Bachelor Agricultural and Biotechnology

Biotechnology - Specialisation

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Yorben Douwen

Promotoren:

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PXL TECH

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Degradation of airborne environmental pollutants using phytoremediation

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As air pollution has been linked to a numerous amount of health problems, research to improve air quality is necessary. Phytoremediation has already shown to be effective in cleaning air as plants are known to scavenge significant amounts of air pollutants on their aboveground plant parts. Despite the complexity in composition of air pollutants, phyllospheric bacteria are promising candidates to help plants to detoxify the pollutants by means of degradation. For this reason selected bacteria from highly polluted sites in Poland and Belgium were inoculated on common ivy (*Hedera helix*) in order to study the ability to degrade naphthalene and benzene. Cuvette systems were setup containing naphthalene and benzene in the air and concentration changes were measured using highly sensitive analytical mass spectrometry techniques. In addition, genome annotation of *Pseudomonas yorbenii 4.1* and *Pseudomonas veronii 4T1* was performed to evaluate their capabilities for aromatic pollutant degradation and plant growth promotion in the field of phytoremediation. Our results indicated that both strains have the genes (NdoA, NahB, XylE, CatA) to degrade different polycyclic aromatic hydrocarbons and can fully degrade naphthalene using the 1,2-dioxygenase pathway. Moreover, the results of the Proton-transfer-reaction mass spectrometry analyses confirmed this by an increased degradation of naphthalene in the cuvettes with ivy inoculated with a consortium of bacteria. This study confirmed that the isolated bacteria are able to degrade airborne environmental pollutants. Further research is needed using these data to improve phytoremediation of airborne pollutants and enhance the degradative activity of bacteria in polluted environments.

Keywords: phytoremediation, leaf microorganisms, ivy, naphthalene, benzene, airborne pollutants

1. Introduction

More people now die from air pollution than malaria and the human immunodeficiency virus (HIV) infection together, this has recently been reported in a high ranked journal (Lelieveld, et al., 2015). The adverse effect of air pollution on human health has also been demonstrated by numerous other epidemiological studies (Saravia, et al., 2014). Air pollution is a combination of a variety of pollutants including gases (NOx, SO_2, CO_2, O_3), particulate matter (PM), volatile organic compounds (VOCs) like benzene, toluene, ethylbenzene, xylene (BTEX) and semi-volatile compounds like polycyclic aromatic hydrocarbons (PAHs). Besides some natural volatile compounds, most of the volatile air pollutants are of human origin and produced via incomplete combustion of carbon containing fuels (wood, oil, coal) from industrial, commercial, vehicular and residential sources. Many of the outdoor air pollutants are also found indoors in concentrations that often can be higher than outdoors (Myers & Maynard, 2005). In addition, several of the PAHs are toxic and/or possible or proven human carcinogens (Nurmatov, et al., 2015).

Despite the complexity of the association between plants and millions of other organisms such as fungi and bacteria, phytoremediation was already shown to be an effective way to detoxify soil and

groundwater pollutants (Weyens, et al., 2015). Phytoremediation uses the synergistic action of plants and their associated microorganisms to transform, degrade or detoxify environmental pollutants. A special type of phyto-remediation is phyllo-remediation which makes use of the aboveground plant parts (leaf canopy) and the phyllosphere associated microorganisms to scavenge and detoxify air pollutants (Montalban Gines, et al., 2016). In total, the estimated leaf surface area comprises 1.017.260.200 km2 on Earth and there are an estimated 10^{26} bacterial cells living on the leaves, which can have potentially a large influence on atmospheric processes (Vorholt, 2012).

To confirm the assumption that soil and phyllospheric bacteria can be involved in the degradation of urban-related volatile pollutants, we designed a series of experiments using static cuvette systems containing a consortium of microorganisms, a common ivy *Hedera helix,* soil and the air pollutants naphthalene and benzene. We measured the concentrations of the compounds in the air phase and the degradation products using state-of-the-art PTR-TOF-MS technique. The microorganisms used in these experiments were collected from a diesel-polluted site in Bóbrka, Poland.

The purpose of our setups was to see if the selectively grown bacteria can degrade naphthalene and benzene in a controlled environment with plants. To have greater insights in the abilities of the microorganisms, they were also tested for the production of volatiles which can induce plant growth promotion effects. Altogether, our results will put us a big step forward to the implementation of phylloremediation in cities threatened by air pollution.

2. Material & methods

2.1. Selection of strains used in this study

Soil samples and samples from leaves were collected from a highly polluted area: a former oil-industry facility in Bóbrka, Poland (Muzeum Przemysłu Naftowego i Gazowniczego, sd). Five different samples from Bóbrka were analysed to isolate PAH-degrading microorganisms. Three other samples were collected from a non-polluted national park from Poland, Białowieża. These were used as a control to select pollution-specific microorganisms. The samples were taken from the soil (around the rhizosphere of plants, around a bucket with crude oil, at a channel..) or washed and collected from the leaves of trees around the park. Four other samples were also washed from leaves and collected from the soil in a diesel contaminated site in Beverlo, Belgium.

Microorganisms were isolated using solid Bushnell-Haas medium (BH, annex 1) with only PAHs (naphthalene, toluene, phenanthrene or diesel) as a carbon source. Each petri dish contained 0,3 ml of naphthalene, toluene or phenanthrene in liquid or air phase. All the different colonies that grew were analysed for the presence of the naphthalene-like dioxygenases and tested for plant promotion. Microorganisms with the best results on all the tests were selected for further experiments (Table 1).

Table 1 PAH-degrading microorganisms isolated from soil or leaves

Strain ID	Colony morphology	Isolation source	Site
Pseudomonas yorbenii VI4.1	Beige round smooth	Soil	Bóbrka, Poland
Pseudomonas veronii VI4T1	Yellow round sticky	Soil	Bóbrka, Poland
Pseudomonas putida VI4.9a	Beige round smooth	Soil	Bóbrka, Poland
Pseudomonas yorbenii VI4N1	Beige round smooth	Soil	Bóbrka, Poland
Pseudomonas veronii VI4T2	Yellow round sticky	Soil	Bóbrka, Poland
Pseudomonas collierea LK2E02	Beige round smooth	Phyllosphere	Bóbrka, Poland
Non_identified strain LK2E03	Beige round smooth	Phyllosphere	Bóbrka, Poland
Exiguobacterium sibiricum LK2E04	White round smooth	Phyllosphere	Bóbrka, Poland
Non_identified strain LK3E05	Beige round smooth	Phyllosphere	Bóbrka, Poland
Non_identified strain LK3E07	Beige round smooth	Phyllosphere	Bóbrka, Poland
Brucella sp. LK3E10	Beige round smooth	Phyllosphere	Bóbrka, Poland
Stenotrophomonas humi AS55	Beige round smooth	Phyllosphere	Bóbrka, Poland
Pseudomonas putida AS12	Beige round smooth	Phyllosphere	Bóbrka, Poland
Bacillus pumilis AS24	White roud smooth	Soil	Beverlo, Belgium
Non_identified strain AS04	White round smooth	Soil	Beverlo, Belgium

