs et al 2017)

CAAAARCACCTGATTYATGGCGACGAAGAGCTTTTCCAACACGAGCTGAAAACCATTTTTGCGCGTAACTG
GCTTTTCCTCACCCATGACAGTCTGATTCCTTCCCCTGGCGACTATGTAACCGCAAAAATGGGAATTGATGA
AGTCATAGTCTCCCGGCAGAACGACGGTTCGATCCGTGCATTTCTTAATGTTTGTCGTCACCGTGGTAAAAC
GCTAGTGAACGCTGAAGCCGGTAATGCCAAAGGTTTCGTTTGCAGCTATCACGGCTGGGGCTTCGGATCCA
ACGGTGAGCTACAGAGCGTCCCGTTTGAAAAAGAACTGTATGGCGAGTCGCTCAACAAAAAATGTATGGG
ATTGAAAGAAGTCSCYCGYRT

1.3. Phenol hydroxylase gene (Imperato et al 2017)

GTGCTGACSAAYCTGYTGTTCGTACCCTTCATGTCCGGTGCCGCCTACAACGGCGATATGGCCACGGTCACC
TTCGGTTTCTCCGCGCAGTCGGACGAGGCGCGGCACATGACCCTGGGTCTGGAAGTGATCAAGTTCATGCT
CGAACAGCATGAAGACAACGTGCCCATCATCCAGCGCTGA

1.4. Catechol dioxygenase gene (Copeland et al 2017)

CGSGTNGCAWANGCAAAGTCATCCCACAGGTACTTGTCCCCCGACAGGTTTATCTGCGTGGTCAGGTGCC
GGTACCCTGGCGCCGAGATAAAGAAGTGCACATGGGCCGGGCGCTGGCCATGACGGCCCAGCAGGTCCA
GGCATTCCTGGGTTGGCCCCTGCGGGTCGCAGCCATAGCCCGATGGCACGATGGAGCGCGCGCGGTAACG
CCCTTGGGCATCGGTAACGATGCGCCGACGCAGGTTGTACGCCGACTGGCTTGGGTCGAAGAACGAATAG
GTACCACGGGTATTGGCATGCCACAGGTCGACCGTGGCACCCGGCAGCGGGTGTCCGTGCGGGTCCAGCA
CCTGGCCTTCCAGGAACATCACCGTGGCCACGCCCTCCTCGCTGCCGTCGTCCATGCGCACTTCACCTTGCG
CAATCGGTGCGCCAGCCACGTACAACGGGCCTTCRATGGTDCGBGGBGT

1.5. Xylene monooxygenase gene (Yano et al 2010)

CTCACCTGGAGTTGCGTACCGCTGGTCCCAATCTTTCAACTTTGGTTTTGCAATGAGAGCGAACCAAAGAG
GCGGAATAAGCCCTAGAAGGAAACACACAAAGAGCGAAGGCATCTGCGGGGCTTCTTTTTCCGGACGCAA
CTCATAGAACCGTGTATAGCCGTCAATATGATGATTGATATGGTTAGTAATTTCGCAACCCAGCGGGCGCA
CAATTGTTCCCATATGATTCCACGCGTGGTGCAGGAGGATAGGCTTATCTAGATCGCGTACTAAACCATAG
TGTTGAAAGTAATTAAAACCCTCGACTATCCCTTTCGCAATAATCATCGAAGCAATCGTAACCAACCCTAAT
GCTGGCCCGCCCAGATAAGCAACCAGGCCAGGCAGAGCGAGCAGGAGTGCGACATATTGATATGTTTTGT
TGGACAAATTCCACGGTGACTGTCCTTTTCTACGTAAAGTTTCAGCCTCA

1.6. Toluene dioxygenase gene (Copeland et al 2017)

CTTCGGTCMAGTAGCTGGTGACCTTTGGCCCCATGATGGCAAGCATCAGATTGGGGTCGCCGACATAGAA
GCCACTTCCATGTCCGCCCCATGACGCACGGTACTGCTTGCCAACTGTCGGCGGAGCAAGGTCGGCCATTT
CAAGGTCTTCTGGCAGGCCTGCCAGGATGCCAGACAGATGCGAGGTCGTCCCGGCATGGTACATGTCGCT
GCAAAACTGCTCTGCGGCGAATTTCCAGTTACAGGGAATGACCCACTTCTGCACGCCCGGGATCGCTTCGG
TGCCGGCCTCGGTGCGGTCGAGCATGTGGTCCATGTAGAACTTCGCCTCGCCCAGATACGTGTCGAGGTCT
ACAGCGTTCTCATCCCAGTTGGCGAAAATCAGGCCCTTGTAGGTTTCTACCCGGGCCTTCAGCGGGCTCCAT
TCCTTCTTGTTCAGGCACGCGAAGGATTCGGCCTCGTAAGGCACATTGACAAGATTGCCGGCGGTGTCGTA
AGCCCACCCGTGGTAGCTGCAAGTGAACGCCTTCGCGTTTCCGGCATCCGCGCGGCAGATGCGCATGCCAC
GGTGGCGGCACTGGTTCAGGAACACGGCAATGCTGGCGTCTTTCTGCCGGACGACCACGACAGGGTCTTC
ACCCATGTAGGTCGTGATGTAATCGCCCGGCTTGCGAATCTGGGTTTCATGCCCCAACAGCAGCCAGGACC
GGGCGAAGACACGCTCCAGTTCGAGTTGGTACAGRTCYTCATCGGT

2. Protocols

2.1. Weighing

2.1.1. Materials

2.1.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Balance	VWR	Measuring substances

2.1.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Spatula	/	Transferring substances
Tinfoil	ALU1145	Measuring substances

2.1.1.3. Overview used fluids

Does not apply.

2.1.2. Methods

- Clean the balance;
- Turn the balance on;
- Take a piece of tinfoil;
- When it is an analytic balance, open the door;
- Place the tinfoil on the balance;
- When it is an analytic balance, close the door;
- Tare;
- Take a spatula;
- Clean the spatula;
- When it is an analytic balance, open the door;
- Measure the amount of substance needed with the use of the spatula and on the tinfoil;
- When it is an analytic balance, close the door for the exact amount;
- Put the measured amount in a volumetric flask, microtube or falcon tube, depending on what is needful;
- Clean everything

2.2. 869 rich-medium

2.2.1. Materials

2.2.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Balance	VWR	Measuring substances
Autoclave	Ceron 500	Sterilize products
Incubator (60°C)	ALIS	Incubating at a certain temperature
Stirring plate	VWR	Stirring

2.2.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Spatula	/	Transferring substances
Volumetric flask	Fisherbrand	Making buffers
Tinfoil	ALU1145	Measuring substances
Autoclave bottle	Schott-Duran	Sterilize product
Autoclave tape	/	Control sterilize product
Stirring stick	/	Stirring

2.2.1.3. Overview used fluids

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Tryptone	LAB Q40489/350	S		/	/	Source for amino acid
Yeast extract powder	Lab M limited Q38407/153	S		/	/	Removing cell walls
NaCl	VWR 27800.360	S		/	/	Osmotic balance
D-(+)-glucose.H_2O	VWR 16C180013	S		/	/	Food source
$CaCl_2.2H_2O$	Fluka 133907323507351	S		/	/	Promote uptake in cells
Agar No.2 Bacteriological	LAB Q40798/063	S		/	/	Creating solid medium
Distilled water	/	L		/	/	Diluting

2.2.2. Methods

- Fill an appropriate flask with distilled water until about 3/4th of the volume. Add a stirring stick;
- Add the appropriate amount of tryptone, yeast extract powder, NaCl, glucose and $CaCl_2$ - like indicated in table 2, following protocol 'Weighing' - and stir until all products are dissolved*;
- Fill the flask with distilled water until the bottle neck;
- Adjust the pH of the medium to 7 with NaOH;
- Fill the flask till the mark with distilled water and let the medium stir thoroughly;

- Divide the medium over autoclavable bottles;
- Autoclave the medium for 10 min;
- Solid medium should be kept at 60°C after autoclaving or poured immediately.

*If solid medium is desired, add the appropriate amount of agar and a stirring stick in every bottle (for 1/10th strength rich medium, 100 mL rich medium and 900 mL distilled water, use also 15 g of agar per liter of medium).

Table 2: Amount of substances per liter

869 (in g)	**1L**	**2L**	**3L**	**5L**	**10L**	**Concentration (g/L)**
Tryptone	10	20	30	50	100	10
Yeast extract powder	5	10	15	25	50	5
NaCl	5	10	15	25	50	5
D-(+)-glucose.H_2O	1	2	3	5	10	1
$CaCl_2.2H_2O$	0,345	0,690	1,035	1,725	3,450	0,345
Agar No.2 Bacteriological	15					15

2.3. Spread plate technique

2.3.1. Materials

2.3.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Incubator (30°C)	Binder	Incubating at a certain temperature
Micropipette (10 µL – 100 µL)	Eppendorf	Transferring fluids
Bunsen burner	/	Disinfecting
Flowcabinet	Telstar BH-100	Sterile air

2.3.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Drigalski spatula	/	Spreading of bacteria
Pipette tips (10 µL – 100 µL)	VWR 633C6	Transferring fluids
Parafilm	Bemis PM-999	Closing plates

2.3.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Ethanol	Ensure 1.00983.1011	L	70%	H225-H319-H371 	P210-P260-P280-P308 + P311-P337 + P313-P403 + P235 	Disinfection

2.3.2. Methods

- Turn the flowcabinet on;
- Disinfect the flow with 70% ethanol;
- Take the bacteriasuspension and a solid plate (see protocol '869 rich medium');
- Label the plate;
- Open the plate;
- Transfer 100 µL of the bacteriasuspension to the plate;
- Close the plate;
- Take the Drigalski spatula and scale it in the ethanol;
- Flame the spatula using the Bunsen burner;
- Cool down the spatula;
- Open the plate;
- Put the spatula slightly down on the plate;
- Spread out the bacteriasuspension in the shape of eights;
- Close the plate;
- Scale the spatula in the ethanol;
- Flame the spatula using the Bunsen burner;
- Lay it down on the flow;
- Close the plate with Parafilm;
- Put it upside down in a 30°C incubator.

2.4. Isolation of bacteria out of soil

2.4.1. Materials

2.4.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Shaking incubator (30°C)	Gerhardt	Incubating at a certain temperature
Micropipette (100 µL – 1000 µL)	Eppendorf	Transferring fluids

2.4.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Falcon tube	Greiner bio-one E160939H	Storage of substances and fluids
Sterile disposable volumetric pipet (10 mL; 50 mL)	Greiner bio-one F1611347	Transferring fluids
Pipette tips (100 µL – 1000 µL)	VWR 633C6	Transferring fluids
Pipetboy	Hirschmann Pipetus	Transferring fluids

2.4.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
869-media	/	L	See protocol 'Rich-media'	/	/	Solvent

2.4.2. Methods

- Measure 5 g of soil in a falcon tube, following protocol 'Weighing';
- Solve the soil in 45 mL of 869-media;
- Incubate the falcon tube 5 minutes at 30°C on a shaking incubator;
- Take three new falcon tubes;
- Fill the falcon tube with 9 mL of 869-media;
- Take 1 mL of the soil-solution;
- Put the soil-solution in the new falcon tube;
- Mix the falcon tube by inversion;
- Repeat the last 4 steps 2 times;
- Spread the soil-solutions in the falcon tubes out, following protocol 'Spread plate technique', on a plate with 1/10 869-media with indole;
- Place the falcon tubes again on the shaking incubator at 30°C for a couple of weeks.

2.5. Cultivation of bacteria in a masterblock

2.5.1. Materials

2.5.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Shaking incubator (30°C)	Gerhardt	Incubating at a certain temperature
Micropipette (100 µL – 1000 µL)	Eppendorf	Transferring fluids
Bunsen burner	/	Disinfecting
Flowcabinet	Telstar BH-100	Sterile air
Freezer (-45°C)	Bosch	Storage of bacteria

2.5.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Masterblock	Greiner Bio-One B160138A	Storage of fluids
Reservoir (25 mL)	Greiner Bio-One 5B10416	Temporary storage of fluids
Pipette tips (100 µL – 1000 µL)	VWR 633C6	Transferring fluids
Multichannel	Eppendorf	Transferring fluids
Sterile toothpick	Party Stars	Picking up colonies
Tweezer	/	Picking up the sterile toothpick

2.5.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
869-media	/	L	See protocol 'Rich-media'	/	/	Solvent

2.5.2. Methods

- Turn the flowcabinet on;
- Disinfect the flow with 70% ethanol;
- Turn on the Bunsen burner;
- Set up the masterblock and the reservoir;
- Open the bottle with 869-media;
- Flame the neck of the bottle using the Bunsen burner;
- Pour the media in the reservoir;
- Flame the neck of the bottle using the Bunsen burner;
- Close the bottle;
- Take 1 mL media using the multichannel;
- Fill the first row of the masterblock with media;
- Repeat the last 2 steps until all the rows are filled or the media in the reservoir is empty. In the last case, repeat the last 7 steps;
- Take the plates with the targeted colony;
- Take the tweezer and scale it in the ethanol;
- Flame the tweezer using the Bunsen burner;
- Cool down the tweezer;
- Take a sterile toothpick using the tweezer;
- Pick up the targeted colony from the plate with the toothpick;

- Put the toothpick in one well of the masterblock;
- Repeat the last 6 steps until all the targeted colonies are cultivated or all the wells are full;
- Close the masterblock with a lid and parafilm;
- Scale the tweezer in the ethanol;
- Flame the tweezer using the Bunsen burner;
- Lay it down on the flow;
- Put the masterblock on the shaking incubator for a couple of weeks;
- When there is a pellet of bacteria, repeat steps 1 until 12 of this protocol;
- Take with the multichannel 1 mL of bacteriasuspension from the first masterblock;
- Pour the bacteriasuspension in the new masterblock;
- Repeat the last 2 steps until the full masterblock is transferred;
- Put the masterblock on the shaking incubator for a couple of weeks;
- Centrifuge the first masterblock 10 minutes at 1200g;
- Discard the supernatant;
- Fill the masterblock with glycerol, following the protocol '15% Glycerol stock';
- Store it in the freezer on -45°C.

2.6. 15 % Glycerol stock

2.6.1. Materials

2.6.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Balance	VWR	Measuring substances
Autoclave	Ceron 500	Sterilize products
Stirring plate	VWR	Stirring

2.6.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Spatula	/	Transferring substances
Volumetric flask	Fisherbrand	Making buffers
Erlenmeyer	Schott-Duran	Measuring substances
Autoclave bottle	Schott-Duran	Sterilize product
Autoclave tape	/	Control sterilize product
Stirring stick	/	Stirring

2.6.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Glycerol	Sigma-Aldrich BCBL2737V	S		/	/	Living of cells
NaCl	VWR 27800.360	S		/	/	Osmotic balance
MilliQ water	/	L		/	/	Diluting

2.6.2. Methods

- Take the substances glycerol and NaCl out of the cupboard;
- Take a erlenmeyer;
- Set the erlenmeyer on the balance;
- Tare;
- Measure 75g of glycerol (37,5M) and 4,25g (2,125M) of NaCl on the balance with the spatula, following the protocol 'Weighing';
- Dilute until 500g with MilliQ water;
- Take a stirring stick;
- Place the stirring stick in the erlenmeyer;
- Place the erlenmeyer on the stirring plate;
- Stir until everything is solved;
- Take a autoclave bottle;
- Pour everything in the autoclave bottle;
- Paste autoclave tape on the autoclave bottle;
- Autoclave the bottle for 10 minutes.

2.7. Purification using quadrat streaking

2.7.1. Materials

2.7.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Incubator (30°C)	Binder	Incubating at a certain temperature
Flowcabinet	Telstar BH-100	Sterile air

2.7.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Disposable sterile inoculation loop	VWR 2DID00	Purification of bacteria
Parafilm	Bemis PM-999	Closing plates

2.7.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Ethanol	Ensure 1.00983.1011	L	70%	H225-H319-H371 	P210-P260-P280-P308 + P311-P337 + P313-P403 + P235 	Disinfection

2.7.2. Methods

- Turn the flowcabinet on;
- Disinfect the flow with 70% ethanol;
- Take the plate with the targeted colony, and a solid plate (see protocol '869 rich medium' and 'Spread plate technique');
- Label the plate;
- Take a disposable sterile inoculation loop;
- Open the plate with the colony;
- Pick up the colony using the inoculation loop;
- Close the plate;
- Take the new solid plate;
- Open the plate;
- Make three lines with the inoculation loop;
- Turn the plate a little bit, until the end of the lines are on the left side of you;
- Make again three lines with the inoculation loop, starting with the end of the previous lines;
- Repeat the last 2 steps 3 times;
- Close the plate;
- Throw away the inoculation loop;
- Close the plate with Parafilm;
- Put it upside down in a 30°C incubator.

2.8. DNA-extraction Qiagen blood and tissue kit

2.8.1. Materials

2.8.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Vortex	VWR Lab Dancer	Mixing
Incubator (37°C)	Binder	Incubating at a certain temperature
Thermomixer (56°C)	Eppendorf compact	Incubating at a certain temperature
Micropipette (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	Eppendorf	Transferring of fluids
Fridge (-4°C)	Bosch	Storage of DNA

2.8.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tip (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	VWR 633C6	Transferring of fluids
Microtubes (1,5 mL; 2 mL)	Greiner bio-one E16063MX	Storage of fluids

2.8.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Lysis solution	/	L	RNase free water Lysozyme Triton X-100 TE-buffer	H316	P332+313	Lysis of cells
Proteinase K	Qiagen 1017738	L		H334 	P261 - P284 - P304 + P341 - P342 + P311	Destruction of proteins
Buffer AL	Qiagen 1014600	L		H315 - H319 	P280	Destruction of celldebris
Buffer AW1	Qiagen 1014797	L		H302 + H332 - H315 - H319 	P280	Washing of DNA
Buffer AW2	Qiagen 1014577	L		/	/	Washing of DNA
Buffer AE	Qiagen 1014591	L		/	/	Elution of DNA
Ethanol	Ensure 1.00983.1011	L	70%	H225-H319-H371 	P210-P260-P280-P308 + P311-P337 + P313-P403 + P235	Create changes in polarity so DNA can bind the column

2.8.2. Methods

- Make lysis solution (see table 3);

Table 3: Protocol lysis solution

Substance	**Amount per sample**	**Concentration**
RNase free water	185,63 µL	
Lysozyme	3,75 mg	14,3 kDa
Triton X-100	2,25 mg	1,2%
TE-buffer	18,75 µL	2x

- Take 1,5 mL bacteriasuspension in a microtube;
- Centrifuge for 10 minutes by 8000g;
- Resuspend bacterial pellet in 180 µL lysis solution;
- Incubate for 30 minutes at 37°C;
- Add 25 µL proteïnase K (11%);
- Add 200 µL buffer AL (89%);
- Vortex;
- Incubate at 56°C for 30 minutes;
- Add 200 µL ethanol (96-100 %);
- Vortex;
- Pipet the mixture into the DNeasy mini spin column placed in a 2 mL collection tube;
- Centrifuge at 9000g for 1 minute;
- Discard flow-through and collection tube;
- Place the DNeasy mini spin column in a new 2 mL collection tube;
- Add 500 µL buffer AW1 (100%);
- Centrifuge for 1 minute at 9000g;
- Discard flow-through and collection tube;
- Place the DNeasy mini spin column in a new 2 mL collection tube;
- Add 500 µL buffer AW2 (100%);
- Centrifuge for 4 minutes at 14000g;
- Discard flow-through and collection tube;
- Place the DNeasy mini spin column in a 1,5 mL or 2 mL microtube;
- Add 100 µL buffer AE (100%);
- Incubate at room temperature for 1 minute;
- Centrifuge for 1 minute at 9000;
- Repeat the last 3 steps;
- Store the DNA extracts in the fridge.

2.9. DNA-extraction E.Z.N.A Bacterial DNA kit

2.9.1. Materials

2.9.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Vortex	VWR Lab Dancer	Mixing
Incubator (37°C)	Binder	Incubating at a certain temperature
Thermomixer (55°C; 65°C)	Eppendorf compact	Incubating at a certain temperature
Micropipette (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	Eppendorf	Transferring of fluids
Freezer (-20°C)	Bosch	Storage of DNA

2.9.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tip (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	VWR 633C6	Transferring of fluids
Microtubes (1,5 mL)	Greiner bio-one E16063MX	Storage of fluids

2.9.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
TE-buffer	Amresco 2055C156	L		H316	P332+313	Destruction of cells
Lysozyme	Omega Bio-Tek LYSP6J89931760	L		/	/	Destruction of cells
TL Buffer	Omega Bio-Tek TL081616QG2347	L		/	/	Destruction of cell debris
Proteinase K Solution	Omega Bio-Tek PKS20061516JC2164	L		H316 - H334 	P261- P280 - P284 - P304 + P340 - P332 + P313 - P342 + P311	Destruction of protein
RNase A	Omega Bio-Tek RG052616JC2108	L		H334 	P261- P280 - P284 - P304 + P340 - P332 + P313 - P342 + P311	Destruction of RNA
BL Buffer	Omega Bio-Tek BL051116JC2070	L		H302 - H315 - H319 - H332 	P261 - P305 + P351 + P338 - P311	Changes in polarity for binding on column
Ethanol	Ensure 1.00983.1011	L	100%	/	/	Changes in polarity for binding on column

HBC buffer	Omega Bio-Tek HBC090716JC M2388	L		H302 - H315 - H319 - H332 	P261 - P305 + P351 + P338 - P311	Equilibration of column
DNA Wash Buffer	Omega Bio-Tek DW060216QG 2147	L		/	/	Washing of DNA
Elution Buffer	Omega Bio-Tek EB032316JC20 13	L		/	/	Elution of DNA

2.9.2. Methods

- Centrifuge the 1,2 mL of bacteriasuspension at 4000g for 10 minutes at room temperature;
- Aspirate and discard the media;
- Add 100 µL TE-buffer (40%);
- Vortex;
- Add 10 µL Lysozyme (4%);
- Incubate at 37°C for 30 minutes;
- Add 100 µL TL buffer (40%);
- Add 20 µL Proteinase K Solution (8%);
- Vortex;
- Incubate at 55 °C for 30 minutes in the thermomixer;
- Add 5 µL RNase A (2%);
- Invert the microtube several times;
- Incubate at room temperature for 5 minutes;
- Centrifuge at 10000g for 2 minutes;
- Transfer the supernatant to a new 1,5 mL microtube;
- Add 220 µL BL buffer (50%);
- Vortex;
- Incubate at 65°C for 10 minutes;
- Add 220 µL 100% ethanol;
- Vortex;
- Insert a HiBind DNA Mini Column into a 2 mL Collection tube;
- Transfer the sample to the column;
- Centrifuge at 10000g for 1 minute;
- Discard the filtrate and the collection tube;
- Insert a HiBind DNA Mini Column into a 2 mL Collection tube;
- Add 500 µL HBC buffer (100%);
- Centrifuge at 10000g for 1 minute;
- Discard the filtrate and reuse the collection tube;

- Add 700 µL DNA Wash Buffer (100%);
- Centrifuge at 10000g for 1 minute;
- Discard the filtrate and reuse the collection tube;
- Repeat the last 3 steps;
- Centrifuge the HiBind DNA Mini Column at maximum speed for 2 minutes;
- Insert a HiBind DNA Mini Column into a 1,5 mL microtube;
- Add 100 µL Elution Buffer heated to 65°C (100%);
- Let sit for 5 minutes at room temperature;
- Centrifuge at 10000g for 1 minute;
- Repeat the last 3 steps;
- Store the DNA at -20°C.

2.10. DNA-extraction Promega Wizard DNA Purification kit

2.10.1. Materials

2.10.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Vortex	VWR Lab Dancer	Mixing
Incubator (37°C)	Binder	Incubating at a certain temperature
Thermomixer (65°C; 80°C)	Eppendorf compact	Incubating at a certain temperature
Micropipette (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	Eppendorf	Transferring of fluids
Fridge (4°C)	Bosch	Storage of DNA

2.10.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tip (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	VWR 633C6	Transferring of fluids
Microtubes (1,5 mL)	Greiner bio-one E16063MX	Storage of fluids

2.10.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Lysis solution	/	L	RNase free water Lysozyme Triton X-100 TE-buffer	H316	P332+313	Lysis of cells
Nuclei Lysis Solution	Promega 0000123561	L		H315 - H319 - H335 	/	Lysis of cells nuclei
RNase Solution	Promega 0000118628	L		/	/	Destruction of RNA
Protein Precipitation Solution	Promega 0000093580	L		/	/	Precipitation of proteins
DNA Rehydration Solution	Promega 0000092209	L		/	/	Rehydration of DNA
Isopropanol	Sigma-Aldrich STBF7471V	L		H225-H319-H336 	P210-P261-P305 + P351 + P338	Precipitation of DNA
Ethanol	Ensure 1.00983.1011	L	70%	H225-H319-H371	P210-P260-P280-P308 + P311-P337 + P313-P403 + P235	Washing of DNA

2.10.2. Methods

- Make lysis solution (see table 4);

Table 4: Protocol lysis solution

Substance	**Amount per sample**	**Concentration**
RNase free water	185,63 µL	
Lysozyme	3,75 mg	14,3 kDa
Triton X-100	2,25 mg	1,2%
TE-buffer	18,75 µL	2x

- Centrifuge 1,2 mL bacteriasuspension for 2 minutes at 13000g;
- Discard the supernatant;
- Add 180 µL of lysis solution (22%);
- Incubate at 37°C for 30 minutes;
- Add 420 µL Nuclei Lysis Solution (52%);
- Vortex;
- Incubate for 5 minutes at 80 °C;
- Cool to room temperature;
- Add 3 µL of RNase Solution (4%);
- Vortex;
- Incubate at 37°C for 15 minutes;
- Cool to room temperature;
- Add 200 µL of Protein Precipitation Solution (24%);
- Vortex;
- Incubate on ice for 5 minutes;
- Centrifuge at 13000g for 3 minutes;
- Transfer the supernatant to a new 1,5 mL microtube, containing 600 µL of room temperature isopropanol (60%);
- Mix by inversion;
- Centrifuge for 2 minutes at 13000g;
- Discard the supernatant;
- Add 600 µL of room temperature 70% ethanol;
- Vortex;
- Centrifuge for 2 minutes at 13000g
- Aspirate the ethanol;

- Air-dry the pellet for 15 minutes;
- Add 100 µL of Rehydration Solution for 1 hour at 65°C or overnight at 4°C.

