THE EFFICACY OF PLANTS TO REMEDIATE INDOOR VOLATILE ORGANIC COMPOUNDS AND THE ROLE OF THE PLANT RHIZOSPHERE DURING PHYTOREMEDIATION

Manoja Dilhani De Silva Delgoda Mudiyanselage

Staffordshire University

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This work is dedicated to my son. You have made me stronger, better and more fulfilled than I could have ever imagined. I love you to the moon and back.

"There's so much pollution in the air now that if it weren't for our lungs there'd be no place to put it all"

- Robert Orben

Abstract

A wide range of volatile organic compounds (VOC) are released from building materials, household products and human activities. These have the potential to reduce indoor air quality (IAQ), poor IAQ remains a serious threat to human health. Whilst the ability of the single plant species to remove VOC from the air through a process called phytoremediation is widely recognised, little evidence is available for the value of mixed plant species (i.e. plant communities) in this respect. The work reported herein explored the potential of plant communities to remove the three most dominant VOCs: benzene, toluene and m-xylene (BTX) from indoor air. During phytoremediation, bacteria in the root zone (rhizosphere) of plants are considered the principal site contributing to the VOC reduction. This project explored BTX degrading bacteria in the rhizosphere through culture-dependent and independent approaches.

This research revealed that mixed plant culture could remove low and high concentrations of BTX from the air. Interestingly, 100 ppm BTX removal rates by single plant species were higher than the removal rate observed for mixed plant species in all cases. At the low concentration (10 ppm), all plants showed higher removal rates of benzene and toluene than m-xylene. Some bacteria in the rhizosphere utilised gaseous BTX as their sole carbon and energy sources were isolated on minimal salt agar. The majority of isolated bacteria were Gram-positive and belonged to the phylum Actinobacteria. Most of the identified bacteria belonged to the genera *Microbacterium, Rhodococcus, Arthrobacter* and *Pseudomonas.* In considering the impact of BTX upon the rhizosphere microbiome, it was shown that overall there were little compositional and functional changes following exposure to 10 ppm gaseous benzene. Findings from this work enhanced our understanding of the benefit of indoor plants in relation to VOC remediation and the consequent improvement of phytoremediation systems for the protection of public health.

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List of abbreviation

A, C, G and T- Adenine, Cytosine, Guanine and Thymine

ABC transporters -ATP-Binding cassette transporters

ANOVA – Analysis of variance

ATD-Automated thermal desorption

ATP-Adenosine triphosphate

BLAST– Basic Local Alignment Search Tool

bp-Base pair

BTX- Benzene, toluene and m-xylene

BTEX- Benzene, toluene, ethylbenzene, and m-xylene

DNA-Deoxyribo-nucleic acid

DW- Distilled water

EDTA-Ethylenediaminetetraacetic acid

F-Forward

FID-Flame ionisation detector

GC-Gas chromatograph/gas chromatography

HLGB- Hugh-Leifson glucose broth

HSE- Health and Safety Executive

IAQ-Indoor air quality

IARC-International Agency for Research on Cancer

IPTG-Isopropyl-b-D-thiogalactopyranoside

ITS-Internal transcribed spacer

KEGG- Kyoto Encyclopedia of Genes and Genomes

LB -Luria Bertani agar

LDPE- Low-density polythene

MSA-Minimal salt agar

NA-Nutrient agar

NB- Nutrient broth

NCBI-National Center for Biotechnology Information

OTU – Operational taxonomic unit

PD- Phylogenetic diversity

PBS-Phosphate buffer saline

PCR – Polymerase chain reaction

PET- Polyethylene terephthalate

PID-Photoionization detector

PICRUST- Phylogenetic investigation of communities by reconstruction of unobserved states

PM-Particulate matter

ppm- Parts per million

ppb- Parts per billion

PAH-Polycyclic aromatic hydrocarbon

PCoA- Principal coordinates analysis

QIIME – Quantitative insights into microbial ecology

R-Reverse

R^2- Correlation coefficient

rRNA – Ribosomal RNA

rpm- rounds per minute

RF- response factor

RT-Retention time

S-svedberg

SBS- Sick building syndrome

SPSS- Statistical package for the social sciences

STAMP-Statistical analysis of metagenomes (STAMP) package

TAE- Tris Acetate EDTA

TD-Thermal desorption/thermal desorber

T_m- Annealing temperature

TVOC-Total volatile organic compound

V1-V9- Hypervariable regions one to nine

VOC-Volatile organic compound

WHO-World Health Organization

Conference paper presentations and posters

Presentations

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Structure of thesis

Chapter 1 presents an introduction to indoor air pollution, phytoremediation by plants, approaches involved during bacterial identifications, techniques used during VOC monitoring, method involved in bacterial community profiling and finally the aims and objectives of the research.

Chapter 2 presents all optimisation and experimental methods used in the study to determine specific objectives in the research.

Chapter 3 presents the development of a test chamber system to monitor VOC removal efficiency by plants. The test chambers were constructed to mimic indoor air conditions. Analysis of VOC level inside the test chambers were optimised using Aeroqual digital VOC monitors (benzene, toluene and n-hexane) and gas chromatography (benzene, toluene and m-xylene). Finally, VOC removal by plants was analysed using the gas chromatography method. Two VOC concentrations: 10 ppm and 100 ppm of each VOC was tested with plants in each experiment.

Chapter 4 presents identification of atmospheric VOC degrading bacteria in plant rhizosphere. This was conducted by isolating VOC degrading bacteria from rhizosphere soil extracted from VOC exposed and non-exposed plants. Identification of bacteria was carried out using classical culture-dependent and culture-independent approaches.

Chapter 5 presents the taxonomical and functional changes of the rhizosphere of three plant species due to exposing to 10 ppm benzene. This was determined by comparing VOC exposed and non-exposed plant rhizosphere bacterial compositions. Community analysis was performed through microbial DNA sequencing coupled with bioinformatic approaches.

Chapter 6 discusses the major findings of this work with previous studies and finally highlights the recommendations for further work.

Chapter 1. Introduction and literature review

1.1. Introduction

Urban residents generally live around 90% of their lives inside buildings such as houses, workplaces and schools (Yang and Liu, 2011). A wide range of volatile organic compounds are emitted from indoor building materials and household products (Salthammer *et al.*, 2010). Additionally, outdoor derived petroleum-based volatile organic compounds (VOC) diffuse indoors and as a consequence, the average VOC level is typically higher indoors than outdoors (Tarran *et al.*, 2007). People who live indoors breathe this polluted air in every second of their lives causing them short-term (e.g. irritation of respiratory system) or long-term (e.g. cancer) health impacts (Brody *et al.*, 2007; Huang *et al.*, 2013). Previous studies have demonstrated that exposure to indoor VOC is associated with "sick building syndrome" (SBS) or building related illness showing at least one or more of its symptoms: nausea, vomiting, headache, stress, allergies, asthma, short term memory, eye, nose and throat irritation, skin rashes, tiredness and loss of concentration (Jenkins *et al.*, 1992; Molhave *et al.*, 1997; Elke *et al.*, 1998; Orwell *et al.*, 2004, 2006; Yang *et al.*, 2009; Yang and Liu, 2011; Russell *et al.*, 2014; Javid *et al.*, 2016; Kim *et al.*, 2016; Lin *et al.*, 2017). As a result of poor indoor air quality (IAQ), average occupational productivity can be decreased (Wijewickrama *et al.*, 2016). A real world study showed an increase of sickness absence of a group of office staff after they moved from a naturally ventilated office to an air conditioned office (Gubéran, 1979). The cost for the loss of productivity due to SBS in a UK office with approximately 2500 occupants were estimated at £400,000 per year if each occupant is sick only one day per year (Burge, 2004). Based on studies across the globe position, the indoor air quality has been becoming a global issue. A number of organisations and governments including the United Kingdom have issued regulations, policies and expressed concerns to maintain and improve good IAQ (DEFRA UK, 2007). Such considerations are focused on notifying the toxicity of indoor VOCs to people, so it raises the awareness of VOC in the indoor environment and also expects to maintain indoor VOC levels less than short term and long term workplace exposure limits (Guo *et al.*, 2004; IARC, 2006; 2007; WHO, 2008; UK/HSE, 2013).

Indoor plants are known as the biofilters to clean the air and several studies reported the capability of some plant species and plant-associated microorganisms to remove VOC from the air through the process called phytoremediation (Daane *et al.*, 2001; Orwell *et al.*, 2004; Wood *et al.*, 2006; Liu *et al.*, 2007; Kim *et al.*, 2008, 2014, 2016; Bisht *et al.*, 2010; Chun *et al.*, 2010; Llewellyn and Dixon, 2011; Irga *et al.*, 2013; Sriprapat and Thiravetyan, 2016; Ite *et al.*, 2016; Lin *et al.*, 2017). Another study demonstrated the capability of twenty-eight indoor plant species to remove 10 ppm concentration of five different VOCs: benzene, toluene, octane, trichloroethylene (TCE), and alpha-pinene from the air. Based on the VOC removal efficiency per leaf area, they categorised those plants with superior VOC removal ability: *Hemigraphis alternata*, *Hedera helix, Asparagus densiflorus*, *Tradescantia pallida* and *Hoya carnosa,* intermediate removal ability: *Ficus benjamina*, *Polyscias fruticosa* and another five species, poor removal ability: *Peperomia clusiifolia*, *Chlorophytum comosum*, *Spathiphyllum wallisii* and another thirteen plant species used in that study (Yang *et al.*, 2009). In another study, Orwell *et al.*, (2004) demonstrated that gaseous phase benzene (25 ppm) removal efficiency by microorganisms in the potting mix and the rhizosphere of *Spathiphyllum floribundum*, *Dracaena deremensis*, *Dracaena marginata* and *Epipremnum aureum*. Chun *et al.*, (2010) showed that the benzene, toluene and xylene removal ability by total bacterial population cultured from the rhizosphere of *S. wallisii*, *Pachira aquatica*, *Ficus elastica*, *Dieffenbachia* sp., and *Chamaedorea elegans*. Also, they noticed that the increase of benzene and toluene removal efficiency by potting mix following inoculation of the total rhizosphere bacterial cultures from *P. aquatica* into the potting mix. In another study *C. comosum* reported the maximum benzene removal efficiency from air followed by *Syngonium podophyllum* and *H. helix* (Sriprapat and Thiravetyan, 2016). In the same study, non-sterilised *C. comosum* performed 1.6 times higher benzene removal efficiency than the sterilized *C. comosum*. Yoo *et al.*, (2006) reported that the highest removal efficiency of gaseous benzene by *S. wallisii* followed by *H. helix*. Their observations were based on the VOC removal per leaf area of the plants. When the plants were exposed to a mixture of 0.5 ppm benzene and 0. 5 ppm toluene, *H. helix* showed the higher removal efficiency than other plants (Yoo *et al.*, 2006). According to Setsungnern *et al,*

(2017), during high concentration VOC removal, *C. comosum* showed 68.77% removal efficiency for 500 ppm benzene removal. In the same study, changes to the light conditions in the day time resulted in different benzene removal rates by *C. comosum*. This literature provides a good foundation for using indoor plants for the phytoremediation of VOC from air.

Most of the above-mentioned studies involved measuring the capacity of plants to remove VOC from the air and are based on test chamber experiments. Methods employed on the analysis of VOC levels in air samples can be determined by commercially developed digital sensors or gas chromatography with a suitable detector such as the flame ionisation detector (FID)(Winkle and Scheff, 2001; Garcia-Jares *et al.*, 2009; Yang *et al.*, 2009; Lee *et al.*, 2013; Prober *et al.*, 2015; Setsungnern *et al.*, 2017; Spinelle *et al.*, 2017a; Torpy *et al.*, 2018). During phytoremediation, above ground part of the plant (leaves, stem and flowers) absorb VOC through the stomata and cuticle followed by metabolism through plant biochemical processes (Weyens *et al.*, 2015). During the phytoremediation process, plant root associated rhizosphere microorganisms, mainly bacteria play a major role to remove VOC from indoor air, a process termed rhizoremediation (Wolverton and Wolverton, 1993; Llewellyn and Dixon, 2011). Therefore, identifying the bacterial composition of the rhizosphere of plant species while they are detoxifying specific VOC is useful to the further optimisation of plant-VOC removal systems in terms of enhancing indoor air quality. Through isolation of gaseous VOC degrading bacteria and profiling the taxonomical and functional diversities of the rhizosphere of those plants, it may be possible to gain an insight into the ability of plant-rhizosphere bacteria to remove VOC from the air. Such identifications can be carried out using traditional microbiological culturing methods and culture-independent molecular techniques (Orphan *et al.*, 2000; Schornsteiner *et al.*, 2014).

This research will focus on three monocyclic volatile organic compounds: benzene, toluene and m-xylene (i.e. BTX) and the capability of plant species: *S. waliisii*, *C. comosum* and *H. helix* to phytoremediate them from the indoor air. Two concentrations, 10 and 100 parts per million (ppm; equal to milligrams per litre, mg/l) of each VOC will be tested with plants to observe the VOC removal rates during the VOC half-lives. The untouched area, how the plant communities: a

mixture of different plant species growing together, detoxifies VOC from the air will be determined. Plant monocultures (single plant species) and the communities will be manipulated and maintained in the plant propagating trays during the study. In addition, the present study will focus on the changes of rhizosphere bacterial community in plant monocultures following exposing to 10 ppm benzene. Finally, gaseous VOC degrading bacteria in plant rhizosphere will be isolated and identified. Results from this study may have applications to optimise the plant phytoremediation system to enhance the indoor air quality focusing to improve the occupant's health, safety and welfare.

1.2. Air pollution and air pollutants

Seinfeld and Pandis (2006) defined air pollution as "*a situation in which substances that result from anthropogenic activities are present at concentrations sufficiently high above their normal ambient levels to produce a measurable effect on humans, animals, vegetation or materials*". Increasing pollutant levels beyond the acceptable standards due to natural or human activities causes adverse or mild effects on humans, animals and plants (IARC, 2015). This also conveys to changes of the climate on the earth and natural cycles such as the rain cycle (Yang and Liu, 2011). A few examples of natural incidents affecting air pollution are; forest fires, pollen dispersal, volcanic eruptions and dust storms (Guieysse *et al.*, 2008). However, these natural incidents do not often happen. Human activities are the main factor affecting air pollution such as; releasing toxic air pollutants through fossil fuel burning, leakages from gasoline storage tanks, chemical emissions from industrial sites and manufacturing activities, emission of toxic pollutants from waste management, VOC emission from indoor building materials and household chemicals (Hayes *et al.*, 2001; Lee *et al.*, 2005; Brody *et al.*, 2007; Garcia-Jares *et al.*, 2009; Xie *et al.*, 2011; Torpy *et al.*, 2013; Jaishankar *et al.*, 2014; Russell *et al.*, 2014; Stefani *et al.*, 2015; Lin *et al.*, 2017). Gaseous air pollutants: methane (CH_4) and carbon dioxide (CO_2) emitted during the fossil fuel combustion may remain in the troposphere for a long period causing global warming which could result in the polar glaciers melting (McMichael, 2003; Barnett *et al.*, 2005; Schmid *et al.*, 2009). Carbon monoxide (CO), nitrogen dioxide (NO_2) and sulphur dioxide (SO_2) released during the

burning of fuel create particulate matters (PM) in the atmosphere and acidic PM causes acid rains (DEFRA UK, 2007). In addition to the environmental changes, air pollutants including volatile hydrocarbons cause acute and chronic diseases in humans (Wolkoff, 2018). Several epidemiological studies revealed that air pollution is one of the main factors causing terminal cancers and breast cancer due to the carcinogenic effect of those pollutants (IARC, 2015). Also some studies suggested that exposure to benzene, formaldehyde and 1,3-butadiene may cause more than 70-75% cancer risks (Labrèche and Goldberg, 1997; Blair *et al.*, 1998; Høyer *et al.*, 2000; Chang *et al.*, 2003; Pavuk *et al.*, 2003; Rudel *et al.*, 2003, 2007; Guo *et al.*, 2004; Rennix *et al.*, 2005; Brody *et al.*, 2007). Such studies strongly suggested an association between childhood leukaemia and exposure to indoor and outdoor volatiles (Raaschou-Nielsen *et al.*, 2001; Guo *et al.*, 2004; Knox, 2005; Raaschou-Nielsen and Reynolds, 2006; Whitworth *et al.*, 2008; Tang *et al.*, 2009; Bailey *et al.*, 2011, 2017; Sheridan *et al.*, 2011; Badaloni *et al.*, 2013; Huang *et al.*, 2013; Gao *et al.*, 2014).

All the air pollutant substances can be divided into three classes based on their nature as criteria pollutants, air toxics and biological pollutants (Department of the Environment and Energy, 2018). According to the National Ambient Air Quality Standards (NAAQS) issued by EPA USA, criteria pollutant group composes of six principal pollutants: CO, lead (Pb), NO_2, ozone (O_3), SO_2 and PM. Criteria pollutants are more dominant outdoor than the indoor air therefore, it is used as the indicator to set the outdoor air quality standards. The second type of air pollutant, air toxic, consists of aliphatic hydrocarbons, benzene, toluene, xylene, formaldehyde and polyaromatic hydrocarbons (WHO, 2010). The third group of pollutants contains biological pollutants such as molds, bacteria, virus, animal saliva, animal dander and pollen (Redlich *et al.*, 1997). Air toxic and biological pollutants are the dominant pollutant types in indoor air rather than outdoors (Lippmann, 1991; Carrington and Darrell, 1993; Wolverton and Wolverton, 1993; Easterly, 1994; Tunnicliffe *et al.*, 2001; Guo *et al.*, 2009; Tang *et al.*, 2009; Salthammer, Mentese and Marutzky, 2010; Huang *et al.*, 2013; Berry *et al.*, 2017).

1.3. Indoor air pollution

Indoor air pollution can be defined as the degradation of indoor air quality, by chemical, biological and physical pollutants (Kankaria *et al.*, 2014). According to the EPA USA, 2008, indoor air quality refers to the air quality inside and surrounding the buildings. In 2016, 3.8 million deaths occurred due to exposing to indoor air pollution and it was 7.7% of the global mortality in that year, thus it is listed as one of the ten greatest threats to human health (WHO, 2018).

As listed below, indoor pollutants can be derived from natural or man-made sources.

- Natural sources
    - Radon (resulted by radioactivity decaying of uranium or radium)
    - Biological contaminant (moulds, virus, fungi and bacteria)
- Man-made sources
    - Combustion and tobacco smoking (CO, SO_2, NO and PM)
    - Asbestos, VOC, formaldehyde and lead

(Liddell, Gilbert and Halliday, 2010; Hamilton *et al.*, 2015; IARC, 2015).

From this pollutant list, VOC is identified for its high abundance in the indoor air, especially in newly constructed buildings (Kim *et al.*, 2008). Exposure to indoor VOC has become one of the main health and safety concerns around the world due to the seriousness of its adverse effects on human health (Finnegan *et al*. 1984; Stolwijk 1991; Molhave *et al*. 1997; Redlich *et al*. 1997; Wargocki *et al*. 1999; Burge 2004; Chun *et al*. 2010; Mosaddegh *et al*. 2014; Belachew *et al*. 2018).

1.3.1. Volatile organic compounds in indoor air

Volatile organic compounds are a group of varied chemicals with different volatility, molecular weight, polarity, solubility, vapour pressure and vapour density (Pubchem, 2018). VOC can be monocyclic or polycyclic aromatic compounds, aliphatic hydrocarbons, halogenated hydrocarbons, terpenes, aldehydes, ketones, esters, ethers and alcohols (UK/HSE, 2000). Usually VOC levels in indoor air can be eight to ten times higher than outdoor levels and they have been identified as one of the main pollutants in indoor air (Molhave *et al*. 1997; Winkle & Scheff 2001;

Prabhat Kumar *et al*. 2011; Goodman *et al*. 2018; WHO 2018). So far, over 900 VOCs are identified in the indoor air and over 350 of them were classified as the causative agents of SBS (EPA USA, 1992; Guo *et al.*, 2004; Shinohara *et al.*, 2004; Burchett *et al.*, 2005; Wood *et al.*, 2006; Orwell *et al.*, 2006; Bessonneau *et al.*, 2013; Torpy *et al.*, 2013; Langer *et al.*, 2015; Weyens *et al.*, 2015). SBS represents a group of skin, mucosal and general symptoms temporally related to working in office and residential (Burge, 2004; Belachew *et al.*, 2018). Also, exposure to VOC for a long period of time, even at low concentrations can lead to acute health conditions like complications in the heart and kidney (EPA USA, 1992; Guo *et al.*, 2004; Shinohara *et al.*, 2004; Bessonneau *et al.*, 2013). Indoor VOC can be generated due to cooking, smoking, dry cleaning, using air fresheners, paint, laminated floors, printing, varnishes used in furniture, fabric, synthetic plastics, rubber, adhesives, cosmetics, wall papers and carpets, and also through the diffusion of outdoor derived petroleum-based VOC to indoor (Guo *et al.*, 2004; Burchett *et al.*, 2005; Wood *et al.*, 2006; Orwell *et al.*, 2006; Tarran *et al.*, 2007; Liddell *et al.*, 2008; Garcia-Jares *et al.*, 2009; Torpy *et al.*, 2013; Soreanu *et al.*, 2013; Mosaddegh *et al.*, 2014; Russell *et al.*, 2014; Hazrati *et al.*, 2016; Wolkoff, 2018). Paints and varnishes used in furniture coatings release approximately 150 different VOCs including aromatic and aliphatic hydrocarbons, ketones, esters and aldehydes (Guieysse *et al.*, 2008). Based on the volatility, organic pollutants can be classified into different classes (Table 1. 1).

Table 1. 1 WHO classification of organic pollutants in indoor air (ISO 16000-5:2007). Modified after (ISO, 2007).

Description	**Abbreviation**	**Boiling point range (°C)**		**Saturation vapour pressures (kPa)**
		From	**To**	
Very volatile organic compounds	VVOC	< 0	50 to 100	> 15
Volatile organic compounds	VOC	50 to 100	240 to 260	> 10^{-2}
Semi-volatile organic compounds	SVOC	240 to 260	380 to 400	10^{-2} to 10^{-8}

Description	Abbreviation	Boiling point range (°C)		Saturation vapour pressures (kPa)
		From	To	
Particulate organic matter	POM	> 380	-	-

(-) Data is not available.

Some of the most abundant VOCs found in indoor air are toluene, formaldehyde, benzene, xylene, 1-butanol, 2-butanone, n-heptane, n-hexane, ethylbenzene, n-decane, limonene, α-pinene and trichlorofluoromethane (Orwell *et al*. 2006; Hori *et al*. 2009; Mo *et al*. 2009; Langer *et al*. 2015). Studies have shown that benzene, formaldehyde, toluene and vinyl chloride levels in indoor air are up to ten times higher than in the outdoor air (Liddell *et al.*, 2008). Also, n-hexane levels in primary schools and kindergarten's can be high due to day to day normal wet cleaning procedure (Mentese *et al.*, 2012). Also, formaldehyde, toluene and benzene levels in newly constructed buildings can be from five to a thousand times higher than in older buildings due to the high emission of those chemicals from building material (Weschler, 2000; Lim *et al.*, 2011; Bessonneau *et al.*, 2013; Gao *et al.*, 2014; Weyens *et al.*, 2015). Based on the toxicity, the short- and long-term approved workplace exposure limits (WEL) of the most abundant VOCs were set in the UK indoor air (Table 1. 2).

Table 1. 2 Approved workplace exposure limits of the most abundant indoor VOC

Substance name and the chemical formula	Workplace exposure limit (WEL)			
	Long-term exposure limit (8-hr reference period)		Short-term exposure limit (15-minutes reference period)	
	ppm	mg.m-3	ppm	mg.m-3
Acetaldehyde (C_2H_4O)	20	37	50	92
Acetone (C_3H_6O)	500	1210	1500	3620
Acrolein (C_3H_4O)	0.02	0.05	0.05	0.12
Benzene (C_6H_6)	1	3.25	-	-
Butane (C_4H_{10})	600	1450	750	1810
Chloroform ($CHCl_3$)	2	9.9	-	-
Ethanol (C_2H_5OH)	1000	1920	-	-

Substance name and the chemical formula	Workplace exposure limit (WEL)			
	Long-term exposure limit (8-hr reference period)		Short-term exposure limit (15-minutes reference period)	
	ppm	mg.m-3	ppm	mg.m-3
Ethylbenzene (C_8H_{10})	100	441	125	552
Methyl ethyl ketone (C_4H_8O)	-	-	0.2	1.5
n-hexane (C_6H_{14})	20	72	-	-
Nitrobenzene ($C_6H_5NO_2$)	0.2	1	-	-
Tetrachloroethylene ($C2Cl_4$)	20	138	40	275
Toluene (C_7H_8)	50	191	100	384
Trichloroethylene C_2HCl_3)	100	550	150	820
Trimethylbenzene (C_9H_{12})	25	125	-	-
(o,m,p) Xylene (C_8H_{10})	50	220	100	441
Formaldehyde (CH_2O)	2	2.5	2	2.5
Ethylbenzene (C_8H_{10})	100	441	125	552

(-) Data is not available. Adapted from HSE, UK *(http://www.hse.gov.uk/pubns/priced/eh40.pdf)*

1.3.1.1.Benzene, toluene and m-xylene (BTX)

Benzene, toluene and m-xylene are three monocyclic aromatic organic compounds which are considered as priority air pollutants in indoor air, thus they are used as markers for exposing VOC and petroleum compounds for two reasons (Schneider *et al*. 2001; Almeida *et al*. 2006; Fan *et al*. 2009). First, high BTX concentrations can be always observed surrounding roads with heavy traffic, petroleum refineries and coal processing plants (Schneider *et al.*, 2001). Secondly, exposure to BTX would result in similar respiratory problems caused by most of other indoor VOCs (Ware *et al.*, 1993). In addition, BTX are identified as three of the main causative agents of SBS symptoms (Huang *et al.*, 2013).

1.3.1.1.1.Benzene

According to Pubchem, 2018 and EPA, 1988, characteristics of benzene are as follows. It (molecular weight: 78.114 g/mol) is an aromatic compound composed of six unsaturated carbon atoms organised into a ring. At liquid phase its density is 874 kg/m^3 at 25 °C, colourless, highly flammable, volatile compound with petroleum odour. Boiling and melting points of benzene are 80.08 °C and 5.5 °C respectively; its relative vapour density is 2.7 (air=1) and the vapour pressure at 25 ^{0}C is 12.7 KPa. Due to its high vapour pressure benzene rapidly evaporates at room temperature. Benzene is used as a solvent for resins, wax, paints, rubber, ink and plastics industries. Also, it is used in pharmaceutical products, detergents and as a constituent in petroleum (Pubchem, 2018; EPA 1988).