2.2. DNA extraction and whole genome sequencing of strains VI4.1 and VI4T1 using Ion Torrent

RNA-free genomic DNA was isolated from stationary phase cells grown in LB (Invitrogen PureLink Genomic DNA Mini Kit, Life Technologies Inc., Burlington, ON) prior to digesting and ligating sequencing adaptors and barcodes using an Ion Xpress Plus Fragment Library Kit (Life Technologies Inc., Burlington, ON). Adaptor-ligated DNA was size selected to 480 bp on a 2% E-Gel SizeSelect agarose gel and Agencourt MAPure XP beads (Beckman Coulter, Mississauga, ON) were used for purification. The library dilution factor was determined using an Ion Library Quantitation Kit prior to amplification and enrichment with an Ion PGM Template Hi-Q OT2 400 kit on an Ion OneTouch 2 system. The enriched Ion Sphere Particles were quantified using an Ion Sphere Quality Control Kit prior to sequencing on a 316v2 chip with an Ion PGM Hi-Q View Sequencing Kit on an IonTorrent PGM (Life Technologies Inc., Carlsbad, CA). Quality filtered reads were assembled using SPAdes 3.8.2 (uniform coverage mode; k-mers 21, 33, 55, 77, 99).

2.3 Genome annotation and search for degradative genes

Genome annotation was completed using RAST (Aziz *et al.*, 2008) and NCBI's PGAP (Abarenkov *et al.*, 2010).

2.4 Accession number of genome sequenced strains

The draft annotated genome sequence of *Pseudomonas yorbenii VI4.1* has been deposited in GenBank under the accession number MULM00000000. The version described in this paper is the first version, MULM00000000.1. The draft annotated genome sequence of *Pseudomonas veronii VI4T1* has been deposited in GenBank under the accession number MULN00000000. The version described in this paper is the first version, MULN00000000.1.

2.5 Degradation of naphthalene in the air by strain VI4.1 and VI4T1 in soil

To test how efficiently the bacteria can degrade PAHs, they were exposed to naphthalene and benzene concentrations in the air-phase and the concentrations of the compounds over time were measured using PTR-TOF-MS. This instrument allows the measurement of concentrations of VOCs in real-time with high sensitivity (into the low pptv range) and a fast response time (in the 40–100 ms time regime) (García-Plazaola, et al., 2017).

The first experiment was performed in sterile 20 ml vials. To triplicate vials we added 1.5 g of sterile potting soil (Asef potting soil osmocote) together with 1 ml of liquid 284 medium (annex 2) (control), or 1 ml of *Pseudomonas yorbenii VI4.1* culture in 284 medium (OD_{600} of 1), or 1 ml of *Pseudomonas veronii VI4T1* culture in 284 medium (OD_{600} of 1). To each of the vials 15 ppmV naphthalene was added in the air phase by spiking 0.2 µl of a 1.5 mg naphthalene per ml ethanol stock solution. Blank vials were empty vials, without soil, without media, without bacteria to detect leakage or a-specific absorption.
The second experiment was performed in sterile 100 ml vials to scale up the test-system. Similarly as described above, to triplicate 100 ml vials, we added 3 g of sterile potting soil (Asef potting soil osmocote) together with 200 µl of liquid 284 medium (annex 2) (control), or 200 µl of a 15-member bacterial consortium in 284 medium including *Pseudomonas yorbenii VI4.1* and *Pseudomonas veronii VI4T1* at an optical density at 600 nm of 1 (*treatment)*. Then the vials were spiked with 52.47 ppmV of naphthalene and 45218 ppmV of benzene (3.7 µl of pure benzene). Four replicas were made.
For both experiments, the vials were moved to the pre-growth chamber for 10 days. The conditions were 16 hour day/ 8 hour night, light intensity of 100 µmol/m^2sec and temperatures of 22°C during the day and 20°C at night and constant humidity of 52 %. After the 10 days, concentrations of naphthalene and benzene in the airspace were measured using PTR-TOF-MS.

2.6 Set-up of a controlled phylloremediation experiment in cuvettes using ivy (*Hedera helix*)

In this experiment, we aimed to increase the degradation of volatile aromatic components such as naphthalene and benzene using degradative bacteria inoculated on the leaves of the plant and in the soil. We chose *Hedera helix* as our plant model because it is a commonly known plant that can be used in many field situations (hedges, green walls, etc.). Moreover ivy is not losing its leaves in winter so it is an ideal plant for year-round phylloremediation.

Figure 1 static cuvette system

The experiment took place in static two-compartment glass cuvette systems assembled like shown in figure 1. Important, when using static systems, is to be sure everything is sealed perfectly so no air-components can leave. If anything can escape the system, the results won't be accurate about what happened inside. To seal the two outlets of the cuvettes, we used Mininert valves (24mm, Supelco) on glass screw thread tubes sealed with Parafilm on the outside.

The cuvette systems consist of two separate parts, an upper part of 1 litres and a lower part of 550 ml. Both parts are completely separated from each other using Teflon plates only the stem of the plant letting through to avoid gas exchange between the two compartments. The space around the stem is sealed off with Monokit paste (Soudal) and wrapped around with Teflon tape. The Teflon plates are covered on the outside with the same Monokit paste and Teflon tape, the glass parts are closed on the plates with Joint Grease (KaWeS). Additional plates on outside are used to create more stability.

The set-up as described was repeated 6 times, three were used as a control and the

other three were inoculated with the consortium of 15 bacteria. In each part (both upper and lower parts), a tube containing $CaCl_2$ was added to control humidity inside the flasks (DOW, 2003). The ivy plants inside were washed three times with 10mM $MgSO_4$ buffer solution under sterile conditions to clean them from bacteria and fungi before adding the consortium. In the lower part, 95 grams of sterilized potting soil was added with 50ml of demineralized water.

After three days of adaption in a pre-growth chamber (bright light, constant humidity and temperature of 23°C), the leaves and soil were inoculated separately by spraying them with 7.5 ml of a 15-member bacterial consortium (OD_{600} of 1). For the consortium preparation, 15 degradative bacteria (Table 1) were grown overnight on solid 869 rich medium (annex 3). The next day we moved 1 colony of each bacterium into one tube containing liquid 284 minimal media and a carbon source. In the controls 7.5 ml of 284 minimal media was added to each part to create the same situation in each flask. After another three days of adaptation, 25 ml of Hoagland medium (annex 4) was added to the lower part and the process was repeated, only this time 3.5 ml of bacteria was added to the top part and 7.5 ml to the lower part. Again, the controls were sprayed with 3,5 ml 284 minimal media to the top part and 7.5 ml of the same media to the lower part.