2.11. Mobio soil DNA-extraction kit

2.11.1. Materials

2.11.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Vortex	VWR Lab Dancer	Mixing
Micropipette (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	Eppendorf	Transferring of fluids
Freezer (-20°C)	Bosch	Storage of DNA
Balance	VWR	Measuring substances
Autoclave	Ceron 500	Sterilize products
Stirring plate	VWR	Stirring
Retsch Mixer	Retsch MM 400	Vortexing

2.11.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tip (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	VWR 633C6	Transferring of fluids
Falcon tubes (15 mL)	Greiner bio-one E160939H	Storage of fluids
Spatula	/	Transferring substances
Volumetric flask	Fisherbrand	Making buffers
Tinfoil	ALU1145	Measuring substances
Falcon tube	Greiner bio-one E160939H	Storage of fluids
Autoclave tape	/	Control sterilize product
Stirring stick	/	Stirring

2.11.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
HCl	VWR 0606C247	S		/	/	Control of pH
NaCl	VWR 27800.360	S		/	/	Osmotic balance
$NaH_2PO_4.H_2O$	Merck 1.06574.1000	S		/	/	Osmotic balance
$Na_2PO_4.7H_2O$	Ensure 1.06580.1000	S		/	/	Osmotic balance
Guanidium isothiocyanate	Promega 0000126343	S		H302 - H312 - H332 - H412	P261 - P280 - P301 + P312 - P304 + P340 - P312 - P363 	Denaturation of proteins
SDS	Fisher 113485	S		H228 - H302 + H332 - H315 - H318 - H335 - H412	P210 - P261 - P273 -. P280 - P305 + P351 + P338 	Denaturation of cells

TRIS	VWR 0606C247	S		/	/	Control of pH
Ammonium acetate	Merck 1.01115.5000	S		H303, H316, H320, H333	P281, P335 	Precipitation of DNA
Aluminum ammonium sulfate dodecahydrate	UCB 1362	S		H315 - H319 - H335 	P261 - P305+P351+P338	Precipitation of DNA
Citric acid anhydrous	Sigma 47H0379	S		H319 	P280-P305 + P351 + P338-P337 + P313	Intermediate in cell metabolism
Trisodium citrate	Merck 244.0500	S		/	/	Controls ion-exchange
Guanidine hydrochloride	Sigma 05645453	S		H302 + H332- H315- H319 	P261-P280-P301 + P312 + P330- P304 + P340 + P312-P305 + P351 + P338-P337 + P313	Chaotrope salt
Phenol:Chloroform:Isoamylalcohol	VWR 2455C099	L	50:49:1 pH 6,8-8	H301 - H311 - H331 - H314 - H351 - H373 	P301 + P310 - P301 + P330 + P331 - P303+P361+P353 - P305 + P351 + P338 - P304 + P340 - P312 - P280 - P271 - P260 - P264 	Denaturation of cells
Isopropanol	Sigma-Aldrich STBF7471V	L	100%	H225- H319- H336 	P210-P261-P305 + P351 + P338	Precipitation of DNA
Ethanol	Ensure 1.00983.1011	L	70%	H225- H319- H371 	P210-P260-P280- P308 + P311-P337 + P313-P403 + P235 	Washing of DNA
RNase-free water	/	L		/	/	Diluting

2.11.2. Methods

1) Making buffers

- Bead solution (181mM $NaPO_4$, 121mM guanidium isothiocyanate):
  - Take a 100 mL volumetric flask;
  - Fill the flask about ¼ with milliQ water;
  - Measure 0,41g of $NaH_2PO_4.H_2O$, 4,05g of $Na_2PO_4.7H_2O$ and 1,4278g guanidium isothiocyanate on the balance with the spatula;
  - Add the substances to the flask;
  - Full the flask with milliQ water until the mark;
  - Decant the fluid, in the volumetric flask, to a falcon tube;
  - Adjust the pH with HCl until the pH is 7,5.
- C1/SR1 (150 mM NaCl, 4% SDS, 0,5M Tris):
  - Take a 100 mL volumetric flask;
  - Fill the flask about ¼ with milliQ water;
  - Measure 0,8766g of NaCl, 4g of SDS and 6,057g TRIS on the balance with the spatula;
  - Add the substances to the flask;
  - Full the flask with milliQ water until the mark;
  - Decant the fluid, in the volumetric flask, to a falcon tube;
  - Adjust the pH with HCl until the pH is 7.
- C2 (133 mM ammonium acetate):
  - Take a 100 mL volumetric flask;
  - Fill the flask about ¼ with milliQ water;
  - Measure 1,0241g ammonium acetate on the balance with the spatula;
  - Add the substances to the flask;
  - Full the flask with milliQ water until the mark;
  - Decant the fluid, in the volumetric flask, to a falcon tube;
  - Adjust the pH with acetate until the pH is 7.
- C3/SR2 (120 mM aluminum ammonium sulfate dodecahydrate):
  - Take a 10 mL volumetric flask;
  - Fill the flask about ¼ with milliQ water;
  - Measure 0,5439g aluminum ammonium sulfate dodecahydrate on the balance with the spatula;
  - Add the substances to the flask;
  - Full the flask with milliQ water until the mark;
  - Decant the fluid, in the volumetric flask, to a falcon tube.
- SR3 (5 M NaCl, 22 mM citric acid anhydrous, 29 mM trisodium citrate):
  - Take a 100 mL volumetric flask;
  - Fill the flask about ¼ with milliQ water;
  - Measure 0,422664g citric acid anhydrous, 0,852g trisodium citrate and 29g NaCl on the balance with the spatula;
  - Add the substances to the flask;
  - Full the flask with milliQ water until the mark;
  - Decant the fluid, in the volumetric flask, to a falcon tube;
  - Adjust the pH with HCl until the pH is 5,0-5,2.
- C4 (5 M guanidine hydrochloride, 30 mM Tris):
  - Take a 100 mL volumetric flask;
  - Fill the flask about ¼ with milliQ water;

- Measure 47,765g guanidine hydrochloride and 0,36342g TRIS on the balance with the spatula;
- Add the substances to the flask;
- Full the flask with milliQ water until 91 mL has been reached;
- Decant the fluid, in the volumetric flask, to a falcon tube;
- Adjust the pH with HCl until the pH is 6,8.

- C5 (10 mM Tris, 100 mM NaCl):
  - Take a 100 mL volumetric flask;
  - Fill the flask about ¼ with milliQ water;
  - Measure 0,5844g NaCl and 0,12114g TRIS on the balance with the spatula;
  - Add the substances to the flask;
  - Full the flask with milliQ water until 50 mL has been reached;
  - Decant the fluid, in the volumetric flask, to a falcon tube;
  - Adjust the pH with HCl until the pH is 7,5.
- C6 (10 mM Tris):
  - Take a 100 mL volumetric flask;
  - Fill the flask about ¼ with milliQ water;
  - Measure 0,12114g TRIS on the balance with the spatula;
  - Add the substances to the flask;
  - Full the flask with milliQ water until 91 mL has been reached;
  - Decant the fluid, in the volumetric flask, to a falcon tube;
  - Adjust the pH until the pH is 8.
- Autoclave all the buffers for 10 minutes.

2) DNA-extraction:

- Add 2g of sample to a 15 mL falcon tube;
- Add 1g of glass beads;
- Add 2,5 mL of Bead Solution;
- Vortex the samples;
- Add 250 µL of SR1;
- Add 800 µL of SR2;
- Vortex;
- Add 3,5 mL of Phenol:Chloroform:Isoamylalcohol;
- Place the falcon tube on a shredder and vortex the tubes at the highest setting for 7 minutes;
- Centrifuge the falcon tube at 2500g for 10 minutes at room temperature;
- Transfer the supernatant to a new falcon tube;
- Add 250 µL 1,5 mL of SR3;
- Vortex to mix;
- Incubate the sample at 4° C for 10 minutes;
- Centrifuge the falcon tube at 2500g for 10 minutes at room temperature;
- Transfer the supernatant to a new falcon tube;
- Add 5 mL of 100% isopropanol;
- Incubate the sample overnight at -20°C;
- Centrifuge the falcon tube at 2500g for 30 minutes at room temperature;
- Decant the supernatant;
- Resuspend the pellet in 150 µL RNase-free water;

3) DNA-purification:

- Add 460 µL of bead solution per 150 µL of DNA;
- Invert to mix;
- Add 140 µL of C1;
- Invert to mix;
- Add 560 µL of C2;
- Invert to mix;
- Incubate at 4°C for 5 minutes;
- Centrifuge at 10000g for 1 minute at room temperature;
- Transfer the supernatant to a clean microtube;
- Add 460 µL of C3;
- Invert to mix;
- Incubate at 4°C for 10 minutes;
- Centrifuge at 10000g for 10 minutes at room temperature;
- Transfer the supernatant to a clean microtube;
- Add 2750 µL of 100% isopropanol;
- Vortex;
- Centrifuge for 2 minutes at 13000g;
- Discard the supernatant;
- Add 600 µL of room temperature 70% ethanol;
- Vortex;
- Centrifuge for 2 minutes at 13000g
- Aspirate the ethanol;
- Air-dry the pellet for 15 minutes;
- Add 200 µL of C6.

2.12. Qubit dsDNA HS Assay

2.12.1. Materials

2.12.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Qubit 2.0 Fluorometer	Molecular probes	Measuring DNA-concentration
Micropipette (1 µL – 10µL; 100 µL – 1000 µL)	Eppendorf	Transferring fluids
Vortex	VWR Lab Dance	Homogenization of substances

2.12.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Qubit assay tubes	Life Technologies Q32856	Measuring DNA-concentration
Pipette tips (1 µL – 10µL; 100 µL – 1000 µL)	VWR 633C6	Transferring fluids

2.12.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Qubit® dsDNA HS Buffer	Invitrogen 1799092	L		/	/	Needed for preparing Qubit® working solution
Qubit® dsDNA HS Reagent	Invitrogen 1799092	L		/	/	Needed for preparing Qubit® working solution
Standard #1	Invitrogen 1799092	L		/	/	Making standard curve for DNA-concentration
Standard #2	Invitrogen 1799092	L		/	/	Making standard curve for DNA-concentration

2.12.2. Methods

1) Preparing samples and standards

- Set up the required number of 0,5 mL Qubit assay tubes for standards and samples;
- Label the tube lids;
- Prepare the Qubit® working solution by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer in a new 0,5 mL microtube;
- Add 190 µL of Qubit® working solution to each of the microtubes used for standards;
- Add 10 µL of each Qubit® standard to the appropriate microtube;

- Vortex;
- Add 190 µL Qubit® working solution to individual assay microtubes;
- Add 10 µL of each DNA-sample to the appropriate microtube;
- Vortex
- Allow all tubes to incubate at room temperature for 2 minutes;
- Press DNA on the Home screen of the Qubit® 2.0 Fluorometer;
- Select dsDNA High Sensitivity as the assay type;
- Press Yes on the Standards screen to read the standards;
- Insert the tube containing Standard #1 into the sample chamber;
- Close the lid;
- Press Read;
- Remove Standard #1;
- Insert the tube containing Standard #2 into the sample chamber;
- Close the lid;
- Press Read;
- Remove Standard #2;
- Insert a sample tube into the sample chamber;
- Close the lid;
- Press Read;
- Remove the sample tube;
- The instrument displays the results on the Sample screen;
- Press Calculate Stock Conc.;
- Select the volume of your original sample that you added to the assay tube;
- Write the concentration of the sample down in the lab notebook;
- Press Read Next Sample;
- Repeat the last 9 steps until all samples have been read.

2.13. NanoDrop

2.13.1. Materials

2.13.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Micropipette (1 µL – 10 µL)	Eppendorf	Transferring fluids
UV-spectrometer	NanoDrop® spectrophotometer ND-1000	Determination of DNA-concentration and purification
Vortex	VWR	Mixing

2.13.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tips (1 µL – 10 µL)	VWR 633C6	Transferring fluids
Paper	/	Cleaning

2.13.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
RNase free water	Omega Bio-Tek NFWD011216J C1856	L		/	/	Setting the NanoDrop to zero

2.13.2. Methods

- Install the software;
- Start the program;
- Choose nucleid acid;
- Lift the arm up;
- Put 1 µL of RNase free water on the bottom of the arm;
- Close the arm;
- Press OK;
- Lift the arm up;
- Clean the bottom of the arm;
- Put 1 µL of the blank on the bottom of the arm;
- Close the arm;
- Press BLANK;
- Lift the arm up;
- Clean the bottom of the arm;
- Vortex the sample;
- Put 1 µL of the sample on the bottom of the arm;
- Close the arm;
- Set the name of the sample;
- Press MEASURE;
- Save the measurement;
- Repeat the last 7 steps until all samples are measured;
- Close the program.

2.14. Cloning reaction

2.14.1. Materials

2.14.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Micropipette (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	Eppendorf	Transferring fluids
Stirring plate	VWR	Stirring
PCR-appliance	Biorad C-1000 Touch	Amplification of DNA
Water bath (42°C)	Grant	Incubating at a certain temperature
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Vortex	VWR Lab Dancer	Mixing

2.14.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tips (1 µL – 10 µL; 10 µL – 100 µL; 100 µL – 1000 µL)	VWR 633C6	Transferring fluids
Cryotubes	Greiner bio-one E16013F4	Storage of bacteria
Microtube (1,5 mL)	Greiner bio-one E16063MX	Storage of fluids or DNA
Stirring stick	/	Stirring
Parafilm	Bemis PM-999	Closing plates
Sterile toothpick	Party stars	Picking up colonies
Falcon tube	Greiner bio-one E160939H	Storage of fluids

2.14.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
X-gal		L		H332 - H302 - H312 	P261 - P264 - P280 - P362+364 - P304+340 - P312 - P301+312 - P330 - P302+352 - P321	Screening recombinants
Ampicillin	/	L		H315- H317- H319- H334-H335 	P261-P280-P305 + P351 + P338- P342 + P311	Building pressure to remain the vector
Alkb-1f-Schulz2013	Biolegio 730980	L	200 nM	/	/	Amplification of DNA
Alkb-1r-Schulz2013	Biolegio 730981	L	200 nM	/	/	Amplification of DNA
alk-H1F-Chen2003	Biolegio 730984	L	200 nM	/	/	Amplification of DNA
alk-H3R-Chen2003	Biolegio 730985	L	200 nM	/	/	Amplification of DNA

NAH-F	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
NAH-R	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
PHE-F	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
PHE-R	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
PCR buffer	Invitrogen 1642490	L	10x	H314 – H335 	P261 - P280 - P305+P351+P338 - P310	Creating good circumstances for the amplification of DNA
$MgSO_4$	Invitrogen 1825424	L		/	/	Co-factor for Taq-polymerase
dNTP-mix	Roche 11580220	L	10µM each	/	/	Building block for the amplification of DNA
Taq High Fidelity-polymerase	Invitrogen 1825424	L	5U/µL	/	/	Amplification of DNA
RNase free water	Omega Bio-Tek NFWD011216J C1856	L		/	/	Diluting
Glycerol	See protocol '15% Glycerol stock'	L	15%	/	/	Storage of bacteria
Rapid Ligation buffer	0000145147	L	2x	/	/	Ligase of PCR-product in vector
Easy Vector	0000139209	L		/	/	Ligase of PCR-product in vector
T4 DNA ligase	000137013	L		/	/	Ligase of PCR-product in vector
Buffer EcoR1	Thermo Scientific 00238529	L	10x	/	/	Restriction of DNA
EcoR1	Thermo Scientific 00238851	L		/	/	Restriction of DNA

869-media	See protocol 'Rich-media'	L		/	/	Growth of bacteria
M13-pUC-forward	/	L		/	/	Sequencing

2.14.2. Methods

- Prepare LB plates + X-gal (final: 80 µg/mL) + Ampicillin (final: 100 µg/mL). Three plates are needed per transformation;
- Prepare 86 mL solid LB Broth and 57 mL liquid LB, according to the protocol: '869 rich medium';
- When cooling down, add to the solid medium 86 µL of ampicillin stock (100 mg/mL water) and 137 µL of X-gal stock (50 mg/ml);
- Stir for mixing;
- Pour plates;
- Store the plates in the cold room until needed;
- The following table is an example of the qPCR-primers used for detecting different ring-hydroxylating-genes;

Table 5: Ring-hydroxylating-genes and associated primers

Gene	Primername	Primersequence	Theoretically Ta (°C)
AlkB	Alkb-1f-Schulz2013	AAYACNGCNCAYGARCTNGGNCAYAA	69,3
	Alkb-1r-Schulz2013	GCITGITGITCISWRTGICGYTG	73,4
	alk-H1F-Chen2003	CIGIICACGAIITIGGIC ACAAGAAGG	76,9
	alk-H3R-Chen2003	IGCITGITGATCIIIGTGICGCTGIAG	78,4
Naphthalene dioxygenase	NAH-F	CAAAARCACCTGATTYATGG	57
	NAH-R	AYRCGRGSGACTTCTTTCAA	62,3
Phenol monooxygenase	PHE-F	GTGCTGACSAAYCTGYTGTTC	62,8
	PHE-R	CGCCAGAACCAYTTRTC	56,9

- Run a gradient-PCR (see table 6 and 7) with the selected primers (see table 5) and, as DNA-templates (see protocol 'DNA-extraction Qiagen Blood and Tissue kit'), *Acinetobacter O9, Rhodococcus D3, Pseudomonas 4.1 and Pseudomonas 4T1* , in a 25 µL reaction volumes;

Table 6: Gradient-PCR

Cycling conditions	
Initial denaturation	180 s at 95°C
30 cycles:	
1. Denaturation	20 s at 95°C
2. Annealing	40 s at 60°C and 58°C* ➔ gradient 10°C
3. Elongation	20 s at 72 °C
Final extension	420 s at 72°C

*For all of the templates, 60°C until 50°C was first tried for the annealingtemperature. For *Rhodococcus D3* and *Pseudomonas 4T1*, there was another gradient-PCR tried with a annealingtemperature from 58°C until 48°C.

Table 7: Mastermix

Components	Final concentration
PCR buffer with MgCl (10x)	1x
$MgSO_4$ **	1,5 mM
dNTP-mix (10µM each)	200 nM
Forward-primer (10µM)	200 nM
Reverse-primer (10µM)	200 nM
Taq High Fidelity-polymerase (5U/µL)	1,25 U
RNase free water	Diluting until 24 µL
DNA-template	1 µL

** Only needed when there is no MgCl added in the buffer.

- Run the PCR-products (5 µL), following the protocol 'Agarose gel-electrophoresis';
- Purify the PCR-product by using the protocol 'PCR purification';
- Measure the concentration of the purified PCR-products, following the protocol 'Qubit dsDNA HS Assay';
- Use pGem®-T Easy Vector System from Promega to ligate the purified PCR-product, following the protocol according to table 8;

Table 8: pGem-T easy vector protocol

Product	Volume
2X Rapid Ligation buffer	5 µL
Easy Vector	1 µL
PCR product	Depending on concentration PCR-product
T4 DNA ligase	1 µL
RNAse Free Water	Depending on concentration PCR-product
	Total: 10 µL

- Vortex;
- Incubate overnight;
- Equilibrate LB + ampicillin + X-gal plates to room temperature;
- Centrifuge ligations tubes;
- Add 2 µL of each ligation reaction to a sterile microtube on ice;
- Thaw competent cells in an ice bath;
- Transfer 50 µL of competent *E. coli* cells to each microtube containing the vectors;
- Gently flick;
- Incubate on ice for 20 minutes;
- Heatshock the cells in a 42°C water bath for 40-50 seconds;
- Return cells to ice for another 2 minutes;
- Add 950 µL LB broth to the microtubes;
- Incubate for 1 h 30 minutes at 37°C with shaking;
- Plate, the microtubes, in duplicate, following the protocol 'Spread plate technique';
- Incubate overnight at 37°C;
- Add 50 µL of [1/100] ampicillin to the remaining LB broth stock;
- Select five white colonies, on the plates, with a sterile toothpick for each gene;
- Streak it on an additional LB + ampicillin plate;

- Drop the toothpick into a falcon tube containing 5 mL of LB broth + ampicillin;
- Secure the falcon tube with parafilm;
- Incubate on an angle, at 37°C overnight with shaking;
- Plates can be incubated at 37°C overnight without shaking;
- Pour 1,5 mL of the falcon tubes into microtubes and set aside for plasmid-extraction;
- Centrifuge the remaining liquid in the falcon tubes for 4 minutes at 11 000g;
- For the making of the glycerol solution, follow protocol '15% Glycerol stock';
- Resuspend pellet in 1mL 15% glycerol solution;
- Place solution in cryotubes for freezing in the -45°C freezer;
- Extract plasmids according to the protocol 'Plasmid-extraction';
- Measure the concentration of the plasmids, following the protocol 'NanoDrop';
- Cut the plasmid-DNA with restriction enzyme EcoR1, following the protocol according to table 9;

Table 9: Restrictionprotocol with EcoR1

Product	**Volume**
10x Buffer EcoR1	2 µL
DNA (0,5-1 µg/µL)	Depending on concentration plasmid-DNA
EcoR1	0,5 µL – 2 µL (Depending on concentration plasmid-DNA)
RNAse Free Water	8 µL
	Total: 20 µL

- Run the restriction-products (5 µL), following the protocol 'Agarose gel-electrophoresis';
- Check the correct insert-size on gel;
- Sent two samples per gene for sequencing to Macrogen. Pipet 10 µL of plasmid-DNA in a PCR-tube. Fill in the Macrogen order sheet. Sequence with M13-pUC-forward and/or M13-PuC-reverse;
- Check sequencing results.

2.15. Agarose gel-electrophoresis

2.15.1. Materials

2.15.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Micropipette (1µL – 10 µL; 10 µL – 100 µL)	Eppendorf	Transferring fluids
Vortex	VWR Lab Dance	Homogenizing of substances
Agarose Gel Electrophoresis system	Sub Cell® GT	Gelelectroforesis
Power source	VWR	Providing power
Balance	VWR	Measuring substances
UV-transilluminator	Biorad	Visualisation DNA
Microwave	Samsung	Heating agarose gel
Centrifuge	Thermo Scientific SL40R	Spinning samples

2.15.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tips (1 µL – 10µL; 10 µL – 100 µL)	VWR 633C6	Transferring fluids
Microtubes (1,5 mL)	Greiner bio-one E16063MX	Storage fluids
Spatula	/	Transferring substances
Parafilm	Bemis PM-999	Measuring substances
Erlenmeyer	Scott-Duran	Making the gel
Graduated cylinder	Vitri	Measuring substances

2.15.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Agarose	Ultrapure Agarose 16500-500	S	/	/	/	Needed for making a gel
Tris-Acetate-EDTA-buffer (TAE) (pH 8,3)	/	L	40 mM Tris 20 mM Acetate 1 mM EDTA	H315, H319, H335, H402 	P261 P305 + P351 + P338	Electric conductans of DNA in the gel
GelRed	Biotium 15G1231-1023138	L	10 µL gelred/100 mL gel	H227, H315, H317, H319, H335, H373	P102, P261, P262, P264, P271, P272, P280	Visualisation of DNA under UV
Loading dye	Thermo Scientific 00436909	L	1 µL loading dye/5 µL DNA	H302 	P301+ P312	Loading & tracking dye
100 bp ladder	Thermo Scientific 00451051	L	/	/	/	Determination of the size of a band

2.15.2. Methods

- Place the gel tray in the casting tray;
- Place the combs in the casting tray;
- Measure 125 mL TAE-buffer in a graduated cylinder;
- Transfer the TAE-buffer to the erlenmeyer;
- Turn the balance on;
- Place a piece of parafilm on the balance;
- Tare;
- Measure 1,8g of agarose using the spatula;
- Transfer the agarose to the erlenmeyer;
- Set the erlenmeyer in the microwave for 2 minutes and 30 seconds;
- Transfer 13 µL of GelRed to the heated erlenmeyer;
- Mix by shaking;
- Cool down until the erlenmeyer is hand warm;
- Pour the warm gel in the gel tray;
- Let the gel coagulate;
- Divide the loading dye on parafilm for all the samples;
- Short-spin the samples;
- Remove the combs from the casting tray;
- Pull the gel tray out of the casting tray;
- Place the gel tray in the electrophoresis system;
- Full the electrophoresis system with TAE-buffer until the gel is under;
- Load the 100 bp ladder on the gel;
- Mix the sample with the loading dye;
- Load the sample on the gel;
- Repeat the last 2 steps until all the samples are loaded;
- Place the lid on the electrophoresis system;
- Connect the power source to the electrophoresis system;
- Set the power source on;
- Set the power source on 100 V for 40 minutes;
- Place the gel under the UV-transilluminator;
- Take a photo of the gel;
- Throw the gel into the bin;
- Clean everything up.

2.16. PCR purification

2.16.1. Materials

2.16.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Micropipette (10 µL – 100 µL; 100 µL – 1000 µL)	Eppendorf	Transferring of fluids
Vortex	VWR Lab Dance	Mixing

2.16.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tips (10 µL – 100 µL; 100 µL – 1000 µL)	VWR 633C6	Transferring of fluids
Microtubes (1,5 mL)	Greiner bio-one E160939H	Storage of fluids

2.16.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Buffer PB	Qiagen 151045845	L	pH 7.0 – 8.5	H225 - H315 - H319 - H336	P210 - P280 	Removing agarose gel
Buffer PE	Qiagen 151038994	L		/	/	Washing of DNA
RNase free water	Omega Bio-Tek NFWD011216J C1856	L		/	/	Elution of DNA

2.16.2. Methods

- Add 5 volumes Buffer PB to 1 volume of the PCR reaction;
- Vortex;
- Place a QIAquick column in a provided 2 mL collection tube;
- Apply the sample to the QIAquick column;
- Centrifuge for 30–60;
- Discard flow-through;
- Place the QIAquick column back in the same tube;
- Add 750 µL Buffer PE to the QIAquick column;
- Centrifuge for 30–60 s;
- Discard flow-through;
- Place the QIAquick column back in the same tube;
- Centrifuge the QIAquick column once more in the provided 2 mL collection tube for 1 minute;
- Place each QIAquick column in a clean 1,5 mL microcentrifuge tube;
- Add 50 µL water to the center of the QIAquick membrane;
- Centrifuge the column for 1 minute
- Discard the column.