Benzene was categorised as a known human carcinogen according to the EPA USA in 1986 (EPA USA, 1988). Following inhalation, benzene can be absorbed and transported in the body and metabolized in the liver and bone marrow producing bipolar metabolites which cause DNA mutations (WHO, 2010). Several epidemiological studies revealed an association between benzene exposure and different types of myeloid leukaemia in workers at petroleum refinery industries (Hamilton, 1922; Rinsky, 1989; Hayes *et al.*, 1997; Elke *et al.*, 1998; Hayes and Travis, 2001; Orwell *et al.*, 2004; Yoo *et al.*, 2006; Guieysse *et al.*, 2008; Kabir and Kim, 2012; Torpy *et al.*, 2013; Mosaddegh *et al.*, 2014; Szulczyński and Gębicki, 2017).

1.3.1.1.2.Toluene

Toluene (molecular weight: 92.15 g/mol) is an aromatic volatile compound. It is a colourless, pungent odour and flammable liquid at 25 °C with -94.9 °C melting point and 110.6 °C boiling point. At liquid phase its density is 862.3 kg/m^3 at 20 °C, relative vapour density (air=1) is 3.1 and the vapour pressure is 3.8 Kpa at 25 °C. Toluene is a constituent of gasoline and used in the production of nylon, pharmaceuticals, plastic, cosmetics, dyes; toluene can also be used to produce benzene (WHO 2000; EPA USA 2012; Pubchem, 2018). Toluene can enter the human body through inhalation, absorption through skin or ingestion. Studies showed that exposure to

toluene cause complications in the central nervous system, immune system, kidney and liver (Phillips and Traxler, 1963; WHO, 2008; EPA USA, 2012).

1.3.1.1.3.m-xylene

m-xylene (molecular weight 106.2 g/mol) is an aromatic volatile compound. It is a colourless, slightly water soluble, aromatic odour and flammable liquid at 25 °C with -48 °C melting point and 139.1 °C boiling point. In the liquid phase the density is 860 kg/m^3, 3.66 relative vapour density (air=1) and 0.8 Kpa vapour pressure at 20 °C. m-xylene is a constituent of gasoline, and used in coal tar production, leather, rubber, varnishes, paints, cleaning products and coating ingredients in the fabric industry; it's also used as a solvent for dye in printing and to produce plastics (Engström *et al*. 1977; Fishbein 1985; EPA USA 1992; ATSDR USA 2007; Pubchem, 2018).

m-xylene can enter the human body through inhalation, skin contact and ingestion. Exposure to m-xylene can lead to complications in the gastrointestinal tract, kidney and liver, central nervous system and respiratory system (Engström *et al*. 1977; Fishbein, 1985; EPA USA, 1992; Zhou, Wu and Lemmon, 2012; Pubchem, 2018).

1.4. Bioremediation of VOC from the environment

Bioremediation is defined as the use of living organisms such as plants, bacteria, fungi and algae to detoxify, degrade, eliminate or reduce pollutants from the environment (McCullough *et al.*, 1999). In the presence of plants and soil, bioremediation takes place in different polluted environments such as petroleum, metal or pesticide-contaminated soil or groundwater and polluted air in outdoor and indoor environments (McCullough *et al*. 1999; Abhilash *et al*. 2009; Bell *et al*. 2011; Das & Chandran 2011; Peixoto *et al*. 2011; Yergeau *et al*. 2012; Bell *et al*. 2014; Truu *et al*. 2015;). Based on the type of living organism (plants or microorganisms) and the method used during remediation, bioremediation can be divided into different processes such as phytoremediation, bioaugmentation and biostimulation (Irga *et al.*, 2018). Phytoremediation is the process of detoxifying pollutants or converting them to harmless levels using plants and plant associated microorganisms (Wolverton and Wolverton, 1993; Truu *et al.*, 2015). The addition of

indigenous or genetically modified microorganisms to enhance the biodegradation process is known as bioaugmentation (Irga *et al.*, 2018). During biostimulation, nutrients, water or electron donors/acceptors are added into the medium to enhance microbial degradation of pollutants (Torpy *et al.*, 2013). Bioaugmentation and biostimulation are extensively used on petroleum-contaminated land to enhance the hydrocarbon degradation process by soil microflora (Grosser *et al*. 1991; McCullough *et al*. 1999; Bell *et al*. 2011; Peixoto *et al*. 2011; Bisht *et al*. 2015; Dealtry *et al*. 2018). Rhizoremediation (i.e. rhizodegradation), phylloremediation and phytovolatilization are sub-divisions of phytoremediation conducted by plants and associated microorganisms (Nwoko, 2010; Etim, 2012; Truu *et al.*, 2015). During rhizoremediation, rhizosphere microflora in plant roots detoxify pollutants into non-toxic compounds (Kuiper *et al.*, 2004). Phylloremediation is the uptake of pollutants through plant leaf followed by metabolism of them inside the plant cells and pollutant detoxification by leaf associated phyllosphere microflora (Wei *et al.*, 2017). In some instances, plants take up contaminants (VOC and inorganic pollutants) in soil or water through the roots and convert them into non-toxic or less toxic gaseous forms followed by releasing into the atmosphere through transpiration known as phytovolatilization (Nwoko, 2010).

1.4.1. Phytoremediation of VOC by indoor plant species

The VOC reducing capability of green plants through phytoremediation has been studied for more than 30 years (Wolverton and Wolverton, 1993; Nwoko, 2010; Douglass, 2011; Dela Cruz *et al.*, 2014). The pioneer investigator of plant-VOC experiments, Bill Wolverton, identified over 50 indoor plant species with different VOC detoxification efficacies (Wolverton and Wolverton, 1993) and most of them are used in many indoors (Fig. 1. 1 and Table 1. 3). An office-based study showed that total VOC compounds in the air ranged from 60-350 ppb without any plants, declined by 75% after introducing *D. deremensis* and *S. wallisii* (Orwell *et al.*, 2006; Wood *et al.*, 2006). Plant species: *S. wallisii*, *C. comosum*, *H. helix*, *H. alternata, D. deremensis, Crassula portulacea, Hydrangea macrophylla, S. podophyllum, Fatsia japonica, Dracaena fragrans, F. benjamina, F. elastica, Kalanchoe blossfeldiana, Pelargonium domesticum* and *T. pallida* are a few common indoor plants identified with detoxification ability of common VOCs (Wolverton and Wolverton,

1993; Cornejo *et al.*, 1999; Liu *et al.*, 2007; Kim *et al.*, 2008, 2014; Yang *et al.*, 2009; Irga *et al.*, 2013). Previous studies showed the capability of plant species: *S. podophyllum, P. domesticum, F. elastica, C. comosum, S. wallisii, H. helix, P. aquatica* and *K. blossfeldiana* to remove indoor benzene from air based on the test chamber experiments (Cornejo *et al.*, 1999; Orwell *et al.*, 2004; Yoo *et al.*, 2006; Song, Kim and Sohn, 2007; Irga, Torpy and Burchett, 2013; Torpy *et al.*, 2013; Mosaddegh *et al.*, 2014).

During phytoremediation, volatiles may penetrate or absorb through plant surfaces (leaves, stem or flowers) and adsorbed volatiles on the surfaces are taken up actively by stomata followed by detoxification inside the plant cells (Ugrekhelidze *et al.*, 1997). Following uptake, volatiles can be detoxified, sequestrated, excreted or stored inside the plant cells (Weyens *et al.*, 2015). Plant-associated phyllosphere and especially rhizosphere microbes, specially bacteria degrade these volatiles into non-harmful compounds through enzymatic detoxification pathways (Behrendt *et al.*, 2001; Gerhardt *et al.*, 2009). During microbial detoxification, volatiles are metabolised into non-volatile compounds. Also, leaf absorbed VOC may translocate downward into the other parts of the plants (roots) to be metabolised (Berg & Smalla 2009; Nwoko 2010; Weyens *et al*. 2015).

Asparagus densiflorus

Crassula portulacea

Hedera helix

Dracaena deremensis

Chamaedorea elegans

Dieffenbachia maculata

Fatsia japonica

Dracaena marginata

Spathiphyllum wallisii

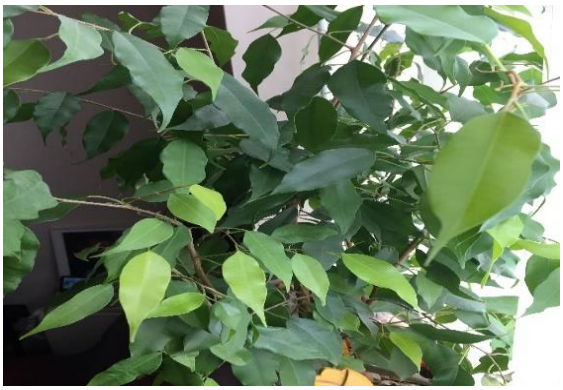
Dracaena fragrans

Ficus elastica

Epipremnum aureum

Chlorophytum comosum

Hydrangea macrophylla



Ficus benjamina

Spathiphyllum floribundum

Ficus benghalensis (Plantnet, 2019)

Nephrolepis exaltata

Kalanchoe blossfeldiana (Plantnet, 2019)

Hemigraphis alternate (Plantnet, 2019)

Pachira aquatica

Peperomia clusiifolia

Opuntia microdasy (Plantnet, 2019)

Dieffenbachia seguine (Plantnet, 2019)

Pelargonium domesticum (Plantnet, 2019)

Schefflera actinophylla (Plantnet, 2019)

Syngonium podophyllum (Plantnet, 2019)

Tradescantia pallida (Plantnet, 2019)

Fig. 1. 1 VOC removing common indoor plant species

Table 1. 3. Common indoor plant species which have VOC phytoremediation ability

Botanical name	**Common name**
Asparagus densiflorus	Foxtail fern
Chamaedorea elegans	Parlour Palm
Chlorophytum comosum	Spider plant
Crassula portulacea	Jade plant
Dieffenbachia seguine	Dumb cane
Dieffenbachia maculate	Dumb cane
Dracaena deremensis	Janet Craig
Dracaena fragrans	Corn plant
Dracaena marginata	Dragon tree
Epipremnum aureum	Devils ivy
Fatsia japonica	Japanese aralia
Ficus benghalensis	Banyan
Ficus benjamina	Weeping fig
Ficus elastica	Red rubber tree
Hemigraphis alternata	Purple waffle

Botanical name	Common name
Hordeum vulgare	Barley
Hydrangea macrophylla	Big Leaf Hydrangea
Kalanchoe blossfeldiana	Flaming Katy
Nephrolepis exaltata	Boston fern
Opuntia microdasy	Bunny Ear Cactus
Pachira aquatica	Malabar chestnut
Pelargonium domesticum	Common geranium
Peperomia clusiifolia	Baby rubber plant
Polyscias fruticosa	Ming aralia
Schefflera actinophylla	Dwarf umbrella tree
Spathiphyllum floribundum	Peace lily
Spathiphyllum wallisii Regal	Peace lily
Syngonium podophyllum	Arrowhead plant
Hedera helix	English ivy
Hoya carnosa	Variegated wax plant
Tradescantia pallida	Purple heart plant

(Yang *et al.*, 2009; Plantnet, 2019)

1.4.1.1. *S. wallisii, C. comosum* and *H. helix* as VOC removing indoor plant species

Three plant species *S. wallisii, C. comosum* and *H. helix* more commonly known as peace lily, English ivy and spider plant are common indoors plants (Kwang *et al.*, 2010). *H. helix* is a woody foliage plant while *S. wallisii* and *C. comosum* are herbaceous foliage plants (Metcalfe, 1958; Kwang *et al.*, 2010). *H. helix* is usually used as a green screen plant species in outdoor environments (Weinmaster, 2009). All three species are easy to propagate and capable of surviving at different climatic conditions such as in low and high-temperature regions, and under drought conditions (Metcalfe, 1958; Schreiber and Riederer, 1996; Laere *et al.*, 2011; Egea *et al.*,

2014; Sandoval-herazo *et al.*, 2018). Furthermore, these three species are popular members of "green walls" due to their medium size, low maintenance requirements and ability to remove different VOCs from the atmosphere (Weinmaster, 2009; Irga *et al.*, 2018; Torpy and Zavattaro, 2018).

During the NASA clean air study in 1973, Wolverton and colleagues evaluated the ability of two of these plant species, *H. helix* and *S. wallisii* to remove benzene, formaldehyde and trichloroethylene from the air within a test chamber setup (Wolverton *et al.*, 1989). The ability of these two species to remove benzene and toluene from test chamber air when the VOC was presented as a single gas or mixture of two gases was also subsequently evaluated in a separate study (Yoo *et al.*, 2006). In a further study, all three species were found to remove gaseous formaldehyde which is considered as one of the dominant VOC in newly constructed building, from indoor air (Schmitz *et al.*, 2000). Bacterial members of the rhizosphere of *S. wallisii* grown in a hydroball cultivation system were found to metabolize the three most common indoor VOCs: benzene, toluene and xylene (Chun *et al.*, 2010). In addition, these plants have shown promising increases in their phytoremediation rate following modification of their growth conditions. For example, *S. wallisii* able to remediate more benzene following the addition of biostimulants (D-cellobiose, D-lactose and L-asparagine) to the plant growth medium (Torpy *et al.*, 2013).

A comprehensive study by Yang and colleagues evaluated the ability of these three plant species to remove a range of different volatile organic compounds including; aromatic hydrocarbons (benzene and toluene), aliphatic hydrocarbons (octane), terpene (alpha-pinene) and halogenated hydrocarbons (trichloroethylene), when placed inside an air-tight glass jar system. During their analysis, based on the VOC removal rate per leaf area, *H. helix* was categorised as a superior removing plant whilst the other two species were classified as having poor removal efficiency (Yang *et al.*, 2009).

C. comosum which known as one of the most efficient benzene removing indoor plants removes high concentrations of benzene (500 ppm) from the air efficiently under different light conditions (Sriprapat and Thiravetyan, 2016; Setsungnern *et al.*, 2017). Also, it has been demonstrated that

VOCs such as formaldehyde are taken up by the *C. comosum* leaves and translocated into the rhizosphere of the plant by transporting VOC molecules through the phloem tissue and in the rhizosphere through oxygenase enzyme reactions VOC are metabolised (Giese *et al.*, 1994; Su and Liang, 2015).

By looking at the literature, it is identified almost all the studies based on the phytoremediation of VOC by plants are based on the single plant species grown in the pots. However, there is no evidence on how mixed plant species (plant community) grown in big pots/ propagating trays perform phytoremediation of VOC from air. A study showed that remediation of MEK (methyl ethyl ketone) contaminated air by sending through a vertical green wall composed with mixed plant species (Torpy *et al.*, 2018). This suggested, plant communities grown in big pots may also enhance the VOC phytoremediation from indoor air.

Therefore, selection of the plant species; *S. wallisii, C. comosum* and *H. helix*, which had shown high phytoremediation efficiencies in previous studies, would make a good combination during plant community manipulation.

1.4.1.2.Mechanism involved during phytoremediation of VOC in plant leaves

Plant leaves absorb VOC *via* stomata or penetrate from cuticle to epidermis of the leaf. This happens in both abaxial and adaxial sides of the leaf (Ugrekhelidze *et al*. 1997; De Nicola *et al*. 2008; Kvesitadze *et al*. 2009). During aromatic VOC detoxification by leaves, through oxidative metabolism the aromatic ring is cleaved into non-volatile compounds such as organic acids, simple sugars, CO_2 or H_2O (Giese *et al.*, 1994; Wei *et al.*, 2017). For example, during benzene degradation, benzene is converted into intermediate form phenol followed by pyrocatechol/catechol (o-diphenols) (Wei *et al.*, 2017). Through a diphenolase enzyme reaction, pyrocatechol is converted into o- quinones (Fig. 1. 2). These reactions are catalysed by copper-containing enzyme group called mono-oxygenase and dioxygenase in the plant leaf (Kvesitadze *et al.*, 2009). Production of these catabolic enzymes is regulated by the group of genes called cytochrome P450 in plant cells. In the next step, o- quinones is hydroxylated resulting in simple

organic acids: muconic acid followed by fumaric acid (Fig. 1. 2) ich enters into the Krebs cycle (tricarboxylic acid cycle) and finally releases as energy, CO_2 and H_2O (Kvesitadze *et al.*, 2009). The above process is mainly conducted in the chloroplast and cytosol in the leaf (Ugrekhelidze *et al*. 1997; Dietz & Schnoor 2001; Kvesitadze *et al*. 2001; Chrikishvili *et al*. 2006; Kim *et al*. 2008; Abhilash *et al*. 2009; Mukherjee *et al*. 2013; Setsungnern *et al*. 2017).

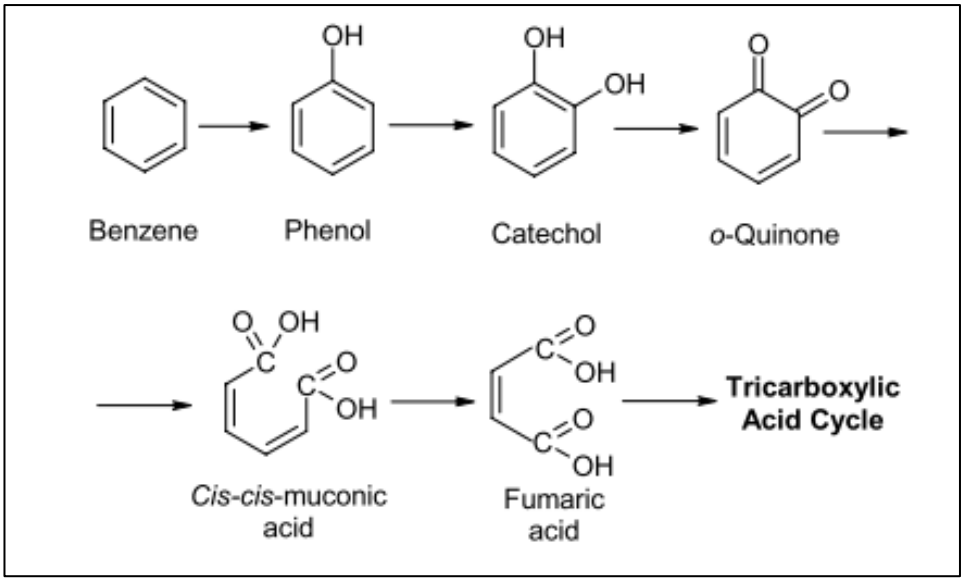


Fig. 1. 2 Oxidative degradation of benzene in plant cells. Adapted from (Kvesitadze *et al.*, 2009).

The intermediate forms produced during the degradation process can be slightly different from one VOC to another (Hamzah *et al.*, 2011). For example, during toluene detoxification, the methyl group can be converted into carboxyl group and then the aromatic ring cleavage occurred similar to the benzene oxidation and hydroxylation explained above or toluene can be hydroxylated without oxidation of the methyl group by producing alpha-carboxymuconic acid and alpha-methylmuconic acid. These intermediates enter into tricarboxylic acid cycle followed by releasing CO_2 and H_2O based on the energy requirement of the plant (Ugrekhelidze *et al.*, 1997; Kvesitadze *et al.*, 2009). CO_2 can also enter into the Calvin cycle to synthesise amino acids, hormones or other cellular components (Ugrekhelidze *et al*. 1997; Schmitz *et al*. 2000; Kim *et al*. 2008; Oikawa & Lerdau 2013; Dela Cruz *et al*. 2014; Weyens *et al*. 2015; Irga *et al*. 2018). However, VOC penetration through the cuticle and stomatal absorption followed by degradation in the plant

leaves is known as the primary pathway of VOC phytoremediation (Schmitz *et al*. 2000; De Kempeneer *et al*. 2004; Yoo *et al*. 2006; Kim *et al*. 2008; Llewellyn & Dixon 2011).

1.4.1.3.Rhizoremediation and mechanism involved during rhizoremediation

VOC degradation by bacteria in the plant rhizosphere is considered as the major pathway in the VOC phytoremediation (Wolverton and Wolverton, 1993; Kuiper *et al.*, 2004; Orwell *et al.*, 2006; Wood *et al.*, 2006; Kim *et al.*, 2008; Abhilash *et al.*, 2009; Chun *et al.*, 2010; Llewellyn and Dixon, 2011; Soreanu *et al.*, 2013). As an example, a test chamber-based study has shown that a higher detoxification level of formaldehyde by the rhizosphere of *F. japonica* and *F. benjamina* than other parts of those two plants species (Kim *et al*. 2008; Chun *et al*. 2010). Rhizosphere bacteria can degrade, detoxify or sequestrate VOC into energy, CO_2 and organic acids or enhance the plant growth by converting VOCs into plant growth hormones (Gopinath and Dhanasekar, 2012; Russell *et al*. 2014; Weyens *et al*. 2015). In addition to bacteria, mycorrhizal fungi in the plant rhizosphere can degrade hydrocarbon containing waste in the polluted soil (Harms *et al.*, 2011). However, bacteria are the most abundant groups in the rhizosphere, also they are identified as the main agent during degradation of hydrocarbon (Das and Chandran, 2011; Mwajita *et al.*, 2013; Yuniati, 2018).

At the initial stage of the rhizoremediation, VOC reaches to the rhizosphere of plant roots by penetration through the plant growing medium or is absorbed through the leaves and translocated through the phloem tissue (De Nicola *et al.*, 2008; Su and Liang, 2015). These processes are regulated by plants synthesizing root exudates and microbial quorum sensing chemicals (Hirsch *et al*. 2003; Bais *et al*. 2006; Faure *et al*. 2009; Berendsen *et al*. 2012; Goh *et al*. 2013; Mendes *et al*. 2013). Root exudates produced by plant roots compose of enzymes, amino acids, water, simple sugars, antimicrobials, proteins and minerals. Root exudates move into the rhizosphere and regulate positive or negative interactions between the plant roots and rhizosphere microorganisms (Schnoor *et al*. 1995; Smalla *et al*. 2001; Bais *et al*. 2006; Berg & Smalla 2009; Mendes *et al*. 2013; Goh *et al*. 2013; Knief 2014; Berg *et al*. 2014; Bisht *et al*. 2015; Venturi & Keel 2016). For example, the root exudate regulates positive interactions (symbiotic

relationship) between root and nitrogen-fixing bacteria while regulating negative interactions (antagonistic relationship) between pathogenic microbes (bacteria, virus or fungi) and insects (Bais *et al.*, 2006). Root exudate "flavonoids" in *Medicago sativa* roots attracts nitrogen fixing rhizobia bacteria in the rhizosphere, thus as a result it enhances the plant growth through high nitrogen fixation in the plants (Szoboszlay *et al.*, 2016; Massalha *et al.*, 2017). Root exudates with antimicrobial and insecticidal properties involve maintaining negative relationships between plants and pathogenic microbes. For example, antifungal phenolic compounds produced by the *Hordeum vulgare* roots system perform negative interaction following infection of *Fusarium graminum* (Berendsen *et al.*, 2012). Production of exudates in plants depends on several factors such as plant species, photosynthetic processes, composition of root microflora, health and age of plant and the availability of nutrients in soil (Miethling *et al*. 2000; Bais *et al*. 2006; Nuruzzaman *et al*. 2006; Asensio *et al*. 2007; Bever *et al*. 2013; Yergeau *et al*. 2014; Bisht *et al*. 2015; Schulz-Bohm *et al*. 2015; Donn *et al*. 2015; Ite *et al*. 2016; Massalha *et al.*, 2017).

In addition to the root exudates, microorganisms in the root zone regulate a signal system known as quorum sensor system which involves regulating microbe-microbe interactions (Lambers *et al*. 2009; Müller *et al*. 2009; Faure *et al*. 2009; Badri *et al*. 2009; Yergeau *et al*. 2014). Gram-negative bacteria in plants produce N-acyl-homoserine lactones (AHLs) while many Gram-positive bacteria synthesise gamma-butyrolactones which are used as the quorum sensing chemicals between microbes. They are diffusible compounds and the production of the quorum chemicals depends on the cell density of different bacteria, the surrounding environment and the presence of plant factors such as root exudates (Gerhardt *et al.*, 2009). Quorum signals control gene expression in the microorganisms in a community, thus it allows them to work as a cooperated group. Therefore, microorganisms in a community (e.g. rhizosphere) possess enzymes focusing on the required functions pre-determined by their quorum system (Hirsch *et al*. 2003; Bais *et al*. 2006; Badri *et al*. 2009; Berg & Smalla 2009; Faure *et al*. 2009; Müller *et al*. 2009; Bakker *et al*. 2013; Kanchiswamy 2015). Thus, through root exudation and quorum systems performing chemical selective strategy, different compositions of bacteria tend to arrange around roots to perform

important functions such as degradation of different pollutants (Faure, Vereecke and Leveau, 2009; Faure *et al*., 2009, Berg & Smalla, 2009; Bever *et al*., 2013).

The primary step during the hydrocarbon degradation process is intracellular uptake of those foreign molecules (VOC) into microbial cells (Gerhardt *et al.*, 2009). Attachment of hydrocarbon onto the cell wall of microbes is regulated by the group of compounds called biosurfactants (Kuiper *et al.*, 2004). Biosurfactants are low molecular weight glycolipids (rhamnolipid and fructosemycolate) or peptidolipid (surfactin and viscosin) and their production depends on the type of microbes (Neu, 1996). Biosurfactants help to solubilize hydrocarbon and uptake into the cells. As an example, *Pseudomonas aeruginosa* possessing rhamnolipid emulsifies the petroleum oil into small size micelles during the intracellular uptake at the initial stage of degradation (Fig. 1. 3) (Whyte *et al*. 2002; Das & Chandran 2011; Marecik *et al*. 2015).