On the same day as last bacterial inoculation, the upper and lower airspace compartments were exposed to benzene and naphthalene with a glass syringe and an inert needle. In each compartment, a concentration of 10.5 ppmV of naphthalene and 9134 ppmV of benzene was added. These concentrations are based on earlier researches of mono- and polycyclic aromatic compounds (Weyens, 2009). However, next time we have to lower the benzene concentration that is spiked. After 10 days of incubation, the cuvettes were analysed using PTR-TOF-MS and GC-MS.

2.7 Set-up of controlled air-pollutant degradation experiment in cuvettes using ivy (Hedera helix) and simulating rain

In the last experiment, we want to analyse the effect of rain in a controlled environment on the degradation of the polluted components. Sterilized 550 ml flasks containing 50 grams of sterile potting soil, 20 ml sterile Hoagland and a clean ivy plant (*Hedera helix*) were used. A tube of $CaCl_2$ was added to control humidity. The ivy plants were washed with the same procedure as described in experiment 2.6. These flasks were moved to the pre-growth chamber, after three days, 7 ml of the consortium of 15 bacteria (described in section 2.6) was sprayed in 6 replicas. To the other 6 replicas, 7 ml of 284 minimal medium was added as controls. This was repeated after another three days of incubation in a pre-growth chamber. The next step was adding the naphthalene and benzene, again with the same concentrations as in section 2.6, to the flasks. All flasks were closed using Mininert valves (24mm, Supelco) on the sidearm and a septum stopper on top, both wrapped around with Parafilm. The septum stopper has a septum which can be punctured with a syringe needle to inject the rain simulation, the septum closes again after injection. Subsequently, these flasks were also analysed using PTR-TOF-MS.

To check if all the bacteria of the consortium can colonise our plants and survive the tests, we added a leaf imprinting experiment. After spraying the leaves a second time with the consortium, one leaf was cut off and both sides were gently pushed on a $1/10^{th}$ concentrated 869 rich medium agar plate with indole. The same was done with the control flasks. After the PTR-TOF-MS test, another leaf is cut off and imprinted on another $1/10^{th}$ concentrated 869 rich medium agar plate with indole to see the difference or similarity between the moment of imprinting but also between the controls and the ones with the consortium.

2.8 PTR-TOF-MS data analysis and data reduction

VOCs are detected in real-time through proton transfer reactions occurring between the H3O+ ions produced within the ion source and the sample gas inserted into the drift tube (Brilli *et al.*, 2014). The drift tube is kept under controlled conditions of pressure (2.3 mbar), temperature (50 °C) and voltage (600 V) resulting in a field density ratio (E/N) of ≈130 Td (E being the electric field strength and N the gas number density; 1 Td = 10−17 V cm2). After a performed proton transfer reaction, protonated ions are extracted from the drift tube and pulsed every 30 µs to the orthogonal time-of-flight region to be separated according to their m/z ratio in the time-of-flight before being detected in conjunction with a multi-channel-plate (MCP) and a time-to-digital converter (TDC) (Burle Industries Inc., Lancaster, PA, USA).

Raw 10 Hz time series of high resolved full mass spectra ranging between 1 and 315 m/z were continuously acquired by the TofDaq software (Tofwerk AG, Switzerland) and stored in hdf5 file format in 6-min time periods (http://www.hdfgroup.org/). After acquisition, each file was post-processed by the routine programs of a software designated for PTR-TOF-MS data analysis (Brilli *et al.*, 2014). This allowed an accurate mass scale calibration and peak detection through peak shape analysis, iterative residual peak analysis to detect multiple isobaric peaks per unit m/z, and quantification of the fitted peak areas corresponding to ion signal intensities based on the 6-min sum spectra. In addition, this software performs post-analysis quality assurance checks of the whole outcome produced.

2.9 Determination of VOC concentrations and calibration

Theoretical absolute concentrations of VOCs were determined from PTR-TOF-MS measurements via the first order kinetic reaction (Lindinger *et al.*, 1998) after applying the correction for sample humidity (Warneke *et al.*, 2001) and for the effect of the duty cycle as proposed by Cappellin et al. (2012).

$$[\mathrm{VOC}] = (1/k\tau)*\left([\mathrm{VOC}\cdot\mathrm{H}^+]_{\mathrm{measured}}/[\mathrm{H_3O}^+]_{\mathrm{measured}}\right)* \left(\sqrt{(m/z)}_{\mathrm{H_3O}^+}/\sqrt{(m/z)}_{\mathrm{VOC}\cdot\mathrm{H}^+}\right)$$

The concentrations [VOC·H+] and [H3O+] are signal ion rates corresponding to the protonated VOC-ions and the primary ion, respectively; τ is the reaction time of [H3O+] in the drift tube; k is the reaction rate coefficient between VOC and H3O+ according to the tabulation provided by Cappellin et al. (2012). When a k for a specific VOC was not available, a standard value of 2*10−9 cm3 s−1 was used (Cappellin *et al.*, 2012). The [H3O16+] concentration at m/z = 19.018 was calculated from the natural isotope [H3O18+] measured at m/z = 21.022.

The background signal of the PTR-TOF-MS was quantified via an automated system of switching valves that introduced VOC-free air, generated by a commercially available gas calibration unit (Ionicon, Innsbruck, Austria) for 6 min every 6 h and the resulting average value was subtracted from all the previously recorded data.

2.10 GC-MS data analysis

VOCs can also be detected using Gas Chromatography Mass Spectrometry analyses. For this we used a 30 meter capillary column DB5-MS, 0.25 mm internal diameter with a film thickness of 0.25 µm (Agilent Technologies). The GC MS parameters used were: 1 µl injection by the TriPlus RSH-autosampler (Thermo Scientific), 30 s splitless mode at 280 °C and split flow at 50 ml min^{-1}. The column temperature was initially 35°C for 1 min, then gradually increased to 245°C at 15°C/min. The MS conditions were a scan at 33-100 in 0.2 seconds for benzene and 33-150 in 0.3 seconds for naphthalene. The GC used was a Trace 1310 gas chromatograph and the MS was a ISQ LT Single Quadrupole Mass Spectrometer (Thermo Scientific). The mass spectra were identified by comparison with the NIST mass spectra database.

3. Results

3.1 Genome annotation and search for degradative genes

For *Pseudomonas yorbenii VI4.1*, sequencing generated a total of 1.24 million reads (mean length 299 bases) and 371 Mb of data (> 344 M Q20 bases) in Torrent Suite 5.0.5. These were assembled using SPAdes 3.8.2 (1, 2) (uniform coverage mode; kmers 21, 33, 55, 77, 99, 127) into 185 contigs greater than 1000 bp, giving a consensus length of 7 346 306 bp at 30 x coverage (GC content 60.0 %; largest contig 285 953 bp; N50 = 22 673 bp). The RAST Server indicated that closely related strains are *Pseudomonas* sp. A3, *P. mandelii* JR-1, *P. mandelii* LMG 21607 and *Pseudomonas fluorescens* NCIMB 11764.