2.17. Plasmid-extraction

2.17.1. Materials

2.17.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Freezer (-20°C)	Bosch	Storage of DNA
Micropipette (10 µL – 100 µL; 100 µL – 1000 µL)	Eppendorf	Transferring fluids

2.17.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tips (10 µL – 100 µL; 100 µL – 1000 µL)	VWR 633C6	Transferring fluids
Microtube (1,5 mL)	Greiner bio-one E16063MX	Storage of fluids and DNA

2.17.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Resuspension Solution	Thermo Scientific 00295526	L		/	/	Resuspension of pellet
Lysis Solution	Thermo Scientific 00306960	L		/	/	Lysis of cells
Neutralization Solution	Thermo Scientific 00302672	L		/	/	To stop the lysis reaction
Wash Solution	Thermo Scientific 00313309	L		/	/	Washing of DNA
Elution Buffer	Thermo Scientific 00295524	L		/	/	Elution of DNA

2.17.2. Methods

- Centrifuge the bacteriasuspension for 10 minutes at maximum speed;
- Discard the supernatant;
- Add 250 µL of 4°C Resuspension Solution;
- Resuspend or vortex until there are no cell clumps remaining;
- Add 250 µL of Lysis Solution;
- Invert the microtube 4-6 times;
- Add 350 µL of Neutralization Solution;
- Invert the tube 4-6 times;
- Centrifuge for 5 minutes at maximum speed;
- Transfer supernatant to the GeneJET spin colomn;
- Centrifuge for 1 minute at maximum speed;
- Discard flow-through;

- Place the column back in the collection tube;
- Add 500 µL of Wash Solution;
- Centrifuge for 30-60 seconds at maximum speed;
- Discard flow-through;
- Place the column back in the collection tube;
- Repeat the last 4 steps;
- Centrifuge for 1 minute at maximum speed;
- Transfer the column in a new 1,5 mL microtube;
- Add 50 µL of Elution Buffer;
- Incubate for 5 minutes at room temperature;
- Centrifuge for 2 minutes at maximum speed;
- Discard the column;
- Store the plasmid-DNA at -20°C.

2.18. Quantitative Polymerase Chain Reaction

2.18.1. Materials

2.18.1.1. Overview used equipment

Hardware	**Brand and type**	**Purpose**
Freezer (-20°C)	Bosch	Storage of qPCR-products
Micropipette (1 µL – 10 µL; 10 µL – 100 µL - 100 µL – 1000 µL)	Eppendorf	Transferring fluids
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Vortex	VWR Lab Dancer	Mixing
Dispenser	Eppendorf	Dividing of mastermix
qPCR-appliance	7500 Fast Real-Time PCR-system	Amplification of DNA

2.18.1.2. Overview used glassware and disposables

Hardware	**Brand and type**	**Purpose**
Pipette tips (100 µL – 1000 µL)	VWR 633C6	Transferring fluids
Microtube (1,5 mL)	Greiner bio-one E16063MX	Storage of fluids
96-wellplate	Applied Biosystem 4346906 MicroAmp Fast Optical 96-well Reaction Plate with barcode (0,1mL)	Storage of fluids
Seal Sheet	Thermo Scientific Optical Clear Adhesive Seal Sheets AB-1170	Closing 96-wellplate

2.18.1.3. Overview used fluids and solutions

Name	**Supplier and batchnumber**	**Solid or liquid**	**Components**	**Risk's H-phrases**	**Safety measures P-phrases**	**Purpose**
EUB-518	Biolegio 345124	L	200 nM	/	/	Amplification of DNA
EUB-338	Biolegio 345123	L	200 nM	/	/	Amplification of DNA
EUK-345F	Biolegio 345131	L	200 nM	/	/	Amplification of DNA
EUK-499R	Biolegio 345132	L	200 nM	/	/	Amplification of DNA
Alkb-1f-Schulz2013	Biolegio 730980	L	200 nM	/	/	Amplification of DNA
Alkb-1r-Schulz2013	Biolegio 730981	L	200 nM	/	/	Amplification of DNA
alk-H1F-Chen2003	Biolegio 730984	L	200 nM	/	/	Amplification of DNA
alk-H3R-Chen2003	Biolegio 730985	L	200 nM	/	/	Amplification of DNA
NAH-F	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
NAH-R	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA

PHE-F	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
PHE-R	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
Yellow Template Dilution Buffer	Qiagen quantinova 1076702	L	1/100	/	/	Diluting of DNA to provide a color reaction when SYBR-Green is added, control of DNA is added in the well
SYBR-Green PCR mastermix	Qiagen quantinova 1076717	L		/	/	Fluorescent labeling of the DNA for measuring the quantity
Rox Reference Dye	Qiagen quantinova 1076698	L		/	/	Normalizing the fluorescent reporter signal
RNase free water	Qiagen quantinova 1076703	L		/	/	Diluting

2.18.2. Methods

1) Setting up standard curves

- Thaw the bacteria for each gene, send to be sequenced in the protocol 'Cloning reaction' and the compounds of the mastermix;
- Diluted them in Rnase free water until there are 10^8 molecules/µL;
- Make a series of dilution for each gene by diluting 5 µL of the 10^8 molecules/µL-bacteria in 45 µL RNase free water in a 8-wellstrip*;

*When, after the PCR, the standard curve is not optimal, make the series of dilution by diluting 5 µL of the 10^8 molecules/µL-bacteria in 20 µL RNase free water.

- Close the strip with the 8-wellcaps;
- Short-spin the strips and the compounds of the mastermix;
- There are five mastermixes needed for the screening, namely with the primers shown in table 10

Table 10: Primers used in the PCR

Gene/region	Primername	Primersequence
AlkB	Alkb-1f-Schulz2013 Alkb-1r-Schulz2013 alk-H1F-Chen2003 alk-H3R-Chen2003	AAYACNGCNCAYGARCTNGGNCAYAA GCITGITGITCISWRTGICGYTG CIGIICACGAIITIGGIC ACAAGAAGG IGCITGITGATCIIIGTGICGCTGIAG
Naphthalene dioxygenase	NAH-F NAH-R	CAAAARCACCTGATTYATGG AYRCGRGSGACTTCTTTCAA
Phenol-monooxygenase	PHE-F PHE-R	GTGCTGACSAAYCTGYTGTTC CGCCAGAACCAYTTRTC
16S	EUB 518 EUB 338	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG
ITS	EUK 345F EUK 499R	AAGGAAGGCAGCAGGCG CACCAGACTTGCCCTCYAAT

- Prepare the mastermix on ice and in microtubes following table 2 and using the formula $c_1 * V_1 = c_2 * V_2$;

Table 11: Mastermix

Components	Final concentration
ROX Dye	50 µM
SYBR-Green	5 mM
Forward-primer (100 µM)*	Depending on gene**
Reverse-primer (100 µM)*	Depending on gene**
RNase free water	Diluting until 8 µL

*For the AlkB-primers, the volume of primer that is calculated has to be divided in two. This because of the fact that two primers are used.

**For the AlkB-gene and 16S-region, the primer concentration need to be 400 µM. For the NAH- and PHE-gene, the primer concentration need to be 300 µM.

- Vortex the mastermix;
- Short-spin the mastermix;
- Divide 8 µL of mastermix over the 96-wellplate using the dispenser;
- Add 2 µL of the bacterial-DNA to the wells;
- Close the 96-wellplate with the seal sheet;
- Short-spin the plate;
- Run following PCR-program:

Cycling conditions	
Stage 1 (1 cycle)	900 s at 95°C
Stage 2 (40 cycles)	
1. Denaturation	15 s at 94°C
2. Annealing	30 s at 54°C
3. Elongation	30 s at 72 °C
Stage 3 (1 cycle)	15 s at 95°C 60 s at 60°C 15 s at 95°C 60 s at 60°C

- Store the PCR-products in the freezer on -20°C.

2) qPCR

- Thaw the bacteria that need to be screened and the compounds of the mastermix;
- Add 2 µL of the bacteria in 18 µL Yellow Template Dilution Buffer;
- Vortex the compounds of the mastermix and the diluted bacteria;
- Short-spin the compounds of the mastermix and the diluted bacteria;
- There are five mastermixes needed for the screening, namely with the primers shown in table 1
- Prepare the mastermix on ice and in microtubes following table 2 and using the formula $c_1*V_1 = c_2*V_2$;
- Vortex the mastermix;
- Short-spin the mastermix;
- Divide 8 µL of mastermix over the 96-wellplate using the dispenser;
- Add 0,8 µL of bacterial-DNA to the wells;
- Close the 96-wellplate with the seal sheet;
- Short-spin the plate;
- Run following PCR-program:

Cycling conditions	
Stage 1 (1 cycle)	900 s at 95°C
Stage 2 (40 cycles)	
1. Denaturation	15 s at 94°C
2. Annealing	30 s at 54°C
3. Elongation	30 s at 72 °C
Stage 3 (1 cycle)	15 s at 95°C 60 s at 60°C 15 s at 95°C 60 s at 60°C

- Store the PCR-products in the freezer on -20°C.

2.19. 284 selective medium

2.19.1. Materials

2.19.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Balance	VWR	Measuring substances
Autoclave	Ceron 500	Sterilize products
Incubator (60°C)	ALIS	Incubating at a certain temperature
Stirring plate	VWR	Stirring

2.19.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Spatula	/	Transferring substances
Volumetric flask	Fisherbrand	Making buffers
Tinfoil	ALU1145	Measuring substances
Autoclave bottle	Schott-Duran	Sterilize product
Autoclave tape	/	Control sterilize product
Stirring stick	/	Stirring

2.19.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Tris/HCl	VWR 0606C247	S		/	/	Control of pH
NaCl	VWR 27800.360	S		/	/	Osmotic balance
KCl	VWR 17B014108	S		/	/	Osmotic balance
NH_4Cl	Fluka 431065/1	S		/	/	Source for amino acid
Na_2SO_4	Merck 1.06649.1000	S		/	/	Osmotic balance
$MgCl_2.6H_2O$	Merck TA523733	S		/	/	Strengthen of the phosphodiester binding
$CaCl_2.2H_2O$	Fluka 133907323507 351	S		/	/	Promote uptake cells
$Na_2HPO_4.2$ H_2O	Merck 1.06580.1000	S		/	/	Source for phosphate molecules
SI7 spore elements	/	L		/	/	Food source
Fe(III)NH_4Ci trate	/	L	48mg/100mL	/	/	Source for amino acid and food source
Sodium lactate	Merck 1.06522.2500	L	50%	/	/	Osmotic balance

D-(+)-glucose	VWR 15G030015	S		/	/	Food source
D-gluconic acid sodium salt	Sigma BCBP6904V	S		/	/	Food source
D-(-)-fructose	Sigma SLBD 4215V	S		/	/	Food source
Sodium succinate.6 H_2O	Sigma BCBP6931V	S		/	/	Food source
Agar No.2 Bacteriological	LAB Q40798/063	S		/	/	Creating solid medium
Distilled water	/	L		/	/	Diluting

2.19.2. Methods

- Fill an appropriate flask with distilled water until about 3/4th of the volume. Add a stirring stick;
- Add the appropriate amount 284 and C-mix products* - like indicated in table 1, following 'Weighing' - and stir until all products are dissolved**;
- Fill the flask with distilled water until the bottle neck;
- Adjust the pH of the medium to 7 with HCl;
- Fill the flask till the mark with distilled water and let the medium stir thoroughly;
- Divide the medium over autoclavable bottles;
- Autoclave the medium for 10 min;
- Solid medium should be kept at 60°C after autoclaving or poured immediately.

*Isn't necessary when an alternative food source will be investigated.

**If solid medium is desired, add the appropriate amount of agar and a stirring stick in every bottle (for 1/10th strength rich medium, 100 mL rich medium and 900 mL distilled water, use also 20g of agar per liter of medium).

Table 12: Amount of substances per liter

284 **in g**	**1L**	**2L**	**3L**	**5L**	**10L**	**Concentration (g/L)**
Tris of HCl	6,06	12,12	18,18	30,3	60,6	6,06
NaCl	4,68	9,36	14,04	23,4	46,8	4,68
KCl	1,49	2,98	4,47	7,45	14,9	1,49
NH_4Cl	1,07	2,14	3,21	5,35	10,7	1,07
Na_2SO_4	0,43	0,86	1,29	2,15	4,3	0,43
$MgCl_2.6H_2O$	0,2	0,4	0,6	1	2	0,2
$CaCl_2.2H_2O$	0,03	0,06	0,09	0,15	0,3	0,03
$Na_2HPO_4.2H_2O$	0,04	0,08	0,12	0,2	0,4	0,04
SI7 spore elements	1mL	2mL	3mL	5mL	10mL	0,1%
Fe(III)NH_4Citrate sol. of 48mg/100mL	10mL	20mL	30mL	50mL	100mL	0,00048%
C-mix **in g**	**1L**	**2L**	**3L**	**5L**	**10L**	
Sodium lactate (sol. 50%)	0,7mL	1,4mL	2,1mL	3,5mL	7mL	0,07%
D-(+)-glucose	0,52	1,04	1,56	2,6	5,2	0,52
D-gluconic acid sodium salt	0,66	1,32	1,98	3,3	6,6	0,66
D-(-)-fructose	0,54	1,08	1,62	2,7	5,4	0,54
Sodium succinate.$6H_2O$	0,81	1,62	2,43	4,05	8,1	0,81
Agar No.2 Bacteriological	20					20

2.20. Cultivation of bacteria on aromatic compounds

2.20.1. Materials

2.20.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Shaking incubator (30°C)	Gerhardt	Incubating at a certain temperature
Micropipette (10 µL – 100 µL)	Eppendorf	Transferring fluids

2.20.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Falcon tube	Greiner bio-one E160939H	Storage of fluids
Test tube	/	Storage of fluids
Sterile disposable volumetric pipet (5 mL)	Greiner bio-one F1611347	Transferring fluids
Pipette tips (10 µL – 100 µL)	VWR 633C6	Transferring fluids
Pipetboy	Hirschmann Pipetus	Transferring fluids

2.20.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
284-media	/	L	See protocol '284 Selective media'	/	/	Solvent
Ethanol	Ensure 1.00983.1011	L	70%	H225-H319-H371 	P210-P260-P280-P308 + P311-P337 + P313-P403 + P235 	Disinfection

2.20.2. Methods

- Turn the flowcabinet on;
- Disinfect the flow with 70% ethanol;
- Turn on the Bunsen burner;
- Sterilizes test tubes in the autoclave for 10 minutes (only if many bacteria are screened);
- Take sterile falcon tubes and the sterilized test tubes;
- Pour 5 mL 284-media in the tubes;
- Add 10 µL of aromatic compounds to the tubes (the aromatic compounds used are toluene, n-xylene, ethylbenzene, benzene or diesel);
- Label the tubes with the bacteria and the aromatic compound added;
- Take the cultivated bacteria (see protocol 'Spread plate technique' and 'Cultivation of bacteria in masterblock');

Bacteria from the plates:

- Take the tweezer and scale it in the ethanol;
- Flame the tweezer using the Bunsen burner;
- Cool down the tweezer;

- Take a sterile toothpick using the tweezer;
- Pick up the targeted colony from the plate with the toothpick;
- Open the tube;
- Let the toothpick fall into tube;
- Close the tube;
- Repeat the last 8 steps until all bacteria are transferred;
- Scale the tweezer in the ethanol;
- Flame the tweezer using the Bunsen burner;
- Lay it down on the flow;
- Set the tubes on a shaking incubator at 30°C until a pellet is formed;

Bacteria from the masterblocks:

- Take 80 µL of the bacteriasuspension;
- Open the tube;
- Add the bacteriasuspension to the tube;
- Close the tube;
- Repeat the last 4 steps until all bacteria are transferred;
- Set the tubes on a shaking incubator at 30°C until a pellet is formed.

2.21. Polymerase Chain Reaction

2.21.1. Materials

2.21.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Fridge (-4°C)	Bosch	Storage of PCR-products
Micropipette (1 µL – 10 µL; 10 µL – 100 µL - 100 µL – 1000 µL)	Eppendorf	Transferring fluids
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Vortex	VWR Lab Dancer	Mixing
Dispenser	Eppendorf	Dividing of mastermix
PCR-appliance	Biorad C-1000 Touch	Amplification of DNA

2.21.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tips (100 µL – 1000 µL)	VWR 633C6	Transferring fluids
Microtube (1,5 mL)	Greiner bio-one E16063MX	Storage of fluids
96-wellplate	Biorad	Storage of fluids
Seal Sheet	Biorad	Closing 96-wellplate

2.21.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
27F	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
1492R	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
Alkb-1f-Schulz2013	Biolegio 730980	L	200 nM	/	/	Amplification of DNA
Alkb-1r-Schulz2013	Biolegio 730981	L	200 nM	/	/	Amplification of DNA
alk-H1F-Chen2003	Biolegio 730984	L	200 nM	/	/	Amplification of DNA
alk-H3R-Chen2003	Biolegio 730985	L	200 nM	/	/	Amplification of DNA
NAH-F	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
NAH-R	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
PHE-F	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
PHE-R	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
CATA-F	Biolegio 759065	L	200 nM	/	/	Amplification of DNA
CATA-R	Biolegio 759066	L	200 nM	/	/	Amplification of DNA
TOL-F	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA

TOL-R	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
TOD-F	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
TOD-R	Macrogen OG160211-003	L	200 nM	/	/	Amplification of DNA
BPH2-F	Biolegio 759067	L	200 nM	/	/	Amplification of DNA
BPH2-R	Biolegio 759068	L	200 nM	/	/	Amplification of DNA
Dreamtaq PCR buffer with MgCl	Thermo Scientific 00311377	L	10x	H314 – H335 	P261 - P280 - P305+P351+P338 - P310	Creating good circumstances for the amplification of DNA
dNTP-mix	Roche 11580220	L	10µM each	/	/	Building block for the amplification of DNA
Dreamtaq-polymerase	Thermo Scientific 00307520	L	5U/µL	/	/	Amplification of DNA
RNase free water	Omega Bio-Tek NFWD011216JC 1856	L		/	/	Diluting

2.21.2. Methods

- Thaw the bacteria that need to be screened and the compounds of the mastermix;
- Vortex the compounds of the mastermix;
- Short-spin the compounds of the mastermix;
- There are four mastermixes needed for the screening, namely with the primers shown in table 14;

Table 13: Primers used in the PCR

Gene	Primername	Primersequence
AlkB	Alkb-1f-Schulz2013 Alkb-1r-Schulz2013 alk-H1F-Chen2003 alk-H3R-Chen2003	AAYACNGCNCAYGARCTNGGNCAYAA GCITGITGITCISWRTGICGYTG CIGIICACGAIITIGGIC ACAAGAAGG IGCITGITGATCIIIGTGICGCTGIAG
Naphthalene dioxygenase	NAH-F NAH-R	CAAAARCACCTGATTYATGG AYRCGRGSGACTTCTTTCAA
Phenol-monooxygenase	PHE-F PHE-R	GTGCTGACSAAYCTGYTGTTC CGCCAGAACCAYTTRTC
Xylene monooxygenase	TOL-F TOL-R	TGAGGCTGAAACTTTACGTAGA CTCACCTGGAGTTGCGTAC
Toluene dioxygenase	TOD-F TOD-R	ACCGATGARGAYCTGTACC CTTCGGTCMAGTAGCTGGTG
Catechol dioxygenase	CATA-F CATA-R	ACVCCVCGHACCATYGAAGG CGSGTNGCAWANGCAAAGT
Biphenyl dioxygenase	BPH2-F BPH2-R	GACGCCCGCCCCTATATGGA AGCCGACGTTGCCAGGAAAAT
16S	27F 1492R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT

- Prepare the mastermix on ice and in microtubes following table 2 and using the formula $c_1*V_1 = c_2*V_2$;

Table 14: Mastermix

Components	Final concentration
PCR buffer with MgCl (10x)	1x
dNTP-mix (10µM each)	200 nM
Forward-primer (10µM)*	200 nM
Reverse-primer (10µM)*	200 nM
Dreamtaq-polymerase (5U/µL)	0,25 U
RNase free water	Diluting until 24 µL

*For the AlkB-primers, the volume of primer that is calculated has to be divided in two. This because of the fact that two primers are used.

- Vortex the samples and the mastermix;
- Short-spin the samples and the mastermix;
- Divide 24,2 µL of mastermix over the 96-wellplate using the dispenser;
- Add 0,8 µL of bacterial-DNA to the wells;
- Close the 96-wellplate with 12-capstrips;
- Short-spin the plate;
- Run following PCR-program:

Cycling conditions	
Initial denaturation	120 s at 95°C
35 cycles:	
1. Denaturation	60 s at 95°C
2. Annealing	30 s at 54°C
3. Elongation	120 s at 72 °C
Final extension	300 s at 72°C

- Store the PCR-products in the fridge on 4°C.

2.22. QIAquick Gel Extraction kit

2.22.1. Materials

2.22.1.1. Overview used equipment

Hardware	Brand and type	Purpose
Centrifuge	Thermo Scientific SL40R	Spinning of fluids
Micropipette (10 µL – 100 µL; 100 µL – 1000 µL)	Eppendorf	Transferring of fluids
Vortex	VWR Lab Dance	Mixing
Thermomixer		Shaking and heating

2.22.1.2. Overview used glassware and disposables

Hardware	Brand and type	Purpose
Pipette tips (10 µL – 100 µL; 100 µL – 1000 µL)	VWR 633C6	Transferring of fluids
Microtubes (1,5 mL)	Greiner bio-one E160939H	Storage of fluids

2.22.1.3. Overview used fluids and solutions

Name	Supplier and batchnumber	Solid or liquid	Components	Risk's H-phrases	Safety measures P-phrases	Purpose
Buffer QG	Qiagen 1014876	L	pH < 7,5	H302 - H318 - H402	P280 - P305 + P351 + P338 - P308 + P311 	Removing agarosegel
Isopropanol	Sigma-Aldrich STBF7471V	L		H225-H319-H336 	P210-P261-P305 + P351 + P338	Creating good circumstances for binding DNA on the column
Buffer PE	Qiagen 151038994	L		/	/	Washing of DNA
Buffer EB	Qiagen 1014608	L		/	/	Elution of DNA

2.22.2. Methods

- Wheigh the gel slice;
- Add 3 volumes Buffer QG to 1 volume of gel;
- Vortex;
- Incubate at 50°C for 10 minutes;
- Vortex every 2 minutes;
- Add 1 volume of isopropanol;
- Vortex;
- Place a QIAquick column in a provided 2 mL collection tube;
- Apply the sample to the QIAquick column;
- Centrifuge for 1 minute;
- Discard flow-through;
- Place the QIAquick column back in the same tube;

- Add 500 µL of QG to the QIAquick column;
- Centrifuge for 1 minute;
- Discard flow-through;
- Add 750 µL Buffer PE to the QIAquick column;
- Centrifuge for 1 minute;
- Discard flow-through;
- Place the QIAquick column back in the same tube;
- Centrifuge the QIAquick column once more in the provided 2 mL collection tube for 1 minute;
- Place each QIAquick column in a clean 1,5 mL microcentrifuge tube;
- Add 50 µL buffer EB to the center of the QIAquick membrane;
- Centrifuge the column for 1 minute
- Discard the column.

2.23 Custom Blast

1. Jon has assembled the Ion Torrent sequencing reads in contigs using Spades;
2. In the drive folder of your genome: search the assembled file, named contigs.fasta;
3. Open the contigs.fasta file in Geneious (program on drive, and on common laptop); Remove the sequences with coverage of 1, and < e.g. 500 bps (or sometimes we also remove contigs < 1000 bp);
4. Save the new file in fasta-format like contigs1000.fasta (this is multi-fasta format, note that for BRIG you need single fasta);
5. To get an idea of the most closely related strains: Blast the 16S rRNA sanger sequence. Additionally blast several other contigs of the contigs.fasta file in NCBI: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch. Copy paste, or write down the closest matches (the accession nr.);
6. GGDC: Determine the best reference genome using GGDC (http://ggdc.dsmz.de/distcalc2.php). Load in your fasta file (contigs1000.fasta), and choose the reference genomes, paste here all the accession nrs. of the closest matches from step 5;
7. Results of GGDC will be sent by e-mail. Save the results: look in formula 2, if the DDH interval > 70 % (same species), and > 79 % (same subspecies). Based on the 16S rRNA gene blast and GGDC you know the most closely related species;
8. RAST: You can now annotate the genome in RAST. Make an account in RAST and upload your genome: http://rast.nmpdr.org/rast.cgi. Note, you need to give the closest reference genome (this you have found in step 7). Finish the upload steps. Look in RAST again at the closest neighbours. Now you are more confident which is the closest reference species;
9. NCBI-WGA submission: Now you are ready to upload the contigs1000.fasta to NCBI WGS submissions: https://submit.ncbi.nlm.nih.gov/subs/wgs/ to get your accession number (note: if you are student, let your supervisor do it);
10. Genoscope: Next thing to do is to upload your genome to Genoscope which annotates the genome and nicely represents it in pathways. Create an account in Genoscope (if you are student, only your supervisor) https://www.genoscope.cns.fr/agc/microscope/ login, and upload your genomes to be annotated + put in pathways (6 weeks waiting time, 10 genomes at same time maximum);
11. Having uploaded your genome to RAST, you can start to annotate/find pathway genes in your strain: Easy is to blast single queries in RAST: click browse genome in server > comparative tools > blast;
12. When you have the NCBI results and RAST results then in both cases download the genbank file (GFF-format):
    a. For NCBI, find the genbank file in your genome record, see figure below);
    b. In RAST click Organism > export, click RAST job detail page for …. (see figure below);
    c. Open the NCBI or RAST genbank file (gb) in Geneious > import as single files when Geneious asks for, concatenate all files, click annotation, export as csv (see figure below);
13. Now you can do quick searches in the excel files for e.g. IAA-pathway genes present, metal transporters etc… this is sufficient for a genome announcement paper.

3. Standard curves

To analyze the copies a qPCR, standard curves are needed. These are represent below for each gene.