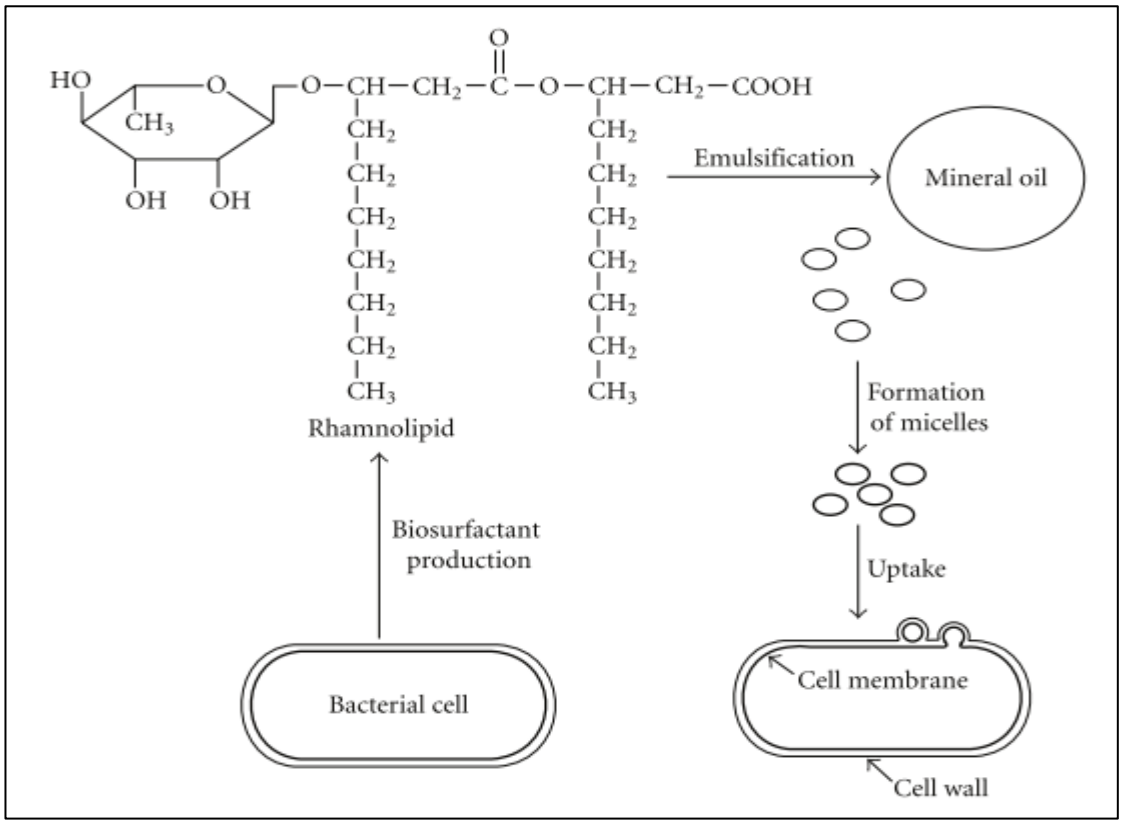


Fig. 1. 3 Intracellular uptake of hydrocarbon by bacteria. Adapted from Fritsche & Hofrichter 2000.

In the aerobic conditions, bacteria possessing oxygenase (monoxygenase and dioxygenase) enzymes catalyse initial oxidation reactions in the hydrocarbon degradation. Synthesis of oxygenase is regulated by the cytochrome P450 genes in the bacteria. During the initial step, the number of oxygen molecules attached to hydrocarbons depends on the length of the carbon chain and number of aromatic rings in the hydrocarbon. Each aromatic ring would be cleaved after the addition of one or two oxygen atoms. When degrading a mixture of hydrocarbons such as complex petroleum compounds in soil, a broad range of enzymes is required for the complete degradation. In the presence of mixed microbial communities in such instance, a high degradation process can be observed due to the presence of a range of metabolic enzymes (Murty *et al*. 1998; Kvesitadze *et al*. 2001; Kvesitadze *et al*. 2009; Nwoko 2010; Das & Chandran 2011; Van Bogaert *et al*. 2011; Peixoto *et al*. 2011; Setsungnern *et al*. 2017). Benzene degrading bacterial species possessing monooxygenase and dioxygenase enzymes to convert benzene into intermediate forms: phenol or trans/cis-dihydro-benzenediol followed by formation of the catechol. These are intermediates of benzene-oxides and during oxidation reaction monooxygenase catalyses attaching one oxygen atom from O_2 molecule to the hydrocarbon (substrate). The remaining oxygen atom joins with two hydrogen (H) atoms released from the substrate by producing H_2O (Fig. 1. 4). Following further oxidations of phenol, the intermediate form, trans-dihydrodiol (trans-dihydro-benzenediol), is produced and it is converted into catechol (Masai *et al.*, 1995; Fritsche and Hofrichter, 2000; Díaz *et al.*, 2001; Lawrence, 2006; Das and Chandran, 2011; Mukherjee *et al.*, 2013) .

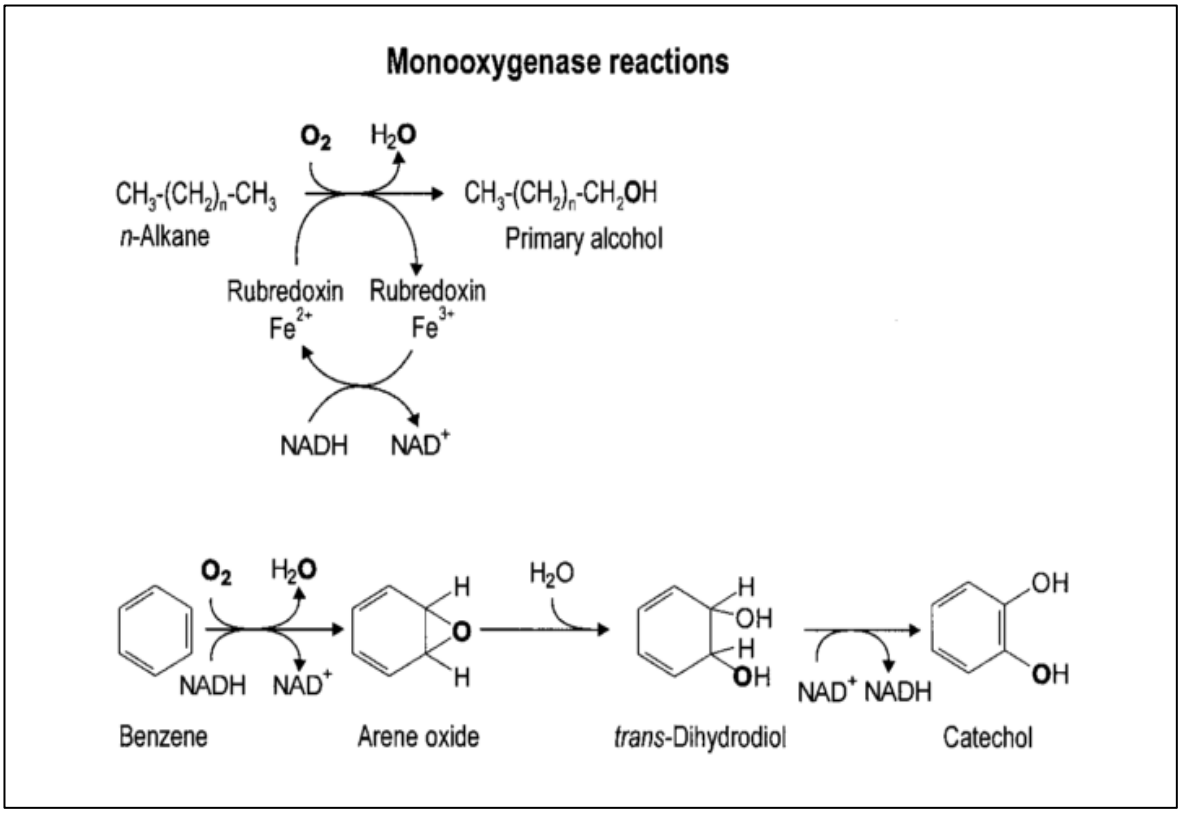


Fig. 1. 4 Monooxygenase reaction involved during rhizodegradation of benzene and alkanes through the oxidative metabolism in microbes. Adapted from Fritsche & Hofrichter 2000

The dioxygenase reaction is slightly different from the monooxygenase. During the initial intermediate formation catalysed by dioxygenase in bacteria, both oxygen atoms in the O_2 molecule attach to the substrate by producing the intermediate form cis-dihydro-diol (cis-dihydro-benzenediol) and through further oxidation reactions, it is converted into catechol (Fig. 1. 5) (Fritsche and Hofrichter, 2000).

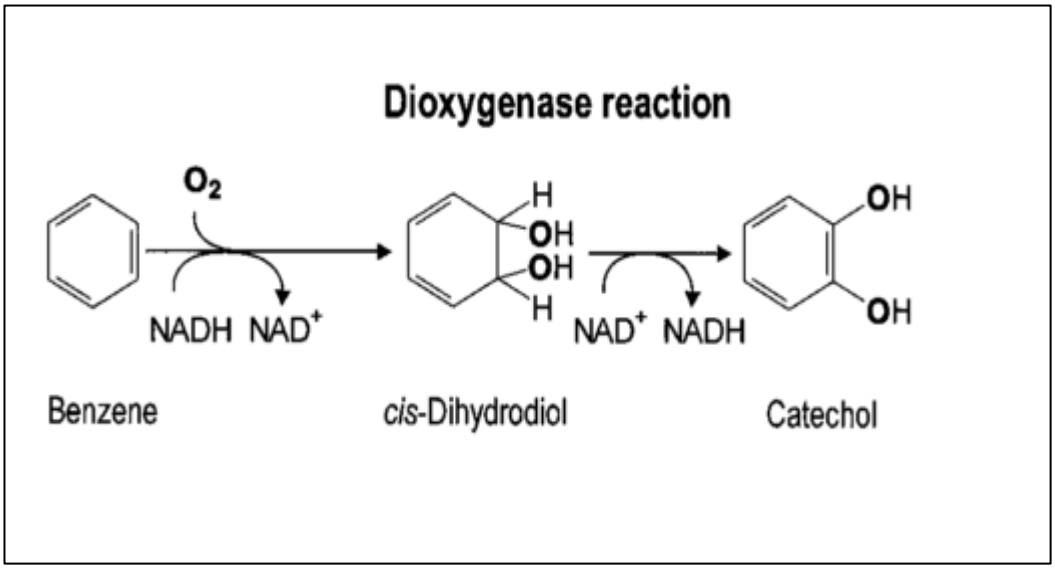


Fig. 1. 5 The Dioxygenase reaction involved in rhizodegradation of benzene by oxidative metabolism in microbes. Adapted from Fritsche & Hofrichter 2000.

Through further oxidation reactions, catechol is converted into cis,cis-muconate, (+)-muconolactone, 3-oxoadipate-enol-lactone, 3-oxoadipate, 3-oxoadipyl-CoA and succinyl-CoA respectively. Finally, succinyl-CoA will join with citrate cycle (Krebs cycle) (Vogt *et al*. 2011).

When the aromatic hydrocarbons contain a substituent group (aliphatic side chains: alkanes) the degradation reaction slightly differs than the ring cleavage reaction explained above. Also, there can be different pathways for the degradation of aromatic compounds which has aliphatic side chains (Hamzah *et al.*, 2011). During intracellular degradation, aliphatic methyl and ethyl groups are oxidised into alcohols, aldehyde or carboxylic groups (Fig. 1. 4). For example, *Pseudomonas* species possess the enzymes required to degrade methyl groups in aromatic hydrocarbons, so they are identified as one of the toluene and xylene degrading bacteria in polluted soil environments (Suyama *et al*. 1996; Panke *et al*. 1998; Fritsche & Hofrichter 2000). A group of toluene dioxygenase enzymes involve catalysing toluene degradation in bacteria (Johnson *et al.*, 2006).

During toluene degradation by *P. putida*, the toluene dioxygenase enzyme converts toluene to an intermediate form cis-toluene dihydrodiol followed by 3-methylcatechol (top reaction in Fig. 1. 6). Through further oxidation, 3-methylcatechol converts to 2-Hydroxy-6-oxohepta-2,4-dienoate.

Through the hydroxylation reaction, the methyl group converts to acetic acid and the aromatic ring section is cleaved to 2-hydroxy-penta-2,4-dienoate (middle reaction in Fig. 1. 6).

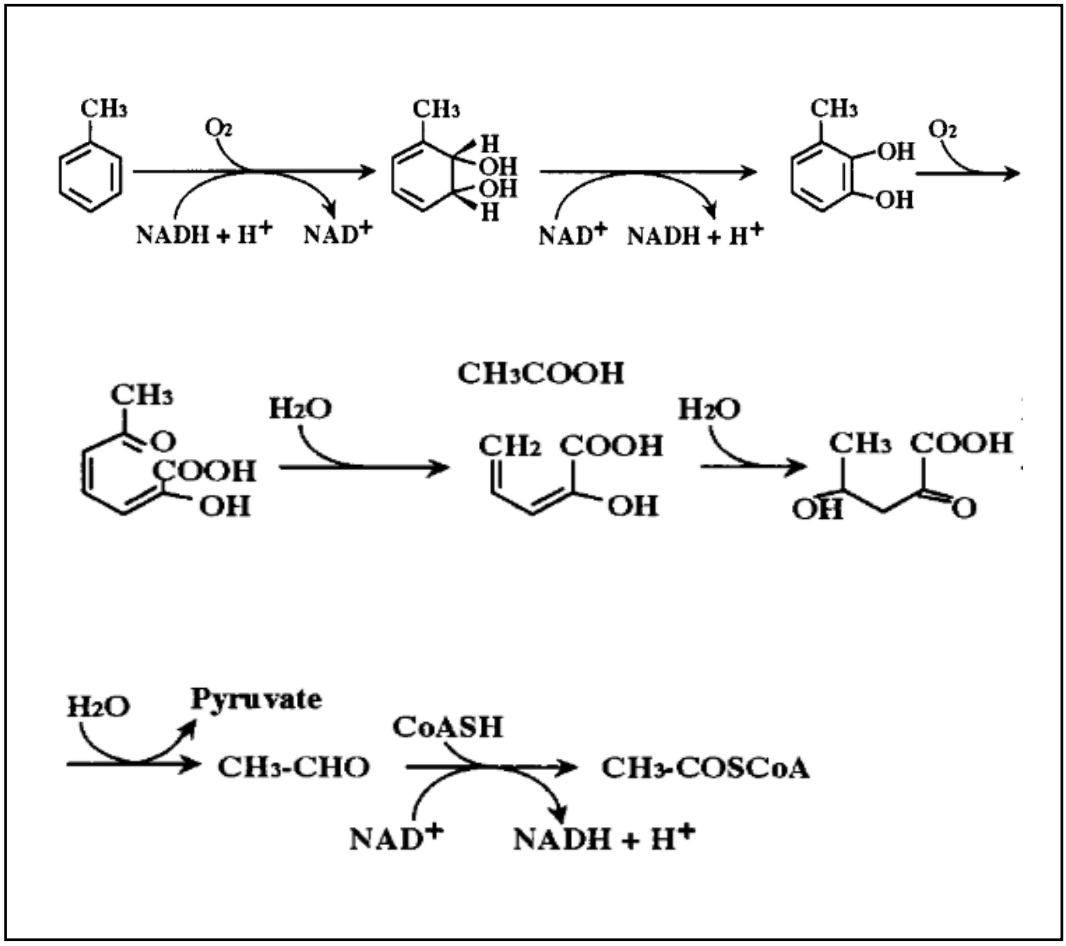


Fig. 1. 6 Toluene and aliphatic hydrocarbon degradation process by *P. putida*. Adapted from (Suyama *et al.*, 1996)

Through further reactions, 2-hydroxy-penta-2,4-dienoate converts to acetaldehyde and pyruvate followed by entering into TCA cycle to produce acetyl coenzyme A (bottom reaction in Fig. 1. 6) (Suyama *et al.*, 1996; Panke *et al.*, 1998; Parales *et al.*, 2000; Johnson *et al.*, 2006; Hamzah *et al.*, 2011).

Therefore, as a summary, once the hydrocarbons enter into the bacterial cells, through different enzymatic reactions, they are degraded into CO_2, H_2O or participate to synthesise cell biomass (Fig. 1. 7) (Fritsche and Hofrichter, 2000; Díaz *et al.*, 2001; Lawrence, 2006).

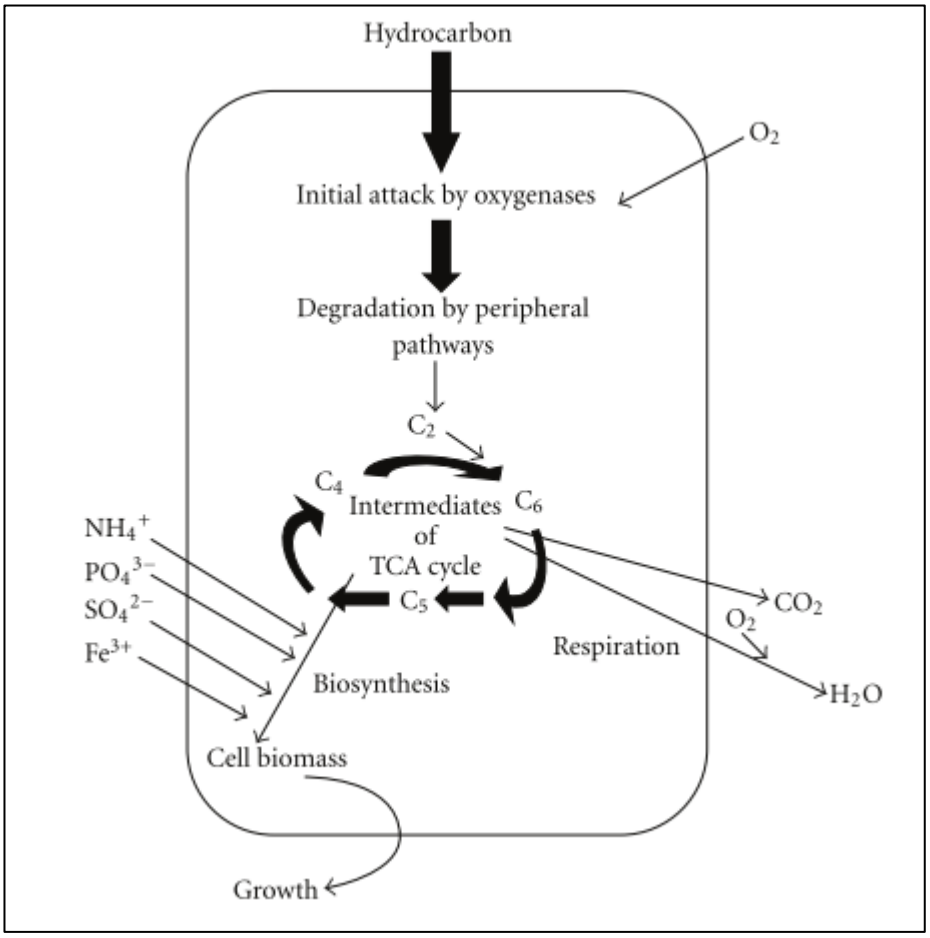


Fig. 1. 7 Schematic diagram to show aerobic degradation of hydrocarbons by bacteria. Adapted from (Fritsche and Hofrichter, 2000).

1.5. Culture-dependent approaches to analyse bacterial community diversity

Culture-dependent classical microbiology methods were dominant during identification of unknown bacteria in the research prior to the advent of PCR and sequencing methods (Orphan *et al.*, 2000). This approach involves direct cultivation of unknown microorganisms (e.g. bacteria) from environmental samples and the classification of them through biochemical and physiological characteristics. Also, individual bacterial isolation through culture-based approaches is an advantage when the DNA extraction of individual bacteria is required (Nielsen *et al.*, 2007; Carraro *et al.*, 2011; Vaz-Moreira *et al.*, 2011; Stefani *et al.*, 2015).

However, the culture-dependent method doesn't provide a comprehensive understanding of a microbial community compared to the culture-independent methods. This is because most of the bacteria are not culturable in the laboratory and only a small fraction of (between 0.1-1%) total

environmental microorganisms can be cultured (Pham & Kim 2012; Mendes *et al*. 2013; Sun *et al* .2014). Some of the possible reasons for unculturability is under the laboratory conditions incapability of environmental bacteria to survive because the medium might contain growth inhibitors or toxic compounds, or they do not grow under high oxygen or nutrient level in the medium (Cho & Giovannoni 2004; Vartoukian *et al*. 2010). Thus, the medium does not supply metabolic and physiological requirements for bacteria, so they can be unculturable. Therefore, culture identifications can be biased research. However, by using combinations of traditional culture-approach and low-resolution culture-independent approaches (Sanger sequencing) would guide for an accurate identification of unknown bacteria (Nielsen *et al.*, 2007).

1.6. Culture-independent approach to analyse bacterial community diversity

In early studies, characterisation of environmental microbiomes relied on the culture-dependent method. It facilitates the identification of microbial taxonomies based on different culture tests, however, the results can be biased and limit the diversity identifications (Srinivasan *et al.*, 2015; Waldor *et al.*, 2015; Ames *et al.*, 2017). Use of culture-independent approaches such as metagenomic analysis and small subunit (SSU) ribosomal RNA screening have been increasing for last few decades since it has many advantages, time efficient, identification of both culturable and unculturable microorganisms, over the traditional culture method (Lebeis, 2014; Schornsteiner *et al.*, 2014).

Culture-independent, whole bacterial community sequencing (profiling of the community) method is playing an important role in the environmental microbiome analysis, because using a small portion of sample such as soil or water, the researcher can get almost all information about the microbial diversity (Orphan *et al*. 2000; Nielsen *et al*. 2007; Carraro *et al*. 2011; Vaz-Moreira *et al*. 2011; Stefani *et al*. 2015). Approximately one gram of bulk soil contains 1 x10^9 bacterial cells (Amann *et al.*, 1995; Roesch *et al.*, 2007; Raynaud and Nunan, 2014; Wagg *et al.*, 2014). Therefore, for a comprehensive analysis of soil microbiome diversity, a culture-independent approach is a better selection compared to the culture-dependent approach.

1.6.1. Molecular techniques involved in community analysis

A community profiling approach consists of a few main steps: DNA extraction from samples, PCR amplicon generation, sequencing library preparation, sequencing and bioinformatic analysis (Sun *et al.*, 2014). Novel DNA extraction kits facilitate whole community DNA extraction from environmental samples directly known as metagenomic DNA extraction. This allows isolating DNA from both culturable and unculturable microbes (Schloss & Handelsman 2008; Bokulich & Mills 2013; Tanase *et al*. 2015; Lear *et al*. 2018). QIAamp DNA stool mini kit and PowerSoil® DNA isolation kit are common DNA isolation kits used in all types of soil experiments (Whitehouse and Hottel, 2007; Santos *et al.*, 2012). One of the main advantages of these kits are, they eliminate PCR inhibitors such as humic acid from the medium thus, it allows successful PCR analysis. Kits containing buffers dissolve and wash out humic acid during DNA extraction (Santos *et al.*, 2012; Lear *et al.*, 2018). Also, the kits perform purification of DNA from non-DNA contaminants: proteins and lipids during the membrane filtration step (Dineen *et al.*, 2010; Vishnivetskaya *et al.*, 2014).

For the separation of bacterial DNA from the total community DNA, polymerase chain reactions can be performed using gene-specific primers on the universal genes of bacteria. The ubiquitous gene of bacteria, the 16S rRNA gene is increasingly being used in studies due to many reasons. The 16S rRNA gene is relatively small with approximately 1,500 bp. It is one of the highly conserved genes in the bacterial domain (Fig. 1. 8).

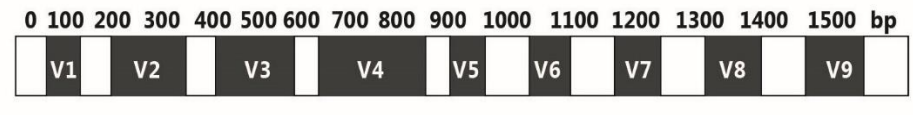


Fig. 1. 8 Schematic diagram of the bacterial 16S rRNA gene. Modified after (Chierico *et al.*, 2015)

It consists of nine hypervariable regions (V1-V9) and those variable regions are flanked by conserved regions. Bacterial universal primers are designed to bind on these highly conserved regions, thus DNA from a broad range of bacteria in a community is amplified during the PCR reaction. Highly variable regions in the 16S rRNA gene provide information to identify different

genera and species in the bacterial domain (Ash *et al*. 1993; Heritage *et al*. 1999; Pham & Kim 2012; Guo *et al*. 2013; Cox *et al*. 2013; Kong *et al*. 2014; Tanase *et al*. 2015; Yang *et al*. 2016; Lear *et al*. 2018).

1.6.2. High through-put sequencing of community DNA

High through-put sequencing known as the next-generation sequencing (NGS) approach is widely used in studies since it enables the research of a comprehensive microbial community analysis. Illumina Miseq and Ion Torrent sequencing approaches enable the sequencing of one or more hypervariable regions of 16S rRNA gene in total bacterial DNA in a community and provide information for the bacterial taxonomical and functional classifications and community diversity analysis (Janda and Abbott, 2007; Milani *et al.*, 2013). Illumina Miseq offers paired-end reads for each DNA amplicon, hence, the nucleotide sequence of one DNA fragment is recorded twice providing high-quality sequence readings of the bacterial genetic material (Vasileiadis *et al*. 2012; Fadrosh *et al*. 2014; Zhang *et al*. 2014). Ion Torrent sequencing provides continuous sequencing of longer amplicon reads, however it doesn't support to paired-end sequencing (Tiemersma *et al.*, 2002; Lahens *et al.*, 2017) Also studies show a higher error rate in the Ion torrent platform than Illumina MiSeq during sequencing the 16S rRNA gene in bacterial community DNA (Salipante *et al.*, 2014). Based on the literature, the Illumina MiSeq sequencing approach proves more suitable in the sequencing of bacterial community DNA in soil samples.

1.6.3. Brief overview of the method involved in Illumina MiSeq sequencing

The Illumina sequencing workflow can be divided into four major steps. These are library preparation, cluster generation, sequencing and data analysis (Walujkar *et al.*, 2014; Lear *et al.*, 2018). During library preparation, DNA samples are fragmented and adapters attached to each side of the fragment for clustering. Library construction in the Illumina workflow can be carried out using a library preparation kit such as Nextera DNA library kit (Illumina) (Quail *et al.*, 2012; Lear *et al.*, 2018). During clustering DNA samples (original strand) along with adapters are hybridized with flow cell primers (lawn primer) through complementary base pairing (Fig. 1. 9).

Then the original DNA strand flips over and makes a bridge by complementary base pairing with an adjacent adapter (lawn adapter) on the flow cell (Knief, 2014).