ORF prediction and gene annotation were completed using the NCBI PGAP pipeline and RAST. The genome of *Pseudomonas yorbenii* VI4.1 has a GC content of 60.0 % and includes 5,951 coding genes, 770 pseudogenes, 7, 3, 2 rRNAs (5S, 16S, 23S), 57 tRNAs, and 4 ncRNAs.

Genes that encode for proteins involved in the naphthalene degradation were located using local blast searches, including naphthalene 1,2-dioxygenase complex: ndoA (2 genes), ndoB (1 gene), ndoC (1 gene) and ndoR (1 gene), and further degradation nahB (1 gene), nahC (1 gene), nahE (1 gene) and nahF (2 genes). All the genes of the naphthalene dioxygenase pathway (shown in Figure 2) are present.

Genes that encode for proteins involved in the toluene degradation were located, including tbuB (1 gene), tbuE (1 gene), todB (1 gene), todF (1 genes), todH (3 genes), todI (4 genes), xylE (2 genes), xylF (1 gene), xylG (2 genes), xylH (2 genes), xylI (4 genes), xylJ (3 genes), xylL (2 genes), xylX (2 genes), xylY (1 gene) and xylZ (2 genes). Furthermore, genes encoding for proteins involved in hydrocarbon (octane) degradation were also located, including alkF (1 gene), alkG (2 genes) and alkJ (3 genes).

Genes that encode for proteins involved in catechol degradation were located including catA (4 genes), catC (6 genes), dmpF (4 genes), dmpG (3 genes), pcaD (1 gene), pcaF (8 genes), pcaI (1 gene), pcaJ (2 genes), salD (2 genes) and xylK (3 genes).

Genes related to plant growth promotion features were localized too. Genes encoding for the biosynthesis of zeatin: LOG (1 gene), for biosynthesis of indol-3-acetate: NIT1 (1 gene), NIT2 (1 gene) and NIT3 (1 gene), biosynthesis of jasmonic acid: PED1 (6 genes), OPR3 (2 genes) and steroid hormone biosynthesis: AKR1C3 (1 gene).

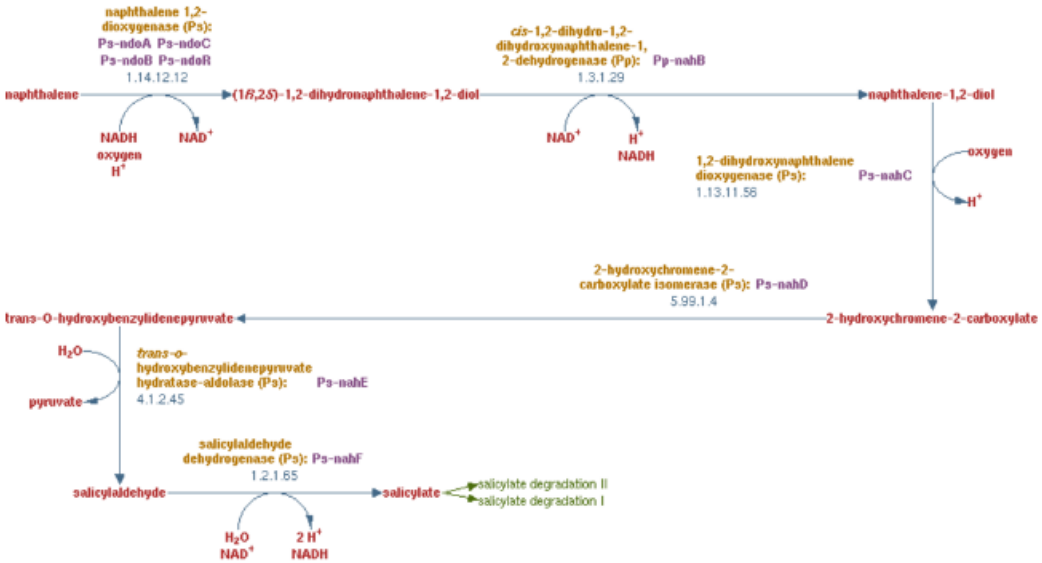

Figure 2 naphthalene degradation pathway

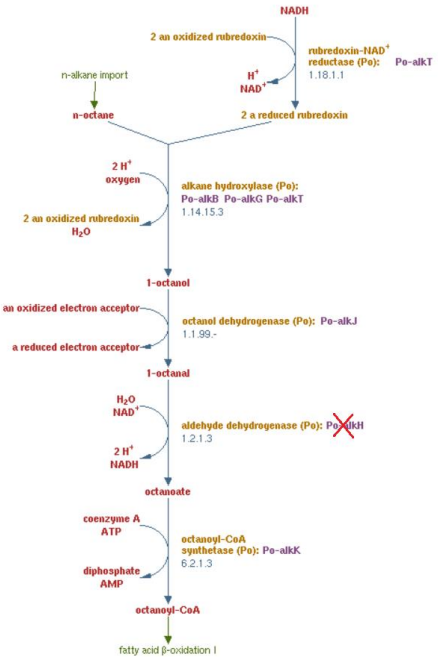

Figure 3 octane oxidation pathway

For *Pseudomonas veronii VI4T1*, in total, 1.1 million reads (mean length 300 bases) generated 331 Mb of data (> 307 M Q20 bases) in Torrent Suite 5.0.5. These were assembled using SPAdes 3.8.2 (uniform coverage mode; kmers 21, 33, 55, 77, 99, 127) in 210 Contigs greater than 1000 bp, giving a consensus length of 7 129 343 bp at 30 X coverage (GC content 60.5 %; largest contig 237 158 bp; N50 = 18 545bp).

The genome of *Pseudomonas veronii* VI4T1 has a GC content of 60.5% and includes 5,744 coding genes, 867 pseudogenes, 7, 1, 2 rRNAs (5S, 16S, 23S), 59 tRNAs, and 4 ncRNAs. Genes that encode for proteins involved in the naphthalene degradation were located including naphthalene 1,2-dioxygenase complex: ndoA (1 gene), ndoB (1 gene), ndoC (1 gene) and ndoR (1 gene), and further degradation nahB (1gene), nahC (1 gene), nahE (1 gene) and nahF (1 gene). All the genes of the naphthalene dioxygenase pathway (shown in Figure 2) are present.

Genes that encode for proteins involved in the toluene degradation were located, including tbuB (1 gene), todF (2 genes), todH (3 genes) and todI (3 genes), xylF (3 genes), xylG (1 gene), xylH (1 gene), xylJ (2 genes), xylX (1 gene) and xylZ (1 gene).
Furthermore, genes encoding for proteins involved in hydrocarbon (octane) oxidation were present including alkB (2 genes), alkF (4 genes), alkG (6 genes), alkJ (3 genes), alkK (1 gene) and alkT (1 gene) shown in Figure 3. Genes that encode for proteins involved in catechol degradation were present including catA (2 genes), cat B (2 genes), dmpF (3 genes), dmpG (3 genes), pcaD (1 gene), pcaF (4 genes), pcaI (1 gene), pcaJ (1 gene), salC (1 gene), salD (1 gene) and xylK (3 genes).