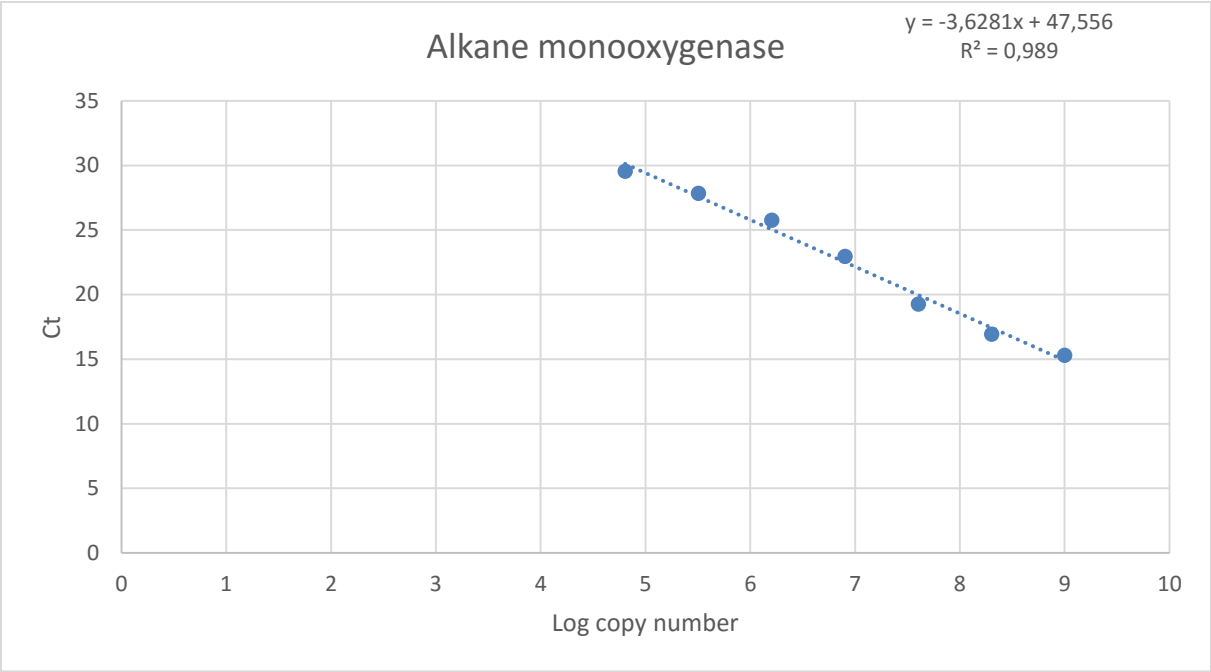


Figure 4a: Standard curve for the alkane monooxygenase gene

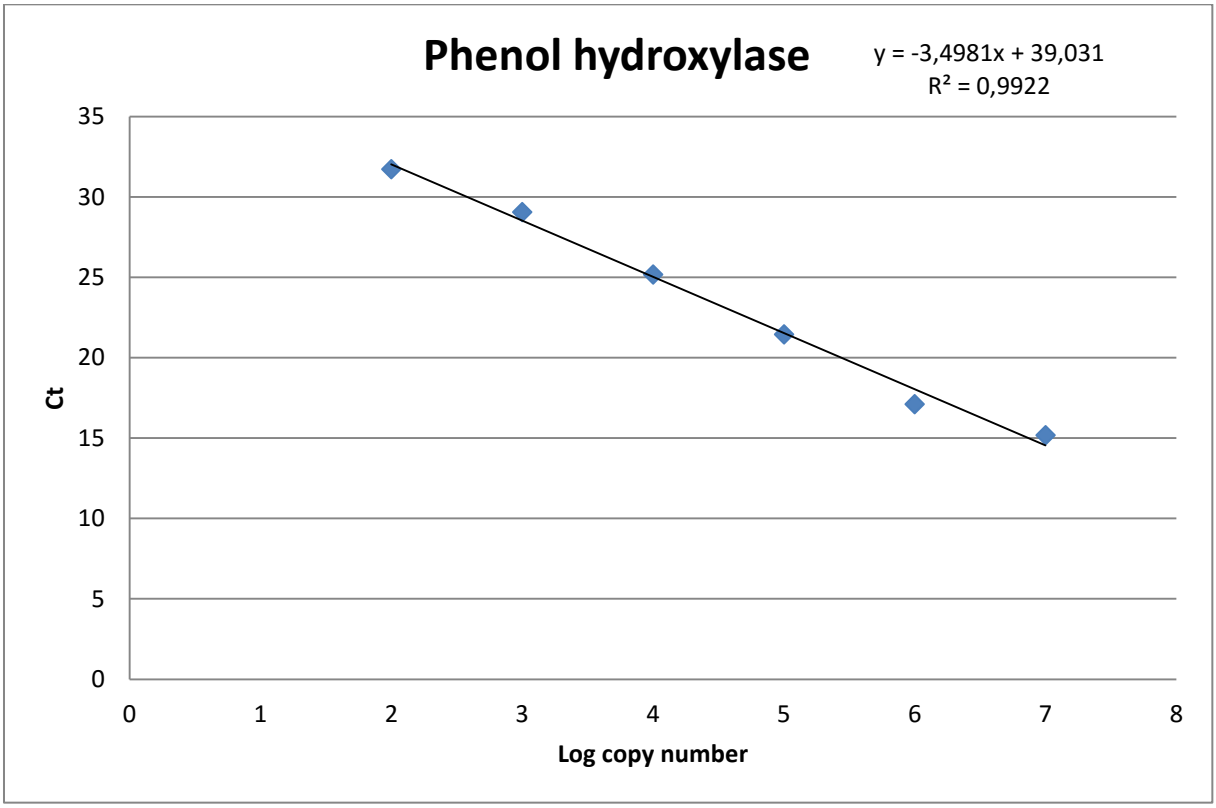


Figure 4b: Standard curve for the phenol hydroxylase gene

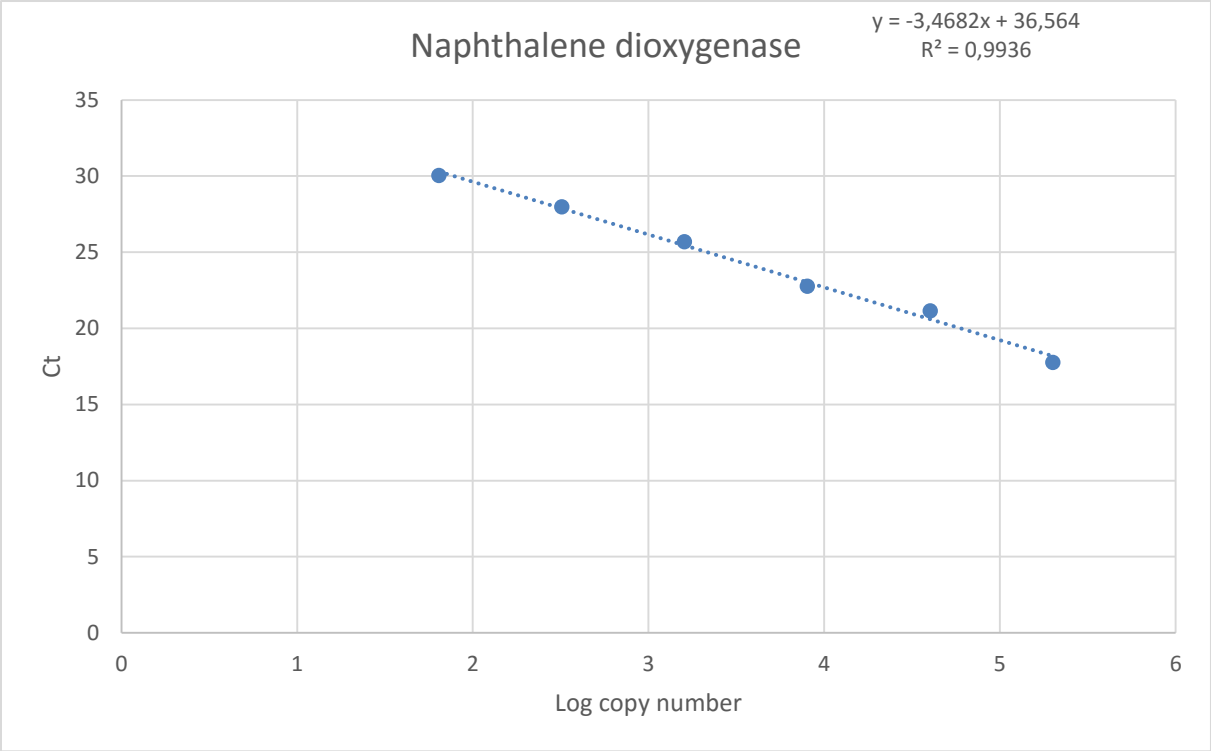


Figure 4c: Standard curve for the naphthalene dioxygenase gene

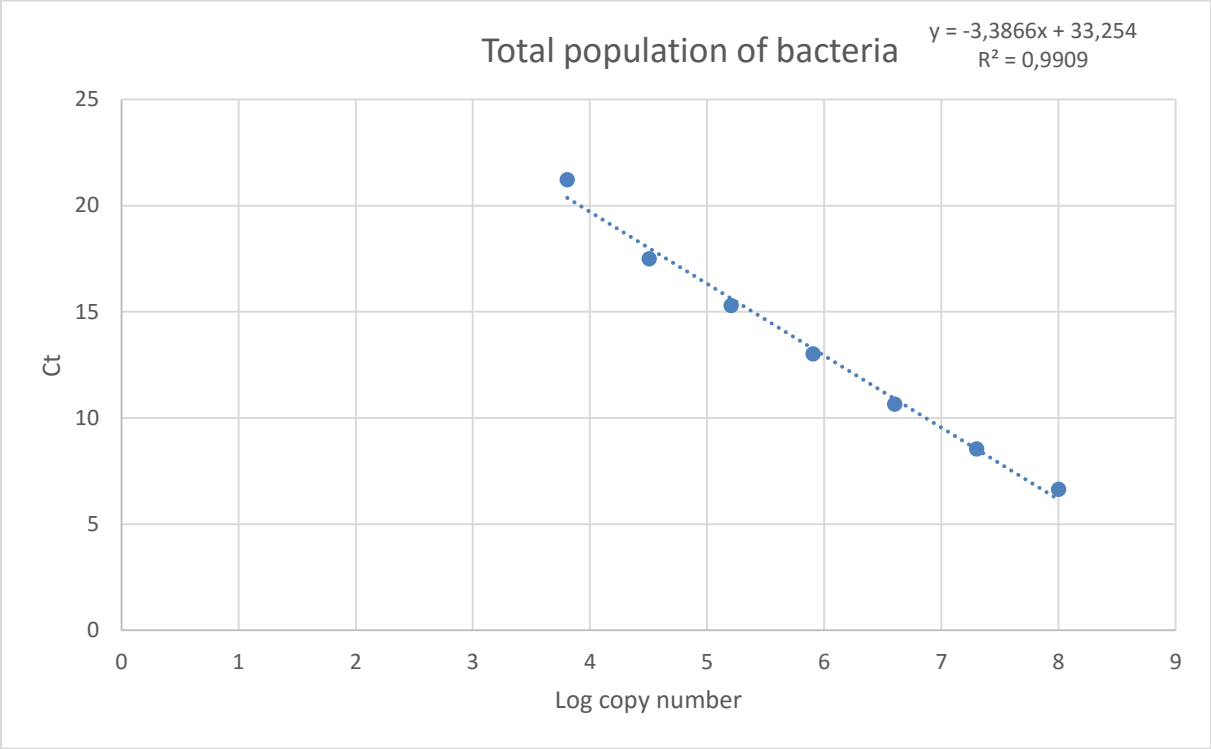


Figure 4d: Standard curve for the total population of bacteria

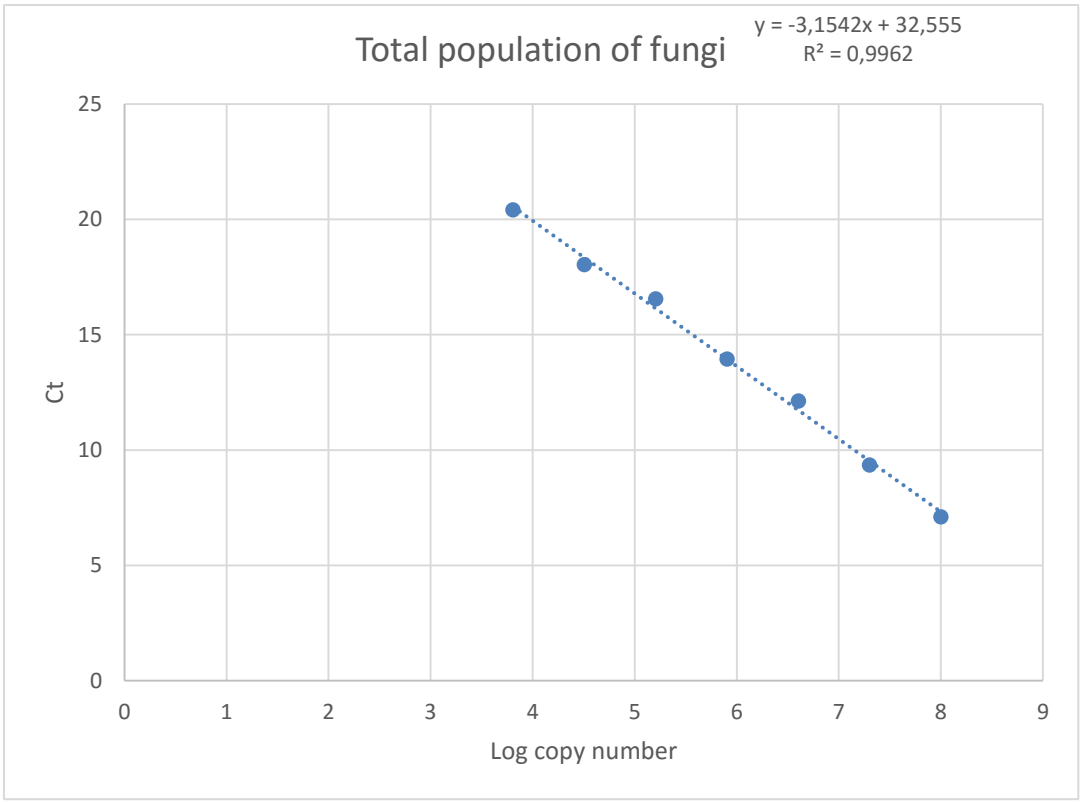


Figure 4e: Standard curve for the total population of fungi

4. Results cloning reaction

4.1. Gradient-PCR

On this gel, the results of the gradient-PCR for AlkB are represent. This to determine the optimum annealingtemperature.

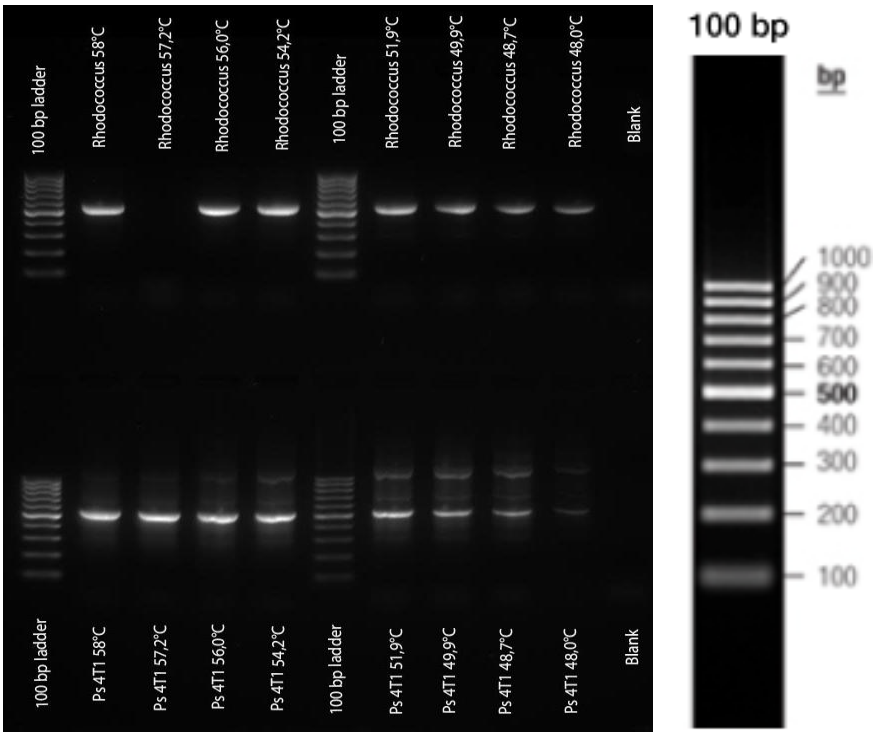


Figure 5: Gradiënt-PCR for the alkane monooxygenase gene

4.2. Restriction-analysis

To results of the restriction-analysis, to find out if the genes are correctly ligated in the vector, are represent in the gel below.

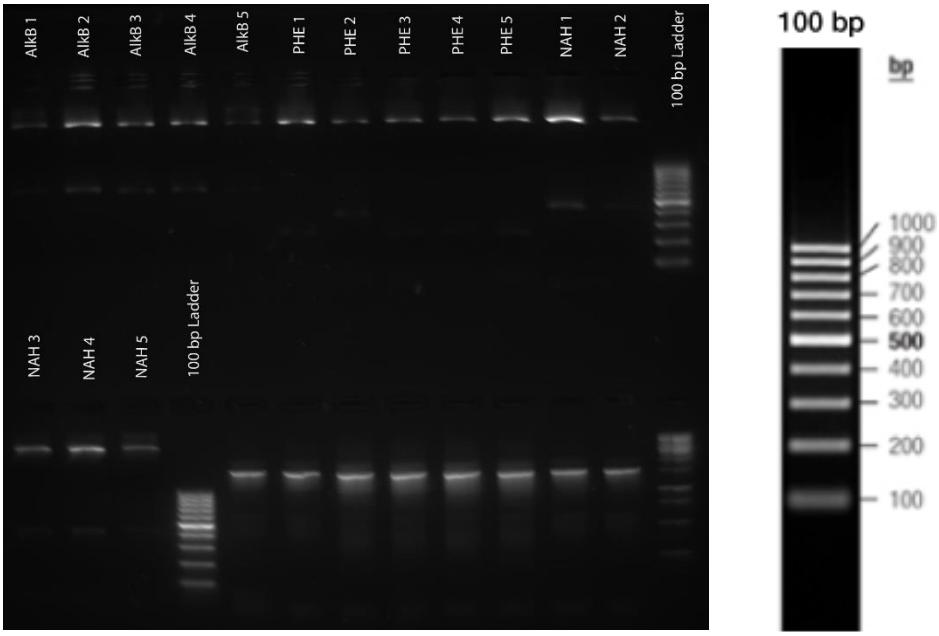


Figure 6: Restriction-analysis of the alkane-monooxgenase-, phenol hydroxylase- and naphthalene dioxygenase gene

5. Results Quantitative Polymerase Chain Reaction

5.1. Raw data of phyllosphere bacteria on epiphytes in Bialowiesa, Bobrka and Warsaw

AlkB	PHE	NAH						
Sample	**Amplicon**	**Ct**	**Log copies/µL 1/10 diluted**	**Log copies/µL**	**Log copies/100µL**	**Mass Leaves**	**Log copies/g**	**Log copies/mg**
epBi11	-	34,89	3,49	34,91	3491,41	0,54	6465,58	6,47
epBi51	-	32,28	4,21	42,11	4210,96	1,35	3119,23	3,12
epBo24	+	34,25	3,67	36,67	3666,85	1,19	3081,39	3,08
epWa21	+	30,22	4,78	47,78	4778,01	2,53	1888,54	1,89
epBi11	+	29,69	2,40	23,97	2396,55	0,54	4438,06	4,44
epBi21	+	30,90	2,06	20,61	2061,19	1,17	1761,70	1,76
epBi22	+	31,06	2,02	20,18	2018,31	0,68	2968,10	2,97
epBi31	+	30,50	2,17	21,71	2170,81	0,83	2615,43	2,62
epBi11			10,54	105,43	10542,64	0,54	19523,42	19,52
epBi51			10,54	105,43	10542,64	1,35	7809,37	7,81
epBo24			10,54	105,43	10542,64	1,19	8859,37	8,86
epWa21			10,54	105,43	10542,64	2,53	4167,05	4,17
epBi21	-	32,98	4,02	40,19	4018,72	1,17	3434,80	3,43
epBi53	-	29,95	4,85	48,51	4851,41	0,72	6738,07	6,74
epBo31	-	32,92	4,03	40,34	4034,04	0,79	5106,38	5,11
epWa22	+	30,97	4,57	45,72	4572,42	1,84	2485,01	2,49
epBi32	+	30,52	2,17	21,67	2166,99	0,85	2549,40	2,55
epBi42	+	29,16	2,54	25,42	2541,73	1,25	2033,38	2,03
epBi51	+	28,58	2,70	27,02	2701,80	1,35	2001,34	2,00
epBi53	+	28,03	2,85	28,55	2854,69	0,72	3964,85	3,96
epBi21			10,54	105,43	10542,64	1,17	9010,81	9,01
epBi53			10,54	105,43	10542,64	0,72	14642,56	14,64
epBo31			10,54	105,43	10542,64	0,79	13345,12	13,35
epWa22	-	32,78	1,09	10,92	1092,41	1,84	593,70	0,59
epBi22	-	33,35	3,91	39,14	3914,31	0,68	5756,33	5,76
epBo11	+	36,32	3,10	30,98	3098,32	1,44	2151,61	2,15
epBo32	+	33,92	3,76	37,59	3759,41	0,74	5080,28	5,08
epWa24	+	30,06	4,82	48,22	4822,06	2,53	1905,95	1,91
epBo11	+	30,70	2,12	21,17	2116,58	1,44	1469,85	1,47
epBo12	+	30,78	2,09	20,95	2094,65	1,6	1309,16	1,31
epBo13	+	31,08	2,01	20,13	2012,58	1,16	1734,98	1,73
epBo14	+	31,17	1,99	19,86	1985,93	1,34	1482,03	1,48
epBi22			10,54	105,43	10542,64	0,68	15503,89	15,50
epBo11			10,54	105,43	10542,64	1,44	7321,28	7,32
epBo32			10,54	105,43	10542,64	0,74	14246,82	14,25
epWa24			10,54	105,43	10542,64	2,53	4167,05	4,17
epBi31	-	30,50	4,70	47,01	4701,36	0,83	5664,29	5,66

epBo12	+	35,08	3,44	34,39	3438,60	1,6	2149,13	2,15
epBo33	+	36,32	3,10	30,98	3097,52	0,78	3971,18	3,97
epWa31	-	33,93	3,76	37,57	3757,04	1,17	3211,14	3,21
epBo21	+	30,47	2,18	21,79	2178,77	0,89	2448,05	2,45
epBo24	+	30,58	2,15	21,50	2149,62	1,19	1806,41	1,81
epBo31	+	30,47	2,18	21,79	2178,77	0,79	2757,94	2,76
epBo32	+	31,18	1,98	19,83	1983,02	0,74	2679,76	2,68
epBi31			10,54	105,43	10542,64	0,83	12701,98	12,70
epBo12			10,54	105,43	10542,64	1,6	6589,15	6,59
epBo33			10,54	105,43	10542,64	0,78	13516,21	13,52
epWa31			10,54	105,43	10542,64	1,17	9010,81	9,01
epBi32	-	32,69	4,10	40,98	4098,40	0,85	4821,65	4,82
epBo13	-	39,41	2,25	22,45	2245,42	1,16	1935,70	1,94
epWa11	+	34,82	3,51	35,10	3510,07	0,09	39000,82	39,00
epBo33	+	31,29	1,95	19,52	1952,41	0,78	2503,09	2,50
epWa11	+	31,11	2,00	20,04	2003,59	0,09	22262,16	22,26
epWa12	+	31,10	2,01	20,05	2005,36	1,12	1790,50	1,79
epWa13	+	28,16	2,82	28,18	2817,77	1,33	2118,63	2,12
epBi32			10,54	105,43	10542,64	0,85	12403,11	12,40
epBo13			10,54	105,43	10542,64	1,16	9088,49	9,09
epWa11			10,54	105,43	10542,64	0,09	117140,50	117,14
epWa33			10,54	105,43	10542,64	1,42	7424,40	7,42
epBi41	-	33,95	3,75	37,50	3750,48	0,96	3906,75	3,91
epBo14	+	36,00	3,19	31,86	3185,58	1,34	2377,30	2,38
epWa12	+	35,23	3,40	33,97	3397,32	1,12	3033,32	3,03
epBi41	-	30,61	2,14	21,40	2140,33	0,96	2229,51	2,23
epWa14	+	31,01	2,03	20,30	2030,20	1	2030,20	2,03
epWa21	+	29,29	2,51	25,07	2506,75	2,53	990,81	0,99
epWa22	+	24,44	3,85	38,49	3848,63	1,84	2091,65	2,09
epBi41			10,54	105,43	10542,64	0,96	10981,92	10,98
epBo14			10,54	105,43	10542,64	1,34	7867,65	7,87
epWa12			10,54	105,43	10542,64	1,12	9413,08	9,41
epWa34			10,54	105,43	10542,64	1,31	8047,82	8,05
epBi42	-	30,78	4,62	46,23	4623,08	1,25	3698,46	3,70
epBo21	-	36,34	3,09	30,92	3091,76	0,89	3473,88	3,47
epWa13	+	34,13	3,70	37,01	3700,72	1,33	2782,50	2,78
epWa24	+	29,79	2,37	23,69	2369,10	2,53	936,40	0,94
epWa31	+	30,99	2,04	20,36	2035,81	1,17	1740,01	1,74
epWa33	+	31,02	2,03	20,29	2028,90	1,42	1428,80	1,43
epWa34	+	30,04	2,30	22,99	2299,00	1,31	1754,96	1,75
epBi42			10,54	105,43	10542,64	1,25	8434,12	8,43
epBo21			10,54	105,43	10542,64	0,89	11845,67	11,85
epWa13		37,19	-0,18	-1,81	-180,53	1,33	-135,73	-0,14
epWa41		37,99	-0,41	-4,13	-412,52	1,51	-273,19	-0,27
epBi43	+	32,15	4,25	42,47	4246,80	0,89	4771,68	4,77

epBo23	+	35,89	3,22	32,16	3216,23	0,3	10720,76	10,72
epWa14	+	34,06	3,72	37,20	3720,16	1	3720,16	3,72
epBi43	-	30,07	2,29	22,90	2290,26	0,89	2573,33	2,57
epBo23	-	30,82	2,08	20,83	2083,37	0,3	6944,57	6,94
epWa41	+	31,15	1,99	19,93	1993,20	1,51	1320,00	1,32
NTC	+	30,53	2,16	21,64	2164,20	0	#DEEL/0!	#DEEL/0!
epBi43			10,54	105,43	10542,64	0,89	11845,67	11,85
epBo23			10,54	105,43	10542,64	0,3	35142,15	35,14
epWa14			10,54	105,43	10542,64	1	10542,64	10,54
NTC			10,54	105,43	10542,64	0	#DEEL/0!	#DEEL/0!