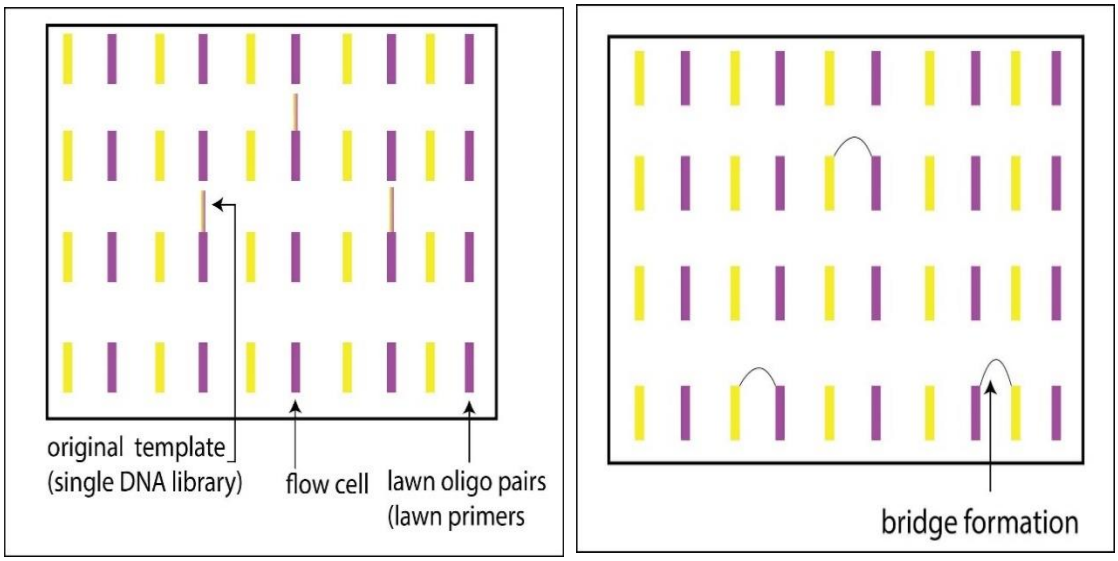


Fig. 1. 9 Schematic diagram of DNA hybridisation on flow cell primers and the single stranded bridge formation involved during Illumina work flow

Then the lawn primer extends in the 3/ direction with bases complementary to the original DNA fragment by adding ddNTPs and polymerases called bridge amplification (Fig. 1. 10). This newly synthesised strand is complementary to the original fragment called sequenced /synthesised strand. Finally, a double-stranded bridge on the flow cell surface is denatured and results in two copies of single-stranded DNA that covalently bound to the flow. Lawn primer extension and bridge formation steps repeat for the all the lawn primers on the flow cell (Duan *et al.*, 2010; Shreepriya Das *et al.*, 2010; Knief, 2014; Zhang *et al.*, 2014). During the linearization, the original DNA strand cleaves and washes away while the synthesized linear strand remains on the flow cell surface (Fig. 1. 10).

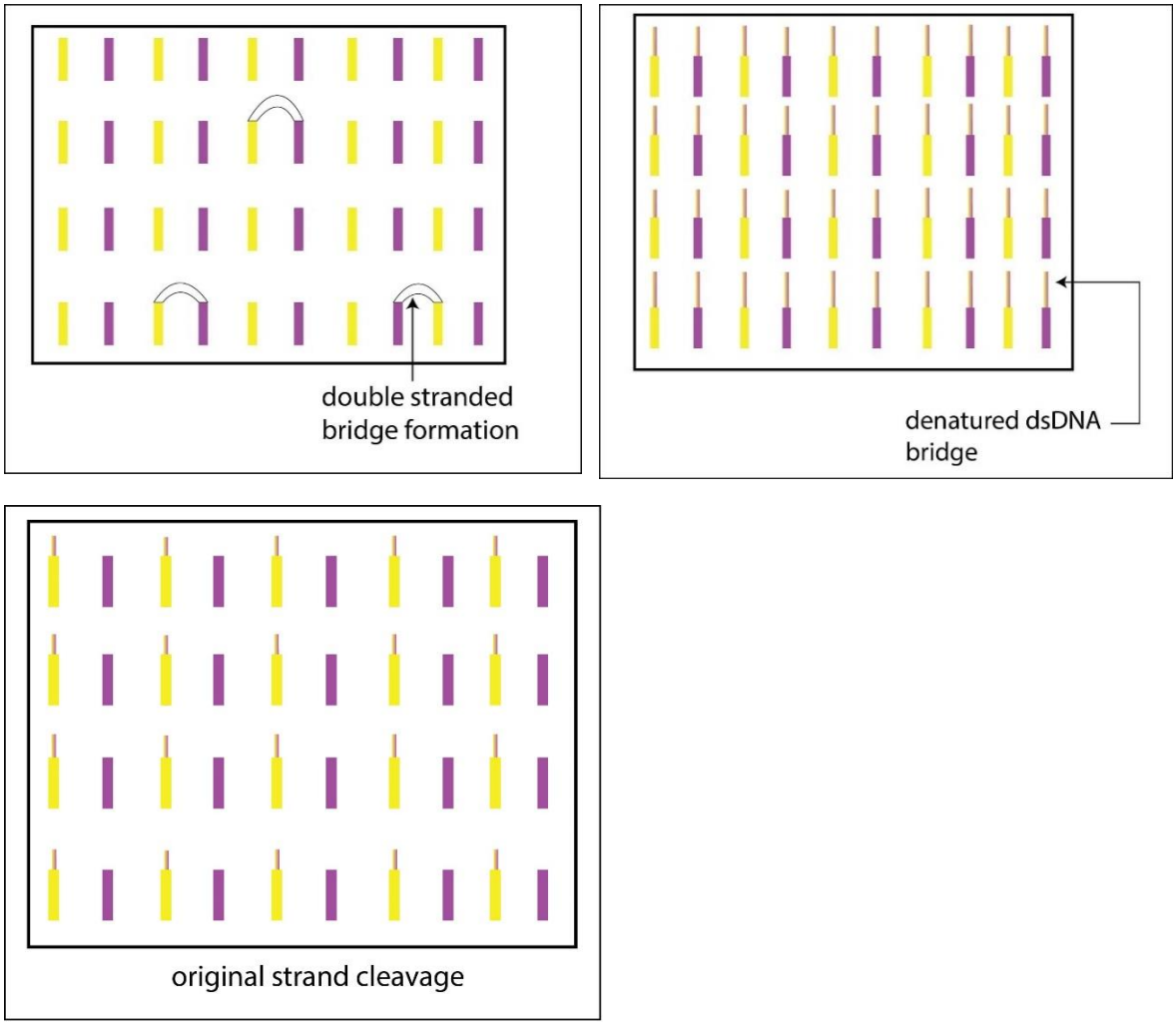


Fig. 1. 10 Schematic diagram of the double stranded bridge formation, denaturation of dsDNA and cleavage of original DNA fragment during Illumina work flow

Then the 3/ end of the lawn primer (where the original fragments attached previously) and synthesized strand will be blocked to avoid the further addition of nucleotides (Fig. 1. 11). During sequencing, the sequencing primer will hybridise with the sequenced fragments (synthesized fragment) and then the original fragment starts to sequence by synthesis (Das and Vikalo, 2011).

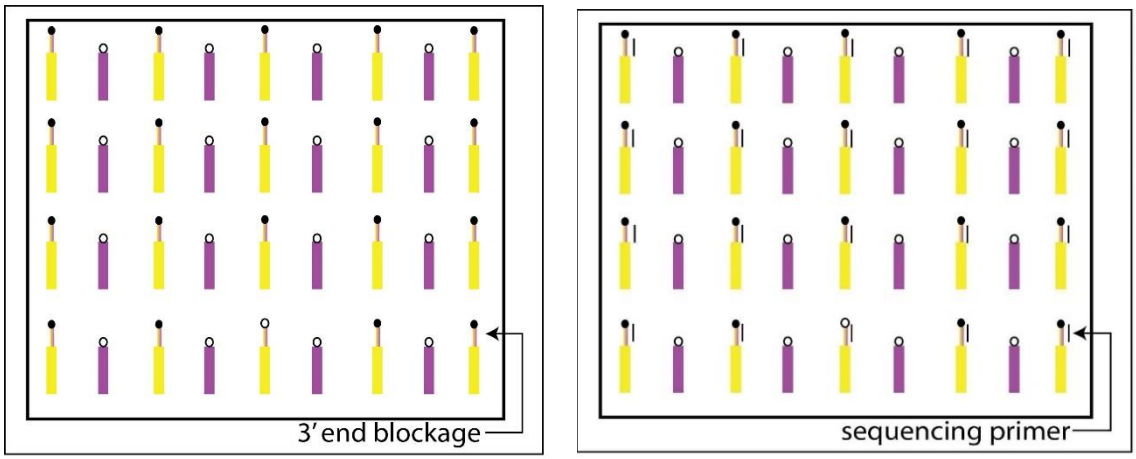


Fig. 1. 11 Schematic diagram of the 3' blockage and initiation sequencing process involved in Illumina work flow

Each of four ddNTPs (dideoxynucleotides) and DNA polymerase carries four different fluorescent molecules that attached to the 3/ position of the nucleotide and it gives a signal when it joins to extend the original DNA fragment (Duan *et al*. 2010). Once one nucleotide is joined and emitted fluorescence, it will be de-blocked (cleaving the fluorescence from 3/ position of base) and the second nucleotide will be joined by its free 3/ position of the previous nucleotide and the signal will be detected (Knief, 2014). In this way, joining nucleotides and signal detection will be repeated until synthesising the full original DNA fragment which is a complete cycle. Illumina MiSeq allows the sequencing of both ends of the fragment called paired-end sequencing which allows sequencing each base twice which will increase the accuracy of sequencing (Das and Vikalo, 2011).

1.6.4. Bioinformatic data analysis

The last step in the culture-independent molecular approach is analysing sequencing data using a suitable software which allows the researcher to interpret the insight of the unknown microbiome. During bioinformatic analysis, based on the information in the DNA/RNA sequencing pool, the members in the community can be classified taxonomically and functionally and also their phylogenetic relationships can be revealed.

Selection of the bioinformatic software can be based on few factors: quality and the type of sequencing data, compatibility of the software with reference databases required by researcher,

quality of interpreting data (e.g. graphs), software installation, memory usage and running time. Quantitative Insights into microbial ecology (QIIME) is one of the popular sequence analysis workflows which allows the user to pre-process the data and perform community diversity analysis (Caporaso *et al.*, 2010). QIIME is compatible with many reference taxonomical databases. Therefore, taxonomical profiling of sequencing reads can be carried out based on the reference taxonomy database such as SILVA (Pruesse *et al.*, 2007) and Greengenes (DeSantis *et al.*, 2006). Using taxonomical classification data, PICRUST (phylogenetic investigation of communities by reconstruction of unobserved states) software (Langille *et al.*, 2013) predicts functional profiling of the microbiomes based on KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology database(Minoru *et al.*, 2004). Therefore, the combination of microbial DNA extraction, DNA amplification, NGS and data analysis through bioinfomatics allows the observation of previously unknown diversity in complex bacterial communities.

1.7. Methods employed in monitoring VOC concentrations in air samples

A suitable analytical approach must be selected to monitor VOC concentration in air samples. Digital monitors are time efficient, automated and easy to handle while gas chromatography-based analysis is known to be accurate and give unique responses to each VOC, though it is more time and labour consuming than the digital method. Two methods employed to monitor VOC level inside test chambers: Aeroqaul digital VOC monitors and automated thermal desorption/gas chromatography with the flame ionisation detector are detailed below.

1.7.1. Background of Aeroqual VOC monitoring method

Aeroqual 500 series VOC monitors are used for monitoring VOC concentrations in both indoor and outdoor environments (Martuzevicius *et al.*, 2014; Berry *et al.*, 2017; Spinelle *et al.*, 2017b; Masiol *et al.*, 2018). The monitor base comprises a rechargeable lithium battery and a display screen. When the battery is fully charged, the unit can be used up to a maximum of 8 hours. This is an advantage when measuring VOC level in outdoor environments such as urban area, roadsides and industrial zones. Another main advantage of this monitor, over other analytical methods, is

measuring real-time VOC level in the environment. The clock on the monitor can be set to the real-time, therefore each data point is recorded with its sampling time. The data logging frequency can be set from 1 minute to a few hours intervals and up to 8188 data points can be saved in the monitor. When the monitor memory is full, the logging data can be downloaded to the computer through Aeroqual PC software (Aeroqual, 2014). The Aeroqual VOC sensor is capable of detecting around 120 different VOCs in air samples and it can be used under a range of humidity level from 10 to 95%. Once the air monitoring is completed, the sensor can be cleaned by warming up to remove any trapped contaminants. After downloading data from the monitor and cleaning the sensor, they are ready for the next analysis (Aeroqual, 2014). These features in series 500 monitor facilitate monitoring VOC level in the air continuously for a few days if it is connected to the main power supply (Aeroqual, 2016). As showing in Fig. 1. 12, the Aeroqual handheld monitor connects to a VOC sensor held on an adapter.

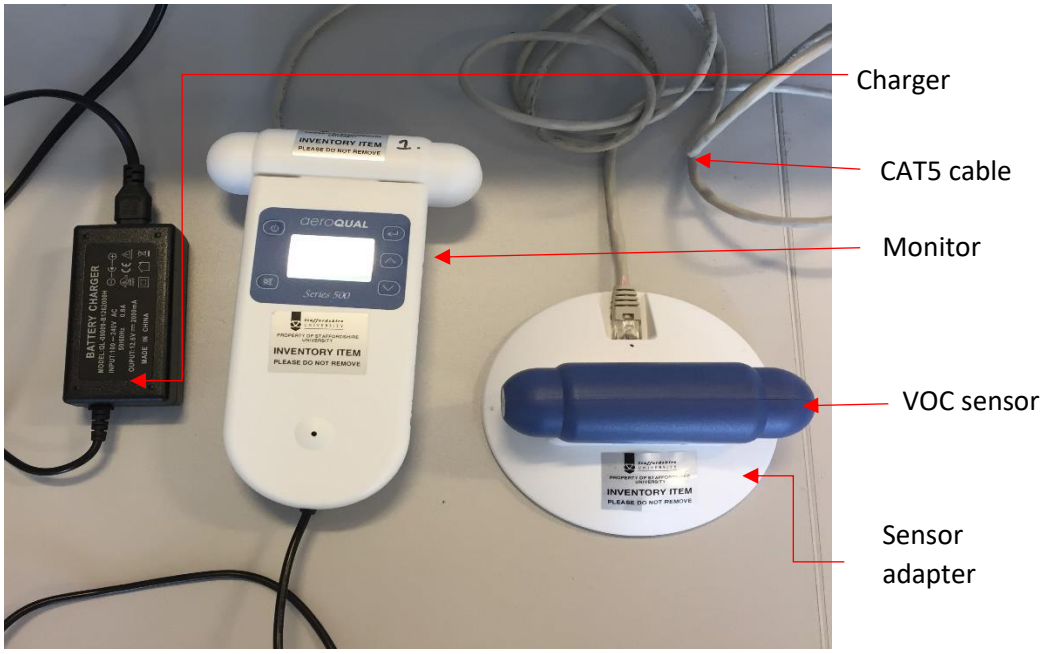


Fig. 1. 12 Aeroqual digital VOC monitor system

1.7.1.1.PID technology in the sensor

Aeroqual VOC sensors detect different VOCs in the air using photoionization detection (PID) technology (Aeroqual, 2016). PID sensor includes a UV lamp which photoionizes the VOC molecules but not non-VOC molecules (Fig. 1. 13). Once a VOC molecule reaches into the UV lamp, it ionizes into negatively and positively charged ions followed by moving ions towards the anode and the cathode respectively (Aeroqual, 2014). Moving ions generate an electric field which is measured by an electrometer and the resulting electric current is proportional to the VOC concentration (Aeroqual, 2016).

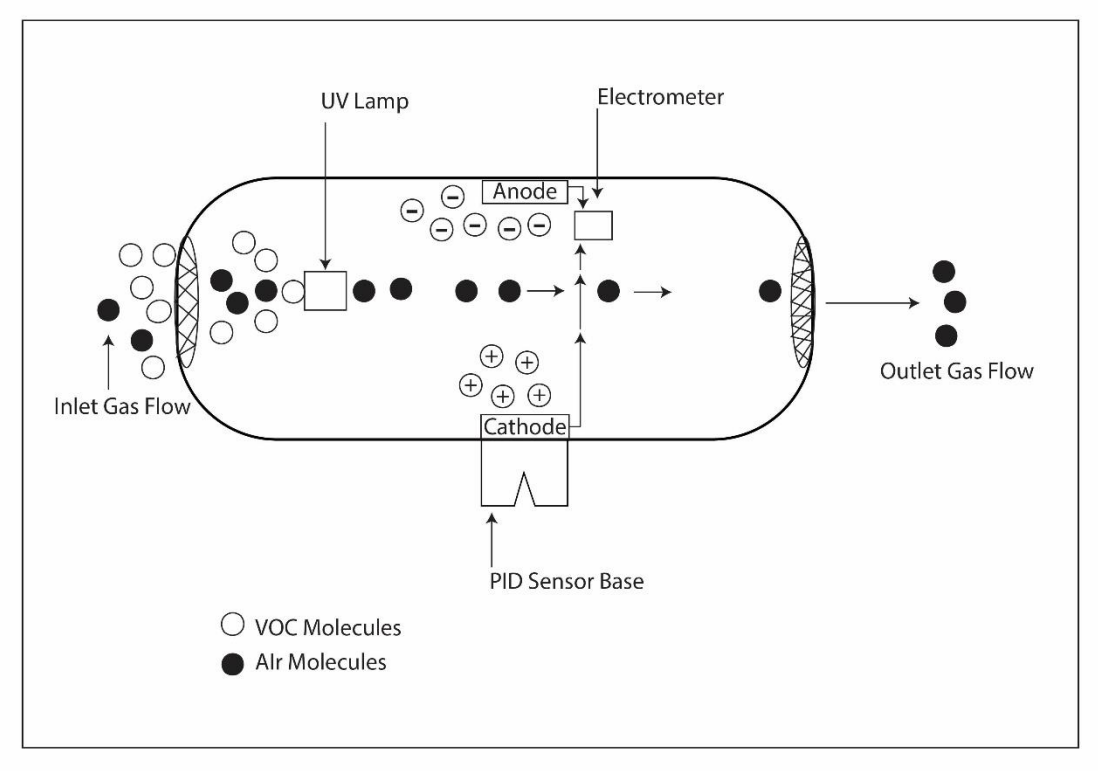


Fig. 1. 13 Schematic diagram of PID sensor technology

PID sensors are nonselective meaning they measure a wide range of VOCs with varying degrees of sensitivity to their concentrations. The Aeroqual PID sensor is capable of detecting VOC concentrations between 0-2000ppm ranges with ±0.2ppm accuracy and provides a minimum detection limit of 0.1ppm. Also, it provides a linear sensitivity to the VOC concentrations within this range (Aeroqual, 2016).

The PID sensor is calibrated against isobutylene which is non-flammable and non-toxic compound within the concentrations used in calibrations (Aeroqual, 2016). The ionization potential of isobutylene is close to the average ionization potential of most VOCs (Aeroqual, 2016). Therefore, it can be used as a representative for most of VOCs. To get the actual concentration of target VOC, sensor detecting VOC level must be multiplied by the response factor (RF) which have been calculated comparatively to the isobutylene concentration (Table 1. 4). For example, if the sensor detects the units of isobutylene is 10 ppm during benzene detection, the actual benzene concentration can be calculated as 10 ppm x 0.53. Here, 0.53 is the RF factor for benzene. Isobutylene conversion factor from ppm to mg/m^3 is 1 ppm = 2.29 mg/m^3. Compounds which have smaller RF factor of isobutylene are more sensitive to PID sensors (Aeroqual, 2016).

Table 1. 4 Aeroqual PID sensor RF value for a few of most abundant VOCs (Adapted from: Aeroqual, 2016).

Compound	**RF values**
1-butanol	3.4
Benzene	0.53
Ethylbenzene	0.51
m- xylene	0.53
methyl tert-butyl ether	0.86
Naphthalene	0.37
n-hexane	4.5
α-pinene	0.4
Tetrachloroethylene	0.50
Toluene	0.53

1.7.2. Analysis of VOC in air using gas chromatography/flame ionisation detection

Determination of VOC from air samples using automated thermal desorption (ATD), gas chromatography (GC) along with flame ionisation detector (FID) is known as a precise and accurate approach in the quantitative examination of outdoor and indoor air analysis (Woolfenden, 1997; Cornejo *et al.*, 1999; Dojahn and Wentworth, 2001; Ahn *et al.*, 2011; Peñuelas *et al.*, 2014; Schulz-Bohm *et al.*, 2015). GC/FID method has a higher sensitivity to all organic compounds (except formaldehyde) than non-organic compounds. Therefore, for the most of VOC analysis, GC/FID is suitable (Tiscione *et al.*, 2011). It has a linear response to a larger range of analyte concentrations (RESTEK, 2003).

The main steps involved during the GC/FID analysis are sorbent material selection, sampling air samples into sorbent material through active or passive sampling, desorption of VOCs into the GC, separation of analytes in the GC and detecting the analytes in the samples respectively (Schieweck *et al.*, 2018). Fig. 1. 14 shows an image of ATD/GC/FID system.

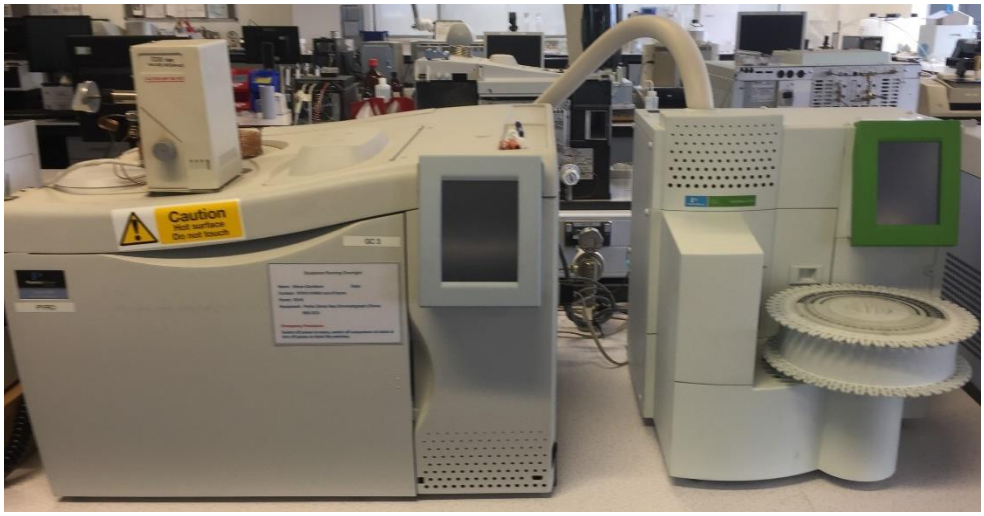


Fig. 1. 14 Perkin-Elmer Turbo Matrix ATD 650 (Perkin-Elmer, UK) desorption unit and Clarus 500 Gas Chromatograph fitted with FID installed in the analytical laboratory

The active sampling of air involves drawing the air through a sorbent material at a constant and low flow rate using a mechanical force such as vacuum pumping. The VOC is trapped and pre-concentrated inside the sorbent material and then desorbed into the GC column through thermal

desorption. Once the sampling is completed, the VOC sample-containing sorbent tube can be capped to avoid contamination (Barro *et al*. 2009; Bessonneau *et al*. 2013; HSE 2016;). In passive sampling, without mechanical air drawn, the sorbent material is exposed to the air for hours or days (Srivastava & Majumdar 1995; Molhave *et al*. 1997; Demeestere *et al*. 2007;Herbarth & Matysik 2013).

1.7.2.1. Choice of Tenax TA as a sorbent material for VOC analysis

There are different sorbents; Tenax TA (2,6-diphenyl- p-phenylene oxide), PDMS (poly-dimethylsiloxane), charcoal sorbent and silica gel sorbent (Flores *et al*. 2007; El-Naggar 2013; HSE 2016; Marcillo *et al*. 2017) which can be used to trap analytes for the GC analysis.

Tenax® TA is popular as a sorbent material in automated thermal desorption tubes for sampling a wide range of volatile hydrocarbons due to its high thermal stability and non-solubility in polar solvents and fast VOC desorption ability (Barro *et al.*, 2009). Tenax TA is extensively used as a sorbent material for VOC analysis in indoor air (Garcia-Jares *et al.*, 2009). It is a porous and highly hydrophobic organic polymer (Woolfenden, 1997). Due to the hydrophobic property of Tenax TA, water molecules don't retain inside the sorbent material (Gallego *et al.*, 2010). It can absorb and provide a good thermal stability for the non-polar hydrocarbons containing less than five carbons and VOCs containing C7-C26 carbons (Woolfenden, 1997). However, it is not suitable to use with highly volatile and polar compounds such as acetone, isopropanol, n-hexane and 1,2-ethanediol (Marcillo *et al*. 2017; Schieweck *et al*. 2018). Tenax TA produces low artefact during the VOC analysis, thus a low interference to the analysis can be observed (Marcillo *et al.*, 2017). Thermally stable property of Tenax TA provides the analysis of compounds with boiling point ranging from 60 °C to 300 °C (Marcillo *et al.*, 2017). Based on these properties, Tenax TA can be used efficiently for the analysis of indoor VOCs such as BTX.

1.7.2.2.Thermal desorption

Thermal desorption technique (TD) is used to desorb VOC samples from the sorbent material to GC column (Demeestere *et al.*, 2007). TD is commonly used when analysing trace levels of VOC in the air such as plant and microbial VOC emissions (Insam and Seewald, 2010; Kim *et al.*, 2014; Wang *et al.*, 2016). During TD, the sample is trapped and concentrated followed by desorbing into the GC column through inert gas flow (Schulz-Bohm *et al.*, 2015). The pre-concentration process of the analytes allows accurate quantitative analysis and provides narrow GC peaks in the chromatogram (Peñuelas *et al.*, 2014). During pre- concentrating process, no external chemicals or solvents are used, thus it minimises interference or artefact in the analysis (Woolfenden, 1997). During desorption, the sample containing material is heated to a pre-programmed temperature, then all the analyte from sorbent material will be released into the capillary gas column (Woolfenden, 2001; Flores *et al.*, 2007; Barro *et al.*, 2009).

Many TD units come with an autosampler device which allows placing over 50 sample tubes and the system automatically insert tubes into the desorption path (PerkinElmer, 2015). At the initial step of ATD, the leak test is performed for each sorbent tube (or trapped sample) followed by elimination of water and oxygen from the sample tube through dry purging using helium (Jia and Fu, 2017). This is useful when the sample contains more moisture (Woolfenden, 1997). Following dry purging, all the analytes in the tubes are transferred into a cold concentrator trap. Once the pre-concentrating is completed, the cold trap is heated rapidly to a high temperature (≈400 °C) inject all the analytes contents into the GC column. Once the desorption of one sample is completed, ATD cools down quickly and returns the sample tube to the autosampler and takes next sample tube for the leak test (Juillet *et al.*, 2005; Gallego *et al.*, 2010; Marotta, 2015).