Genes related to plant growth promotion features were localized too. Genes encoding for the biosynthesis of zeatin: LOG (1 gene), for biosynthesis of indol-3-acetate: NIT1 (1 gene), NIT2 (1 gene), NIT3 (1 gene) and AMT1 (1 gene) and biosynthesis of jasmonic acid: PED1 (4 genes), OPR3 (3 genes).

3.2 Degradation of naphthalene in the air by 4.1 and 4T1 in soil

Adding *Pseudomonas veronii VI4T1* or *Pseudomonas yorbenii VI4.1* to the 20ml vials resulted in a decrease of naphthalene concentration (Figure 4). Taking into account that the soil absorbed an average of 72.56% of the total naphthalene spiked, we can analyse the remaining contaminant degradation by our bacteria. Inoculation of the soil with *Pseudomonas veronii VI4T1* resulted in a further degradation of 88.38 % in comparison to the non-inoculated control and 96.82% degradation by *Pseudomonas yorbenii VI4.1*.

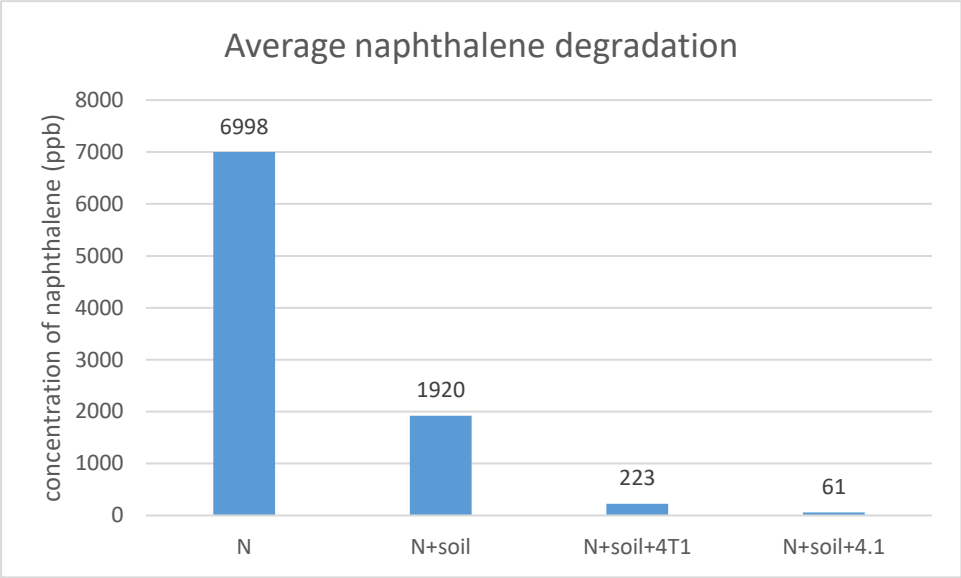

Figure 4 Results of naphthalene degradation in 20 ml vials in parts per billion (ppb) comparing only naphthalene (N), naphthalene + soil (N+soil), naphthalene + soil and pseudomonas veronii 4T1 (N+soil+4T1) and naphthalene + soil + pseudomonas yorbenii 4.1 (N+soil+4.1).

The results of the concentrations in the 100ml vials are presented in parts per billion by volume (ppbV). For the naphthalene present in the vials at the moment of spiking, we measured an average concentration of 1865 ppbV in the vials with bacteria and 5769 ppbV in the controls. There is thus a significant decrease in the amount of naphthalene in the vials with the bacteria, the same trend is found in the average concentration of the naphthalene degradation products (Figure 5).

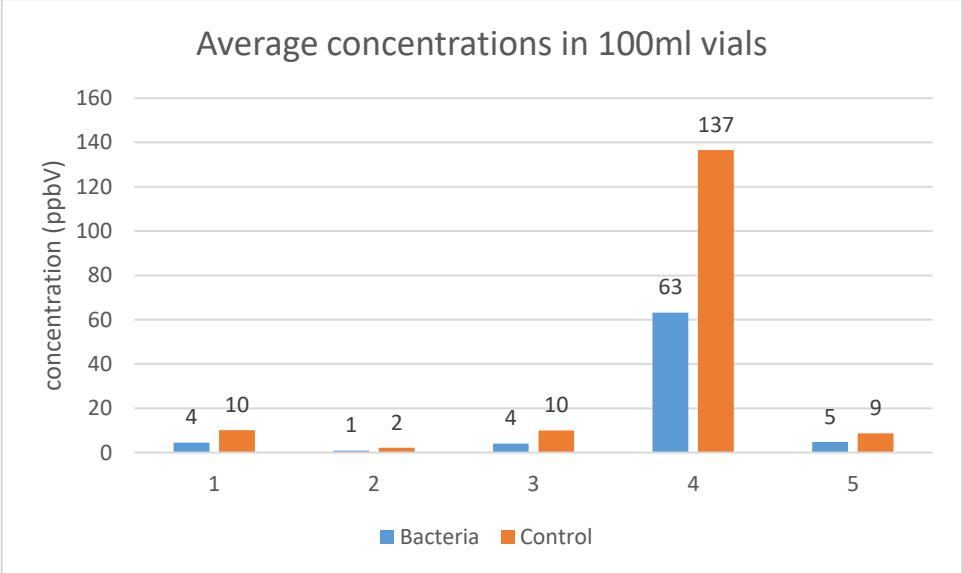

Figure 5 Results of the average concentration degradation products of naphthalene (in ppbv) in the 100 ml vials: dihydronaphthalene diol (1), naphthalene diol (2), 2-hydroxychromene-2-carboxylate + trans-o-hydroxybenzylidenepyruvate (3), salicylaldehyde (4) and hydroxybenzoate + salicylate (5).

Statistical analyses were performed using a Two Factor ANOVA with Replication test to detect a significant difference between the samples with bacteria and the controls. Since the p-value was 3,54E-16 < 0,05, we can reject the null hypothesis and conclude (with 95% confidence) that there are significant differences between bacteria and controls.

3.3 Controlled air-pollutant degradation experiment in cuvettes using ivy (*Hedera helix*)

Figure 6 shows the average concentrations of naphthalene degradation products produced in the big cuvettes. The same applies to the *Hedera helix* plants inoculated with the consortium of bacteria and the controls. No significant differences were measured between the upper part and lower part of the cuvettes. An average of 6383 ppbV of naphthalene was left at the time of measurement in each part of all the cuvettes.