5.2. Statistical analysis of phyllosphere bacteria on epiphytes in Bialowiesa, Bobrka and Warsaw

5.2.1. Alkane monooxygenase gene

In this statistical analysis, an ANOVA is performed to determine a significant difference of the expression of alkane monooxygenase between epiphytes. These epiphytes were planted on the nature reserve Bialowiesa, the oil-polluted site Bobrka and the city of Warsaw.

One Way Analysis of Variance woensdag, mei 31, 2017, 8:43:57

Data source: Data 1 in 010317 juist

Normality Test: Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks woensdag, mei 31, 2017, 8:43:57

Data source: Data 1 in 010317 juist

Group	N	Missing	Median	25%	75%
Epiphytes Bialowiesa	3	2	4,772	4,772	4,772
Epiphytes Bobrka	9	2	3,081	2,208	4,803
Epiphytes Warsaw	9	2	2,782	2,051	3,548

H = 1,214 with 2 degrees of freedom. (P = 0,545)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,545)

5.2.2. Phenol hydroxylase gene

An ANOVA is performed to determine if there is a significant difference between the expression of phenol hydroxylase in epiphytes. These epiphytes were planted on the nature reserve Bialowiesa, the oil-polluted site Bobrka and the city of Warsaw.

One Way Analysis of Variance woensdag, mei 31, 2017, 8:45:14

Data source: Data 1 in 010317 juist

Normality Test: Passed (P = 0,547)

Equal Variance Test: Passed (P = 0,482)

Group Name	N	Missing	Mean	Std Dev	SEM
Epiphytes Bialowiesa	8	2	2,934	1,025	0,419
Epiphytes Bobrka	4	2	2,603	0,219	0,155
Epiphytes Warsaw	7	2	1,578	0,580	0,259

Source of Variation	DF	SS	MS	F	P
Between Groups	2	5,147	2,573	3,872	0,057
Residual	10	6,647	0,665		
Total	12	11,794			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,057).

Power of performed test with alpha = 0,050: 0,431

The power of the performed test (0,431) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

5.3. Raw data of phyllosphere bacteria on ivy in Hasselt and De Maten

5.3.1. Alkane monooxygenase- and phenol hydroxylase gene

AlkB	PHE							
Sample	Amplicon	Ct	Log copies/µl 1/10 diluted	Log copies/µl	Log copies/100µL	Mass Leaves	Log copies/g	Log copies/mg
CW 1.1	-	34,08	3,72	37,15	3715,44	1,60	2322,15	2,32
CW 3.3	-	32,04	4,28	42,77	4276,67	1,36	3144,61	3,14
C+	+	30,18	4,79	47,89	4789,31	1,48	3226,86	3,23
PW 3.2	-	31,99	4,29	42,89	4289,35	2,98	1439,38	1,44
A+	-	30,70	4,65	46,47	4646,73	0,97	4777,64	4,78
CW 1.1	-	30,21	2,52	25,23	2522,94	1,60	1576,84	1,58
A+	+	28,92	2,89	28,90	2889,54	0,97	2970,94	2,97
B+	+	29,96	2,59	25,93	2592,95	1,30	2000,27	2,00
PW 3.2	-	30,02	2,58	25,75	2575,03	2,98	864,10	0,86
C+	+	30,38	2,47	24,72	2472,14	1,48	1665,64	1,67
CW 1.2	-	32,51	4,15	41,47	4146,77	1,34	3094,61	3,09
CW 4.1	-	32,52	4,14	41,44	4143,71	2,17	1909,54	1,91
PW 1.1	-	32,67	4,10	41,03	4103,33	3,70	1109,01	1,11
CW 2.3	+	33,17	3,96	39,64	3964,31	1,59	2493,27	2,49
B+	-	32,67	4,10	41,03	4103,30	1,30	3165,40	3,17
CW 1.2	+	30,85	2,34	23,38	2337,90	1,34	1744,70	1,74
CW 4.1	-	31,17	2,25	22,48	2248,39	2,17	1036,13	1,04
CW 1.3	+	30,41	2,46	24,65	2464,60	2,00	1232,30	1,23
CW 2.1	+	29,10	2,84	28,40	2839,54	3,11	913,04	0,91
CW 2.2	+	29,81	2,64	26,36	2635,86	2,35	1121,64	1,12
CW 1.3	-	33,98	3,74	37,42	3741,79	2,00	1870,90	1,87
CW 4.2	-	33,33	3,92	39,21	3921,47	1,77	2215,52	2,22
PW 1.2	-	38,37	2,53	25,33	2532,59	3,27	775,68	0,78
PW 4.1	-	33,27	3,94	39,37	3937,05	2,15	1831,18	1,83
NTC	-	36,84	2,95	29,55	2954,52	0,00	#DEEL/0!	#DEEL/0!
CW 2.3	+	26,58	3,56	35,60	3559,70	1,59	2238,81	2,24
CW 4.2	-	30,71	2,38	23,78	2378,15	1,77	1343,59	1,34
CW 3.1	+	19,10	5,70	56,99	5699,01	2,22	2567,12	2,57
PW 4.1	-	31,19	2,24	22,43	2242,85	2,15	1043,18	1,04
NTC	-	32,99	1,73	17,27	1727,42	0,00	#DEEL/0!	#DEEL/0!
CW 2.1	-	32,41	4,17	41,74	4173,92	3,11	1342,10	1,34
CW 3.1	+	28,52	5,25	52,47	5247,48	2,22	2363,73	2,36
PW 1.3	-	33,51	3,87	38,70	3870,15	2,83	1367,55	1,37
CW 3.2	+	32,03	4,28	42,80	4280,48	1,79	2391,33	2,39
CW 3.2	+	30,46	2,45	24,52	2451,56	1,79	1369,59	1,37
CW 4.3	-	30,84	2,34	23,41	2340,70	2,34	1000,30	1,00
PW 1.3	-	30,52	2,43	24,33	2432,78	2,83	859,64	0,86
CW 3.3	+	24,40	4,18	41,81	4181,18	1,36	3074,40	3,07

CW 2.2	-	31,02	4,56	45,57	4556,79	2,35	1939,06	1,94
CW 5.1	-	33,47	3,88	38,82	3881,67	1,57	2472,40	2,47
CW 4.3	+	30,26	4,77	47,67	4766,85	2,34	2037,11	2,04
CW 5.2	+	33,61	3,85	38,45	3845,15	2,10	1831,03	1,83
CW 5.1	+	30,05	2,57	25,69	2568,82	1,57	1636,19	1,64
CW 5.2	+	30,13	2,55	25,45	2545,12	2,10	1211,96	1,21
CW 5.3	+	28,47	3,02	30,19	3018,72	1,84	1645,08	1,65
D+	+	29,79	2,64	26,40	2640,32	1,39	1904,44	1,90
D+	+	29,81	4,89	48,90	4890,16	1,39	3527,24	3,53
PW 2.1	+	34,26	3,67	36,66	3666,08	2,63	1393,95	1,39
PW 2.2	-	31,26	4,49	44,91	4490,56	5,98	750,93	0,75
PW 5.1	-	34,61	3,57	35,68	3567,79	3,76	948,88	0,95
PW 1.1	+	30,96	2,31	23,08	2307,94	3,70	623,77	0,62
PW 1.2	+	30,56	2,42	24,23	2422,66	3,27	742,01	0,74
PW 2.1	+	30,37	2,48	24,77	2477,12	2,63	941,87	0,94
PW 2.2	+	29,94	2,60	25,98	2598,27	5,98	434,49	0,43
PW 2.3	+	31,07	4,54	45,43	4543,48	4,51	1007,42	1,01
CW 5.3	-	34,41	3,62	36,24	3623,83	1,84	1974,84	1,97
PW 3.3	+	33,19	3,96	39,60	3960,45	2,91	1360,98	1,36
PW 5.2	-	33,90	3,77	37,65	3765,08	2,77	1359,24	1,36
PW 2.3	+	28,74	2,94	29,41	2941,43	4,51	652,20	0,65
PW 3.1	+	31,34	2,20	21,98	2198,19	2,88	763,26	0,76
PW 3.3	+	30,14	2,54	25,41	2540,78	2,91	873,12	0,87
PW 4.2	+	31,09	2,27	22,70	2270,03	2,93	774,76	0,77
PW 4.2	+	33,37	3,91	39,09	3909,32	2,93	1334,24	1,33
PW 4.3	+	33,49	3,88	38,76	3876,13	1,57	2468,87	2,47
PW 3.1	-	32,64	4,11	41,11	4110,64	2,88	1427,30	1,43
PW 5.3	-	35,53	3,31	33,15	3314,88	1,31	2530,44	2,53
PW 4.3	+	31,72	2,09	20,90	2089,51	1,57	1330,90	1,33
PW 5.1	+	31,13	2,26	22,60	2260,00	3,76	601,06	0,60
PW 5.2	+	31,12	2,26	22,62	2262,43	2,77	816,76	0,82
PW 5.3	-	31,54	2,14	21,43	2142,59	1,31	1635,57	1,64

5.3.2. Naphthalene dioxygenase gene and total population of bacteria

NAH	Bacteria							
Sample	Amplicon	Ct	Log copies/µl 1/10 diluted	Log copies/µl	Log copies/100µL	Mass Leaves	Log copies/g	Log copies/mg
CW 1.1			9,82	98,19	9819,29	1,60	6137,05	6,14
CW 3.3			9,82	98,19	9819,29	1,36	7220,06	7,22
D+	-	39,51	-1,85	-18,47	-1847,34	1,39	-1332,47	-1,33
PW 3.2	-	35,26	-0,59	-5,91	-591,04	2,98	-198,33	-0,20
A+	+	32,51	0,22	2,21	220,84	0,97	227,06	0,23
A+	+	17,10	5,61	56,13	5612,94	0,97	5771,07	5,77
B+	+	17,73	5,43	54,32	5431,64	1,30	4190,11	4,19
C+	+	17,47	5,51	55,06	5506,31	1,48	3709,95	3,71
CW 1.1	+	18,19	5,30	52,97	5296,52	1,60	3310,33	3,31
CW 1.2	+	18,76	5,13	51,34	5134,10	1,34	3831,42	3,83
CW 1.2			9,82	98,19	9819,29	1,34	7327,83	7,33
CW 4.1	-	37,44	-1,24	-12,35	-1235,40	2,17	-569,31	-0,57
PW 1.1			9,82	98,19	9819,29	3,70	2653,86	2,65
PW 3.3			9,82	98,19	9819,29	2,91	3374,33	3,37
B+	-	38,87	-1,66	-16,58	-1657,53	1,30	-1278,66	-1,28
CW 1.3	+	18,03	5,34	53,43	5342,83	2,00	2671,41	2,67
CW 2.1	+	17,63	5,46	54,59	5458,97	3,11	1755,30	1,76
CW 2.2	+	16,74	5,72	57,17	5716,74	2,35	2432,66	2,43
CW 2.3	+	18,32	5,26	52,59	5259,44	1,59	3307,83	3,31
CW 3.1	+	15,71	6,01	60,12	6012,08	2,22	2708,14	2,71
CW 1.3	-		9,82	98,19	9819,29	2,00	4909,64	4,91
CW 4.2	-		9,82	98,19	9819,29	1,77	5547,62	5,55
PW 1.2	-	39,12	-1,73	-17,32	-1732,15	3,27	-530,52	-0,53
PW 4.1	-	34,11	-0,25	-2,54	-253,91	2,15	-118,10	-0,12
NTC	-		9,82	98,19	9819,29	24548,22	0,40	0,00
CW 3.2	+	18,82	5,12	51,18	5117,55	1,79	2858,97	2,86
CW 3.3	+	17,39	5,53	55,28	5527,80	1,36	4064,56	4,06
CW 4.1	+	17,88	5,39	53,87	5386,68	2,17	2482,34	2,48
CW 4.2	+	18,49	5,21	52,11	5210,97	1,77	2944,05	2,94
CW 4.3	+	18,55	5,19	51,93	5192,92	2,34	2219,20	2,22
CW 2.1	-	37,76	-1,33	-13,31	-1331,28	3,11	-428,06	-0,43
CW 4.3	-		9,82	98,19	9819,29	2,34	4196,28	4,20
PW 1.3	-	37,28	-1,19	-11,88	-1187,95	2,83	-419,77	-0,42
CW 2.2	+	31,34	0,57	5,67	566,65	2,35	241,13	0,24
CW 5.1	+	18,56	5,19	51,91	5190,93	1,57	3306,33	3,31
CW 5.2	+	18,36	5,25	52,48	5247,59	2,10	2498,85	2,50
CW 5.3	+	17,84	5,40	53,99	5398,62	1,84	2942,03	2,94
D+	+	16,94	5,66	56,58	5657,63	1,39	4080,81	4,08
CW 3.1	+	35,52	-0,67	-6,70	-670,38	2,22	-301,97	-0,30
CW 5.1	-	40,00	-1,99	-19,91	-1991,02	1,57	-1268,17	-1,27

PW 2.1			9,82	98,19	9819,29	2,63	3733,57	3,73
PW 4.3	-	39,73	-1,91	-19,11	-1911,03	1,57	-1217,22	-1,22
NTC	+	26,12	3,01	30,12	3012,34	7530,85	0,40	0,00
PW 1.1	+	16,69	5,73	57,32	5731,76	3,70	1549,13	1,55
PW 1.2	+	19,13	5,03	50,28	5028,26	3,27	1540,05	1,54
PW 1.3	+	18,66	5,16	51,62	5161,70	2,83	1823,92	1,82
CW 2.3			9,82	98,19	9819,29	1,59	6175,65	6,18
CW 5.2			9,82	98,19	9819,29	2,10	4675,85	4,68
PW 2.2			9,82	98,19	9819,29	5,98	1642,02	1,64
PW 5.1			9,82	98,19	9819,29	3,76	2611,51	2,61
PW 2.1	+	19,22	5,00	50,01	5000,75	2,63	1901,43	1,90
PW 2.2	+	18,08	5,33	53,30	5329,71	5,98	891,26	0,89
PW 2.3	+	17,03	5,63	56,34	5633,56	4,51	1249,13	1,25
PW 3.1	+	19,22	5,00	50,01	5000,87	2,88	1736,41	1,74
CW 5.3	+	31,88	0,41	4,06	405,78	1,84	221,13	0,22
PW 4.2	+	32,84	0,12	1,21	121,24	2,93	41,38	0,04
PW 2.3			9,82	98,19	9819,29	4,51	2177,23	2,18
PW 5.2			9,82	98,19	9819,29	2,77	3544,87	3,54
PW 3.2	+	17,30	5,55	55,54	5554,00	2,98	1863,76	1,86
PW 3.3	+	18,07	5,33	53,33	5332,74	2,91	1832,56	1,83
PW 4.1	+	18,59	5,18	51,84	5183,61	2,15	2410,98	2,41
PW 4.2	+	18,20	5,30	52,96	5295,54	2,93	1807,35	1,81
CW 3.2			9,82	98,19	9819,29	1,79	5485,64	5,49
C+	-	39,45	-1,83	-18,30	-1830,45	1,48	-1233,29	-1,23
PW 3.1	-	39,23	-1,77	-17,66	-1766,05	2,88	-613,21	-0,61
PW 5.3			9,82	98,19	9819,29	1,31	7495,64	7,50
PW 4.3	+	17,89	5,38	53,85	5384,72	1,57	3429,76	3,43
PW 5.1	+	19,32	4,97	49,73	4973,04	3,76	1322,62	1,32
PW 5.2	+	18,16	5,31	53,05	5305,09	2,77	1915,19	1,92
PW 5.3	+	21,44	4,36	43,61	4361,40	1,31	3329,31	3,33

5.3.3. Total population of fungi

Fungi								
Sample	Amplicon	Ct	Log copies/µl 1/10 diluted	Log copies/µl	Log copies/100µL	Mass Leaves	Log copies/g	Log copies/mg
CW 1.1	+	17,95	4,63	46,31	4631,06	1,60	2894,41	2,89
CW 3.3	+	16,77	5,01	50,05	5005,07	1,36	3680,20	3,68
D+	+	17,04	4,92	49,20	4919,57	1,39	3548,45	3,55
PW 3.2	+	16,03	5,24	52,39	5238,73	2,98	1757,96	1,76
A+	+	16,35	5,14	51,36	5136,33	0,97	5281,03	5,28
CW 1.2	+	17,54	4,76	47,59	4759,40	1,34	3551,79	3,55
CW 4.1	+	17,91	4,64	46,43	4643,33	2,17	2139,78	2,14
PW 1.1	+	16,47	5,10	50,98	5098,44	3,70	1377,96	1,38
PW 3.3	+	17,70	4,71	47,11	4710,96	2,91	1618,89	1,62
B+	+	16,85	4,98	49,80	4980,31	1,30	3841,94	3,84
CW 1.3	+	17,47	4,78	47,83	4783,11	2,00	2391,56	2,39
CW 4.2	+	17,63	4,73	47,31	4730,96	1,77	2672,86	2,67
PW 1.2	+	17,27	4,85	48,47	4846,87	3,27	1484,49	1,48
PW 4.1	+	17,77	4,69	46,87	4686,64	2,15	2179,83	2,18
NTC	+	30,21	0,74	7,43	742,53	1856,33	0,40	0,00
CW 2.1	+	16,43	5,11	51,12	5111,72	3,11	1643,64	1,64
CW 4.3	+	17,46	4,79	47,86	4785,78	2,34	2045,20	2,05
PW 1.3	+	17,17	4,88	48,79	4878,51	2,83	1723,86	1,72
PW 4.2	+	17,01	4,93	49,29	4928,98	2,93	1682,25	1,68
CW 2.2	+	15,92	5,27	52,75	5274,94	2,35	2244,65	2,24
CW 5.1	+	18,75	4,38	43,77	4377,46	1,57	2788,19	2,79
PW 2.1	+	18,24	4,54	45,40	4539,92	2,63	1726,20	1,73
PW 4.3	+	17,05	4,91	49,15	4914,84	1,57	3130,47	3,13
CW 2.3	+	17,66	4,72	47,24	4723,70	1,59	2970,88	2,97
CW 5.2	+	17,79	4,68	46,82	4681,79	2,10	2229,42	2,23
PW 2.2	+	17,41	4,80	48,02	4802,11	5,98	803,03	0,80
PW 5.1	+	18,90	4,33	43,28	4328,01	3,76	1151,07	1,15
CW 3.1	+	16,29	5,16	51,58	5158,20	2,22	2323,51	2,32
CW 5.3	+	17,39	4,81	48,07	4807,15	1,84	2619,70	2,62
PW 2.3	+	16,04	5,23	52,34	5234,42	4,51	1160,62	1,16
PW 5.2	+	17,71	4,71	47,06	4706,36	2,77	1699,05	1,70
CW 3.2	+	17,84	4,66	46,64	4664,07	1,79	2605,62	2,61
C+	+	16,97	4,94	49,40	4940,27	1,48	3328,57	3,33
PW 3.1	+	17,08	4,91	49,07	4907,04	2,88	1703,83	1,70
PW 5.3	+	19,01	4,29	42,94	4294,08	1,31	3277,93	3,28

5.4. Statistical analysis of phyllosphere bacteria on ivy in Hasselt and De Maten

5.4.1. Alkane monooxygenase gene

An ANOVA is performed to see if there is a significant difference between the expression of alkane monooxygenase by phyllosphere bacteria on ivy. The ivy was planted on the non-polluted site De Maten and the city of Hasselt.

One Way Analysis of Variance woensdag, mei 31, 2017, 12:51:45

Data source: Data 1 in 270317_juist

Normality Test: Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks woensdag, mei 31, 2017, 12:51:45

Data source: Data 1 in 270317_juist

Group	N	Missing	Median	25%	75%
De Maten	9	2	2,391	2,119	3,043
Hasselt	7	2	1,361	1,253	1,663

H = 4,807 with 1 degrees of freedom. P(est.)= 0,028 P(exact)= 0,030

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,030)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0,05
De Maten vs Hasselt	4,629	2,192	Yes

5.4.2. Phenol hydroxylase gene

In this statistical analysis, an ANOVA is performed to determine a significant difference of the expression of phenol hydroxylase between phyllosphere bacteria on ivy. The ivy was planted on the non-polluted site De Maten and the city of Hasselt.

One Way Analysis of Variance woensdag, mei 31, 2017, 12:52:20

Data source: Data 1 in 270317_juist

Normality Test: Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks woensdag, mei 31, 2017, 12:52:20

Data source: Data 1 in 270317_juist

Group	N	Missing	Median	25%	75%
De Maten	15	2	1,645	1,227	1,988
Hasselt	15	2	0,775	0,645	1,039

H = 8,695 with 1 degrees of freedom. (P = 0,003)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,003)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0,05
De Maten vs Hasselt	115,000	4,170	Yes

5.4.3. Naphthalene dioxygenase gene

The ANOVA will prove the significance difference between the expression of naphthalene dioxygenase in phyllosphere bacteria on ivy. The ivy was planted on the non-polluted site De Maten and the city of Hasselt.

One Way Analysis of Variance woensdag, mei 31, 2017, 12:53:04

Data source: Data 1 in 270317_juist

Normality Test: Passed (P = 0,920)

Equal Variance Test: Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks woensdag, mei 31, 2017, 12:53:04

Data source: Data 1 in 270317_juist

Group	N	Missing	Median	25%	75%
De Maten	4	2	0,231	0,221	0,241
Hasselt	4	2	0,134	0,0414	0,227

H = 0,600 with 1 degrees of freedom. P(est.)= 0,439 P(exact)= 0,667

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,667)

5.4.4. Total population of bacteria

An ANOVA is performed to see if there is a significant difference between total population of bacteria on ivy. The ivy was planted on the non-polluted site De Maten and the city of Hasselt.

One Way Analysis of Variance woensdag, mei 31, 2017, 12:53:41

Data source: Data 1 in 270317_juist

Normality Test: Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks woensdag, mei 31, 2017, 12:53:41

Data source: Data 1 in 270317_juist

Group	N	Missing	Median	25%	75%
De Maten	19	2	2,942	2,495	3,410
Hasselt	19	2	1,833	1,547	2,641

H = 6,943 with 1 degrees of freedom. (P = 0,008)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,008)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0,05
De Maten vs Hasselt	153,000	3,726	Yes

5.4.5. Total population of fungi

In this statistical analysis, an ANOVA is performed to determine a significant difference between total population of fungi on ivy. The ivy was planted on the non-polluted site De Maten and the city of Hasselt.