Once the analytes enter into the GC column, it separates all the different analytes in the sample. The GC column consists of the mobile phase and the stationary phase (Srivastava and Majumdar, 2011). Injected samples travel through the mobile phase (carrier gas nitrogen) are separated based on their degree of interactions with the stationary phase and degree of volatility (Demeestere *et al.*, 2007). Different VOCs are separated at different times called their retention

times (Harborne, 1998). Retention time is the time between sample injection and the analyte entering into the detector which creates a peak area, and each VOC has a unique value. FID produces a very low noise, therefore based on the retention time, analytes can be easily detected (EPA USA, 1999). Once the analytes separate, carrier gas transports them into the FID to detect the concentrations (Molhave *et al*. 1997; EPA USA 1999). Once the analytes enter into the FID, it ionizes the analytes into charged particles (electrons) and they move to the electrodes producing an electric current (Fig. 1. 15). FID repeats detecting each analyte continuously and provides the electric current proportional to the quantity of analyte (concentration)(Akihiro *et al.*, 2001). Thus, the greater the number of ions, a greater current is produced. This quantitative estimation is represented by the peak area in the chromatograms (Srivastava and Majumdar, 1995; Molhave *et al.*, 1997; Akihiro, Shozo and Mika, 2001; Marotta, 2015).

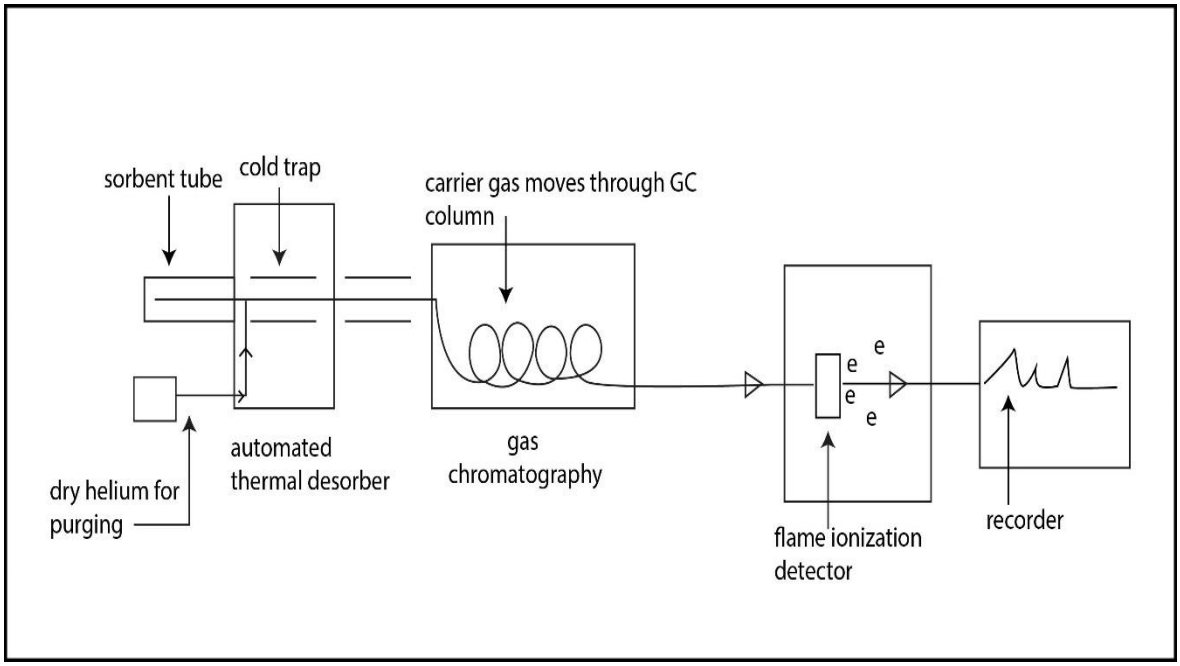


Fig. 1. 15 Schematic diagram of the ATD/GC/FID analysis workflow**.** Modified after (Akihiro *et al.*, 2001)**.**

1.8. Aims of the project

The aims of this project are whether the three plants species and plant communities are able to remediate high and low concentration of BTX from air in a chamber setup, understand which plant species perform best and whether plant communities are able to enhance the phytoremediation process. Therefore, it is hypothesised that there is no significant difference between the phytoremediation rates of BTX by single plant species and plant communities. To work towards these aims, the following objectives were set,

- Single plant species*: S. wallisii*, *C. comosum* and *H. helix* are studied for their benzene, toluene and m-xylene removal rates
- Plant communities (mix culture) composed with the above three plant species are studied for the removal of the above VOC separately
- This study compares, how the plant potting medium: compost removes BTX from indoor air
- Compare taxonomical and functional composition in the bacterial population in the rhizosphere of single plant species exposed and non-exposed to benzene
- Identify BTX degrading bacteria in rhizosphere of single plant species exposed and non-exposed to BTX

The knowledge from this study will be useful to enhance the efficiency of phytoremediation system to remove volatile organic compounds from the indoor air.

Chapter 2. Materials and method

2.1. Media and reagents

The composition of the standard buffers and reagents used in the forthcoming research are detailed in Table 2. 1. All the growth media and buffers, with the exception of the TAE, were sterilised at 121 °C for 15 minutes under 15 lbs pressure prior to use.

Table 2. 1 Growth media and buffers used in the study

Growth media or buffer	**Constituents**
Nutrient agar	One litre agar contains: Lab-Lemco' powder 1.0g, yeast extract 2.0g, peptone 5.0g, NaCl 5.0g, agar 15.0g, distilled water 1 litre, pH 7.4 ± 0.2 at 25 °C
Nutrient broth	One litre broth contains: Lab-Lemco' powder 1.0g, yeast extract 2.0g, peptone 5.0g, NaCl 5.0g, distilled water 1 litre, pH 7.4 ± 0.2 at 25 °C
Defined minimal source medium (Shen *et al.*, 1998)	$NaNO_3$ 4g, KH_2PO_4 1.5g, Na_2HPO_4 0.5g, $FeSO_4.7H_2O$ 0.0011g, $MgSO_4.7H_2O$ 0.2g, $CaCl_2$ 0.01g, bacteriological agar number 01 15g, distilled water 1 litre, pH 7±0.2
LB agar	One litre agar contains: Pancreatic digest of casein 10.0 g, NaCl 10.0g, yeast extract 5.0g, bacteriological agar number 01 15g, distilled water 1 litre
Bacterial motility agar	Brain heart infusion powder 10g, Tryptose 10g, Gelatin 30g, NaCl 5g, K_2HPO_4 2g, KNO_3 2g, bacteriological agar number 01 1g, distilled water 1 litre
Hugh-Leifson glucose broth (HLGB) (Hugh and Leifson, 1953)	Bromocresol purple 0.0015g, peptone 0.2g, yeast extract 0.5g, NaCl 0.5g, glucose 1.0g, bacteriological agar number 01 0.3g, distilled water 100 ml
Glucose acid test broth	Proteose peptone 10g, NaCl 5g, beef extract 1g, phenol red 0.018g, glucose 10g, distilled water 1 litre
Phosphate buffer saline (PBS)	0.01 M phosphate buffer: 0.0027 M KCl, 0.147M NaCl, pH7.4, distilled water 200 ml
50 x Tris Acetate EDTA buffer (TAE)	Tris base 242g, Glacial acetic acid 57.1 ml, 0.5M EDTA (pH 8.0) 100ml, distilled water 1 litre

2.2. Plant species selection

Though there were several studies based on single plant species VOC removal efficacy, there was a lack of knowledge about how mixed plant species (plant communities) perform during indoor VOC removal. To study this, it was important to manipulate a plant community using different plant species. Three plant species: *S. wallisii*, *C. comosum* and *H. helix* were chosen to form the plant community since they showed good VOC removal capacity including BTX removal in previous literature (Yoo *et al.*, 2006; Yang *et al.*, 2009; Chun *et al.*, 2010; Dela Cruz *et al.*, 2014; Sriprapat and Thiravetyan, 2016; Irga *et al.*, 2018).

Ten-month old *C. comosum, H. helix* and *S. wallisii* maintained under greenhouse conditions, which averaged 24±5 °C were used in all experiments. *C. comosum* and *S. wallisii* were approximately 20– 30cm in height at the time of use whilst *H. helix* had spread as a vine. At the end of the seventh month of age, 3-4 plants from the same species were transferred into 0.8 litre clay pots, in a standard potting mixture and acclimated for another three months in the greenhouse. Standard potting mixture (9-gallon bag): John Innes number 02 compost (Westland Horticulture, UK) was composed of John Innes soil mix (7:3:2 of medium sterilized loam: peat substitute: coarse sand), 56g of John Innes Base (2:2:1 of hoof and horn meal: superphosphate: sulphate of potash) and 10g of ground chalk. At the end of the tenth month, two replicates (two potted plants) per each VOC treatment were used for the batch experiment (detailed in section 2.3).

During the monitoring of VOC removal by plants (detailed in section 2.12), plants with the same conditions as explained above were used. At the seventh month of age, plant monocultures (single species) composed 12-15 *C. comosum*, 7-9 *H. helix* and 8-10 *S. wallisii* in plastic trays (plant's propagating trays) (0.38 x 0.24 x 0.12 m^3) and the plant community (mixed culture) composed approximately 5-8 *C. comosum,* 3-5 *H. helix* and 4-6 *S. wallisii* in single tray (Fig. 2. 1) with standard potting mixture were prepared. Equal volumes of potting mixtures (≈4 litres) were used for the monocultures, communities and the compost tray (tray containing only unused

potting mixture). Plant monocultures, communities and compost trays were maintained under greenhouse conditions for another three months.

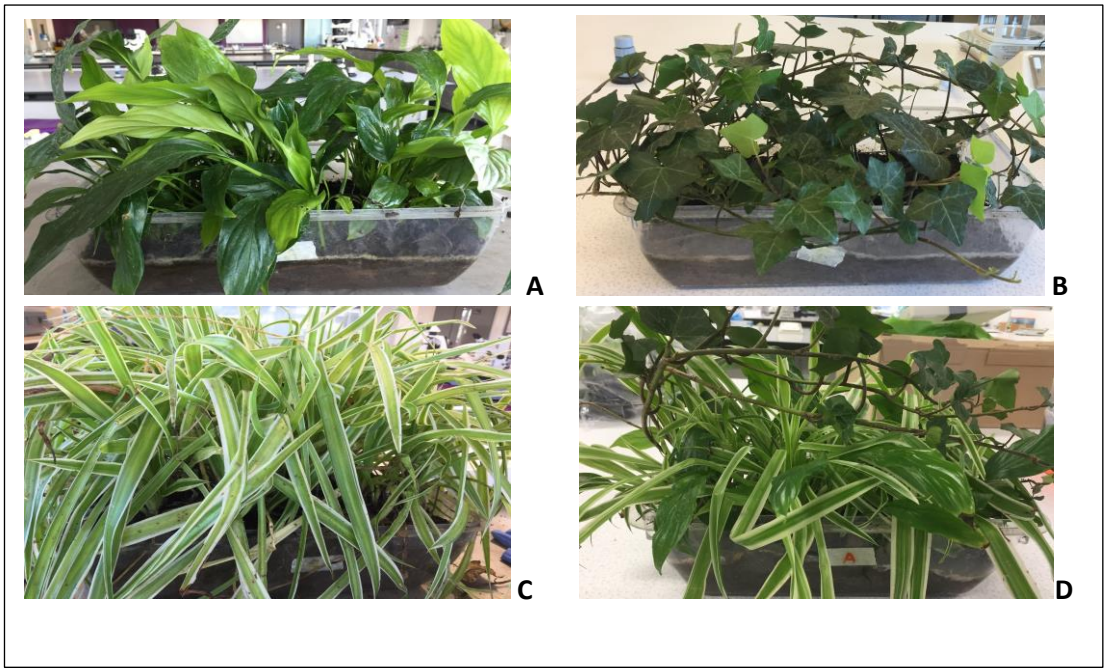


Fig. 2. 1 Potted *S. wallisii* (A)*, H. helix* (B), *C. comosum* (C) and plant community (D) in plastic plant propagating trays

After each VOC monitoring experiments (detailed in section 2.12), characteristics of the plant-propagating tray units (Appendix 1 for plant-propagating tray characteristics) were recorded. Total leaf area per propagating tray was measured using ImageJ 1.52a software (Abràmoff *et al.*, 2004). Dry weight of the plant-tray unit was measured following the drying of shoot, roots and the potting mixture in an oven at 70 °C for 24 hours.

2.3. Exposure of plants to VOC to investigate possible changes to the rhizosphere community

Through exposing plants to VOCs, it was expected to observe taxonomical and functional changes in the bacterial community in the rhizosphere of plants. During batch experiments, plant monocultures were exposed to VOCs as follows. *C. comosum, H. helix* and *S. wallisii* in clay pots (n=2) (detailed in section 2.2) were placed inside the glass test chambers (76.2 cm x 45.7 cm x 30.5 cm) (Fig. 2. 2). Chambers comprised of upturned aquarium tanks, positioned such that the

open portion faced downwards and rested on a wooden surface, this allowed for the taking of plants out during watering. Initially benzene was used as the test VOC. The daily dosage of 10 ppm benzene was added into a watch glass placed inside the chamber using an automated pipette for four weeks. Every Monday and Thursday before adding benzene, pots were taken out and watered to saturation, drained for 1 hour and placed back in the chamber. Another two potted plants were maintained in a separate test chamber under the same conditions without benzene administration as experimental controls. The same procedure was followed when exposing plants to 100 ppm benzene, toluene and m-xylene.

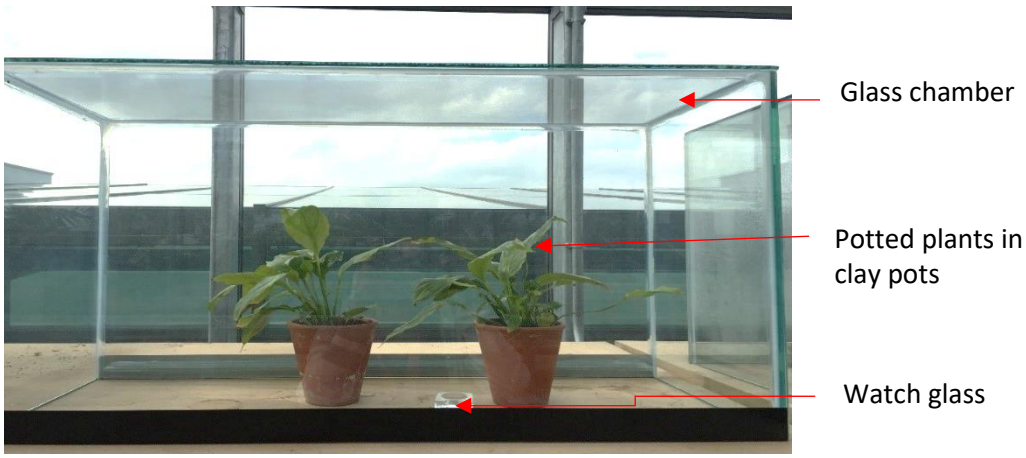


Fig. 2. 2 Plant test chamber

2.4. Rhizosphere soil sample collection

Collection of rhizosphere soil samples was required to extract total community DNA. At the end of the four weeks, plants were removed from the test chamber and uprooted from the pots. Plant roots were shaken vigorously to remove the soil not tightly adhering to the roots. The soil firmly adhered to the roots (Rhizosphere) was extracted by placing roots inside a 15 ml tube containing 10 ml of sterilised phosphate buffer saline (PBS) followed by vortexing for 10 minutes. After vortexing the soil-PBS suspension was centrifuged (Sigma Laboratories, Germany) at 5000 rpm for 10 minutes at 4°C, the supernatant was discarded, and particulate pellet was collected. The pellet was stored at 4 °C for maximum 24 hours until DNA extraction had taken place.

2.5. Total DNA extraction from rhizosphere

Two commercially available kits: QiAmp Fast DNA stool mini Kit (Qiagen, UK) and the MO BIO PowerSoil® DNA isolation kit (MoBio Laboratories, Inc) were first evaluated for selecting a suitable method for total DNA extraction from rhizosphere soil. Both kits have been used in numerous other studies (Whitehouse and Hottel, 2007; Madhaiyan *et al.*, 2010; Kostka *et al.*, 2011; Donn *et al.*, 2015; Prober *et al.*, 2015). In addition to the DNA extraction, both kits have a DNA purification step by sending the sample through a spin column membrane followed by membrane washing steps to yield a high purity DNA. During the evaluation study, total community DNA samples extracted from the rhizosphere of VOC untreated *S. wallisii* using both kits were quantified by the fluorometric method (detailed in section 2.5.3). The commercial kit which on average, yielded the highest concentration of DNA was used throughout the study.

2.5.1. QIAamp fast DNA stool mini kit

QIAamp fast DNA stool mini kit is designed to extract DNA from human stool samples. However, the modified protocol which involved incubating the samples in InhibitEx buffer at 70 °C allowed efficient cell lysis of bacteria and other microbial cells from non-stool samples (QIAamp, 2014). All manufacturer's guidelines were followed throughout the DNA extracting procedure except the above-mentioned modification.

This commercial kit enables the extraction of total DNA from 0.18-0.22 g soil. Benzene untreated *S. wallisii* rhizosphere (0.20 g) was added to the 2 ml microcentrifuge tube with InhibitEx buffer (2 ml). The soil sample was vortexed thoroughly for 1 minute and then the suspension was heated for 5 minutes at 70°C. Following another vortexing for 15 seconds, the sample was centrifuged at 20,000 x g for 1 minute until the pellet separated from the supernatant. The supernatant (200 µl) was transferred into a new 1.5 microcentrifuge tube containing proteinase K (15 µl). Buffer AL (200 µl) was added and vortexed for 15 seconds. Following incubation at 70 °C for 10 minutes, of 100% ethanol (200 µl) was added and vortexed. Approximately 600 µl of lysate was loaded into the QIAamp spin column and centrifuged at 20,000 x g for 1 minute. The spin column was placed

in a new collection tube and buffer AW1 (500 µl) was added into it. Following centrifugation at 20,000 x g for 1 minute, the spin column was placed again in a new collection tube and buffer AW2 (500 µl) was added. Following centrifugation at 20,000 x g for 3 minutes, the spin column was placed in a new collection tube and centrifuged for 3 minutes at 20,000 x g. The spin column was placed in a 1.5 ml microcentrifuge tube and buffer ATE (200 µl) was added to the column membrane. Following incubation for 1 minute at room temperature, the tube was centrifuged at 20,000 x g for 1 minute. Eluted DNA was quantified using a Qubit™ fluorometer (Invitrogen, UK) and stored at -20 °C.

2.5.2. MO BIO PowerSoil® DNA isolation kit

MO BIO PowerSoil® DNA isolation kit enables the extraction of total DNA from about 0.25 g soil. The kit was used according to the manufacturer's guidelines as follows. VOC untreated *S. wallisii* rhizosphere soil samples (0.25 g) were added to the power bead tube and gently vortexed. Solution C1 (60 µl) was added and the tube was secured horizontally on a Clifton™ Cyclone Vortex Mixer (UK) using tape. Following vortexing at maximum speed for 10 minutes, the tubes were centrifuged at 10,000 x g for 30 s. Then the supernatant was transferred to a sterile 2 ml collection tube. Solution C2 (250 µl) was added and vortexed for 5 seconds. The tube was then incubated at 4 °C for 5 minutes. Following centrifugation at 10,000 x g for 1 minute, approximately 600 µl of the supernatant was transferred to a collection tube. Then solution C3 (200 µl) was added and vortexed. Following incubation at 4 °C for 5 minutes, tubes were centrifuged at 10,000 x g for 1 minute. Approximately 750 µl of supernatant was added into a collection tube containing 1.2 ml of solution C4. The supernatant-C4 solution (675 µl) was transferred into the provided spin filter and centrifuged at 10,000 x g for 1 minute. This step was repeated until the entire sample had been passed through the column. Then solution C5 (500 µl) was added into the spin column and centrifuged 10,000 x g for 30 seconds. After discarding flow through, the tube was centrifuged again at 10,000 x g for 1 minute. The spin filter was placed in a collection tube and solution C6 (100 µl) was transferred into the centre of the column. Following

centrifugation at 10,000 x g for 30 s the spin filter was discarded, DNA was quantified using Qubit™ fluorometer. All DNA samples were stored at -20 °C until used.

2.5.3. Determination of DNA concentration

Analysis of soil DNA concentrations was required for selection of a suitable DNA extraction method for the study. Soil DNA quantification in this study was carried out using a Qubit™ fluorometer and the dS DNA BR (broad range) assay kit (Invitrogen, Life Technologies). Prior to starting, DNA samples and fluorometer standards were thawed at room temperature. The Qubit™ working solution was prepared by diluting Qubit™ reagent 1:200 in Qubit™ buffer. The working solution was used as the master mix for the DNA extracts and the DNA standards. According to the manufacturer's guidelines, DNA samples, working solution and the standards were mixed in clear 0.5 ml PCR tubes and incubated at room temperature for 2 minutes. Tubes were inserted into the fluorometer to obtain the DNA concentration.

2.6. Amplification of 16S rRNA gene from rhizosphere community

Total community DNA extracted from rhizosphere of VOC untreated *S. wallisii* using MO BIO PowerSoil® DNA isolation kit was amplified using different primer pairs that annealed to different hypervariable regions of 16S rRNA gene. During primer selection, primer pairs that successfully annealed and amplified V3/V4 region and the V1-V9 region of 16S rRNA gene were chosen to quality check the DNA samples prior to Illumina MiSeq and amplify DNA samples for Sanger sequencing respectively (detailed in sections 2.9.1 and 2.11.2.1 respectively).

In addition to the amplification of bacterial 16S rRNA gene, ITS1 (Internal transcribed spacer) and ITS2 regions of fungi were amplified to observe the presence of fungi DNA in the metagenomic DNA pool extracted from soil samples. This verified the efficiency of total community DNA extraction carried out by MO BIO PowerSoil® DNA isolation kit.

PCR amplifications were carried out in a MyCycler™ thermal cycler (Bio-Rad Laboratories, UK) as follows. Each of PCR mixture (25 µl) consisted of 2 x GoTaq® G2 Hot Start Master Mix (12.5 µl) (Promega, UK), 10 µM primer pair mix (1.0 µl), PCR water (9.5 µl) and 10 ng. μl^{-1} concentration of

rhizosphere soil DNA (2.0 µl). The thermal cycler conditions were as follows (with specific primer annealing temperatures mentioned in the Table 2.2): 95 °C for 2 minutes followed by 30 cycles of denaturing at 95 °C for 15 seconds, primer annealing for 1 minute, DNA extension at 72 °C for 1 minute and the final extension at 72 °C for 5 minutes.

Table 2. 2 Details of primers used in the study

Primer pair	Sequence	T_a	Expected amplicon size (bp)	Target region	reference
Probio uni	5/-CCT ACG GGR SGC AGC AG-3/	57	194	V3 region of 16s rRNA gene	Milani *et al.*, 2013
Probio R	5/-ATT ACC GCG GCT GCT -3/				
1492R	5/-GGT TAC CTT GTT ACG ACT T -3/	54	1485	Entire gene	Tam and Diep, 2015
27F	5/-AGA GTT TGA TCC TGG CTC AG-3/				
EUB 518	5/-ATT ACC GCG GCT GCT GG-3/	54	200	V3-V4 region 16s rRNA gene	Bell *et al.,* 2011
EUB338	5/-ACT CCT ACG GGA GGC AGC AG -3/				
ITS1-F	5/-TCC GTA GGT GAA CCT GCG G -3/	55	200-400	ITS1 region of fungi	Fujita *et al.*, 2001
ITS2-R	5/-GCT GCG TTC TTC ATC GAT GC-3/				
ITS3-F	5/-GCA TCG AGT AAG AAG GCA GC-3/	55	250-420	ITS2 region of fungi	Fujita *et al.*, 2001
ITS4-R	5/-TCC TCC GCT TAT TGA TAT GC-3/				

T_a-primer annealing temperature (°C), R is A or G and S is G or C

2.7. Agarose gel electrophoresis

PCR products were analysed by agarose gel electrophoresis and UV spectroscopy. The gel comprised of agarose powder (Bioline, UK), 1 x TAE (Tris Acetate EDTA) and ethidium bromide (0.5 µg ml^{-1}). An aliquot (4.0 µl) of each PCR product was loaded on the gel with 100bp DNA ladder (6 µl) (New England BioLabs®, UK) as the molecular size marker and 1 x TAE buffer containing 0.5 µg ml^{-1} ethidium bromide was used as the running buffer. Agarose gels with 1.5-2.0% and 0.8-1.2% density were used to separate the expected amplicon size of 190-1000 bp and >1000 bp respectively. Each gel was run at 90 volts (Bio-Rad PowerPac Basic, UK) in an electrophoresis gel tank (Bio-Rad Laboratories, UK) until the amplicons were separated (35 to 60

minutes). Once the dye front had travelled ¾ of the distance, the gels were examined using UVP-GelDoc-It™ TS Imaging system (UVP, Cambridge, UK). After analysing the gel images, Probio uni/R and 27F/1492R primer sets were chosen to amplify V3/V4 region from rhizosphere bacterial community and the entire 16S rRNA gene from bacterial genomic DNA respectively.

2.8. Purification of PCR products

DNA (PCR) amplicons were purified prior to cloning into the pGEM ® -T Easy vector and sequenced through the Sanger sequencing approach (detailed in section 2.11.2). PCR purification was conducted to remove primer dimers and any unincorporated primers. This was done using QIAquick PCR purification kit (Qiagen, UK) according to the manufacturer's guidelines as follows. PCR (20 µl) product was mixed with buffer PB (100 µL). The mixture was applied to the QIAquick spin column in a 2 ml collection tube. Following centrifugation at 17,900 x g for 1 minute, flow-through was discarded and the spin column was placed in a new collection tube. Buffer PE (750 µl) was added to the spin column and centrifuged at 17,900 x g for 1 minute. After discarding the flow through, the spin column was placed in the same collection tube and centrifuged at 17,900 x g for 1 minute. The spin column was placed in a new 1.5 ml microcentrifuge tube and buffer EB (50 µl) was added to the centre of the column. Tubes were centrifuged at 17,900 x g for 1 minute to collect DNA and all purified DNA amplicons were stored at -20 °C.

2.9. Rhizosphere bacterial community profiling using Illumina MiSeq Sequence platform

Total community DNA from plant rhizosphere was extracted using MO BIO PowerSoil® DNA isolation kit (refer Appendix 4 for the concentration of DNA). Illumina sequencing platform was used to characterise the bacterial community in the rhizosphere of 10 ppm benzene treated and untreated *S, wallisii*, *C. comosum* and *H. helix* and benzene untreated compost. This involved sequencing 16S rRNA amplicons, preparation of sequencing reads for the analysis and taxonomical and functional profiling of the rhizosphere and compost communities.