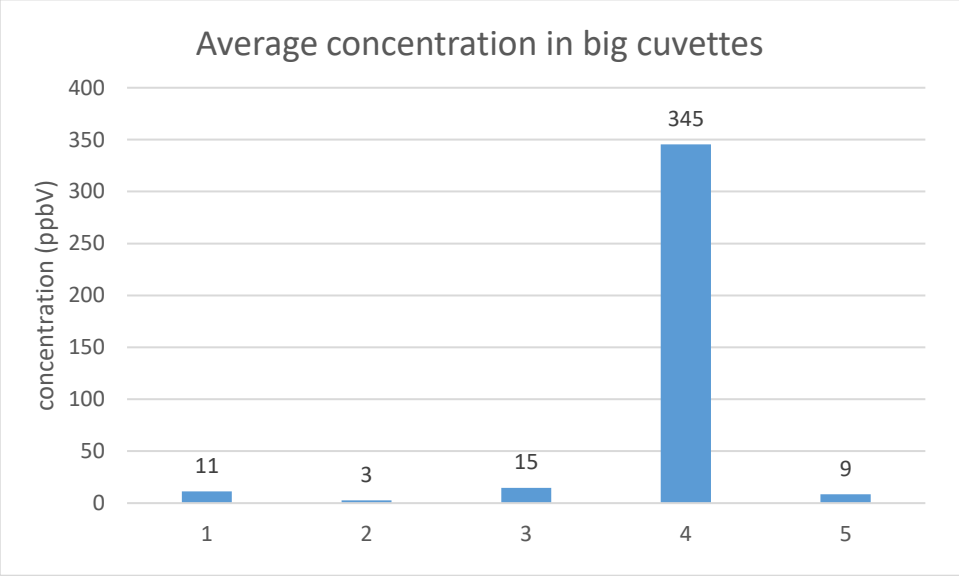

Figure 6 Results of the average concentration degradation products of naphthalene (in ppbv) in all of the big cuvette systems: dihydronaphthalene diol (1), naphthalene diol (2), 2-hydroxychromene-2-carboxylate + trans-o-hydroxybenzylidenepyruvate (3), salicylaldehyde (4) and hydroxybenzoate + salicylate (5).

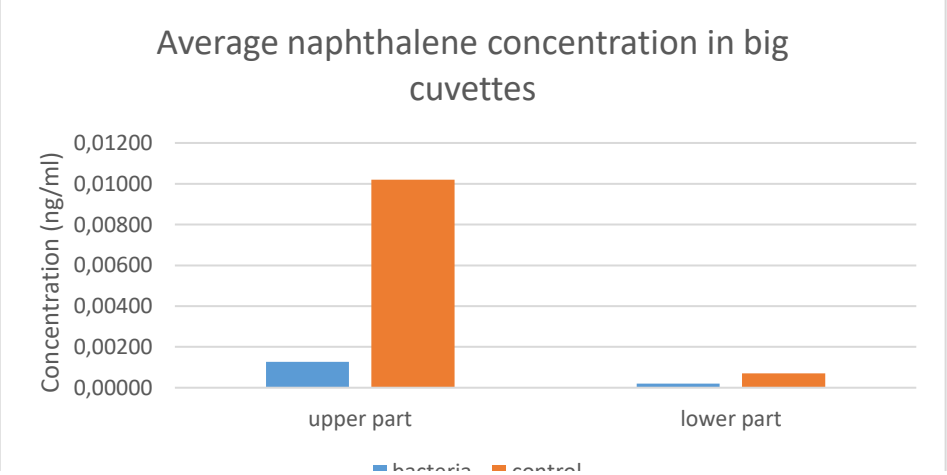

Figure 7 Results of the average concentration of naphthalene (ng/ml) using GC-MS of the big cuvette systems

Re-analyses of the big cuvette systems in GC-MS revealed differences between the inoculated cuvettes and the controls as shown in Figure 7. The concentrations of naphthalene (ng/ml) are lower than the controls for both the upper and lower part of the cuvettes.

Discussion

In this study, we used different microorganisms isolated from sites polluted with volatile organic compounds to test their ability to degrade these pollutants in the presence of a common ivy plant *Hedera helix*. We combined phyllospheric bacteria with soil bacteria because the degradation of aromatic compounds involves more than one gene to degrade it. Tests regarding the dioxygenase gene and plant growth promotion were used to carefully select the best possible candidates (i) to degrade organic compounds in the air, (ii) associate with plants without having a negative impact on them and (iii) to identify potential interesting bacteria in regard to phylloremediation applications. The sampling locations, a with diesel polluted site in Bóbrka (Poland) and Beverlo (Belgium), were carefully selected in order to be relevant in the context of phytoremediation of air pollution.

Multiple catabolic genes were found in the genome of both *Pseudomonas yorbenii VI4.1* and *Pseudomonas veronii VI4T1*. Both have very similar genes for the degradation of naphthalene, toluene, hydrocarbons and catechol. Together with genes encoding for plant growth promotion this makes them suiTable candidates to use in phylloremediation.

From the results of the 20ml vials, it is clear that both of the bacteria are capable of removing naphthalene vapours from air. The degradation mechanism probably is through the dioxygenase pathway (Maigari & Maigari, 2015). The decrease of naphthalene concentration in the vials with soil added are due to the absorption factor of the soil and in part by the air humidity effect in a closed system. Clearly, there is a further decrease of naphthalene concentrations in the vials with bacteria added. This further decrease occurred by the degradation capacity of the bacteria. Too high concentrations of benzene were spiked in all of the experiments which resulted in no valuable data considering benzene degradation.

The results of the 100ml vials are also promising, the concentration of naphthalene as well as its degradation products are lower in the vials inoculated with the bacteria in comparison to the controls. This could mean that the bacteria can use each degradation component of naphthalene as a carbon source.

The values of the degradation products in the big cuvettes are in similar orders as in the 100 ml vials. There are no differences between the concentrations measured in the cuvettes inoculated with the consortium of bacteria and the controls. The main reason for this could be that the plant is interrupting in the degradation of naphthalene by the bacteria. Plants produce other products such as methanol (Nemecek-Marshall, et al., 1995) that can be used as a carbon source instead of the naphthalene and benzene spiked in the cuvettes.

The reason that salicylaldehyde concentrations are very high could be explained by the fact that also hydroxybenzoate, having the same molecular mass, was highly present in the samples. P-hydroxybenzoate, or p-hydroxybenzoic acid is a phenolic compound that is produced by many plants and could be the reason why the concentration is so high in our samples (Kähkönen, et al., 1999).

In the re-analysation of this experiment using GC-MS a more clear result was achieved. Concentrations of naphthalene are lower in the cuvette systems with bacteria resulting from a degradation of this compound.

Conclusion

After analysing our results, we can conclude that *Pseudomonas putida VI4T1*, *Pseudomonas yorbenii VI4.1* and our consortium of bacteria are able to degrade airborne environmental pollutants such as naphthalene. There is a significant difference between the concentration of naphthalene in the samples inoculated with the bacteria and the control samples.
To analyse the degradation abilities more in depth, further research is needed using different analytical and molecular techniques in different experimental set-ups. Suggestions might be using plasmids with genes of a part of the naphthalene degradation process and analyse the degradation of each step separately to confirm the complete degradation pathway. Plasmids of *Escherichia coli* can be used as cloning vectors for this kind of experiments (Bolivar & Backman, 1979). A step further for the plant experiments is to use dynamic cuvette systems to decrease the air humidity effect and perform further analyses on the degradation of a wider variety of airborne pollutants.