One Way Analysis of Variance woensdag, mei 31, 2017, 12:54:27

Data source: Data 1 in 270317_juist

Normality Test: Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks woensdag, mei 31, 2017, 12:54:27

Data source: Data 1 in 270317_juist

Group	N	Missing	Median	25%	75%
De Maten	19	2	2,620	2,241	3,060
Hasselt	19	2	1,704	1,458	2,417

H = 6,763 with 1 degrees of freedom. (P = 0,009)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,009)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0,05
De Maten vs Hasselt	151,000	3,678	Yes

5.5. Raw data of soil-bacteria in Bialowiesa and Bobrka

5.5.1. Aromatic degrading genes

AlkB	PHE	NAH					
Sample	**Amplicon**	**Ct**	**Log copies/µl 1/10 diluted**	**Log copies/µl**	**Log copies/100µl**	**Log copies/g**	**Log copies/mg**
10a	+	24,33	6,40	64,01	6401,01	16002,52	16,00
10b	+	24,05	6,48	64,78	6477,91	16194,77	16,19
10c	+	24,63	6,32	63,18	6317,99	15794,98	15,79
1a	+	25,29	6,14	61,38	6138,01	15345,02	15,35
10a	+	22,61	4,35	43,53	4352,78	10881,95	10,88
10b	+	22,21	4,47	44,65	4465,08	11162,69	11,16
10c	+	22,52	4,38	43,79	4379,24	10948,11	10,95
1a	+	31,52	1,89	18,91	1891,08	4727,70	4,73
1a			10,54	105,43	10542,64	26356,61	26,36
3c			10,54	105,43	10542,64	26356,61	26,36
10a	+	32,54	1,16	11,60	1159,88	2899,70	2,90
10b	+	32,49	1,17	11,73	1173,46	2933,65	2,93
1b	+	25,59	6,06	60,56	6055,76	15139,40	15,14
1c	+	24,77	6,28	62,81	6281,33	15703,33	15,70
2a	+	25,89	5,97	59,71	5971,39	14928,47	14,93
2b	+	25,69	6,03	60,26	6026,16	15065,39	15,07
1b	+	33,79	1,26	12,62	1261,61	3154,03	3,15
3c	+	32,80	1,54	15,35	1535,45	3838,62	3,84
4a	+	32,78	1,54	15,42	1541,67	3854,18	3,85
4b	+	23,87	4,01	40,06	4005,53	10013,83	10,01
1b			10,54	105,43	10542,64	26356,61	26,36
4a			10,54	105,43	10542,64	26356,61	26,36
10c	+	34,38	0,63	6,29	629,26	1573,15	1,57
4b	+	30,93	1,63	16,26	1625,51	4063,78	4,06
2c	+	25,63	6,04	60,43	6043,33	15108,32	15,11
3a	+	23,94	6,51	65,09	6509,33	16273,32	16,27
3b	+	27,04	5,65	56,54	5653,59	14133,98	14,13
3c	+	23,91	6,52	65,18	6517,76	16294,41	16,29
1c	+	34,20	1,15	11,49	1149,07	2872,68	2,87
4c	+	23,87	4,00	40,04	4004,40	10010,99	10,01
5a	+	26,21	3,36	33,59	3359,20	8397,99	8,40
5b	+	27,18	3,09	30,91	3091,03	7727,57	7,73
1c			10,54	105,43	10542,64	26356,61	26,36
4c	+	31,20	1,55	15,47	1546,62	3866,56	3,87
6a	+	28,73	2,26	22,58	2258,40	5646,01	5,65
6b	+	29,12	2,15	21,47	2147,40	5368,49	5,37
4a	+	24,41	6,38	63,79	6378,90	15947,26	15,95
4b	+	25,25	6,15	61,48	6147,98	15369,96	15,37
4c	+	24,53	6,35	63,47	6347,23	15868,09	15,87

2a	+	36,27	0,58	5,76	576,48	1441,21	1,44
5c	+	27,94	2,88	28,80	2879,77	7199,42	7,20
6a	+	25,78	3,48	34,77	3477,08	8692,69	8,69
2a			10,54	105,43	10542,64	26356,61	26,36
6c	+	28,73	2,26	22,59	2259,04	5647,60	5,65
8a	+	36,87	-0,09	-0,89	-89,12	-222,81	-0,22
6a	+	26,05	5,93	59,27	5927,18	14817,95	14,82
5a	-	25,03	6,21	62,08	6208,18	15520,45	15,52
6b	+	25,41	6,10	61,03	6102,81	15257,02	15,26
2b	+	38,06	0,08	0,82	81,96	204,90	0,20
6b	+	27,29	3,06	30,58	3058,26	7645,66	7,65
6c	+	28,09	2,84	28,38	2837,74	7094,35	7,09
2b			10,54	105,43	10542,64	26356,61	26,36
5a			10,54	105,43	10542,64	26356,61	26,36
8b	+	38,37	-0,52	-5,21	-520,93	-1302,33	-1,30
6c	+	25,80	6,00	59,95	5995,20	14988,01	14,99
5b	-	24,54	6,34	63,43	6342,96	15857,40	15,86
8a	+	23,83	6,54	65,39	6539,24	16348,09	16,35
2c	+	37,99	0,10	1,01	100,98	252,46	0,25
8a	+	28,06	2,85	28,47	2847,17	7117,92	7,12
8b	+	29,88	2,34	23,43	2342,88	5857,21	5,86
2c			10,54	105,43	10542,64	26356,61	26,36
5b	-	39,21	-0,76	-7,64	-763,85	-1909,64	-1,91
8c	+	36,34	0,06	0,64	63,75	159,38	0,16
8b	+	24,26	6,42	64,20	6420,28	16050,69	16,05
5c	-	24,34	6,40	63,98	6398,47	15996,18	16,00
8c	+	25,47	6,09	60,86	6086,49	15216,23	15,22
3a	+	35,78	0,71	7,11	711,48	1778,70	1,78
8c	+	22,62	4,35	43,52	4351,98	10879,95	10,88
9a	+	20,14	5,04	50,36	5035,75	12589,38	12,59
3a			10,54	105,43	10542,64	26356,61	26,36
5c			10,54	105,43	10542,64	26356,61	26,36
9a	+	33,75	0,81	8,12	811,78	2029,44	2,03
9a	+	22,41	6,93	69,32	6932,11	17330,28	17,33
9b	+	23,06	6,75	67,50	6750,42	16876,05	16,88
9c	+	23,26	6,70	66,96	6696,09	16740,24	16,74
NTC	-		13,11	131,08	13107,69	32769,22	32,77
3b	+	37,49	0,24	2,39	239,44	598,59	0,60
9b	+	20,48	4,94	49,42	4942,35	12355,86	12,36
9c	+	20,45	4,95	49,50	4949,78	12374,46	12,37
NTC	+	33,26	1,41	14,08	1407,95	3519,87	3,52
3b			10,54	105,43	10542,64	26356,61	26,36
9b	+	33,78	0,80	8,04	803,62	2009,04	2,01
9c	+	35,10	0,42	4,22	422,03	1055,09	1,06
NTC	-		10,54	105,43	10542,64	26356,61	26,36

5.5.2. Total population of bacteria and fungi

Bacteria	Fungi	AlkB					
Sample	Amplicon	Ct	Log copies/µl 1/10 diluted	Log copies/µl	Log copies/100µl	Log copies/g	Log copies/mg
10a	+	24,33	2,63	26,34	2634,35	6585,88	6,59
10b	+	24,05	2,72	27,17	2716,74	6791,84	6,79
10c	+	24,63	2,55	25,45	2545,41	6363,54	6,36
1a	+	25,29	2,35	23,53	2352,60	5881,49	5,88
10a	+	22,61	3,15	31,52	3152,11	7880,29	7,88
10b	+	22,21	3,28	32,81	3280,86	8202,16	8,20
10c	+	22,52	3,18	31,82	3182,46	7956,14	7,96
1a	+	31,52	0,33	3,30	329,69	824,22	0,82
1b	+	25,59	2,26	22,64	2264,48	5661,21	5,66
1c	+	24,77	2,51	25,06	2506,14	6265,35	6,27
2a	+	25,89	2,17	21,74	2174,10	5435,24	5,44
2b	+	25,69	2,23	22,33	2232,77	5581,93	5,58
1b		33,79	-0,39	-3,92	-392,02	-980,04	-0,98
3c	+	32,80	-0,08	-0,78	-78,05	-195,14	-0,20
4a	+	32,78	-0,07	-0,71	-70,92	-177,30	-0,18
4b	+	23,87	2,75	27,54	2753,98	6884,95	6,88
2c	+	25,63	2,25	22,51	2251,17	5627,92	5,63
3a	+	23,94	2,75	27,50	2750,40	6876,00	6,88
3b	+	27,04	1,83	18,34	1833,64	4584,10	4,58
3c	+	23,91	2,76	27,59	2759,43	6898,59	6,90
1c	+	34,20	-0,52	-5,21	-521,05	-1302,63	-1,30
4c	+	23,87	2,75	27,53	2752,68	6881,70	6,88
5a	+	26,21	2,01	20,13	2012,94	5032,34	5,03
5b	+	27,18	1,71	17,05	1705,47	4263,68	4,26
4a	+	24,41	2,61	26,11	2610,67	6526,68	6,53
4b	+	25,25	2,36	23,63	2363,28	5908,21	5,91
4c	+	24,53	2,58	25,77	2576,74	6441,86	6,44
2a		36,27	-1,18	-11,78	-1177,54	-2943,85	-2,94
5c	+	27,94	1,46	14,63	1463,26	3658,14	3,66
6a	+	25,78	2,15	21,48	2148,09	5370,22	5,37
epWa33	-	31,20	4,51	45,06	4506,49	11266,23	11,27
5a	+	25,03	2,43	24,28	2427,77	6069,44	6,07
5b	+	24,54	2,57	25,72	2572,17	6430,42	6,43
5c	+	24,34	2,63	26,32	2631,64	6579,09	6,58
2b	+	38,06	-1,74	-17,45	-1744,53	-4361,33	-4,36
6b	+	27,29	1,67	16,68	1667,90	4169,76	4,17
6c	+	28,09	1,42	14,15	1415,07	3537,66	3,54
epWa34	-		13,11	131,06	13106,03	32765,08	32,77
6a	+	26,05	2,13	21,27	2126,73	5316,84	5,32
6b	+	25,41	2,31	23,15	2314,89	5787,22	5,79

6c	+	25,80	2,20	22,00	2199,61	5499,03	5,50
2c	+	37,99	-1,72	-17,23	-1722,72	-4306,80	-4,31
8a	+	28,06	1,43	14,26	1425,88	3564,69	3,56
8b	+	29,88	0,85	8,48	847,70	2119,24	2,12
epWa41	-	39,21	2,30	22,98	2297,84	5744,60	5,74
8a	+	23,83	2,78	27,82	2782,44	6956,09	6,96
8b	+	24,26	2,65	26,55	2654,99	6637,48	6,64
8c	+	25,47	2,30	22,97	2297,41	5743,52	5,74
3a	+	35,78	-1,02	-10,23	-1022,76	-2556,91	-2,56
8c	+	22,62	3,15	31,51	3151,20	7877,99	7,88
9a	+	20,14	3,94	39,35	3935,17	9837,91	9,84
NTC	-		13,11	131,06	13106,03	32765,08	32,77
9a	+	22,41	3,20	32,03	3203,33	8008,33	8,01
9b	+	23,06	3,01	30,09	3008,68	7521,70	7,52
9c	+	23,26	2,95	29,50	2950,48	7376,20	7,38
NTC	-		9,82	98,19	9819,29	24548,22	24,55
3b	+	37,49	-1,56	-15,64	-1563,98	-3909,95	-3,91
9b	+	20,48	3,83	38,28	3828,07	9570,18	9,57
9c	+	20,45	3,84	38,37	3836,60	9591,50	9,59
NTC	+	33,26	-0,22	-2,24	-224,24	-560,60	-0,56

5.6. Statistical analysis of soil-bacteria in Bialowiesa and Bobrka

5.6.1. Alkane monooxygenase gene

An ANOVA is performed to see if there is a significant difference between the expression of alkane monooxygenase by soil-bacteria. The soil is coming from the nature reserve Bialowiesa and the oil-polluted site Bobrka.

One Way Analysis of Variance woensdag, mei 31, 2017, 9:00:28

Data source: Data 2 in 010317 juist

Normality Test: Passed (P = 0,796)

Equal Variance Test: Passed (P = 0,841)

Group Name	N	Missing	Mean	Std Dev	SEM
Bialowiesa	11	2	15,332	0,681	0,227
Bobrka	17	2	15,920	0,717	0,185

Source of Variation	DF	SS	MS	F	P
Between Groups	1	1,943	1,943	3,920	0,060
Residual	22	10,905	0,496		
Total	23	12,848			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,060).

Power of performed test with alpha = 0,050: 0,359

The power of the performed test (0,359) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

5.6.2. Phenol hydroxylase gene

In this statistical analysis, an ANOVA is performed to determine a significant difference of the expression of phenol hydroxylase between soil-bacteria. The soil is coming from the nature reserve Bialowiesa and the oil-polluted site Bobrka.

One Way Analysis of Variance woensdag, mei 31, 2017, 9:00:51

Data source: Data 2 in 010317 juist

Normality Test: Passed (P = 0,569)

Equal Variance Test: Passed (P = 0,079)

Group Name	N	Missing	Mean	Std Dev	SEM
Bialowiesa	4	2	4,283	0,629	0,445
Bobrka	20	2	9,156	2,466	0,581

Source of Variation	DF	SS	MS	F	P
Between Groups	1	42,737	42,737	7,413	0,014
Residual	18	103,772	5,765		
Total	19	146,508			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,014).

Power of performed test with alpha = 0,050: 0,672

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Bobrka vs. Bialowiesa	4,873	2,723	0,014	0,050	Yes

5.6.3. Total population of bacteria

An ANOVA is performed to see if there is a significant difference between total population of bacteria in the soil. The soil is coming from the nature reserve Bialowiesa and the oil-polluted site Bobrka.

One Way Analysis of Variance woensdag, mei 31, 2017, 9:02:35

Data source: Data 2 in 010317 juist

Normality Test: Passed (P = 0,768)

Equal Variance Test: Passed (P = 0,757)

Group Name	N	Missing	Mean	Std Dev	SEM
Bialowiesa	11	2	5,868	0,729	0,243
Bobrka	20	2	6,475	0,705	0,166

Source of Variation	DF	SS	MS	F	P
Between Groups	1	2,208	2,208	4,347	0,047
Residual	25	12,700	0,508		
Total	26	14,908			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,047).

Power of performed test with alpha = 0,050: 0,408

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Bobrka vs. Bialowiesa	0,607	2,085	0,047	0,050	Yes

5.6.4. Total population of fungi

In this statistical analysis, an ANOVA is performed to determine a significant difference between total population of fungi in soil. The soil is coming from the nature reserve Bialowiesa and the oil-polluted site Bobrka.

One Way Analysis of Variance woensdag, mei 31, 2017, 9:03:14

Data source: Data 2 in 010317 juist

Normality Test: Passed (P = 0,413)

Equal Variance Test: Passed (P = 0,052)

Group Name	N	Missing	Mean	Std Dev	SEM
Bialowiesa	4	2	0,412	0,583	0,412
Bobrka	19	2	6,259	2,459	0,596

Source of Variation	DF	SS	MS	F	P
Between Groups	1	61,170	61,170	10,708	0,004
Residual	17	97,111	5,712		
Total	18	158,281			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,004).

Power of performed test with alpha = 0,050: 0,850

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Bobrka vs. Bialowiesa	5,847	3,272	0,004	0,050	Yes

5.7. Raw data of soil-bacteria in Beverlo before and after inoculation

5.7.1. Aromatic degrading genes and total population of bacteria

AlkB	PHE	NAH	Bacteria				
Sample	Amplicon	Ct	Log copies/µl 1/10 diluted	Log copies/µl	Log copies/100µL	Log copies/g	Log copies/mg
1a			10,10	100,99	10098,74	25246,86	25,25
1ai	-	27,14	5,63	56,28	5627,85	14069,62	14,07
NTC	-	38,45	2,51	25,10	2509,72	6274,29	6,27
1a	-	37,38	0,47	4,71	471,40	1178,50	1,18
1ai	+	25,41	3,89	38,93	3893,40	9733,50	9,73
NTC	-	30,18	2,53	25,30	2529,80	6324,50	6,32
1a			10,26	102,64	10264,30	25660,74	25,66
1ai			10,26	102,64	10264,30	25660,74	25,66
NTC			10,21	102,07	10207,13	25517,82	25,52
1a	-	20,20	3,85	38,54	3854,46	9636,14	9,64
1ai	-	16,41	4,97	49,74	4973,96	12434,89	12,43
NTC	-	27,08	1,82	18,22	1821,97	4554,94	4,55
1b			10,10	100,99	10098,74	25246,86	25,25
1bi	+	26,34	5,85	58,48	5847,66	14619,15	14,62
1b			10,16	101,58	10157,63	25394,07	25,39
1bi	+	24,76	4,08	40,80	4079,50	10198,75	10,20
1b			10,26	102,64	10264,30	25660,74	25,66
1bi	-	32,88	1,06	10,62	1062,08	2655,20	2,66
1b			10,21	102,07	10207,13	25517,82	25,52
1bi	+	15,41	5,27	52,69	5268,85	13172,13	13,17
2a			10,10	100,99	10098,74	25246,86	25,25
2ai	+	23,67	6,58	65,83	6583,00	16457,51	16,46
2a			10,16	101,58	10157,63	25394,07	25,39
2ai	+	25,38	3,90	39,03	3903,35	9758,37	9,76
2a			10,26	102,64	10264,30	25660,74	25,66
2ai			10,26	102,64	10264,30	25660,74	25,66
2a		31,17	0,62	6,16	615,81	1539,52	1,54
2ai	-	15,62	5,21	52,07	5206,84	13017,11	13,02
2b			10,10	100,99	10098,74	25246,86	25,25
2bi	+	27,20	5,61	56,12	5612,00	14030,00	14,03
2b		39,28	-0,07	-0,70	-70,18	-175,45	-0,18
2bi	+	25,94	3,74	37,41	3740,92	9352,29	9,35
2b			10,10	100,99	10098,74	25246,86	25,25
2bi			10,16	101,58	10157,63	25394,07	25,39
2b	-	18,77	4,28	42,77	4276,89	10692,21	10,69
2bi	-	16,67	4,90	48,97	4896,98	12242,44	12,24
4a			10,10	100,99	10098,74	25246,86	25,25
5ai			10,16	101,58	10157,63	25394,07	25,39
4a			10,26	102,64	10264,30	25660,74	25,66

5ai			10,21	102,07	10207,13	25517,82	25,52
4a			10,10	100,99	10098,74	25246,86	25,25
5ai			10,16	101,58	10157,63	25394,07	25,39
4a			10,26	102,64	10264,30	25660,74	25,66
5ai			10,21	102,07	10207,13	25517,82	25,52
4b			10,10	100,99	10098,74	25246,86	25,25
5bi			10,10	100,99	10098,74	25246,86	25,25
4b			10,16	101,58	10157,63	25394,07	25,39
5bi			12,69	126,88	12688,03	31720,06	31,72
4b			10,26	102,64	10264,30	25660,74	25,66
5bi			10,26	102,64	10264,30	25660,74	25,66
4b	-	25,28	2,36	23,55	2355,49	5888,72	5,89
5bi			9,89	98,85	9885,14	24712,84	24,71
6a	+	21,60	7,15	71,53	7152,89	17882,22	17,88
6ai	-	30,20	4,78	47,83	4782,70	11956,74	11,96
6a	+	23,04	4,57	45,71	4570,60	11426,49	11,43
6ai	-	21,52	5,00	50,05	5004,63	12511,58	12,51
6a	-	37,13	-0,16	-1,63	-162,82	-407,06	-0,41
6ai	+	33,87	0,78	7,78	777,90	1944,74	1,94
6a	-	14,47	5,55	55,46	5545,71	13864,27	13,86
6ai	+	14,56	5,52	55,20	5520,05	13800,12	13,80
6b	+	21,25	7,25	72,49	7249,36	18123,40	18,12
6bi	+	30,27	4,77	47,65	4765,14	11912,85	11,91
6b	+	22,77	4,65	46,48	4647,78	11619,45	11,62
6bi	-	20,37	5,33	53,35	5334,78	13336,95	13,34
6b			9,89	98,85	9885,14	24712,84	24,71
6bi	+	37,05	-0,14	-1,41	-140,91	-352,27	-0,35
6b	-	14,32	5,59	55,90	5589,71	13974,27	13,97
6bi	-	14,32	5,59	55,91	5590,92	13977,29	13,98

5.7.2. Total population of fungi

Fungi							
Sample	Amplicon	Ct	Log copies/µl 1/10 diluted	Log copies/µl	Log copies/100µL	Log copies/g	Log copies/mg
1a			10,32	103,21	10321,16	25802,90	25,80
1ai	+	21,01	3,66	36,59	3658,65	9146,61	9,15
1bi	+	19,73	4,07	40,67	4067,02	10167,55	10,17
1b	-	36,02	-1,10	-10,97	-1097,20	-2743,01	-2,74
2a	+	32,30	0,08	0,82	82,02	205,04	0,21
2ai	+	21,34	3,56	35,56	3555,74	8889,34	8,89
2bi	+	21,67	3,45	34,52	3451,65	8629,13	8,63
2b			10,32	103,21	10321,16	25802,90	25,80
6a	+	21,24	3,59	35,88	3587,72	8969,31	8,97
4a			10,32	103,21	10321,16	25802,90	25,80
5ai			10,32	103,21	10321,16	25802,90	25,80
4b			10,32	103,21	10321,16	25802,90	25,80
5bi			10,32	103,21	10321,16	25802,90	25,80
6ai	+	25,82	2,14	21,36	2136,29	5340,74	5,34
6b	+	21,37	3,55	35,46	3546,03	8865,08	8,87
6bi	+	23,23	2,96	29,58	2957,77	7394,43	7,39
NTC	+	31,79	0,24	2,42	242,22	605,54	0,61

5.7.3. Extra samples

AlkB	PHE	NAH	Bacteria	Fungi			
Sample	Amplicon	Ct	Log copies/µl 1/10 diluted	Log copies/µl	Log copies/100µL	Log copies/g	Log copies/mg
1b				0	0	0	0
NTC	-			0	0	0	0
1b	-	36,79	0,64	6,39	639,43	1598,58	1,60
NTC	-	34,12	1,40	14,04	1403,79	3509,48	3,51
1b				0,00	0,00	0,00	0,00
NTC	-			0,00	0,00	0,00	0,00
1b	+	18,60	4,33	43,28	4327,76	10819,41	10,82
NTC	-	27,37	1,74	17,36	1736,23	4340,56	4,34
1b	-	31,47	0,34	3,44	344,37	860,92	0,86
NTC	-			0,00	0,00	0,00	0,00
2b				0,00	0,00	0,00	0,00
2b	-	27,68	3,25	32,46	3246,25	8115,62	8,12
2b				0,00	0,00	0,00	0,00
2b	+	16,36	4,99	49,88	4988,28	12470,69	12,47
2b	-	23,33	2,92	29,25	2924,93	7312,31	7,31
4b	-	37,23	2,85	28,45	2845,18	7112,95	7,11
4b	+	25,21	3,95	39,52	3951,52	9878,79	9,88
4b	-	39,32	-0,08	-0,82	-82,27	-205,68	-0,21
4b	+	15,06	5,37	53,73	5373,24	13433,09	13,43
4b	+	26,79	1,83	18,27	1827,21	4568,04	4,57
1ib	+	30,10	4,81	48,10	4810,48	12026,20	12,03
6b	+	25,55	3,85	38,53	3852,61	9631,51	9,63
6b				0,00	0,00	0,00	0,00
6b	+	15,80	5,16	51,55	5155,29	12888,22	12,89
6b	+	25,27	2,31	23,08	2308,35	5770,88	5,77
6b	+	24,14	6,45	64,55	6454,87	16137,17	16,14
1ib	-	27,54	3,29	32,86	3286,16	8215,39	8,22
1ib				0,00	0,00	0,00	0,00
1ib	+	18,27	4,42	44,23	4423,34	11058,36	11,06
1ib	+	23,73	2,80	27,98	2797,67	6994,17	6,99
2ib				0,00	0,00	0,00	0,00
2ib	-	25,25	3,94	39,39	3939,05	9847,63	9,85
2ib	-	35,44	0,32	3,24	323,77	809,43	0,81
2ib	+	15,37	5,28	52,80	5280,43	13201,07	13,20
2ib	+	20,47	3,83	38,31	3831,08	9577,71	9,58
5ib				0,00	0,00	0,00	0,00
5ib				0,00	0,00	0,00	0,00
5ib				0,00	0,00	0,00	0,00
5ib				0,00	0,00	0,00	0,00
5ib				0,00	0,00	0,00	0,00

6ib	-	36,27	3,11	31,09	3109,45	7773,63	7,77
6ib	-	24,37	4,19	41,93	4192,50	10481,26	10,48
6ib				0,00	0,00	0,00	0,00
6ib	+	16,59	4,92	49,22	4921,87	12304,67	12,30
6ib	+	27,56	1,58	15,83	1583,48	3958,69	3,96

5.8. Statistical analysis of soil-bacteria in Beverlo before and after inoculation

5.8.1. Alkane monooxygenase gene

An ANOVA is performed to see if there is a significant difference between the expression of alkane monooxygenase by soil-bacteria. The soil samples are from the polluted soil of Beverlo, before and after the inoculation with aromatic degrading bacteria.

One Way Analysis of Variance woensdag, mei 31, 2017, 10:32:25

Data source: Data 1 in Notebook1

Normality Test: Passed (P = 0,419)

Equal Variance Test: Passed (P = 0,360)

Group Name	N	Missing	Mean	Std Dev	SEM
Before inoculation	5	2	17,381	1,084	0,626
After inoculation	7	2	13,809	1,904	0,851

Source of Variation	DF	SS	MS	F	P
Between Groups	1	23,921	23,921	8,521	0,027
Residual	6	16,843	2,807		
Total	7	40,764			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,027).

Power of performed test with alpha = 0,050: 0,633

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Before vs. After	3,572	2,919	0,027	0,050	Yes

5.8.2. Phenol hydroxylase gene

In this statistical analysis, an ANOVA is performed to determine a significant difference of the expression of phenol hydroxylase between soil-bacteria. The soil samples are from the polluted soil of Beverlo, before and after the inoculation with aromatic degrading bacteria.

One Way Analysis of Variance woensdag, mei 31, 2017, 10:33:17

Data source: Data 1 in Notebook1

Normality Test: Passed (P = 0,811)

Equal Variance Test: Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks woensdag, mei 31, 2017, 10:33:17

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Before inoculation	6	2	10,653	9,755	11,523
After inoculation	6	2	9,746	9,543	9,979

H = 1,333 with 1 degrees of freedom. P(est.)= 0,248 P(exact)= 0,343

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,343)

5.8.3. Total population of bacteria

An ANOVA is performed to see if there is a significant difference between total population of bacteria in the soil. The soil samples are from the polluted soil of Beverlo, before and after the inoculation with aromatic degrading bacteria.

One Way Analysis of Variance woensdag, mei 31, 2017, 10:33:56

Data source: Data 1 in Notebook1

Normality Test: Passed (P = 0,096)

Equal Variance Test: Passed (P = 0,804)

Group Name	N	Missing	Mean	Std Dev	SEM
Before inoculation	6	2	12,403	1,127	0,563
After inoculation	7	2	12,707	1,065	0,476

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0,206	0,206	0,173	0,690
Residual	7	8,344	1,192		
Total	8	8,550			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,690).

Power of performed test with alpha = 0,050: 0,049

The power of the performed test (0,049) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

5.8.4. Total population of fungi

In this statistical analysis, an ANOVA is performed to determine a significant difference between total population of fungi in soil. The soil samples are from the polluted soil of Beverlo, before and after the inoculation with aromatic degrading bacteria.

One Way Analysis of Variance woensdag, mei 31, 2017, 10:34:14

Data source: Data 1 in Notebook1

Normality Test: Passed (P = 0,173)

Equal Variance Test: Passed (P = 0,890)

Group Name	N	Missing	Mean	Std Dev	SEM
Before inoculation	6	2	7,043	2,219	1,110
After inoculation	11	2	7,789	2,064	0,688

Source of Variation	DF	SS	MS	F	P
Between Groups	1	1,539	1,539	0,346	0,568
Residual	11	48,844	4,440		
Total	12	50,382			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,568).

Power of performed test with alpha = 0,050: 0,048

The power of the performed test (0,048) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

6. Results screening of bacteria

6.1. Explanation sample code

6.1.1. Suffix

Without suffix → grown on toluene
X → grown on xylene
B → grown on ethylbenzene
benzene → grown on benzene
diesel → grown on diesel
(-*) → Dilution

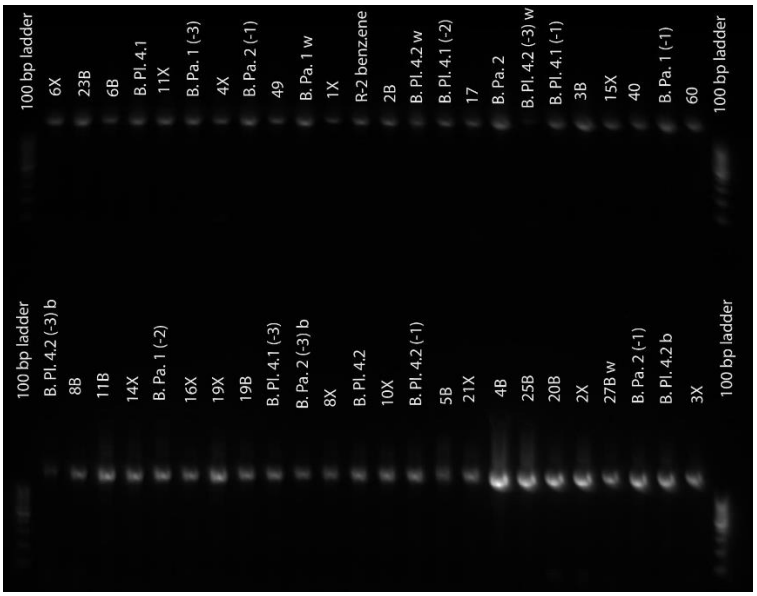
6.1.2. Prefix

B. Pa. → Bodem Panos
B. Pl. 4 → Bodem Polen place 4
Pt → Plate

6.2. Sequencing of bacteria

The bacteria are sequenced to know which bacteria exactly is isolated. The 16S-gene, amplificated by a PCR-reaction, are send to sequencing.