2.9.1. Illumina MiSeq Sequencing

The V3/V4 region amplicons of 16S rRNA gene were barcoded using Nextera DNA library kit and multiplexed sequencing performed using the Illumina MiSeq sequencing platform (Eurofins Genomics, Germany). De-multiplexing (fastq_splitter) was used to split the entire population of sequencing reads into individual samples using the unique barcode added as part of the library preparation procedure. Demultiplexed paired-end FASTQ reads were received as raw sequencing data. Both forward and reverse reads were quality checked using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and all the reads had the expected fragment length distribution. Since the V3/V4 region reads comprised long reads of approximately 430 bp, it created a large distance matrix during bioinformatic data processing. Therefore, to reduce the computational data processing time and memory usage, the V3 region was removed leaving just the V4 region using cutadapt version 1.22 (Martin, 2011). The nucleotide sequence before "CCGCGGTAA" in V3/V4 forward read and sequence after "TTACCGC" in V3/V4 reverse read were clipped out using the commands "cutadapt-g" and "cutadapt-a" respectively. To reduce the distance matrix issues due to the very large size of data sets, all FASTQ files were randomly subsampled to the smallest sample size (133,106 reads in compost sample) cut off level using SEQTK coding software (https://github.com/lh3/seqtk). These down-sampled V4 read datasets were used for the bioinformatic analysis.

2.9.2. Taxonomical classification of soil bacterial community

Quality checked and trimmed FASTQ files (detailed in section 2.9.1) were used for the analysis. Taxonomical classification of rhizosphere community was performed using QIIME (Quantitative Insights into Microbial Ecology) (Caporaso *et al.*, 2010) bioinformatic software which runs in the cloud-based open source microbiome analysis platform: "NEPHELE" (https://nephele.niaid.nih.gov/). During the analysis, reads with more than 275 bp, homopolymer longer than 6 bp and ambiguous bases were removed from the sequencing pool. Unique sequences were clustered into OTUs (operational taxonomic units) based on the open reference clustering algorithm and the taxonomy was assigned to each OTU based on the SILVA database at

the 99% sequence identity (Pruesse *et al.*, 2007). All other parameters were left as default. In order to standardize the number of sequences per sample during the diversity analysis, all the samples were rarefied randomly to 120,000 reads sampling depth.

Alpha diversity metrics including observed species richness, Chao1 estimator (Chao, 1984), Shannon index (Shannon, 1948) and phylogenetic diversity (PD) (Faith, 1992) were calculated to each sample. Beta diversity metrics were estimated based on weighted UniFrac distances (Lozupone and Knight, 2005) and visualised in principal coordinate analysis (PcoA) plot. To observe differentially abundant bacterial groups: phylum, class and genus between different samples, group comparison was performed for the mean percentage relative abundance of each taxonomy using descriptive statistics (SPSS, version 20.0).

2.9.3. Functional classification of soil bacterial community

For the functional classification, down-sampled FASTQ files used in 2.9.1 were reanalysed as follows. Taxonomical classification of 16S rRNA reads was re-conducted in the QIIME software implemented NEPHELE open source platform based on the GreenGene 99 reference database (DeSantis *et al.*, 2006). Taxonomies were assigned based on open reference OTU picking strategy for the sequencing reads using "pick_open_reference_otus.py" in QIIME. Following the taxonomical classifications, PICRUST (version 1.1.3) predicted the functions into microbiome data using QIIME command "predict_metagenomes.py" using the relative abundance of each bacterial taxonomy. Then these functional predictions were assembled into the KEGG Orthology database using the "categorize_by_function.py" (Minoru *et al.*, 2004). Functional predictions were assigned to three KEGG levels. Based on the abundance of functions, PCoA plot was created and statistical analysis and graphs were produced for the KEGG modules using the STAMP (Statistical Analysis of Metagenomic Profiles), v2.1.3 software (Parks and Beiko, 2010). Group comparison for the percentage relative abundance of different functions were compared based on descriptive statistics (mean with SEM).

2.10. Isolating VOC degrading bacteria from rhizosphere soil

Bacteria were isolated from the rhizosphere of *C. comosum, H. helix* and *S. wallisii* of both the unexposed (control) and exposed to VOC (100ppm of benzene, toluene and m-xylene) in batch experiments (detailed in section 2.3). In addition to the rhizosphere soil, VOC degrading bacteria were isolated from the VOC unexposed compost samples. Approximately 0.25 g of soil (plant rhizosphere or compost) was suspended in PBS (1 ml) followed by vortexing. Tubes were centrifuged at 1000 x g at 4 °C for 10 minutes to remove soil particles and separate the supernatant containing bacterial cells. Soil supernatant (100 µl) was inoculated on minimal salt agar (MSA) (Shen *et al.*, 1998) and the plates were placed in a jar supplied with VOC (100µl) containing a microcentrifuge tube (Fig. 2. 3). As an experimental control, another set of inoculated MSA plates were incubated in the sealed jars under the same conditions without supplying VOC.

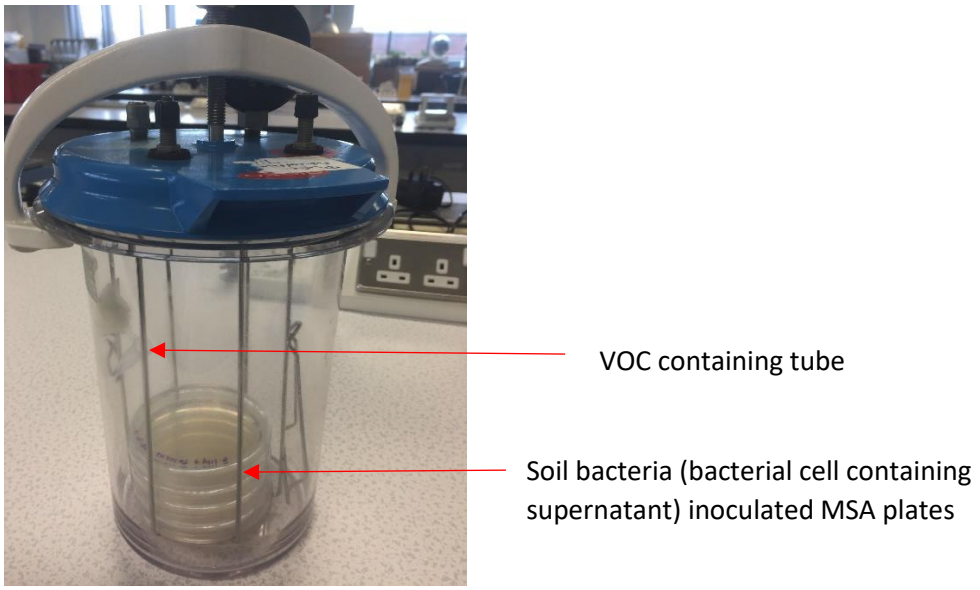


Fig. 2. 3 Jar containing MSA plates and VOC

The sealed jars were incubated at 25 °C for 7-10 days. Following the incubation, bacterial colonies were picked using a sterile toothpick and re-inoculated on MSA medium. Plates were placed in the jar along with VOC and incubated under the same conditions detailed above. After this second incubation on MSA, repeated sub-culturing of bacterial strains was carried out to ensure isolation of pure cultures. This was done aerobically on nutrient agar at 25 °C. In addition, the strains were inoculated on nutrient agar slant for long term storage.

2.11. Characterisation and identification of bacterial isolates

Identification of isolated VOC degrading rhizosphere bacteria was performed using both traditional microbiological culture methods and molecular methods. Freshly cultured bacterial culture in nutrient broth (NB)or nutrient agar (NA) plates were used in all morphological and biochemical tests and DNA extraction.

2.11.1. Classical approach to identify bacteria

Fresh bacterial cultures grown on NA and NB for 24-48 hours at 25 °C were used throughout the classical identifications. Gram staining, oxidase test, catalase test, motility test, glucose acid test, anaerobic growth (Cowan and Steel, 2010) acid fast test (Ziehl-Neelsen, 1882) and glucose oxidation fermentation test (Hugh and Leifson, 1953) were conducted to identify unknown bacterial isolates (refer to Appendix 02 for the methods used in culture approach). Cell morphology was examined using a high-power light microscope (Nikon, UK) under 1000 x magnification. Based on the culture-characteristics, bacteria were classified into the genus level using Bergey's manual of determinative bacteriology, 9th edition (Bergey *et al.*, 1994).

2.11.2. Molecular approach to identify bacteria

Molecular approaches were used to identify isolated VOC degrading rhizosphere bacteria up to the genus or species level. Molecular methods such as extracting genomic DNA from fresh bacterial cultures, amplification of entire 16S rRNA gene from these bacteria using 27F/1492R primer set, purification of amplicons and sequencing through Sanger sequencing approach were conducted respectively to each bacterial strain (as a quality control step of initial PCR, only a selected list of purified PCR amplicons were cloned into pGEM-T® Easy vectors followed by conducting colony PCR). Sequencing reads were compared to the Gene bank data base (NCBI).

2.11.2.1. Genomic DNA extraction and amplification of entire 16S rRNA gene

In order to identify bacteria at or above the genus level through Sanger sequencing, genomic DNA extraction from bacterial isolates was conducted using Qiagen DNeasy DNA extraction kit (Qiagen, UK) following manufacturer's instructions. Overnight grown bacterial culture (1.75 ml) on NB at

25 °C was added into a 2 ml microcentrifuge tube. Following centrifugation at 20,000 x g for 5 minutes, the supernatant was discarded. Lysis buffer (180 µl) was added into the bacterial pellet and vortexed for 20 seconds. Following incubation at 37 °C for 30 minutes, Proteinase K (25µl) and buffer AL (200µl) were added to the tube. The tubes were vortexed briefly and incubated at 56 °C for 30 minutes. Then,100% ethanol (200 µl) was added to the tube. Following brief vortexing, approximately 600µl of solution from the tube was added to a Qiagen spin column. The spin column was centrifugated at 10,000 x g for 1 minute. The spin column was placed in a new 2 ml collection tube and buffer AW1 (500µl) was added. Following centrifugation at 10,000 x g for 1 minute, the spin column was placed in a new 2 ml collection tube and buffer AW2 (500µl) was added. Then the tubes were centrifuged at 20,000 x g for 3 minutes. The spin column was transferred into a 1.5ml microcentrifuge tube and buffer AE (200µl) was added. After incubation at room temperature for 1 minute, the tube was centrifuged to collect DNA at 10,000 x g for 1 minute.

Primer pair 27F/1492R was used to amplify entire 16S rRNA gene of bacterial isolates (detailed in section 2.6). PCR amplification for all individual isolates were performed (detailed in section 2.6) and the PCR products were purified using QIAquick PCR purification kit (detailed in section 2.8).

2.11.2.2. 16S rRNA gene transformation into competent cells and colony PCR

Purified PCR amplicons of the entire 16S rRNA gene were cloned using pGEM®- T East vector system-I cloning kit ((Promega, UK) according to the manufacturer guidelines. In addition to the standard ligation reaction which carried 16S rRNA gene amplicons, the positive control and the background control reactions were set up as quality controlling of the gene transformation experiments (Table 2. 3).

Table 2. 3 Composition of ligation mixture

Reagents	**Standard reaction (µl)**	**Positive control (µl)**	**Background control (µl)**
2 x Rapid ligation buffer	5	5	5
pGEM ® -T Easy vector	1	1	1
Test DNA	3	-	-
Control insert DNA	-	2	-
T4 DNA ligase	1	1	1
Deionized water	-	1	3

Following incubation of the ligation mixture at room temperature for 1 hour, the mixture was centrifuged briefly and ligation reaction (2 µl) was added into 1.5ml micro-centrifuge tube placed on ice. A tube containing *Escherichia coli* JM109 high-efficiency competent cells (Promega, UK) was placed in an ice bath until thawed. Following mixing the cells by flicking gently, competent cells (50 µl) were transferred into each tube containing ligation reaction (2µl). The tubes were incubated on ice for 20 minutes and then the cells were heat shocked at 42 °C for 50 seconds. Then these ligation-transformation reaction tubes were transferred onto ice for 2 minutes incubation. LB medium (950µl) was added into the tube and incubated at 37 °C for 1.5 hours in a shaker at 150 rpm. The transformation culture (100µl) was plated on LB agar plates containing ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl- b-D-galactopyranoside) and IPTG (isopropyl-b-D-thiogalactopyranoside). Following incubation at 37 °C for overnight, successful transformations were selected as white colonies on the plate.

To perform colony PCR, single white colonies were picked and added into individual PCR tubes containing PCR water. PCR reaction was set up using 27F/1492R primer pair targeting entire 16SrRNA gene according to the method detailed in section 2.6. Following PCR amplification, PCR product was visualised *via* agarose gel electrophoresis (detailed in section 2.7). Gene transformation and colony PCR were performed to ensure the successful amplification of 16S rRNA gene during the initial PCR reaction performed for the genomic DNA extracted from isolated

bacterial colonies (quality checking). Then the forward strand of PCR amplicons was sequenced *via* Sanger sequencing using 27F primer (Eurofins genomics, UK).

2.11.2.3. Species level identification of unknown bacteria

Taxonomic classification was performed by comparing the quality clipped Sanger sequences (query sequence) to the Gene bank database in the national Centre for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using blastn algorithm against 16S ribosomal RNA (Bacteria and Archaea) database. During the blastn search, "the uncultivated bacterium (taxid:77133)" hits were excluded by adding ENTREZ option. Top significant alignment with the maximum identity obtained from nucleotide blast were picked as the reference species of the query sequence. For a query sequence, when more than one hit was obtained (multiple hits) with the similar highest percent identities, at least first three hits were picked. Finally, using the nucleotide sequence of the 16S rRNA gene in these reference species and the test species, a phylogenetic tree was created using Archaeopteryx software (0.972 version).

2.12. Monitoring VOC removal by plant monocultures and communities

Similar size glass test chambers that were used in batch experiments (detailed in section 2.3) were used to monitor VOC detoxification by plants under the indoor conditions. Plants were kept in the laboratory for one and half months to habituate to indoor conditions. The room temperature was at 20±3 °C and the light/dark period was 12/12 during the experiments. VOC monitoring experiments involved injecting VOCs into the test chamber containing the plant monocultures, communities and compost in plastic propagating trays (detailed in section 2.2) and then monitoring the reduction of test chamber VOC concentration resulting from phytoremediation. Every Monday, plants and compost were watered to saturation, drained for 1 hour and placed in the sealed chamber. Aeroqual digital VOC monitor (Aeroqual, UK) (for benzene, toluene and n-hexane) and GC/FID (for benzene, toluene and m-xylene) methods were studied during method optimisation. Different chamber sealing materials were used to seal the tank's open face and the VOC leakage was monitored from the empty tank during sealing material selection.

2.12.1. Optimisation of VOC monitoring method with Aeroqual digital monitors

Aeroqual series 500 VOC monitors are designed to monitor a wide range of VOCs in a concentration range of 0-2000 ppm (Aeroqual, 2014). The monitor was used following the manufacturer guidelines. Aeroqual photoionization detector head (sensor) was inserted into the sensor head adapter which connected to the monitor through a CAT5 cable. The monitor was coupled with the charger which connected to the main power supply. The new sensors and sensors unused for a long time were warmed up for 24 hours to burn off potential contaminants. After the sensor was connected to the monitor, the VOC monitoring frequency was set to an interval of 10 minutes. During VOC monitoring, the sensor was warmed up for three minutes or until the "sensor warming up" message disappeared. Then the sensor started to detect real-time VOC level in the air and all the data was stored in the monitor (Aeroqual, 2014).

Aeroqual sensors are calibrated against isobutylene as the reference VOC, therefore default sensor concentration is in the units of parts per million (ppm) of isobutylene (Aeroqual, 2016). To get the actual concentration of another VOC, this default concentration should be multiplied by the response factor (RF) provided by Aeroqual (Table 2. 4).

Table 2. 4 Response factor of Aeroqual sensor. Adapted from (Aeroqual, 2016).

VOC	**RF value**
Benzene	0.53
Toluene	0.53
n-hexane	4.5

2.12.1.1. Sealing chamber using wooden board

The Aeroqual sensor connected to the sensor adapter was placed inside the tank. The sensor adapter was connected to the monitor through the cable and switched on for sensor warming up process. Then a pine wood board (Homebase, UK) of 76.2 length x 30.5 width cm^2 was used to cover the upturned tank's open side (Fig. 2. 4). The thickness of wooden board was approximately 1.5 cm.

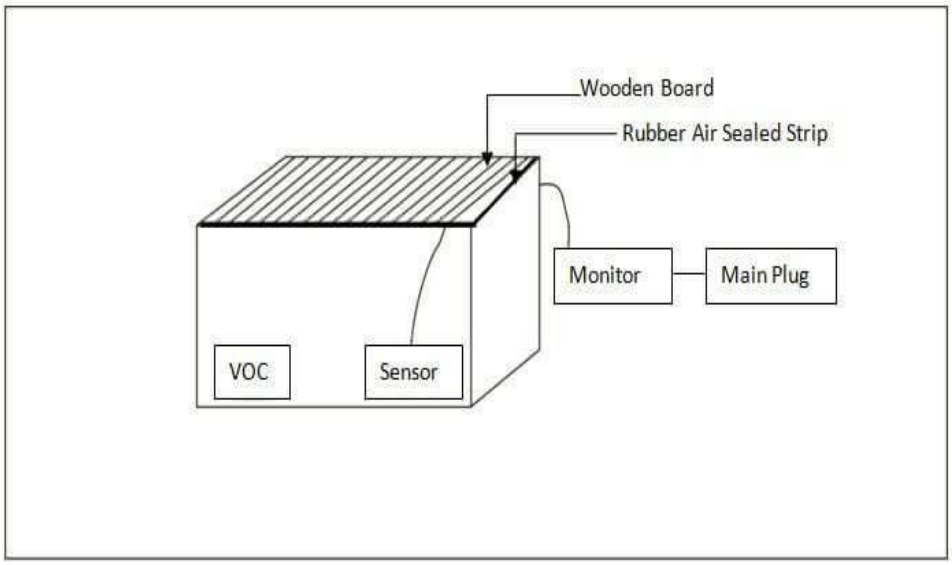


Fig. 2. 4 Schematic diagram of test chamber sealed with wooden board

After the wooden board was placed on the tank, there were no visible gaps between the tank wall and the board. Tank- wooden board edges were sealed using rubber air-sealed strips. After the tank was sealed halfway, 100 ppm benzene (37.4 µl liquid volume) was injected into the empty chamber and the tank was sealed immediately. Then the sensor was started to monitor real-time VOC concentration and the data was logged every 10 minutes. The wooden- board method was optimised only for benzene.

2.12.1.2. Sealing chamber using thin low-density polythene (LDPE-Cling films)

The Aeroqual sensor was placed inside the test chamber and connected to the monitor (detailed in section 2.12.1.1). Then the open side of the tank was sealed using 3 layers of low-density polythene (Sainsbury's, UK) (Fig. 2. 5). After the tank edges were almost covered and sealed with thin polythene, VOC was added into the chamber and the rest of the tank edge was sealed immediately. After the experiment, logged data for the VOC concentration was obtained from the Aeroqual monitor. Sealing material testing was carried out for 100 ppm benzene (37.4 µl), toluene (43.0 µl) and n-hexane (53.9 µl) respectively.

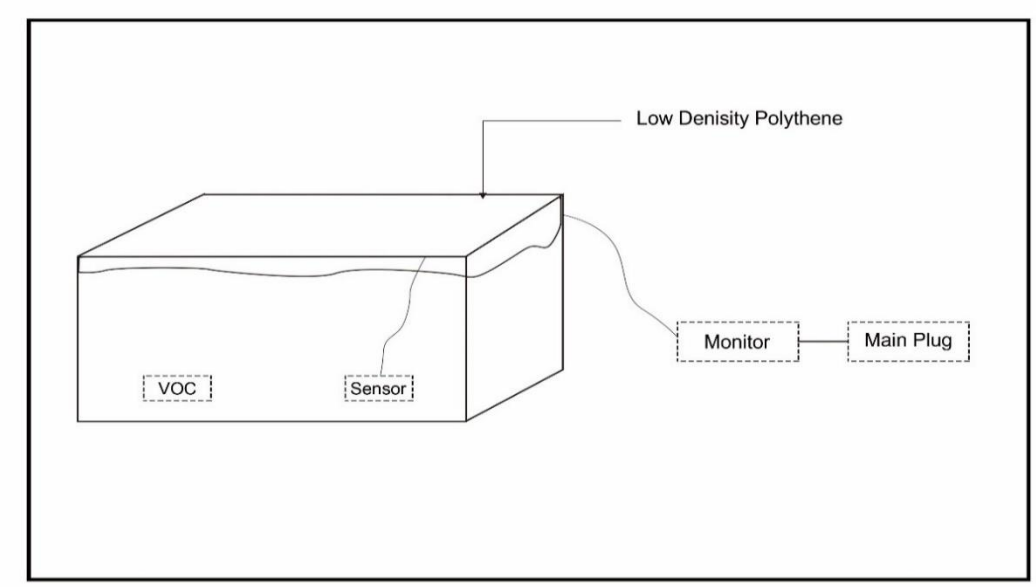


Fig. 2. 5 Schematic diagram of test chamber sealed with low density polythene

2.12.1.3. Sealing chamber open surface with polyurethane foam sheet (rigid foam)

The test chamber was in upside-down position by facing its open side into a polyurethane foam sheet (rigid foam) (Easy foam, UK) placed on a laboratory bench. The Aeroqual sensor was placed inside the tank and the cables were taken out between the tank and polyurethane sheet (Fig. 2. 6). The cable was secured by the tank weight since the foam sheet reduces the pressure coming from the tank to cable. Changes of VOC concentration was measured for 100 ppm benzene, toluene and n-hexane separately using Aeroqual sensors (detailed in section 2.12.1).

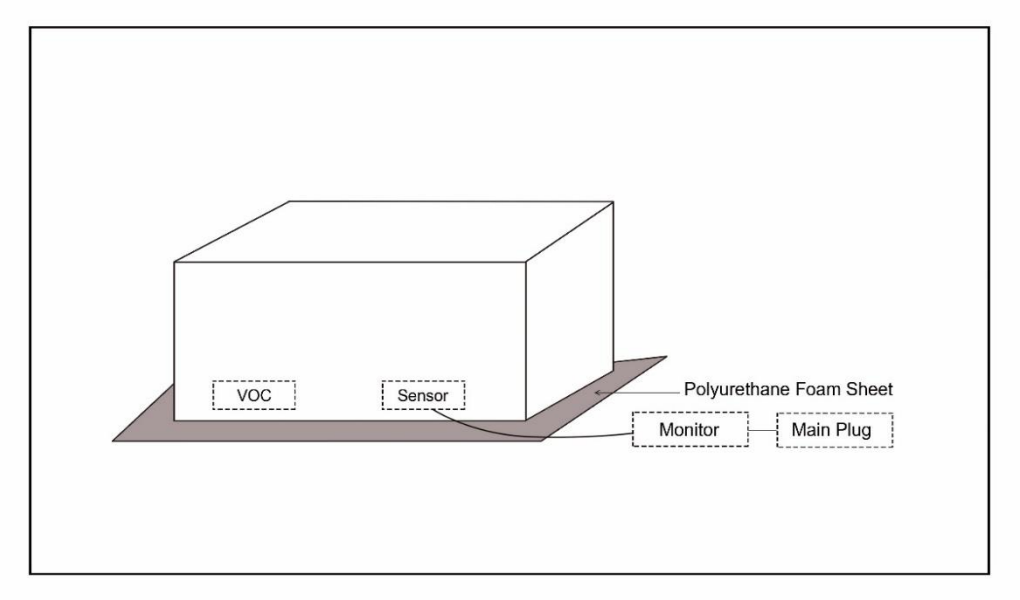


Fig. 2. 6 Schematic diagram of test chamber sealed with polyurethane foam sheet

2.12.1.4. Sealing chamber open surface with adhesive covering film

The Aeroqual sensor was placed inside the upturned tank (detailed in section 2.12.1.1). The open side of the chamber was sealed using adhesive covering film (WH Smith, UK). The whole open side was covered using adhesive film and then VOC was injected into the chamber through a tiny hole made on the sheet (VOC inlet) (Fig. 2. 7). Immediately after injecting VOC into the chamber, the hole was sealed using a section of adhesive polythene and real-time VOC concentration was measured using the Aeroqual sensor.

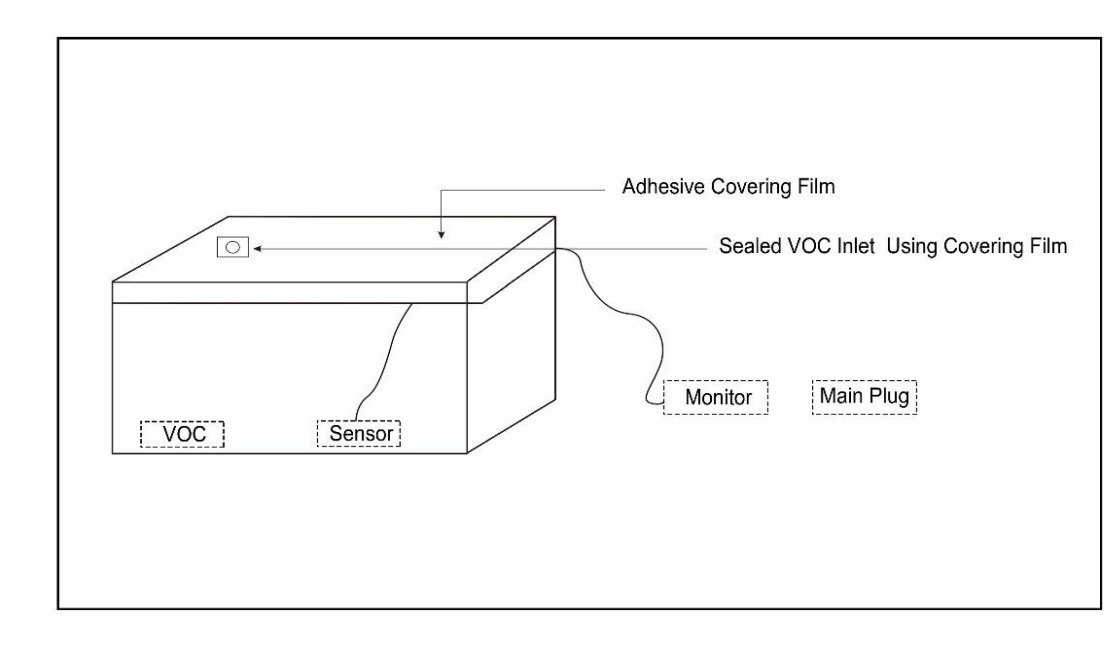


Fig. 2. 7 Schematic diagram of test chamber sealed with adhesive covering film

VOC loss rates inside the chambers based on the sealing materials were analysed using non-parametric one-way ANOVA (Kruskal-wallis test) (SPSS, version 20.0 SPSS).