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Annexes

Annex 1: Bushnell-Haas medium

Bushnell Haas Agar M349

Bushnell Haas Agar is recommended for the microbial examination of fuels and for studying microbial hydrocarbon
deterioration.

Composition**

Ingredients Gms / Litre
Magnesium sulphate 0.200
Calcium chloride 0.020
Monopotassium phosphate 1.000
Dipotassium phosphate 1.000
Ammonium nitrate 1.000
Ferric chloride 0.050
Agar 20.000
Final pH (at 25°C) 7.0±0.2
**Formula adjusted, standardized to suit performance parameters

Directions

Suspend 23.27 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at
15 lbs pressure (121°C) for 15 minutes. A white precipitate prior to sterilization becoming yellow to orange after sterilization
is normal.

Principle And Interpretation

Bushnell Haas Agar is prepared as per the formula of Bushnell and Haas (1) and recommended for the microbiological
examination of fuels by the SIM Committee on microbiological deteriorations of fuels (2). These media contain all nutrients
except carbon source, necessary for the growth of bacteria. Only those bacteria that are able to decompose hydrocarbon will
grow in these media. Specific carbon source i.e. hydrocarbon can be added to this medium and their utilization by different
microorganisms can be studied.
These bacteria can decompose a variety of hydrocarbons like kerosene, mineral oil, paraffin wax and gasoline. For liquid
hydrocarbon the hydrocarbon is layered on the surface of inoculated agar. For testing volatile hydrocarbons such as gasoline
the Petri-plates containing the medium are inverted and the hydrocarbon is poured into the lid. Magnesium sulphate, calcium
chloride and ferric chloride provide trace elements. Ammonium nitrate is a nitrogen source while monopotassium phosphate
and potassium phosphate buffers the medium.

Quality Control

Appearance
White to cream homogeneous free flowing powder
Gelling
Firm, comparable with 2.0% agar gel.
Colour and Clarity of prepared medium
Light amber coloured, clear to slightly opalescent gel forms in Petri plates.
Reaction
Reaction of 2.33% w/v aqueous solution at 25°C. pH : 7.0±0.2
pH
6.80-7.20
Cultural Response
M349: Cultural characteristics observed after an incubation at 25-30°C within 1 week.

Organism	**Inoculum (CFU)**	**Growth (Plain)**	**Growth w/**

minerals
Cultural Response
HiMedia Laboratories Technical Data
Pseudomonas aeruginosa
ATCC 27853
50-100 poor good-luxuriant
Pseudomonas aeruginosa
ATCC 9027
50-100 poor good-luxuriant

Storage and Shelf Life

Store below 30°C in tightly closed container and the prepared medium at 2 - 8°C. Use before expiry date on the label.

Reference

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2. Allred, DeGray, Edwards, Hedrick, Klemme, Rogers, Wulf and Hodge, 1963, Proposed Procedures for Microbiological
Examination of Fuels, SIM Special Publications, No. 1. Merck, Sharp & Dohme Research Laboratories, Rahway, N.J.
Revision : 2 / 2015
HiMedia Laboratories Pvt. Ltd. A-516,Swastik Disha Business Park,Via Vadhani Ind. Est., LBS Marg, Mumbai-400086, India. Customer care No.: 022-6147
1919 Email: techhelp@himedialabs.com

Annex 2: 284 selective medium

284 selective medium

Name: Jolien Janssen
Original protocol/article: Schlegel *et al.*, 1961
Original protocol modified: /
Date last modified: /

Special safety and hazardous waste disposal remarks + lab zone:
LAB ZONE: Lab Microbiology G.10: main laboratory area Lab Functional Biology G.72: main laboratory area
SAFETY:
Relevant MSDS sheets: MSDS-Tris of HCl MSDS-NaCl MSDS-KCl MSDS-NH_4Cl MSDS-Na_2SO_4 MSDS-$MgCl_2.6H_2O$ MSDS-$CaCl_2.2H_2O$ MSDS-$Na_2HPO_4.2H_2O$ MSDS-Fe(III)NH_4Citrate MSDS-Sodium lactate MSDS-Glucose MSDS-Gluconic acid sodium salt MSDS-Fructose MSDS-Sodium succinate.$6H_2O$ MSDS-Agar No.2 **Tris of HCl, NaCl, KCl, NH_4Cl, Na_2SO_4, $MgCl_2$, $CaCl_2$, Na_2HPO_4, Fe(III)NH_4Citrate, lactate, glucose, gluconic acid, fructose, succinate, agar, $ZnSO_4$**: no particular risks , in case of eye or skin contact, rinse with plenty of water for several minutes. In case of respiratory irritation, move to fresh air. **$CdSO_4$**: very hazardous in case of ingestion or inhalation. Evacuate the victim to a safe area as soon as possible. Loosen tight clothing such as a collar, tie, belt or waistband. If breathing is difficult, administer oxygen. If the victim is not breathing, perform mouth-to-mouth resuscitation. Examine the lips and mouth to ascertain whether the tissues are damaged, a possible indication that the toxic material was ingested; the absence of such signs, however, is not conclusive. Do not induce vomiting. Seek immediate medical attention. **During normal execution of the protocol: Wear lab coat and gloves.**
WASTE
/
EQUIPMENT OPERATION: be familiar with operation of:
Micro(balances) Autoclaves

MATERIALS

284	**in g**	**1L**	**2L**	**3L**	**5L**	**10L**
Tris of HCl	T019	6.06	12.12	18.18	30.3	60.6
NaCl	N013	4.68	9.36	14.04	23.4	46.8
KCl	K005	1.49	2.98	4.47	7.45	14.9
NH_4Cl	A025	1.07	2.14	3.21	5.35	10.7
Na_2SO_4	N035	0.43	0.86	1.29	2.15	4.3
$MgCl_2.6H_2O$	M002	0.2	0.4	0.6	1	2
$CaCl_2.2H_2O$	C007	0.03	0.06	0.09	0.15	0.3
$Na_2HPO_4.2H_2O$	N082	0.04	0.08	0.12	0.2	0.4
SI7 spore elements	fridge	1ml	2ml	3ml	5ml	10ml
Fe(III)NH_4Citrate sol. of 48mg/100ml	A051	10ml	20ml	30ml	50ml	100ml
C-mix	**in g**	**1L**	**2L**	**3L**	**5L**	**10L**
Sodium lactate (sol. 50%)	N056	0.7ml	1.4ml	2.1ml	3.5ml	7ml
D-(+)-glucose	G003	0.52	1.04	1.56	2.6	5.2
D-gluconic acid sodium salt	G019	0.66	1.32	1.98	3.3	6.6
D-(-)-fructose	F014	0.54	1.08	1.62	2.7	5.4
Sodium succinate.$6H_2O$	N089	0.81	1.62	2.43	4.05	8.1
Agar No.2 Bacteriological		20				

IMPORTANT EXPERIMENTAL NOTES

/

PROCEDURE

1. Fill an appropriate flask with distilled water until about half of the volume. Add a stirring stick.
2. Add the 284 and C-mix products and stir until all products are dissolved.
3. Fill the flask with distilled water until the bottle neck.
4. Adjust the pH of the medium to 7 with HCl.
5. Fill the flask till the mark with distilled water and let the medium stir thoroughly.
6. Divide the medium over autoclavable bottles.