6.2.1. 16S-gene



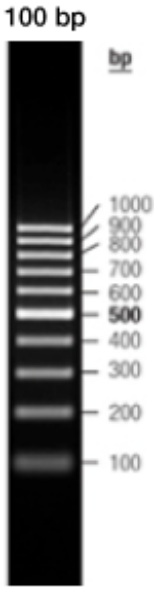
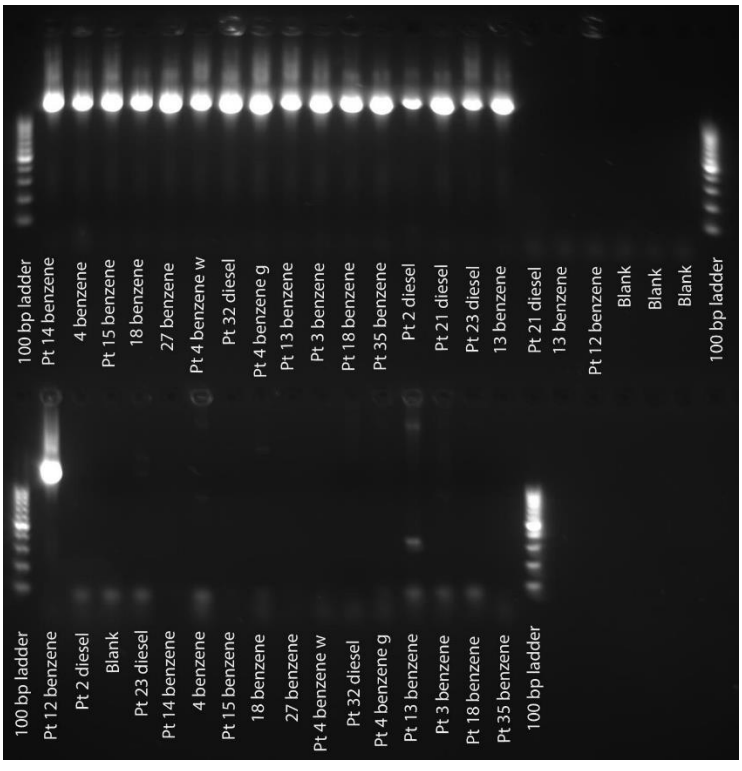


Figure 7a: Results of the PCR-amplification of the 16S-gene



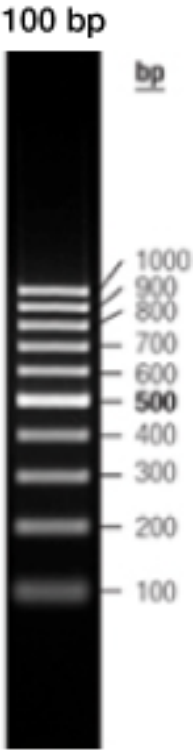


Figure 7b: Results of the PCR-amplification of the 16S-gene in the entire first comb and the second lane of the second comb

6.2.2. Results of the 16S-sequencing

Code	Tracking number	Sample code	Sequencing results 16S
A01	AS_01	6x	Rhodococcus erythropolis strain N11
A02	AS_02	23B	Rhodococcus erythropolis strain N11
A04	AS_03	8B	Nocardia coeliaca strain DSM 44595
A05	AS_04	B. Pa. 2	Bacillus aerius strain 24K
A06	AS_05	4B	Paenibacillus tundrae strain A10b
A08	AS_06	4 benzene	Pseudomonas putida strain NBRC 14164
A09	AS_07	Pt 12 benzene	Pseudomonas japonica strain NBRC 103040
B01	AS_08	6B	Pseudomonas oryzihabitans strain L-1
B02	AS_09	B. Pl. 4.1	Serratia liquefaciens strain ATCC 27592
B03	AS_10	11B	Pseudomonas graminis strain DSM 11363
B04	AS_11	14x	Pseudomonas brenneri strain CFML 97-391
B06	AS_12	25B	Pseudomonas putida strain NBRC 14164
B07	AS_13	Pt 15 benzene	Bacillus cereus ATCC 14579
B08	AS_14	18 benzene	Pseudomonas japonica strain NBRC 103040
C01	AS_15	11x	Nocardia coeliaca strain DSM 44595
C02	AS_16	B. Pa. 1 (-3)	Rhodanobacter thiooxydans strain LCS2
C03	AS_17	B. Pa. 1 (-2)	Rhodanobacter ginsenosidimutans strain Gsoil 3054
C04	AS_18	16x	Arthrobacter bambusae strain THG-GM18
C05	AS_19	B. Pl. 4.1 (-1)	Serratia liquefaciens strain ATCC 27592
C06	AS_20	20B	Rhodococcus erythropolis strain N11
C07	AS_21	27 benzene	Stenotrophomonas humi strain R-32729
C08	AS_22	Pt 4 benzene wit	Stenotrophomonas humi strain R-32729
D01	AS_23	4x	Agromyces allii strain UMS-62
D02	AS_24	B. Pa. 2 (-1)	Bacillus aerius strain 24K
D03	AS_25	19x	Paenibacillus tundrae strain A10b
D04	AS_26	19B	Paenibacillus tundrae strain A10b
D05	AS_27	3B	Paenibacillus tundrae strain A10b
D06	AS_28	2x	Nocardia coeliaca strain DSM 44595
D07	AS_29	Pt 32 diesel	Yersinia kristensenii strain ATCC 33638
D08	AS_30	Pt 4 benzene geel	Ochrobactrum anthropi strain ATCC 49188
E01	AS_31	49	Stenotrophomonas maltophilia strain ATCC 13637
E02	AS_32	B. Pa. 1 wit	Bacillus thermotolerans strain SgZ-8
E03	AS_33	B. Pl. 4.1 (-3)	Bacillus aerius strain 24K
E04	AS_34	B. Pa. 2 (-3) bruin	Rhodanobacter ginsenosidimutans strain Gsoil 3054
E05	AS_35	15x	Arthrobacter bambusae strain THG-GM18
E06	AS_36	27B wit	Stenotrophomonas rhizophila strain e-p10
E07	AS_37	Pt 13 benzene	Yersinia kristensenii strain ATCC 33638
F01	AS_38	1x	Nocardia coeliaca strain DSM 44595
F02	AS_39	R-2 benzene	Bacillus mycoides strain NBRC 101228
F03	AS_40	8x	Serratia fonticola strain NBRC 102597

F04	AS_41	B. Pl. 4.2	Serratia liquefaciens strain ATCC 27592
F05	AS_42	40	Pseudomonas japonica strain NBRC 103040
F06	AS_43	B. Pa. 2 (-1)	Burkholderia diffusa strain R-15930
F07	AS_44	Pt 18 benzene	Stenotrophomonas rhizophila strain e-p10
F08	AS_45	Pt 35 benzene	Pseudomonas japonica strain NBRC 103040
G01	AS_46	2B	Paenibacillus tundrae strain A10b
G02	AS_47	B. Pl. 4.2 wit	Bacillus thuringiensis strain NBRC 101235
G03	AS_48	10x	Bacillus tequilensis strain 10b
G04	AS_49	B. Pl. 4.2 (-1)	Bacillus mycoides strain NBRC 101228
G05	AS_50	B. Pa. 1 (-1)	Bacillus oleronius strain ATCC 700005
G06	AS_51	B. Pl. 4.2 bruin	Bacillus simplex strain LMG 11160
G07	AS_52	Pt 2 diesel	Pseudomonas japonica strain NBRC 103040
G08	AS_53	Pt 21 diesel	Serratia proteamaculans strain DSM 4543
H01	AS_54	B. Pl. 4.1 (-2)	Bacillus toyonensis strain BCT-7112
H02	AS_55	17	Stenotrophomonas humi strain R-32729
H03	AS_56	5B	Pseudomonas graminis strain DSM 11363
H04	AS_57	21x	Paenibacillus tundrae strain A10b
H05	AS_58	60	Pseudomonas japonica strain NBRC 103040
H06	AS_59	3x	Nocardia coeliaca strain DSM 44595
H08	AS_60	13 benzene	Pseudomonas helmanticensis strain OHA11

6.3. Results first screening

The sequenced bacteria are screened on the presence of different ring-hydroxylating oxygenase genes.

6.3.1. Alkane monooxygenase gene

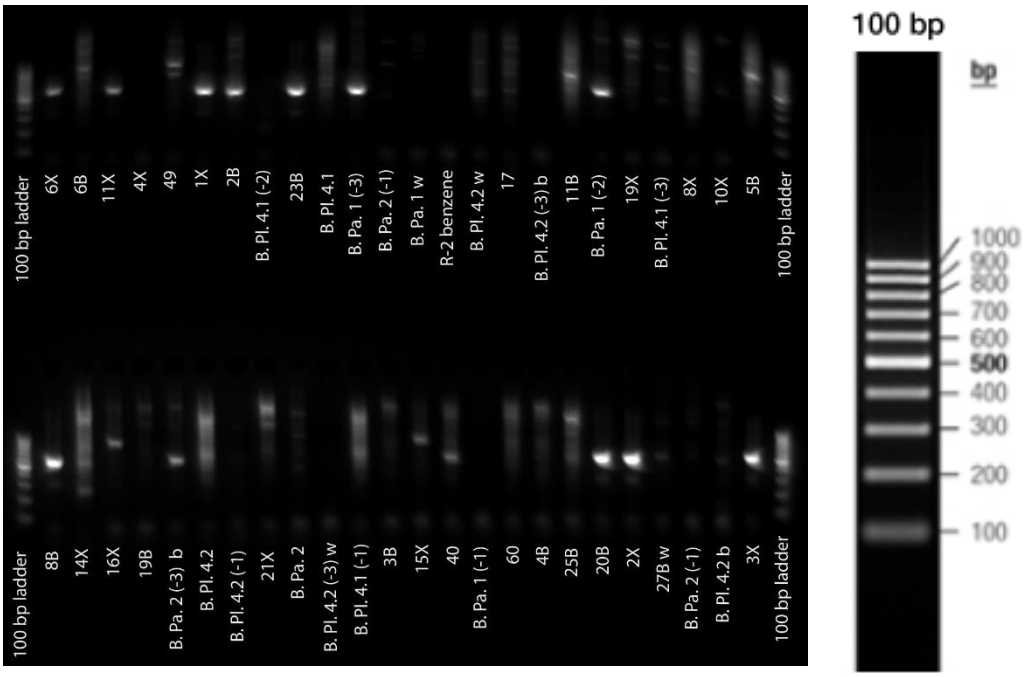


Figure 8a: Results of the PCR-amplification of the alkane monooxygenase gene

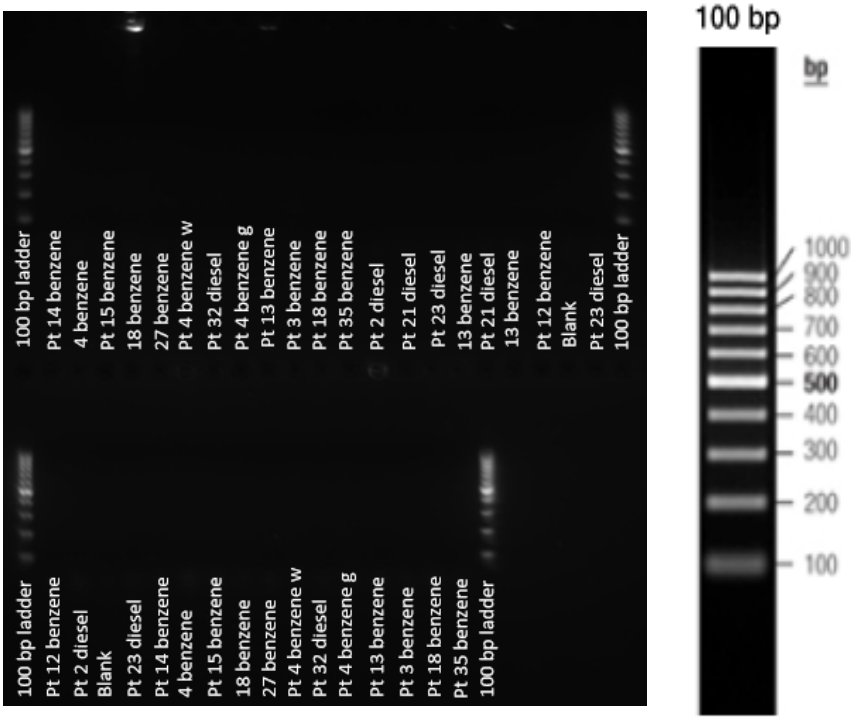


Figure 8b: Results of the PCR-amplification of the alkane monooxygenase gene in the entire first comb and the second lane of the second comb

6.3.2. Phenol hydroxylase gene

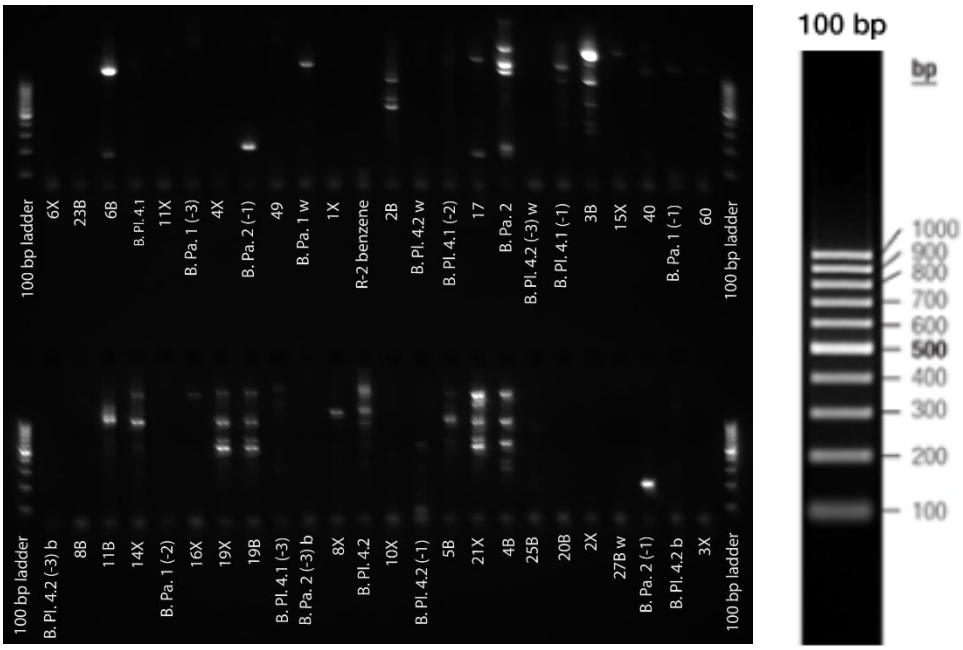


Figure 9a: Results of the PCR-amplification of the phenol hydroxylase gene

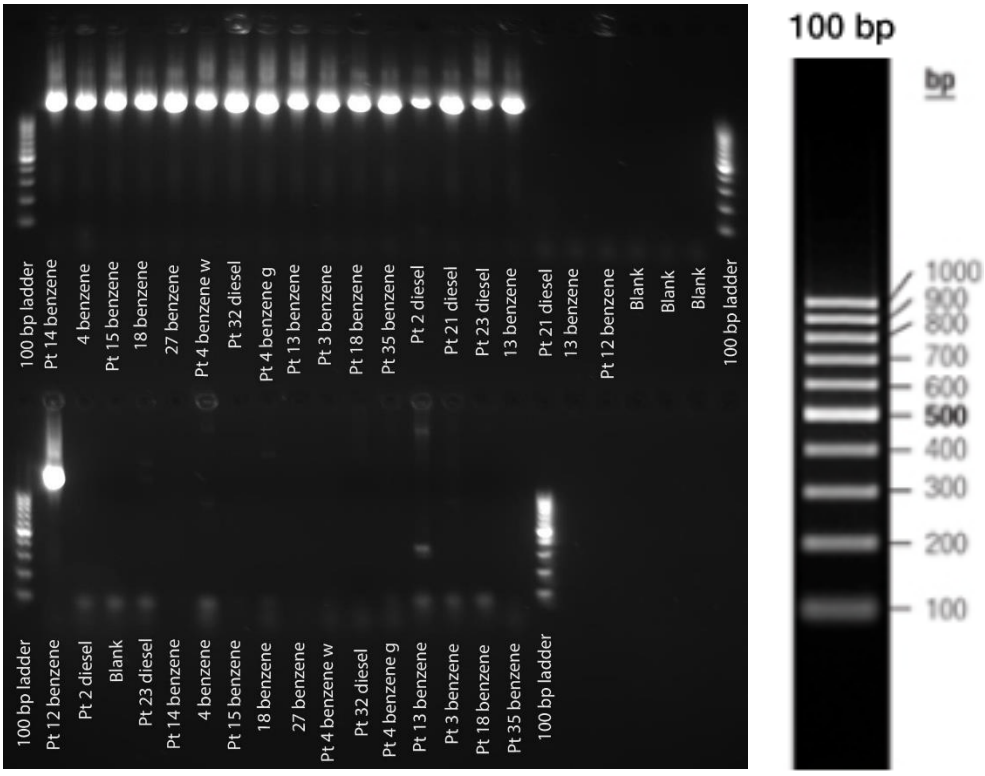


Figure 9b: Results of the PCR-amplification of the phenol hydroxylase gene in lane 20, 21, 22 of the first comb and 3 until 17 of the second comb

6.3.3. Naphthalene dioxygenase gene

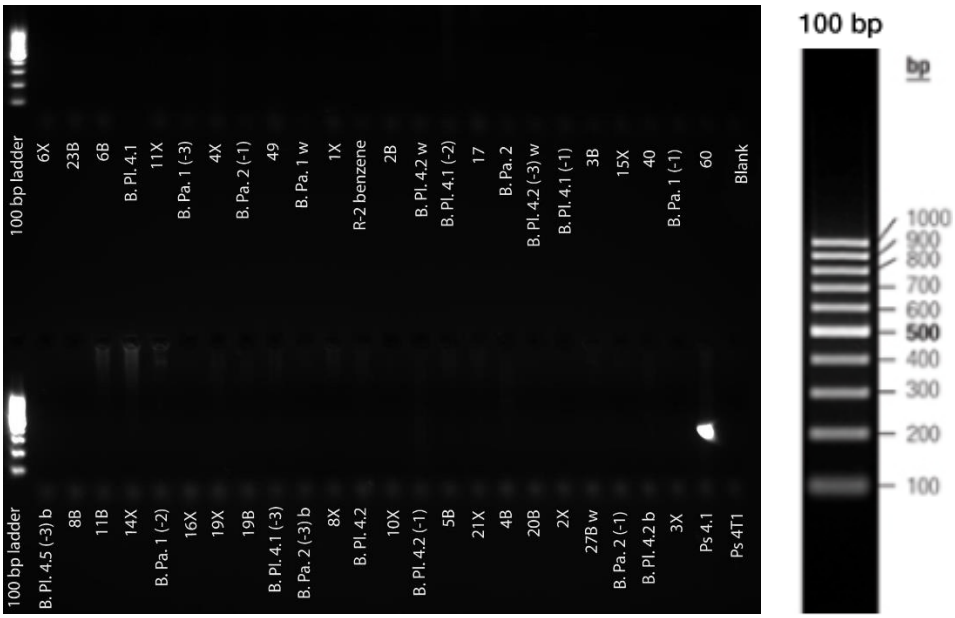


Figure 10a: Results of the PCR-amplification of the naphthalene dioxygenase gene

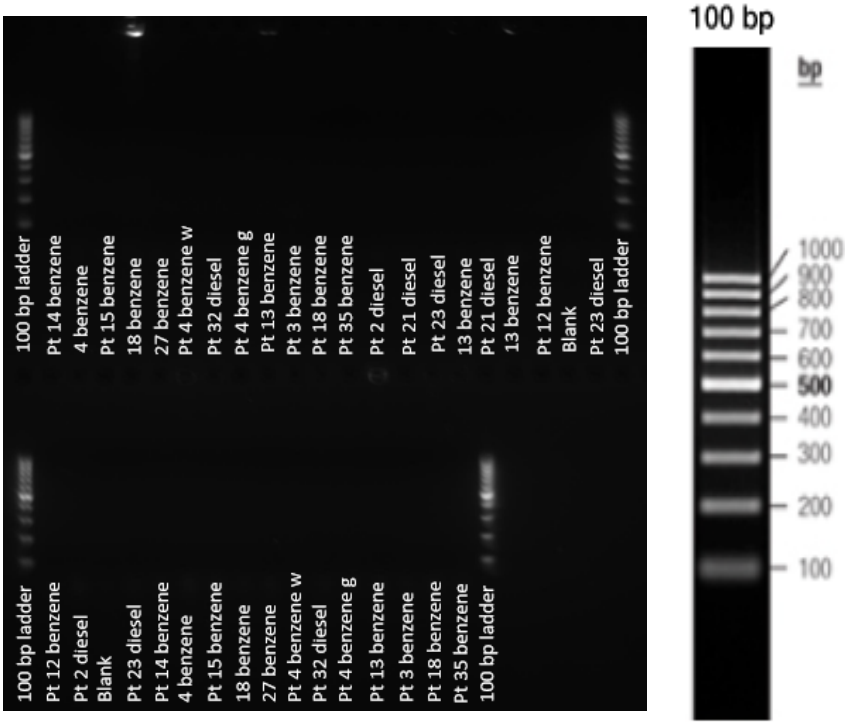


Figure 10b: Results of the PCR-amplification of the naphthalene dioxygenase gene in lane 20, 21, 22 of the first comb and 3 until 17 of the second comb

6.3.4. Catechol dioxygenase gene

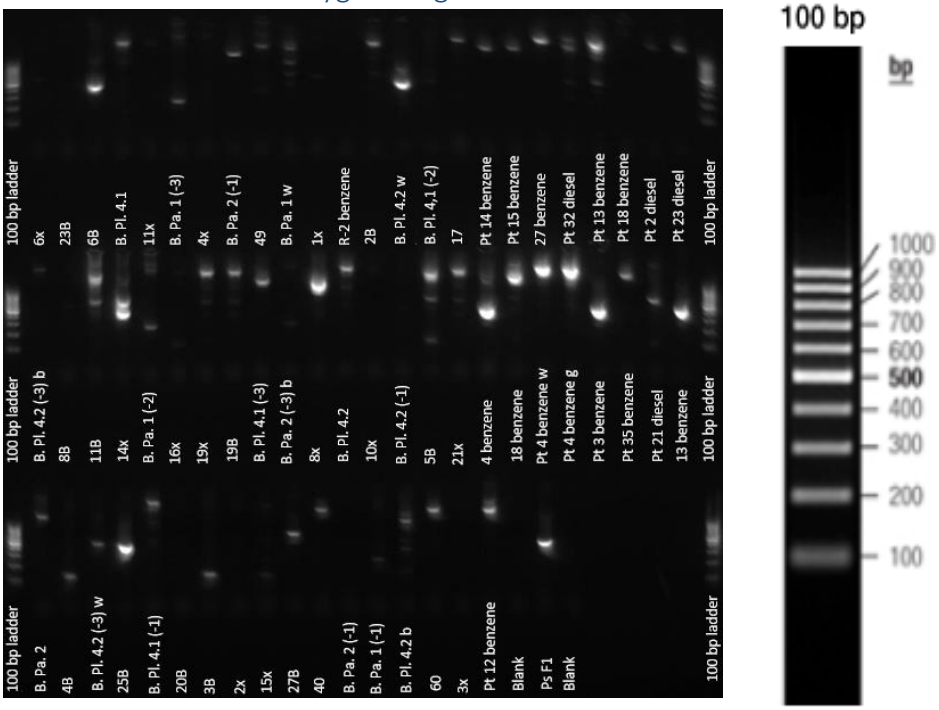


Figure 11: Results of the PCR-amplification of the catechol dioxygenase gene

6.3.5. Xylene monooxygenase- and toluene dioxygenase gene

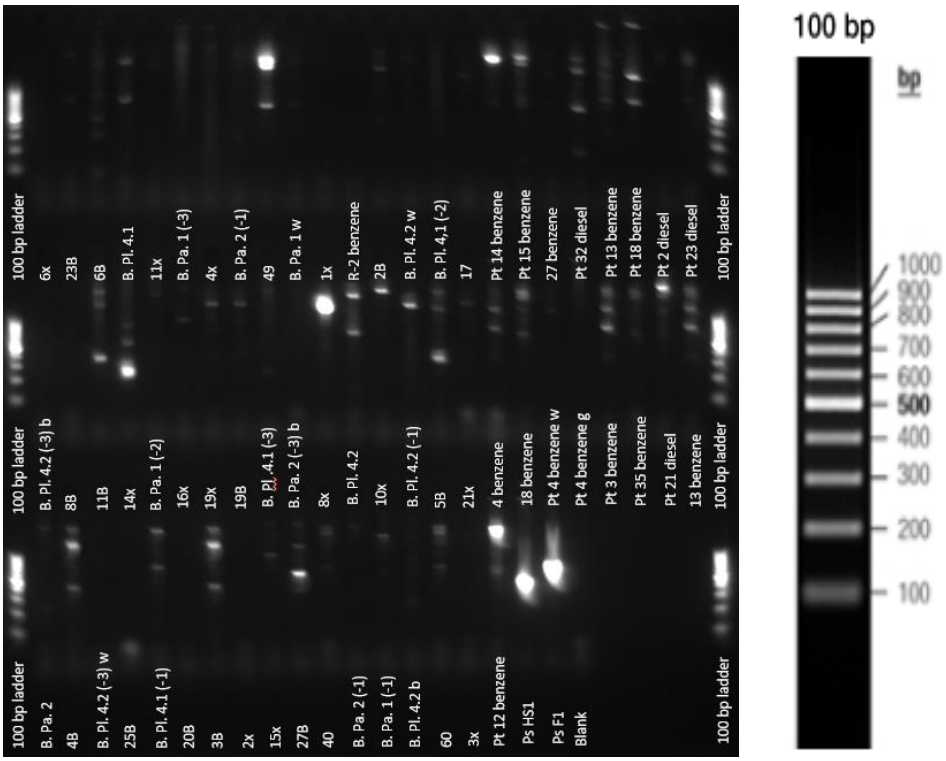


Figure 12: Results of the PCR-amplification of the xylene monooxygenase- and toluene dioxygenase gene