2.12.1.5. Design and optimisation of dual test chamber system

Due to some problematic errors of the Aeroqual sensors during monitoring VOC level inside the single chamber containing plants, it was decided to design a dual chamber system. Through this design, it was expected to improve the air circulation and reduce humidity level inside the chamber, thus the design reduced sensor contacting with moisture which might be the issue in previous chamber systems. A dual chamber set up was designed to place plants in one chamber

and the sensor in one chamber. A set of new Aeroqual sensors was used in the analysis carried out in the dual chamber system. Two chambers were connected *via* a rubber tube (Fig. 2. 8). In addition to the sensor, a linear air pump (Charles Austen Ltd, UK) was placed inside the same chamber (chamber A) to circulate air between two chambers. Cables were taken out through a sealed silicon rubber septum. Plant chamber (chamber B) was sealed using adhesive covering film and Perspex while the chamber containing the sensor and the air pump was sealed using only a Perspex sheet. All the edges were sealed with silicon sealant.

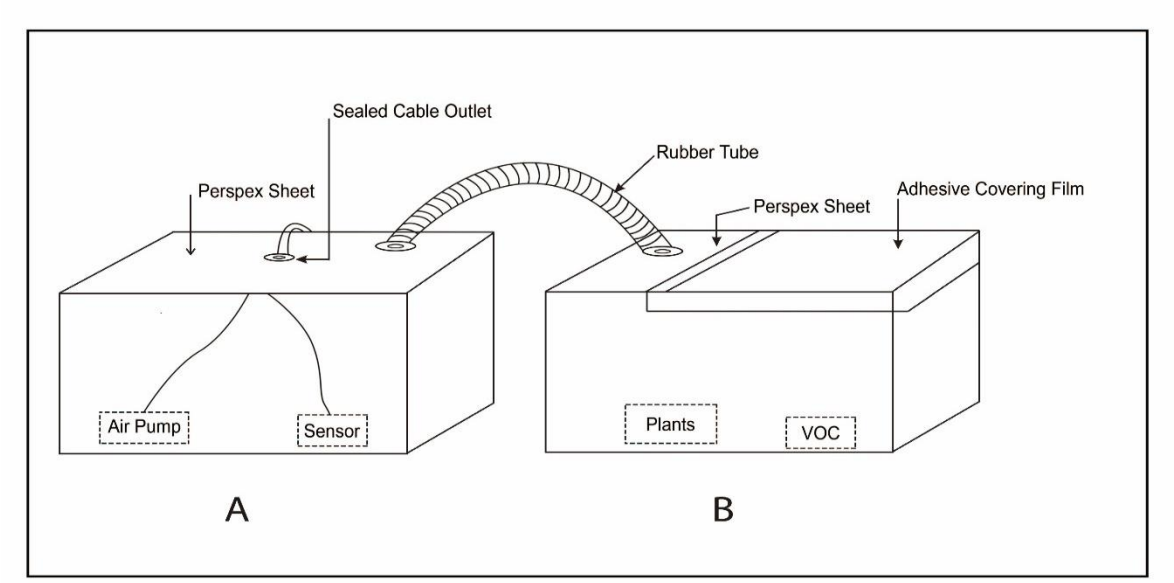


Fig. 2. 8 schematic diagram of two chambers connected with a tube

After injecting VOC into the empty chamber B through the adhesive covering film, VOC injecting hole was sealed immediately sticking a piece of adhesive covering film. Then the air was circulated for 10 minutes at 100 l/min flow rate to get an equilibrium between two chambers. Data was collected from the Aeroqual digital monitor (detailed in section 2.12.1) until the chamber air became VOC half-life.

2.12.1.6. Modification of dual chamber system

Since the Aeroqual sensor still reported problematic errors during VOC monitoring, the dual chamber system was re-modified. An extra tube was fixed between the two chambers to maximise the air circulation after VOC injection and during VOC monitoring (Fig. 2. 9 and Fig. 2. 10). Air was not circulated continuously throughout the experiment to avoid the heating effect of

the pump. Therefore, following initial 10 minutes of air circulation, the linear air pump was operated for 2 minutes in every two hours to ensure an equilibrium between plant chamber (B) and sensor containing chamber (A). VOC monitoring was carried out using the Aeroqual digital monitors as explained in previous sections.

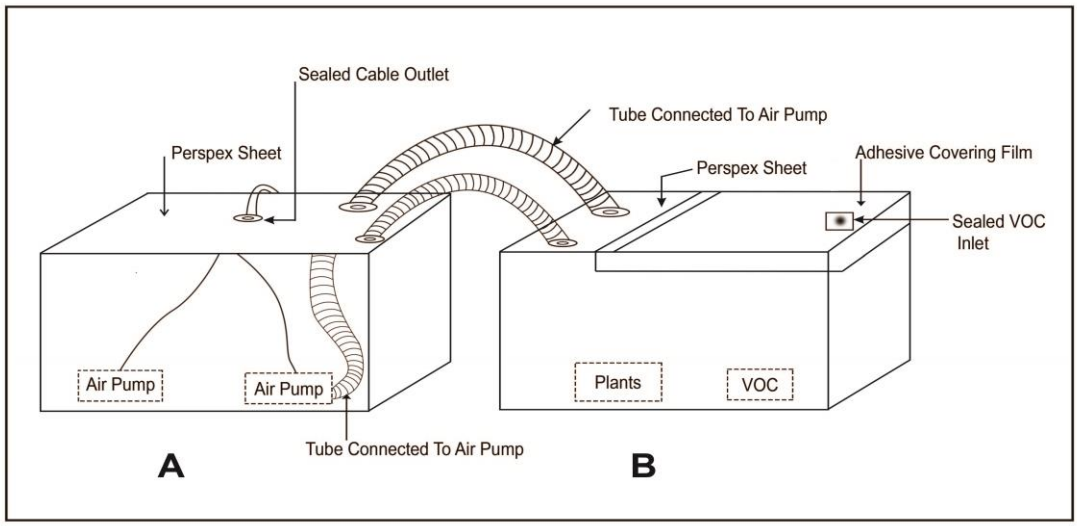


Fig. 2. 9 Schematic diagram of modified dual chamber set up with extra tube

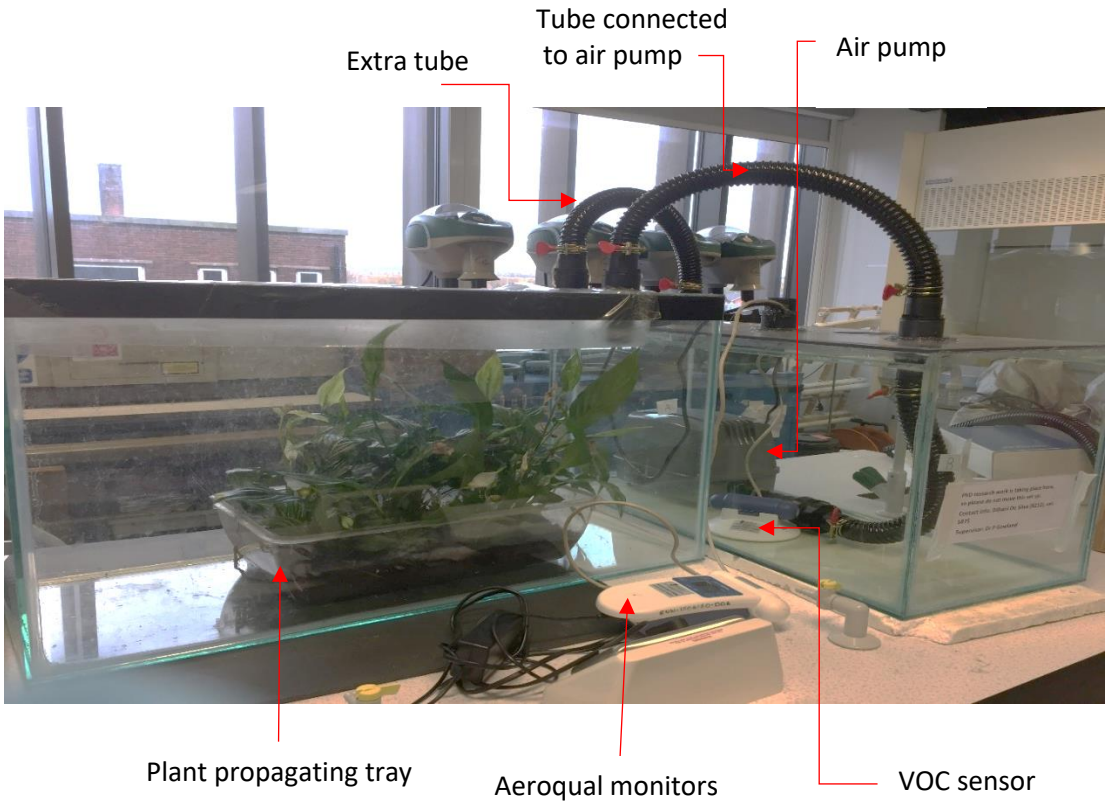


Fig. 2. 10 Modified dual chamber set up with extra tube

2.12.1.7. Comparison of new and used Aeroqual sensors

Due to issues in monitoring VOC level inside the plant test chambers using the Aeroqual digital monitors, it was decided to evaluate the accuracy of Aeroqual sensor. A new and a used sensor were placed inside a single test chamber sealed using adhesive covering film. 100 ppm benzene was injected into the chamber and real-time VOC concentration was measured using both sensors. Different readings obtained from two sensors were evaluated to move for selection of another VOC monitoring method.

2.12.2. Optimisation of GC/FID method to monitor VOC detoxification by plants

Since the Aeroqual sensors were reported a less sensitivity for the test VOCs during monitoring VOC removal by plants experiments, a new analytical method was required to continue the study. This method involved analysis of VOCs in test chamber air using automated thermal desorption (ATD) and gas chromatography (GC) using Flame Ionisation Detector (FID). The instruments used for the analysis are Perkin-Elmer Turbo Matrix ATD 650 (Perkin-Elmer, UK) desorption unit, Clarus 500 Gas Chromatograph fitted with FID. Prior to starting the ATD, the mass gas flow controllers were checked and adjusted to provide correct flow rates for the system.

During air sample analysis, test chamber air (50 ml) was drawn into Tenax® TA tube (Perkin-Elmer, UK) using a 100 ml of plastic syringe through vacuum pumping (Fig. 2. 11). A needle connected to the Tenax® tube was sent through the chamber sealing material and air was withdrawn. Immediately after the air was withdrawn, the sampling hole was re-sealed using the same sealing material. After sampling, the needle and the syringe were removed and the Tenax® TA tubes were recapped using plastic caps.

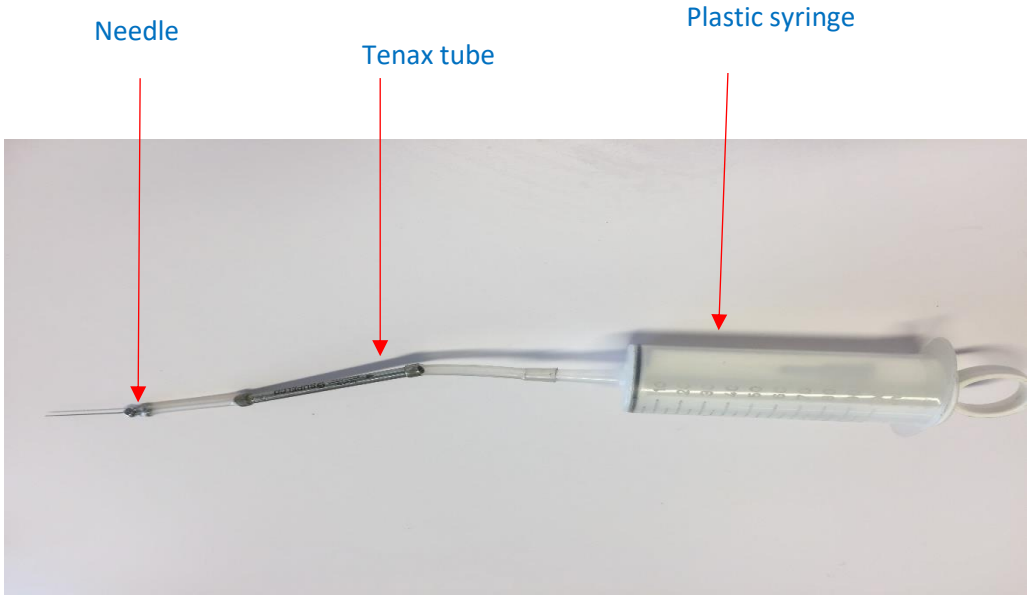


Fig. 2. 11 Tenax® TA tube connected to the needle and plastic syringe

Using TurboMatrix software program, the sequence of tube runs were programmed in the ATD. In addition to the analysing VOC in each tube, another sequence row was edited for each tube for checking contaminants. Tenax® TA tubes were loaded into the ATD carousel according to the sequence. The same sequence file was uploaded into the GC software "TcNav" for GC analysis. Once the ATD showed ready for run, the "Run sequence" button was pressed for tube analysis. Following the desorption of VOC containing Tenax TA tubes, samples were separated in the GC column and the results were obtained as hard and soft copies. All ATD, GC and FID conditions were set to achieve the maximum detection limit for the tested VOCs (Table 2. 5).

Table 2. 5 Conditions used during ATD, GC and FID analysis

Thermal desorption conditions	
Tube desorb temperature	250 °C
Tube desorption time	5.0 minutes
Tube desorb flow	30 ml/minute
Trap low	-30 °C
Trap high	250 °C
Concentrator trap hold	2.0 minute
Purge temperature	50 °C

Purge time	1.0 minute
Dry purge flow	50 ml/minute
Column flow	2.0 ml/minute
Inlet split	35 ml/minute
Outlet split	25 ml/minute
Column flow during trap desorb	1.0 ml/minute
Run time	36 minutes
GC /FID conditions	
Column	30.0 m DB 320 µm
Inlet	20:1 split ratio
Purge flow to split vent 1 ml minute^{-1}	999.00 minutes
Flow rate at	8 PSIG
Initial oven temperature	40 °C
Initial hold	4.0 minutes
Ramp 1	10.0 °C/minute to 235 °C
Final hold	4.0 minutes
Maximum temperature	320 °C
Carrier gas	He
FID temperature	300 °C
Run time	27.5 minutes

Samples which were not analysed in the same day following sampling were placed inside sealed Nylon bags and stored in 4 °C for maximum of 24 hours until analysis was undertaken.

2.12.2.1. Tube conditioning procedure

All the Tenax® tubes used during VOC monitoring were supplied by Perkin-Elmer. There were 10 newly packed tubes and two used tubes. All newly packed tubes and the used tubes which had not been used for a long time were conditioned before using them. During tube conditioning, tubes were cleaned to remove any volatile organic contaminants resulting from storage, transport or manufacturing. New tube conditioning was performed by sending the carrier gas flow through the tube sorbent material at 10.0 ml/minute flow rate at 250 °C for 35 minutes. During conditioning of the used tubes, the carrier gas flow was sent for 10 minutes at same rate and temperature used for new tube conditioning. In addition to the new and the old tube

conditioning, all the tubes were conditioned after each subsequent analysis. This was done to remove any residual contaminants trapped inside the Tenax. For conditioning the tube following thermal desorption of analyte (sample), carrier gas flow was sent through the tube for 5 minutes at 250 °C at 10.0 ml/minute flow rate. After tube conditioning, capped cleaned tubes were placed inside the sealed nylon bags and placed in a desiccator at room temperature until next usage.

2.12.2.2. Retention time analysis and chamber sealing material selection

Although n-hexane was used as a one of the testing VOCs during Aeroqual method, n-hexane analysis was excluded from using GC/FID method due to its high volatility and its use as a solvent for different VOCs in GC analysis (Demeestere *et al.*, 2007; Salimon *et al.*, 2014). Therefore, another indoor dominant VOC: m-xylene was chosen to study with the plants. Prior to start analysis, the GC retention time of each compound (VOC) should be investigated. Therefore, pure compound (≈15µL) of three testing VOCs (benzene, toluene and m-xylene) were injected into the sealed (sealed using adhesive polythene) chambers separately and 50 ml of air sample containing each VOC was analysed (detailed in section 2.12.2) using ATD/GC/FID. Retention time for each VOC was identified based on the chromatogram peak separation time.

Since the data produced using Aeroqual sensor was not reliable, it was essential to study sealing material efficiency based on the ATD-GC-FID method. Therefore, following the retention time analysis, selection of test chamber sealing material was conducted as follows. Four different sealing materials; nylon, PET (polyethylene terephthalate) sheet, adhesive covering film and adhesive covering film with its paperback were used during the selection. Following sealing the test chambers using four different materials, 100 ppm of VOC was injected into each chamber. VOC inlet was sealed again using same sealing material and after 30 minutes 50ml air samples were withdrawn through Tenax® TA tubes and analysed using ATD-GC-FID (detailed in section 2.12.2). Sealed chambers containing VOC were maintained at room temperature for 48 hours. Four replicates of each sealing material experiments were conducted by getting air samples throughout the test period to observe which chamber would maintain the maximum average VOC

concentration. VOC loss rates inside the chambers based on the sealing materials were analysed using non-parametric one-way ANOVA (Kruskal-wallis test) (SPSS, version 20.0 SPSS).

2.12.2.3. GC calibration for different VOC and Shewhart test

External calibration of GC was carried out by running seven-point calibration standards for benzene, toluene and m-xylene ranging from 2.5 to 125 ppm. Calibration standards were prepared using neat chemicals. The required volume from each neat VOC to get the standard concentration was calculated and injected into the test chamber sealed with adhesive covering film along with the paper back. VOC inlet was sealed immediately and allowed to equilibrate VOC inside the chamber for 30 minutes at room temperature. 50ml of chamber air was passed through the Tenax® TA tube and analysed using ATD-GC-FID (detailed in section 2.12.2). The FID response: the peak area of analyte was plotted against the concentration of VOC used to produce the calibration curve. The correlation coefficient (R^2) was calculated for each standard curve to show the linearity of the method for the concentration range used.

During the plant-VOC detoxification analysis, one-point calibration: Shewhart test (Shewhart, 1939) was carried out for 25 ppm of each VOC for every experiment. This was done by injecting the required neat VOC volume to get 25 ppm in the test chamber, following sampling chamber air through the Tenax® TA tube, samples were analysed in GC/FID (detailed in section 2.12.2). This was done to show the accuracy of the GC method.

2.12.2.4. Analysing VOC concentration in the test chamber containing plants

After sealing material selection and calibrations were completed, VOC monitoring in the test chambers containing plants and compost was carried out. All the experiments were conducted at room temperature 20±3 °C and the light/dark period was 12/12 hours. The test chamber was sealed using adhesive covering film along with the paper left on the inside covering the area which was exposed to the inside of the tank. Following injecting 10 ppm or 100 ppm of each VOCs into the chamber at 0 hours, the VOC inlet was sealed using a section of adhesive covering film. VOC reduction by plants and compost were analysed using ATD-GC-FID method. During the

analysis, air samples (50 ml) were taken using Tenax® TA tube connected to a needle and syringe. After sampling, the tubes were recapped to seal them and placed in the ATD carousel for sample analysis (detailed in section 2.12.2). Samples were taken from the test chamber once in every 24 hours until the chamber VOC concentration reduction was less than 2.5ppm. Actual concentration inside the chamber air was calculated according to the calibration curve. In addition to the VOC analysis in plant and compost chambers, leak tests were carried out in the chambers without plants. Finally, the VOC removal rate per plant monocultures, community and compost in plant propagating trays (i.e. per chamber) were calculated. Different VOC removal rates achieved by plants during the VOC half-life was analysed using non-parametric one-way ANOVA (Kruskal-Wallis test) by setting the statistical significance of ≤ 0.05 (SPSS, version 20.0 SPSS).

Chapter 3. Investigation of VOC removal efficiency by plant monocultures, communities and compost

3.1. Method development and investigation of VOC removal by plants using Aeroqual digital VOC monitors

The aim of this section is to present the development of the single and dual test chamber systems, test chamber sealing material selection and optimisation of VOC monitoring method using Aeroqual digital monitors. This section will also discuss the suitability of the Aeroqual VOC monitoring method for the plant test chamber experiments.

3.1.1. Introduction

Plant research can be done using environmental test chambers which are available from the commercial suppliers, but they are expensive, large in scale, not easily portable and use high electrical power. The literature contains several examples of plant experiments being carried out in simple bench-top test chamber systems made from glass, stainless steel or Perspex (Tarran *et al.*, 2007; Kim *et al.*, 2008). Since this work aims to carry out plant-VOC exposure experiments in comparison to experimental controls with replicates, several experimental chambers were required. Therefore, at the beginning of the project, there was a requirement to design a cost-effective and easily maintainable test chamber set-up for carrying out the experiments. To address this, we based our initial designs on glass fish tanks (0.1 m^3) which had an optimal volume for housing our selected plant species, were cost effective. Our original design is detailed in section 2.3.

In order to monitor the concentration of VOC within our experimental chambers, we purchased a set of Aeroqual series 500 VOC monitors which measured the range of VOCs wished to study, were portable, and enabled continuous data logging. Given their small size, they could be easily used in any environment. In addition, it was a user-friendly, time efficient, durable and reusable method which could be used in research studies. The sensor and the monitor could be programmed as required, thus VOC sampling and monitoring process could be operated automatically. This means the sampling frequency could be programmed as required by the user to get samples ranging from every one minute to a few times a day. Therefore, this continuous

monitoring facility of Aeroqual VOC monitor provided more accurate graphs than the point sampling GC approach which allows analysis of only a limited number of samples. In addition, Aeroqual performed the real-time and *in situ* air sample detection, thus there was no requirement to draw out samples from the test chambers. Also, they were compatible to use with selected fish tank model test chambers. Based on the above features of the Aeroqual VOC monitors, the monitor along with the PID sensor was used to develop a method to investigate the VOC removal efficacy of plant monocultures and communities in glass test chambers.

3.1.2. Test chamber sealing material selection

The main aim of carrying out the test chamber optimisation was selecting the most effective sealing method to reduce VOC loss that can happen as a result of leakage and adsorption onto the chamber walls and chamber sealing material. The following sections will explain VOC loss rates inside the test chambers sealed using different sealing materials: pine wood board, thin low-density polyethylene (LDPE), polyurethane foam sheet and adhesive covering film (detailed from 2.12.1.1 to 2.12.1.4 respectively).

During the chamber optimisation carried out using pine wooden board, (Fig. 3. 1), it took 12 minutes to reach the maximum concentration of benzene which was 84.25 ppm (theoretical maximum 100ppm). The difference between the injected concentration (100ppm) and the sensor recorded concentration (84.25 ppm) might be caused due to a few factors such as sensitivity of the Aeroqual sensor to detect benzene, benzene may not have uniformly distributed within the tank or there could have been a VOC loss during injecting procedures.

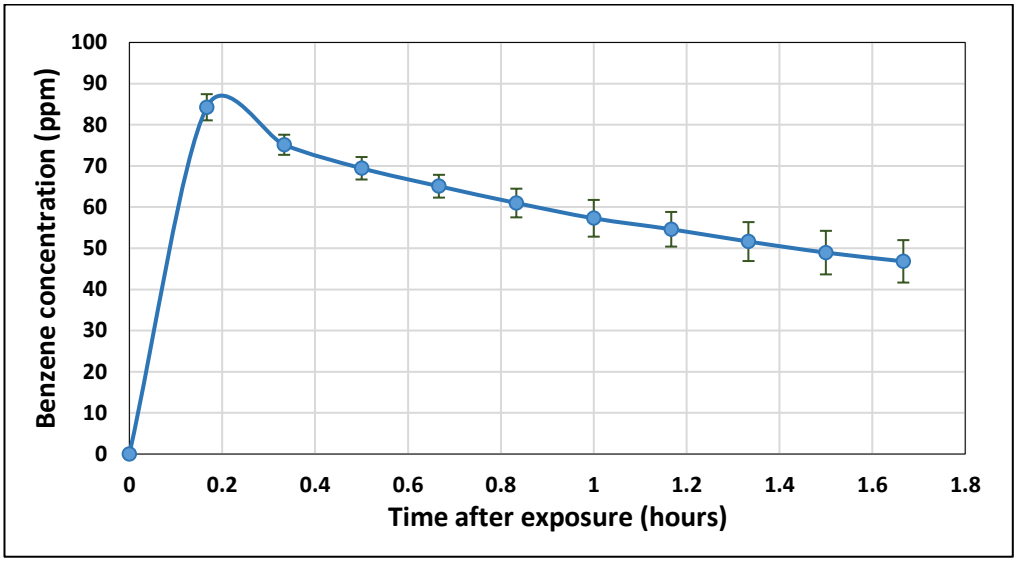


Fig. 3. 1 Benzene loss in the single chamber sealed using wooden board. Decline of initial benzene concentration (100 ppm) injected at 0 hour, expressed as the actual benzene concentration monitored. The half-life of benzene was 1.38±0.15 hours and the average rate of benzene loss is 24.95 ± 3.95 ppm/hours. Data is presented as the mean ± SEM (n=3).

However, immediately after reaching the maximum concentration, the VOC loss through the chamber had been started and within 1.38 hours it declined to 50% concentration. These observations concluded that the pine wooden board did not afford the level of sealing required to conduct the experiments over the required timeframe, hence it was not optimised with the other two testing VOCs: toluene and n-hexane.

Therefore, the chamber optimisation experiment was carried out for another sealing material: low-density polyethylene film (LDPE-cling film) (detailed in section 2.12.1.2). The maximum benzene, toluene and n-hexane concentrations inside the chambers were 87.25, 92.18 and 96.10 ppm (theoretical maximum 100 ppm) reached within 20 minutes of initial injection (Fig. 3. 2). The average benzene loss rate: 18.81± 2.189 ppm/hours was less than the loss rate observed in the previous chamber system sealed using pine wooden board. The average toluene and n-hexane loss rates were 12.99± 0.450 and 13.19 ± 0.263 ppm/hours. The half-lives were 1.66, 2.61 and 2.81 hours for benzene, toluene and n-hexane respectively.