    ✓ If solid medium is desired, add the appropriate amount of agar and a stirring stick in every bottle

7. Autoclave the medium for 10 min.
8. Solid medium should be kept at 60°C after autoclaving or poured immediately.

Annex 3: 869 rich medium

869 Rich medium

Name: Jolien Janssen
Original protocol/article: Mergeay *et al.*, 1985
Original protocol modified: /
Date last modified: /

Special safety and hazardous waste disposal remarks + lab zone:
LAB ZONE: Lab Microbiology G.10: main laboratory area Lab Functional Biology G.72: main laboratory area
SAFETY:
Relevant MSDS sheets: MSDS-Tryptone MSDS-Yeast extract powder MSDS-NaCl MSDS-D-(+)-glucose monohydrate MSDS-$CaCl_2.2H_2O$ MSDS-NaOH MSDS-Agar No.2 **Tryptone, yeast extract powder, NaCl, glucose, $CaCl_2$, NaOH, agar**: no particular risks , in case of eye or skin contact, rinse with plenty of water for several minutes. In case of respiratory irritation, move to fresh air. **During normal execution of the protocol: Wear lab coat.**
WASTE
/
EQUIPMENT OPERATION: be familiar with operation of:
Micro(balances) Autoclaves

MATERIALS

869	**in g**	**1L**	**2L**	**3L**	**5L**	**10L**
Tryptone		10	20	30	50	100
Yeast extract powder		5	10	15	25	50
NaCl	N013	5	10	15	25	50
D-(+)-glucose.H_2O	G018	1	2	3	5	10

$CaCl_2.2H_2O$	C007	0.345	0.690	1.035	1.725	3.450
Agar No.2 Bacteriological		15				

IMPORTANT EXPERIMENTAL NOTES

/

PROCEDURE

9. Fill an appropriate flask with distilled water until about 3/4th of the volume. Add a stirring stick.
10. Add the tryptone, yeast extract powder, NaCl, glucose and $CaCl_2$ and stir until all products are dissolved.
11. Fill the flask with distilled water until the bottle neck.
12. Adjust the pH of the medium to 7 with NaOH.
13. Fill the flask till the mark with distilled water and let the medium stir thoroughly.
14. Divide the medium over autoclavable bottles.

    - ✓ If solid medium is desired, add the appropriate amount of agar and a stirring stick in every bottle (for 1/10th strength rich medium, use also 15 g of agar per liter of medium)

15. Autoclave the medium for 10 min.
16. Solid medium should be kept at 60°C after autoclaving or poured immediately.

Annex 4: Hoagland medium

Hoagland solution

Name: Stefanie De Smet/ Marijke Gielen
Original protocol/article: - Hoagland, D.R. and D.I. Arnon. 1950. The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular 347:1-32
Original protocol modified: - K. Smeets, J. Ruytinx, F. Van Belleghem, B. Semane, D. Lin, J. Vangronsveld, A. Cuypers, Critical evaluation and statistical validation of a hydroponic culture system for Arabidopsis thaliana, Plant Physiol. Biochem. 46 (2008) 212-218 - Remans 21/12/2012, modified Smeets (2008) : final $ZnSO_4.7H_2O$ concentration = 0.08µM (instead of 0.16µM)
Last date modified: 14/04/2014

Special safety and hazardous waste disposal remarks + lab zone: **Labzone:** Greenhouse/climate chamber Field lab
Safety: **MSDS sheets** MSDS-A021 Ammonium dihydrogen phosphate MSDS-C010 Calcium Nitrate Tetrahydrate MSDS-E010 EDTA Disodium Salt MSDS-K012 Potassium Nitrate MSDS-K029 Copper Sulphate Pentahydrate MSDS-M005 Magnesium Sulphate Heptahydrate MSDS-M012 Manganese Chloride Tetrahydrate MSDS-M028 Molybdic Acid MSDS-Z004 Zinc Sylphate Heptahydrate **Extract from MSDS sheets** First aid measures: Inhalation → move to fresh air Skin → wash with plenty of water and soap Eye → rinse with plenty of water, remove contact lenses. Roll eye during rinsing Ingestion → drink water, consult a physician, for Ammonium Dihydrogen Phosphate and Zinc Sulphate Hepatahydrate rinse mouth with water, DO NOT VOMIT/EAT/DRINK and obtain immediate medical attention.

MATERIALS

- Several products
- Distilled water
- Flasks
- Stirrer
- Balance

PROCEDURE

Make 3 separate stock solutions (macro, micro en Fe) in ratios below:

Hoagland stock solutions							1x Hoagland	**Our diluted Hoagland**
						conc	final	**final**
Macro elements		**10x**		For 2L	*Mr*	mM	mM	**µM**
KNO_3	**K012**	10,2	g/l	20,4 g	101,11	100,88	10,1	**505**
$Ca(NO_3)_2.4H_2O$	**C010**	7,08		14,16 g	236,15	29,98	3,0	**150**
$NH_4H_2PO_4$	**A021**	2,3		4,6 g	115,03	19,99	2,0	**100**
$MgSO_4.7H_2O$	**M005**	4,9		9,8 g	246,48	19,88	2,0	**100**
						conc	final	
Fe		**1667x**		For 250 mL	*Mr*	mM	µM	
$FeSO_4.7H_2O$	**I002**	7,6	g/l	1,9 g	278,02	27,34	16,4	**1,64**
Na_2-EDTA	**E010**	5,0		1,25 g	372,24	13,43	8,1	**0,81**
						conc	final	
Micro elementen		**1000x**			*Mr*	mM	µM	
H_3BO_3	**B010**	2,86	g/l		61,83	46,26	46,3	**4,63**
$MnCl_2.4H_2O$	**M012**	1,81			197,91	9,15	9,1	**0,91**
$CuSO_4.5H_2O$	**K029**	0,08			249,68	0,32	0,3	**0,03**
$H_2MoO_4.H_2O$	**M028** (or N029)	0,09			161,95	0,56	0,6	**0,06**
$ZnSO_4.7H_2O$	**Z004**	0,22			287,54	0,76	0,8	**0,08**

Add $FeSO_4.7H_2O$ and dissolve first, then add Na_2-EDTA and dissolve, otherwise the solution will oxidize.

Hoagland solution

1x Hoagland	**½ Hoagland**	**1/10 Hoagland**	
1000ml	500ml	50 ml?	**10x Macro elements**
10ml	5ml	1 ml	**1000x Micro elements**
6ml	3ml	600 µl	**1667x Fe**

In 10l dH_2O