6.3.6. Biphenyl dioxygenase gene

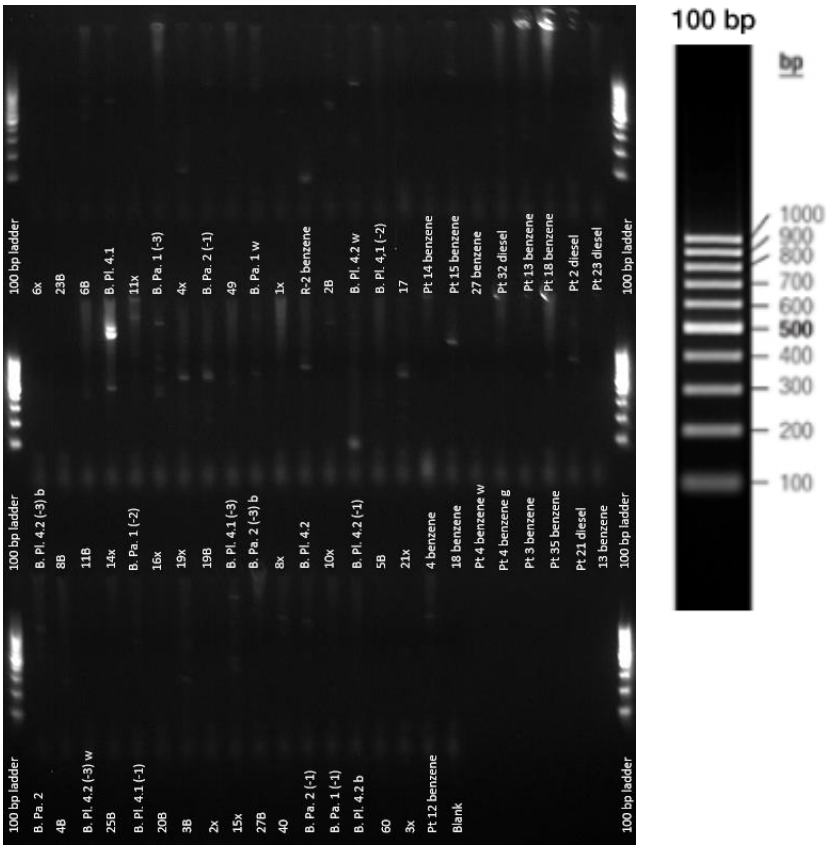


Figure 13: Results of the PCR-amplification of the biphenyl dioxygenase gene

6.3.7. Summary of the positive and negative bacteria for the different aromatic degrading genes after the first screening

Sample code	16S	AlkB	PHE	NAH	CATA	TOL	TOD	BPH
6x	+	+	-	-	-	-	-	-
6B	+	-	+	-	+	+	-	+
11x	+	+	-	-	-	-	-	-
4x	+	-	-	-	-	-	-	-
49	+	-	-	-	+	-	-	-
1x	+	+	-	-	-	-	-	-
2B	+	+	-	-	-	-	-	-
B. Pl. 4.1 (-2)	+	-	-	-	+	-	-	-
23B	+	+	-	-	-	-	-	-
B. Pl. 4.1	+	+	-	-	-	-	+	+
B. Pa. 1 (-3)	+	+	-	-	-	-	-	-
B. Pa. 2 (-1)	+	-	+	-	-	-	-	-
B. Pa. 1 wit	+	-	-	-	-	-	+	-
R-2 benzene	+	-	-	-	-	-	-	+
B. Pl. 4.2 wit	+	+	-	-	+	-	-	+
17	+	+	+	-	-	-	-	-
B. Pl. 4.2 (-3) bruin	+	-	-	-	-	-	-	-
11B	+	+	-	-	+	+	-	-
B. Pa. 1 (-2)	+	+	-	-	-	-	-	-
19x	+	+	-	-	+	-	-	+
B. Pl. 4.1 (-3)	+	-	-	-	-	-	-	+
8x	+	-	-	-	-	-	-	-
10x	+	+	-	-	-	-	-	-
5B	+	+	-	-	+	+	-	-
8B	+	+	-	-	-	-	-	-
14x	+	+	-	-	+	+	+	+
16x	+	-	-	-	-	-	-	+
19B	+	-	-	-	+	-	-	+
B. Pa. 2 (-3) bruin	+	+	-	-	-	-	-	+
B. Pl. 4.2	+	+	-	-	-	-	-	+
B. Pl. 4.2 (-1)	+	+	-	-	-	-	-	-
21x	+	+	-	-	+	-	-	+
B. Pa. 2	+	-	+	-	-	-	-	-
B. Pl. 4.2 (-3) wit	+	-	-	-	+	-	-	-
B. Pl. 4.1 (-1)	+	+	-	-	-	-	+	+
3B	+	-	-	-	-	+	-	+
15x	+	-	-	-	-	-	-	+
40	+	+	-	-	-	-	-	-
B. Pa. 1 (-1)	+	-	-	-	-	-	-	-

60	+	-	-	-	-	-	-	-
4B	+	-	-	-	-	+	-	-
25B	+	+	+	-	+	-	-	-
20B	+	+	-	-	-	-	-	-
2x	+	+	-	-	-	-	-	-
27B wit	+	+	-	-	-	+	+	-
B. Pa. 2 (-1)	+	+	+	-	-	-	-	-
B. Pl. 4.2 bruin	+	+	-	-	+	-	-	-
3x	+	+	-	-	-	-	-	-
Pt 14 benzene	+	-	-	-	-	-	-	-
Pt 15 benzene	+	-	-	-	-	-	-	-
27 benzene	+	-	-	-	-	-	-	-
Pt 32 diesel	+	-	-	-	+	-	+	-
Pt 13 benzene	+	-	+	-	+	+	+	-
Pt 18 benzene	+	-	-	-	-	-	+	-
Pt 2 Diesel	+	-	-	-	-	-	-	-
Pt 23 Diesel	+	-	-	-	-	+	-	-
4 benzene	+	-	-	-	-	-	-	-
18 benzene	+	+	-	-	+	-	-	-
Pt 4 benzene wit	+	-	-	-	-	-	-	-
Pt 4 benzene geel	+	-	-	-	-	-	-	-
Pt 3 benzene	+	-	-	-	-	-	-	+
Pt 35 benzene	+	-	-	-	-	-	-	+
Pt 21 diesel	+	-	-	-	+	-	-	-
13 benzene	+	-	-	-	-	-	-	-
Pt 12 benzene	+	-	-	-	-	-	+	-

6.4. Second screening

The bacteria, that are positive for a certain aromatic degrading-gene out of the first screening, are screened again on a agarose gel with a higher resolution.

6.4.1. Phenol hydroxylase gene

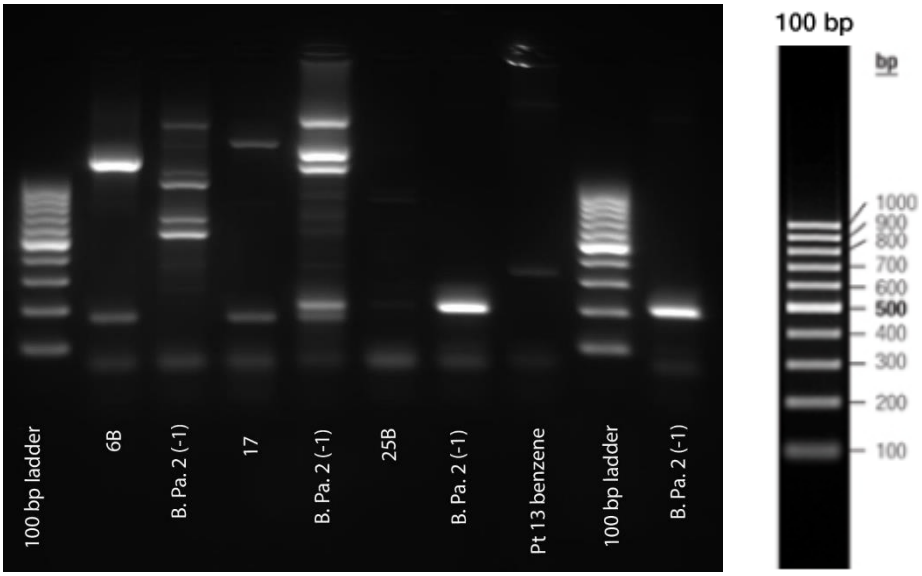


Figure 14: Results of the PCR-amplification of the phenol hydroxylase gene, the bacterium in the third lane is contaminated so this bacteria is again loaded on lane 10

6.4.2. Catechol dioxygenase gene

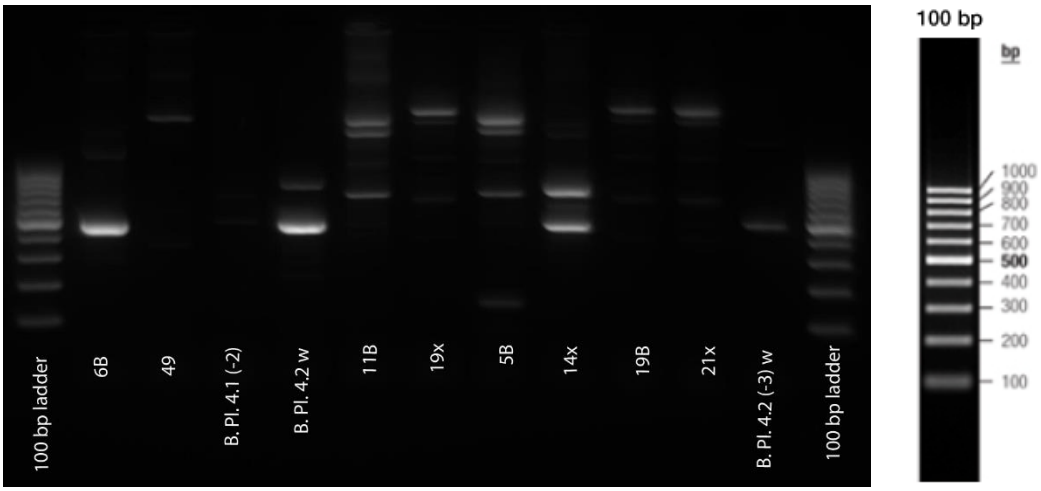


Figure 15a: Results of the PCR-amplification of the catechol dioxygenase gene

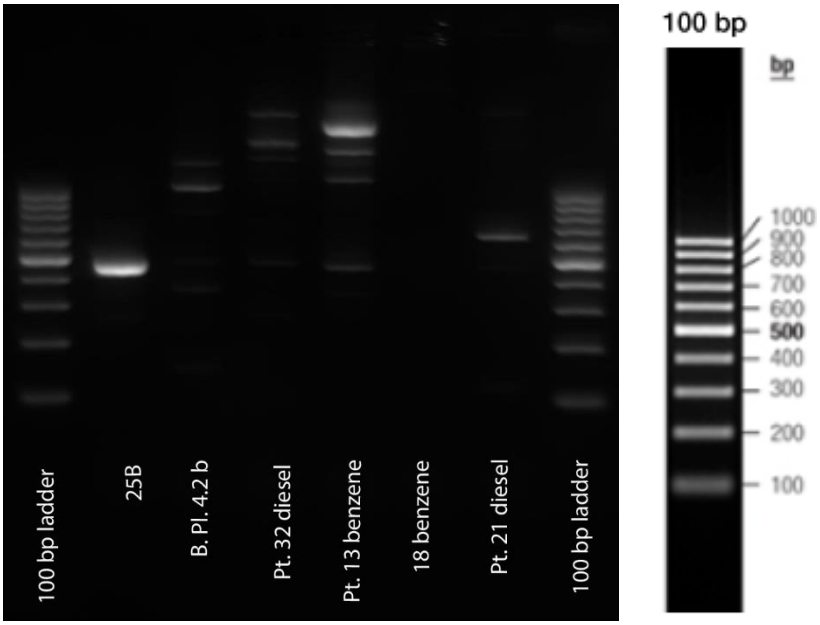


Figure 15b: Results of the PCR-amplification of the catechol dioxygenase gene

6.4.3. Biphenyl dioxygenase gene

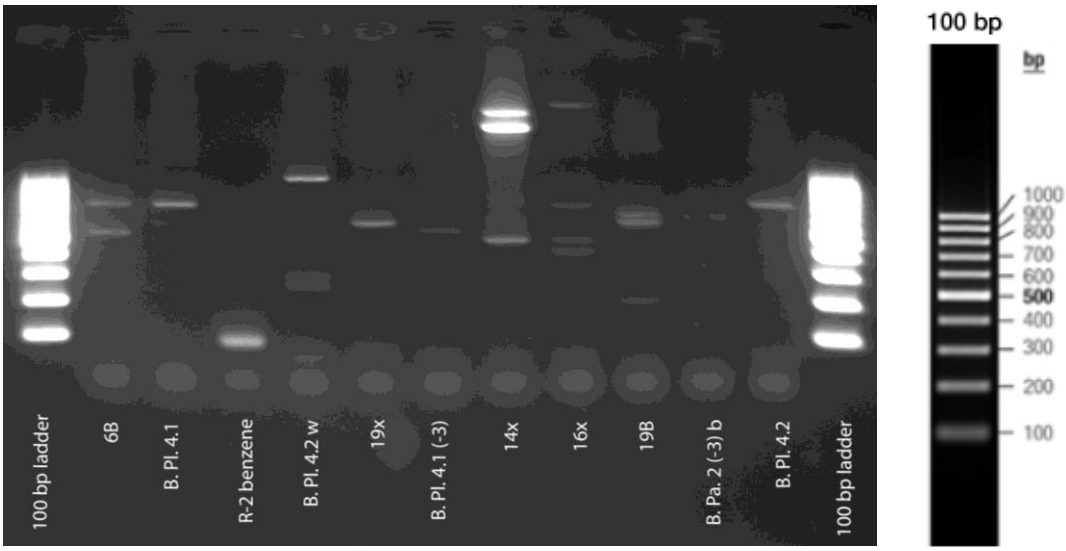


Figure 16a: Results of the PCR-amplification of the biphenyl dioxygenase gene

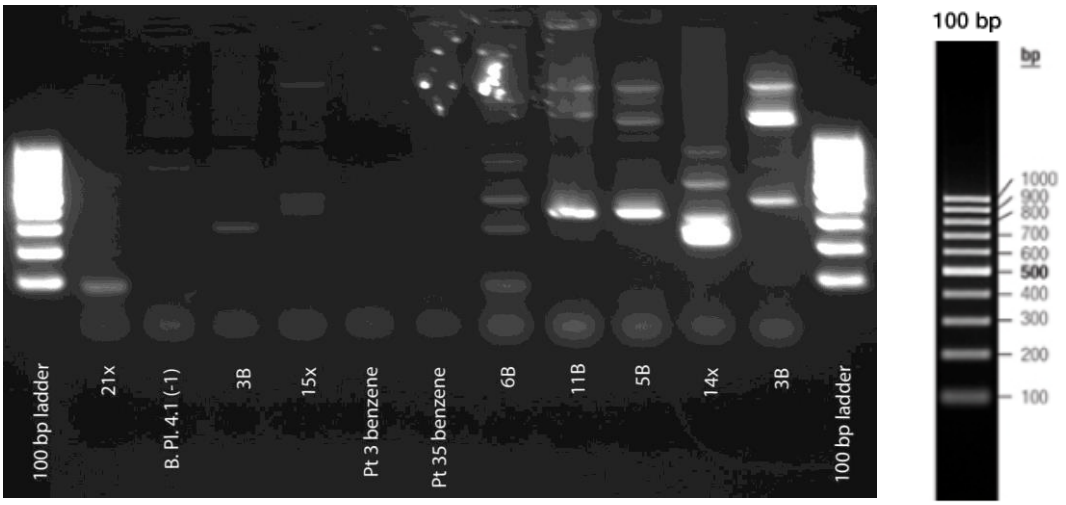


Figure 16b: Results of the PCR-amplification of the biphenyl dioxygenase gene from lane 2 until 7

6.4.4. Xylene monooxygenase gene and toluene dioxygenase gene

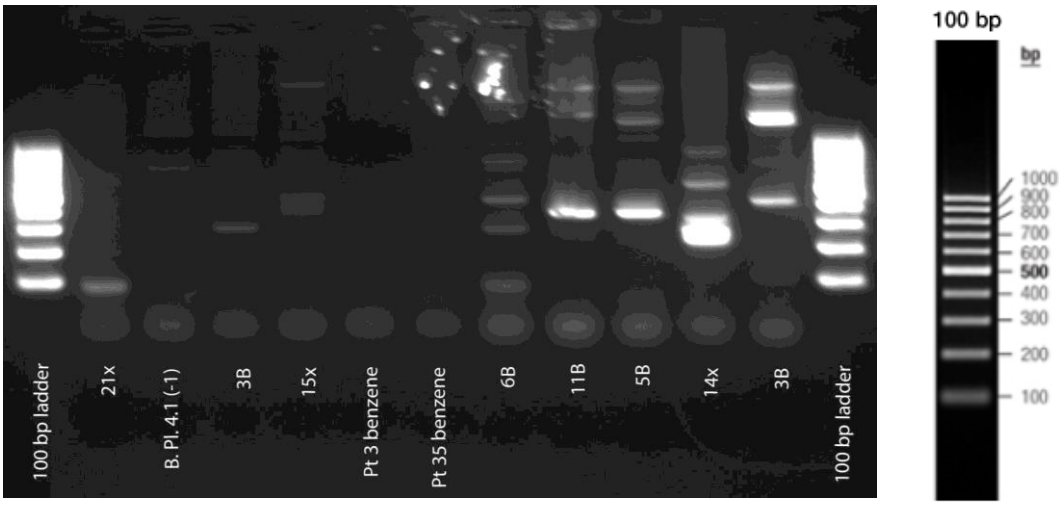


Figure 17a: Results of the PCR-amplification of the xylene monooxygenase- and toluene dioxygenase gene from lane 8 until 12

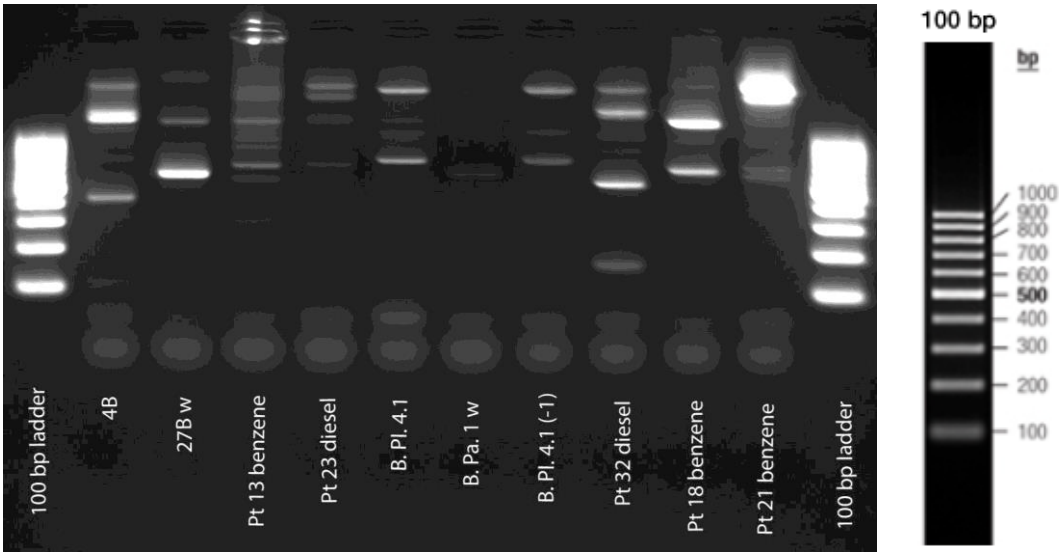


Figure 17b: Results of the PCR-amplification of the xylene monooxygenase- and toluene dioxygenase gene

6.4.5. Summary of the positive and negative bacteria for the different aromatic degrading genes after the second screening

Staal	PHE	CATA	TOL	TOD	BPH
6x	-	-	-	-	-
6B	+	+	+	+	+
11x	-	-	-	-	-
4x	-	-	-	-	-
49	-	-	-	-	-
1x	-	-	-	-	-
2B	-	-	-	-	-
B. Pl. 4.1 (-2)	-	+	-	-	-
23B	-	-	-	-	-
B. Pl. 4.1	-	-	-	+	+
B. Pa. 1 (-3)	-	-	-	-	-
B. Pa. 2 (-1)	+	-	-	-	-
B. Pa. 1 wit	-	-	-	-	-
R-2 benzene	-	-	-	-	-
B. Pl. 4.2 wit	-	+	-	-	-
17	+	-	-	-	-
B. Pl. 4.2 (-3) bruin	-	-	-	-	-
11B	-	+	-	-	-
B. Pa. 1 (-2)	-	-	-	-	-
19x	-	-	-	-	-
B. Pl. 4.1 (-3)	-	-	-	-	+
8x	-	-	-	-	-
10x	-	-	-	-	-
5B	-	+	-	+	-
8B	-	-	-	-	-
14x	-	+	-	-	-
16x	-	-	-	-	+
19B	-	-	-	-	-
B. Pa. 2 (-3) bruin	-	-	-	-	-
B. Pl. 4.2	-	-	-	-	+
B. Pl. 4.2 (-1)	-	-	-	-	-
21x	-	-	-	-	-
B. Pa. 2	+	-	-	-	-
B. Pl. 4.2 (-3) wit	-	+	-	-	-
B. Pl. 4.1 (-1)	-	-	-	+	-
3B	-	-	+	+	+
15x	-	-	-	-	+
40	-	-	-	-	-
B. Pa. 1 (-1)	-	-	-	-	-

60	-	-	-	-	-
4B	-	-	+	+	-
25B	+	+	-	-	-
20B	-	-	-	-	-
2x	-	-	-	-	-
27B wit	-	-	-	-	-
B. Pa. 2 (-1)	+	-	-	-	-
B. Pl. 4.2 bruin	-	+	-	-	-
3x	-	-	-	-	-
Pt 14 benzene	-	-	-	-	-
Pt 15 benzene	-	-	-	-	-
27 benzene	-	-	-	-	-
Pt 32 diesel	-	+	-	-	-
Pt 13 benzene	-	+	-	-	-
Pt 18 benzene	-	-	-	-	-
Pt 2 Diesel	-	-	-	-	-
Pt 23 Diesel	-	-	-	-	-
4 benzene	-	-	-	-	-
18 benzene	-	-	-	-	-
Pt 4 benzene wit	-	-	-	-	-
Pt 4 benzene geel	-	-	-	-	-
Pt 3 benzene	-	-	-	-	-
Pt 35 benzene	-	-	-	-	-
Pt 21 diesel	-	+	-	-	-
13 benzene	-	-	-	-	-
Pt 12 benzene	-	-	-	-	-

6.5. Sequences of ring-hydroxylating oxygenase genes

The positive ring-hydroxylating oxygenase genes, out of the screened bacteria, were send to sequencing. The gene-sequence is hereby determined.

Tracking number	Sample code	Gene	Accession number	Sequence	Reference
AS_08	6B	Catechol dioxygenase	SKC04095.1	MTVKISHTADIQAFFNRVAGLDHAEGNPRFKQIILRVLQDTARLIEDLEITEDEFWHAVDYLNRLGGRNE AGLLAAGLGIEHFLDLLQDAKDAEAGLGGGTPRTIEGPLYVAGAPLAQGEARMDDGTDPGVVMFLQGQVF DADGKPLAGATVDLWHANTQGTYSYFDSTQSEFNLRRRIITDAEGRYRARSIVPSGYGCDPQGPTQECLD LLGRHGQRPAHVHFFISAPGHRHLTTQINFAGDKYLWDDFAYATRDGLIGELRFVEDAAAARDRGVQGER FAELSFDFRLQGAKSPDAEARSHRPRALQEG	(Varghese 2017)
AS_11	14x	Catechol dioxygenase	AMW86009.1	MTIRLSQTAHAQQFLEEASGNLNDGGNPRAKALIYRILRDTVNIIEDLEVTPEEFWKAVNYLNELGKNQE AGLLAAGLGLEHYLDMLMDAADEEAGKSGGTPRTIEGPLYVAGAPLSKYEARLDDGQDPGVPLFMQGQVR DTNGKPLAGAIVDVWQANTGGTYSWFDGTQSEFNLRRRIETDAQGNYRFRSIVPSGYGCPPTGPTQQLLD QLGRHGQRPAHIHFFISAPGHRHLTTQINLSDDPYLHDDFAYATRDELIAEIRFSEDANLAKEFGVDGQF AQIDFDFELQPAAAPVEQKRMQRVRALED	(Morrison et al 2016)
AS_37	Pt 13 benzene	Catechol dioxygenase	AMR16191.1	MSNAFVQQEAVQKLLREGAGLHVPGGNERFKAIIHRLLENICTLIDDYNITEEEFWHAVNYLHALGGRQE AALLAAGLGLEHFLDLRQDAIDAAARRETGTPRTIEGPLYVANAPLAEGHARMDDGADPGEVMWLHGQVK DTEGRPVANAIVDIWHANTLGNYSFFDPSQSEYNLRRRIRTGADGRYSVRSILPSGYGCPPDGPTQKLLD QLGRHGNRPAHIHFFVSAPGHKHLTSQINLSGDKYLWDDFAFATRDGLIADPIKVTDRETIAQRDLEGEH TEVCFDFTLCKALSADEEQRGARLRAKA	(Elliott et al 2016)
AS_47	B. Pl. 4.2 wit	Catechol dioxygenase	ARQ57195.1	MSVKIFGRPDIQDFLSVLSGLDKDGGNPRVKLIVHRIMSDLFKAIDDLDITPDEYWSGIAWLNEIGAAGQ AGLISPGLGLDHFLDERLDAIDEALGIENPTPRTIEGPLYVAGAPVCHGFARLDDGTDANGHTLIMHGTV YGSDGKPQPGATVEVWHCDTRGFYSHFDPTGKQAPFNMRRTIVTDEQGCYKFQSIVPHGYGVPPGSPTEQ LLSALGRHGQRPAHIHLFISADGHRKLTTQINIEGDPLVNDDFAYATRDGLVPALIERSDEASIKANGLS GPFAEITFDIHLTALVDGIDNQINEQRKRAAA	(Santamaria et al 2017)