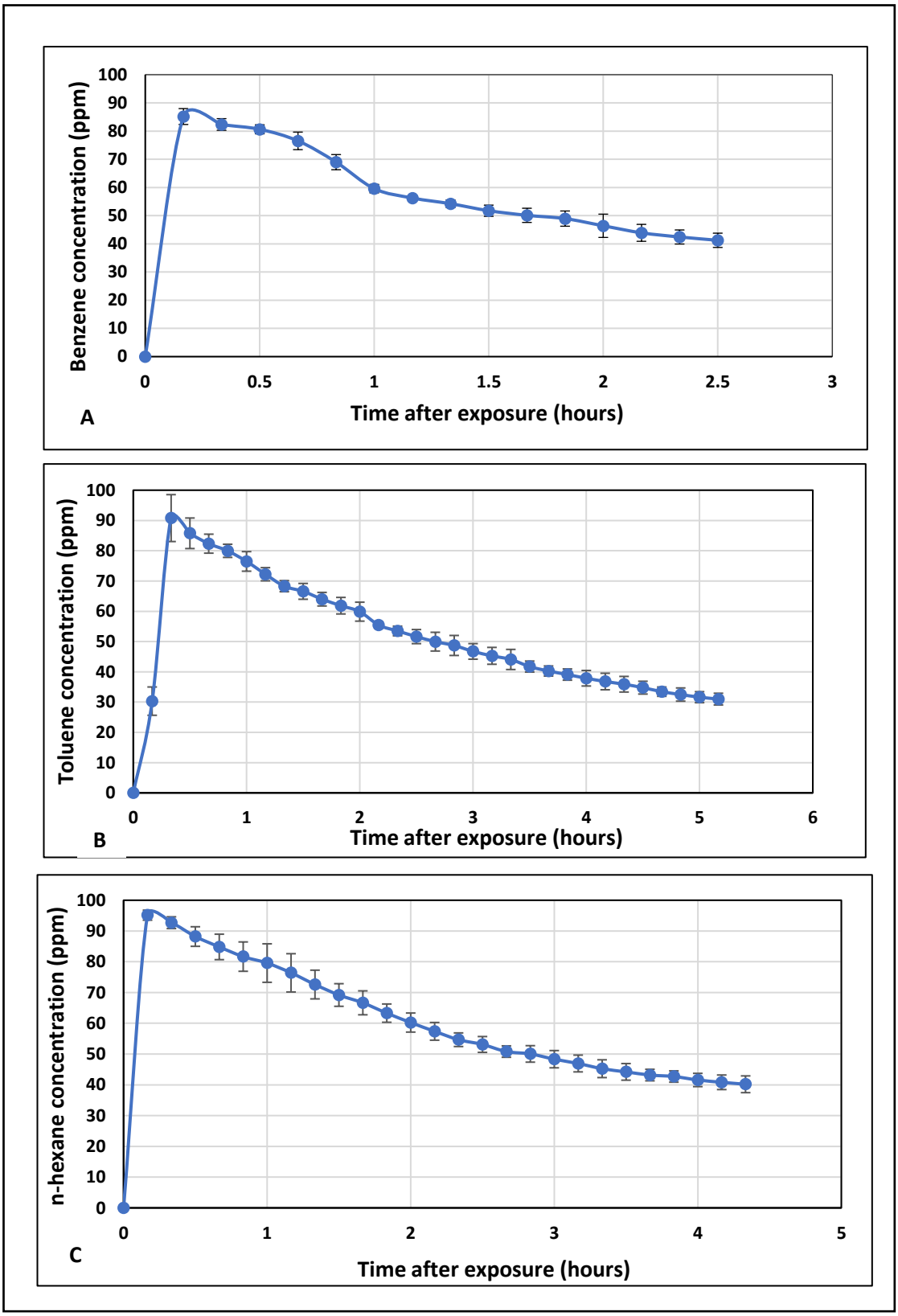


Fig. 3. 2 Benzene (A), toluene (B) and n-hexane (C) losses in the single chamber sealed using thin low-density polyethylene film (LDPE). The half-lives of three VOCs were 1.66±0.170, 2.61±0.280 and 2.81±0.150 hours and the average rates of VOC losses were 18.81 ± 2.189, 12.99± 0.450 and 13.19 ± 0.263 ppm/hours. Data is presented as the mean ± SEM (n=3).

Although a higher initial concentration for toluene and n-hexane (nearly 100 ppm) was achieved in the chambers at the beginning, due to the observation of high VOC loss rates and short half-lives for all three VOC tested, LDPE was not an efficient method to seal the test chambers in the proposed plant-VOC removal monitoring experiments. Therefore, the optimisation experiment was carried out to test the sealing efficiency of the polyurethane foam sheet (detailed in section 2.12.1.3).

The maximum VOC concentrations achieved during polyurethane foam sheet method were 87.90, 93.10 and 82.36 ppm for benzene, toluene and n-hexane respectively (Fig. 3. 3). The longest half-life, 7.9 hours was observed for toluene which was a good indication for the sealing efficiency of polyurethane foam sheet. The VOC half-lives for benzene and n-hexane were observed at fairly similar rates (4.2 and 4.5 respectively).

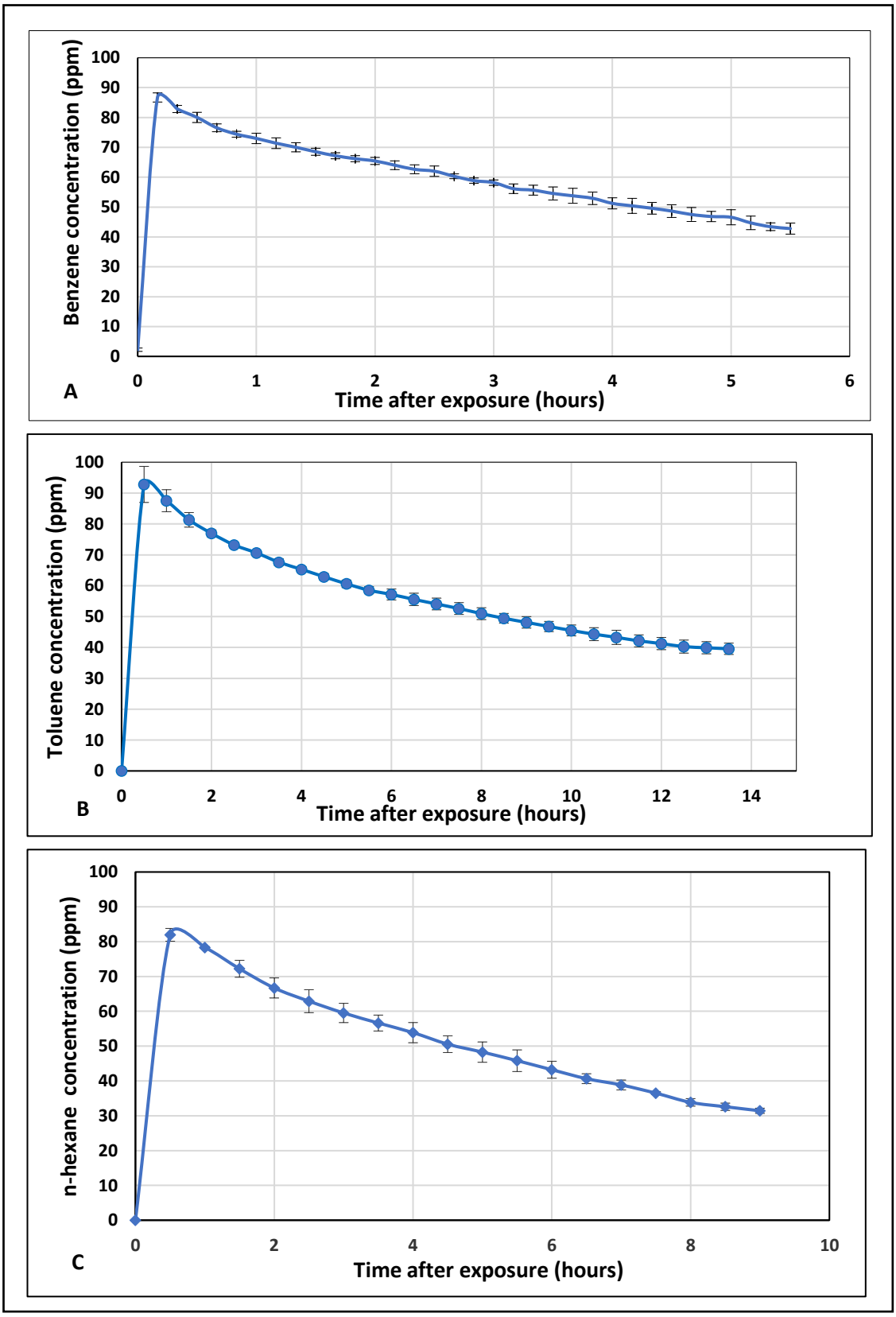


Fig. 3. 3 Benzene (A), toluene (B) and n-hexane (C) losses in the single chamber sealed using polyurethane foam sheet. The half-lives of VOCs were 4.2±0.231, 7.9±0.402 and 4.5±0.311 hours and the average loss rates were 8.56 ± 0.345, 4.42 ±0.253 and 5.94± 0.353 ppm/hours respectively. Data is presented as the mean ± SEM (n=3).

VOC loss rates in the chamber sealed using polyurethane foam sheet (rigid foam) were less than the previous method by maintaining 8.56±0.345, 4.42±0.253 and 5.94±0.353 ppm/hours for benzene, toluene and n-hexane respectively, however, for the proposed study, it was essential to further reduce VOC loss. Therefore, the sealing efficiency of the adhesive covering film was tested using the same VOCs (detailed in section 2.12.1.4).

Following sealing the chambers using adhesive covering film, the maximum benzene, toluene and n-hexane concentrations achieved inside the chambers were 87.5, 83.4 and 91.3 ppm respectively (Fig. 3. 4) while the VOC loss rates accounted for 3.60 ± 0.442, 2.47±0.867and 3.59 ± 0.315 ppm per hour for the three VOCs respectively. The half-lives of VOC were 10.0, 11.9 and 11.5 for benzene, toluene and n-hexane indicated more than 50% of each VOC remained in the chamber during this time. Therefore, this sealing method reduced VOC loss rate better than all previous methods tested. Thus, compared to all other sealing methods used previously, adhesive covering film showed the best achievable efficiency for sealing the test chamber.

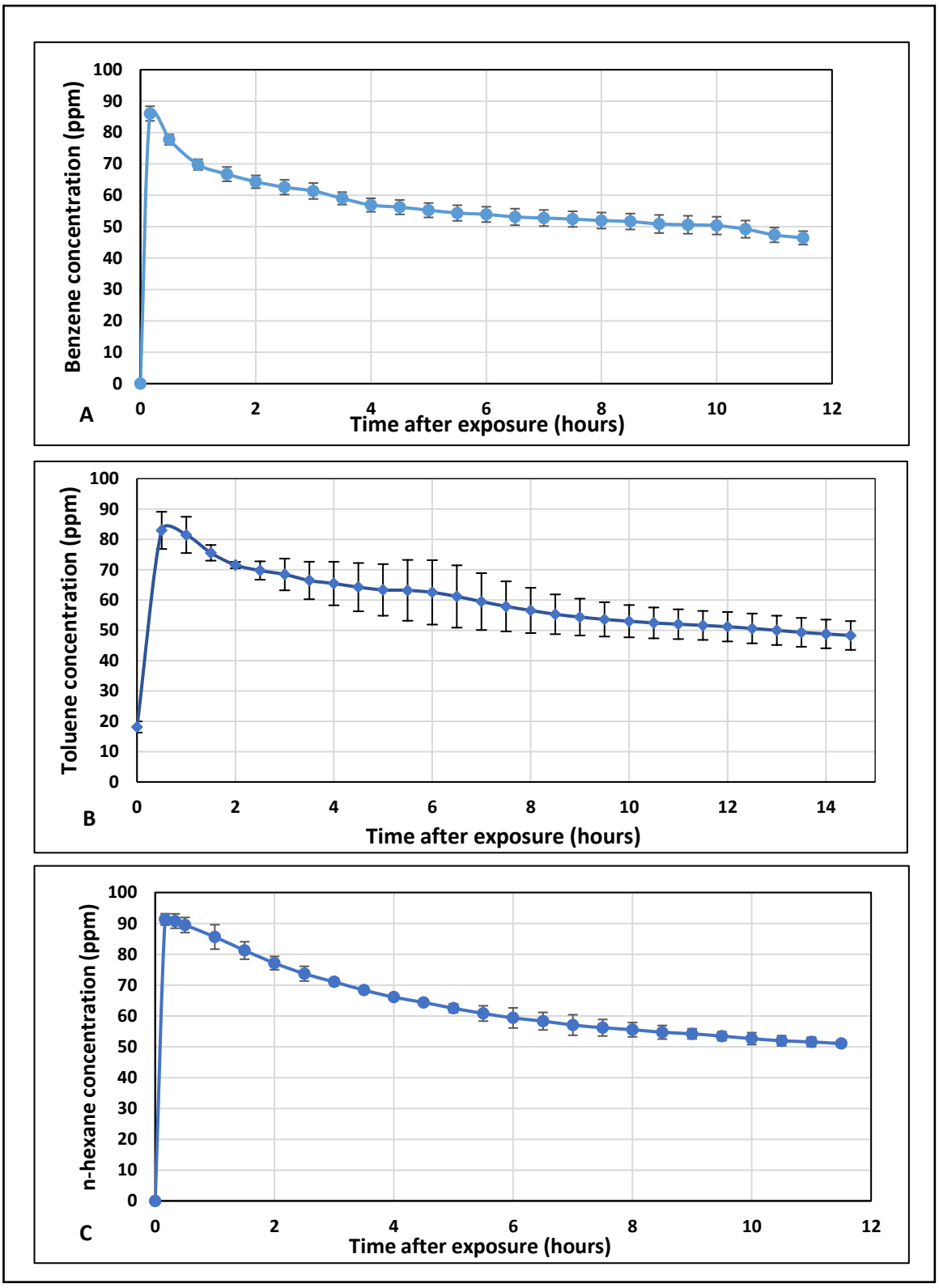


Fig. 3. 4 Benzene (A), toluene (B) and n-hexane (C) losses in the single chamber sealed using adhesive covering film. The halves-life of VOCs were 10.0±1.401, 12.0±2.430 and 11.5±0.112 hours and the average loss rates were 3.60 ± 0.442, 2.47±0.867 and 3.59 ± 0.315 ppm/hour. Data is presented as the mean ± SEM (n=3).

Mean VOC loss rates were compared for the sealing methods using non-parametric one-way ANOVA excluding wooden board method, since it was tested only with benzene. According to the nonparametric one-way ANOVA (kruskal wallis test), there were statistically significant differences (p=0.027) between the mean VOC loss rate of adhesive covering film and the LDPE for all three VOC tested. No statistically significant difference between adhesive covering film and polyurethane foam sheet was observed (Table 3. 1). However, based on the minimum loss rate and the longest VOC half-life, adhesive covering film demonstrated that the rate of VOC decline inside the chamber was manageable compared to the other two sealing materials.

Table 3. 2 Summary of half-lives and VOC loss rates in the sealed single chambers

Chamber sealing method (n=3)	**VOC loss rate (ppm/hour)**			**Half-life of VOC (hours)**		
	Benzene	**Toluene**	**n-hexane**	**Benzene**	**Toluene**	**n-hexane**
Wooden board*	24.95 ± 3.95	N/A	N/A	1.38± 0.15	N/A	N/A
LDPE	18.81 ± 2.189	12.99± 0.450	13.19 ± 0.263	1.66± 0.170	2.61± 0.280	2.81± 0.150
Polyurethane foam sheet	8.56 ± 0.345	4.42 ± 0.253	5.94± 0.353	4.2± 0.231	7.9± 0.402	4.5± 0.311
Adhesive covering film	3.60 ± 0.442	2.47± 0.867	3.59 ± 0.315	10± 1.401	12± 2.430	11.5± 0.112
p value	0.027	0.027	0.027	N/A	N/A	N/A

*Excluded from statistical analysis

3.1.3. Conclusion

According to the results obtained after sealing the chamber using three different materials, adhesive covering film showed the minimum loss rate and maintained the longest half-life of benzene, toluene and n-hexane (Table 3. 2). This indicated that adhesive covering film supplied an adequate sealing to create a gas-tight condition inside the chamber, thus this was selected as the sealing material of plant test chamber.

3.1.4. Determination of VOC removal by plants using Aeroqual digital VOC monitors

Considering the workplace exposure limit of VOCs in the indoor air, the exposure limit concentration ranges between 1 and 100 ppm for most common VOCs (Table 1. 2). For example, the short term and long term exposure limit of toluene is 50 ppm and 100 ppm respectively (UK/HSE, 2013). Phytoremediation of some VOCs at the low concentrations such as 1-10 ppm is quite well studied, but very little is known about the potential of plants to remediate higher concentration of VOC, such as 100 ppm level from the indoor air. Therefore, the initial 100 ppm VOC level was selected as the testing concentration in these experiments. After selecting the best chamber sealing material, the first set of experiments to monitor VOC removal by plants were conducted using 100 ppm benzene (detailed in section 2. 2 and 2.12.1.4). In the first experiments "early experiment", the sensor recorded maximum benzene concentration (≈78.5 ppm) for the initial 100 ppm benzene injected declined in the last experiments (≈29.5 ppm) "later experiments" (Fig. 3. 5).

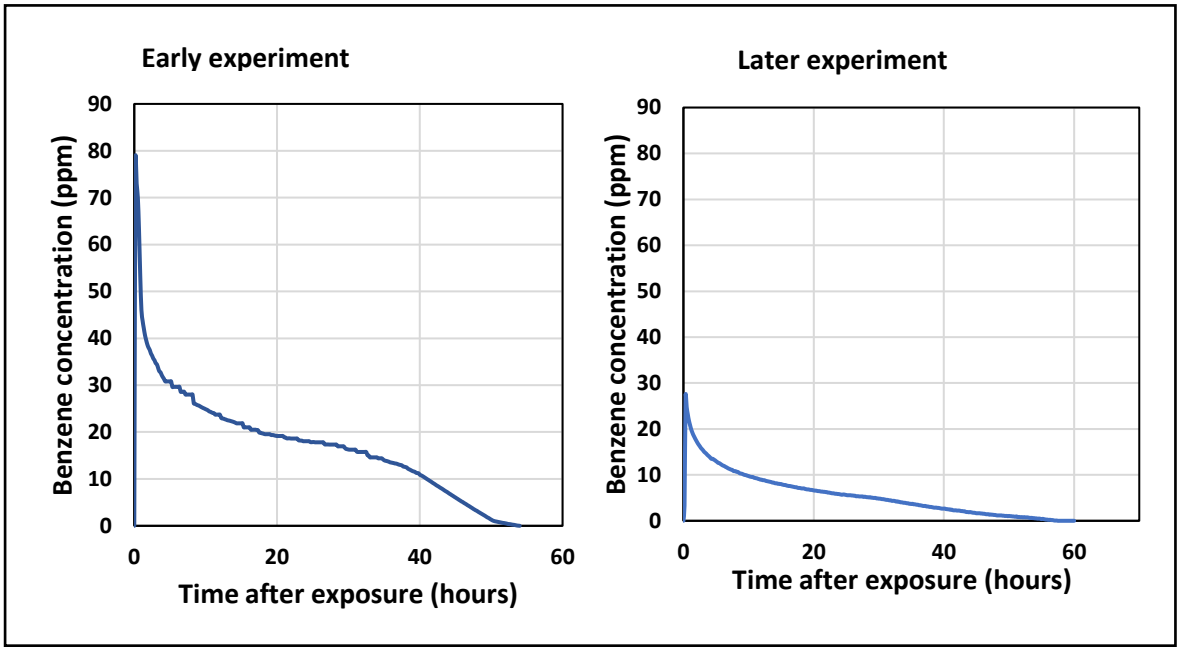


Fig. 3. 5 Sample graph of early and later experiments to show the benzene removal capacity of potted *C. comosum* measured using Aeroqual VOC sensors. Decline of initial benzene concentration (100 ppm) injected at 0 hour, expressed as the actual benzene concentration monitored. The maximum concentrations are ≈78.5 and ≈29.5 ppm in early and later experiments respectively.

In earlier experiments, the initial benzene level inside the chamber reached ≈ 78.5 ppm, however, this is a lower concentration than was obtained during the sealing material selection (87.5 ppm) (Fig. 3. 4). The reason for the reduction of achieving maximum benzene concentration in the presence of plants was thought to be that the plants might be causing a disturbance to create a benzene equilibrium inside the chamber. However, initially obtained maximum benzene concentration which was ≈78.5 ppm was reduced further in later experiments with *C. comosum.* It was suspected that the sensors might be affected by conditions such as humidity level changes or poor air circulation inside the chamber due to the presence of plants, so their efficiency of working reduced with time. According to the manual, the Aeroqual sensor is certified for conditions up to 95% humidity. There was no visible water condensation inside the chamber. Since the cause of this issue was not clear, it was decided to modify the chamber system by doubling the chamber volume by using a dual tank system.

3.1.5. Design and optimisation of dual test chamber system

Through the development of the dual chamber system (detailed in section 2.12.1.5), it was expected to improve air circulation from the plant chamber to the sensor through air pumping. By expanding the total volume of the chamber than the previous single chamber system, it reduced sensor exposure to possible water vapours. Then the time required to make a benzene equilibrium between the dual chambers was analysed (Fig. 3. 6). By achieving the maximum stationary phase concentration within 6 minutes of induction, this system showed its capability to reduce the time required for making benzene equilibrium inside the plant chamber which was essential in the experiment. However, according to the same figure, the maximum benzene concentration achieved was ≈ 55 ppm indicated the chamber containing the sensor was not receiving the 100 ppm concentrated air from the plant chamber. This happened probably because there was only one tube connected between the two chambers (detailed in 2.12.1.5), thus both inlet and outlet air flow from plant chamber to sensor containing chamber and *vice versa* had to move through it.

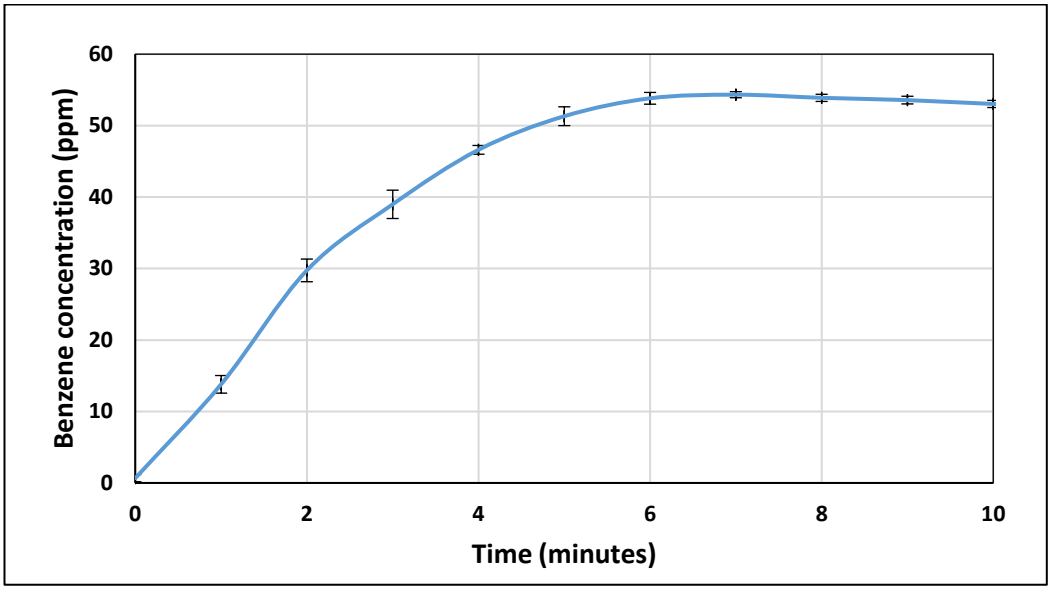


Fig. 3. 6 Time required to establish benzene equilibrium between dual-chamber system. Decline of initial benzene concentration (100 ppm) injected at 0 hour, expressed as the actual benzene concentration monitored. After 5 minutes, the system achieved and maintained the maximum concentration. Data is presented as the mean ± SEM (n=3).

Therefore, to improve the air circulation, this chamber system was modified by including an additional tube (detailed in section 2.12.1.6). Following this modification, sensor achieved maximum benzene concentration ≈ 90 ppm within 5 minutes air circulation (Fig. 3. 7).

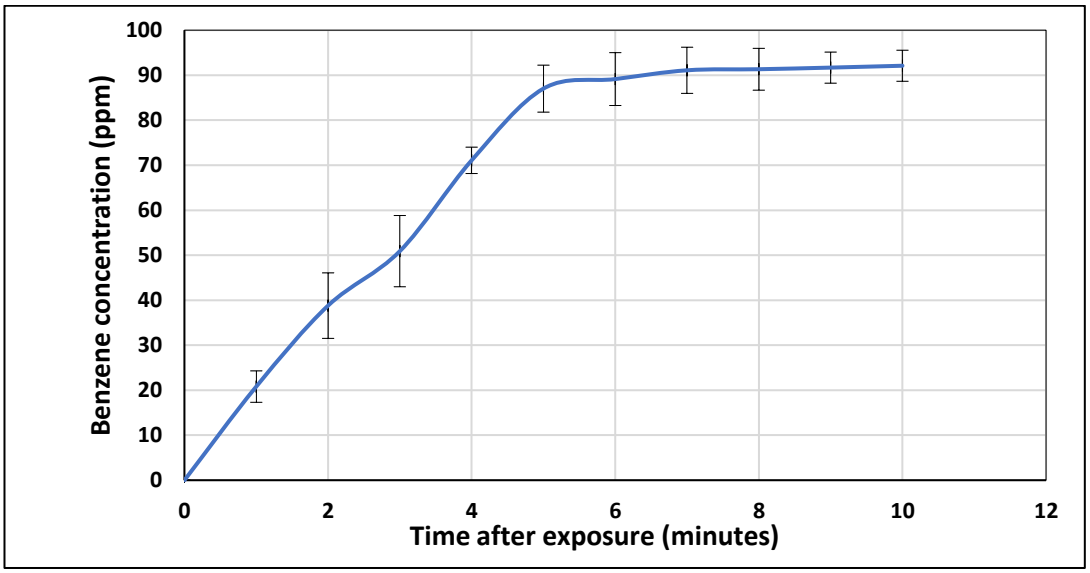


Fig. 3. 7 Time required to establish benzene equilibrium in modified dual chamber system. Decline of initial benzene concentration (100 ppm) injected at 0 hour, expressed as the actual

benzene concentration monitored. After 5 minutes of air circulation, the system achieved and maintained the maximum concentration. Data is presented as the mean ± SEM (n=3).

Prior to starting the experiment with plants, this chamber system was tested again by keeping a VOC sensor in each chamber and monitoring the benzene concentration separately. This was done to estimate the benzene losses during plant experiments due to leakage or adsorption to the chamber surface or possible chemical reactions.

The maximum benzene concentration ≈90 ppm was observed in the sensor-containing chamber (A) and the plant chamber (B) and both chambers maintained ≈70 ppm for 10 hours (Fig. 3. 8). The benzene loss rates were 2.84 and 2.13 ppm/hour in chamber A and B respectively. Thus, this optimisation results indicated this modified system supplied adequate gas-tight condition inside the test chambers to conduct testing plant VOC removal experiments.

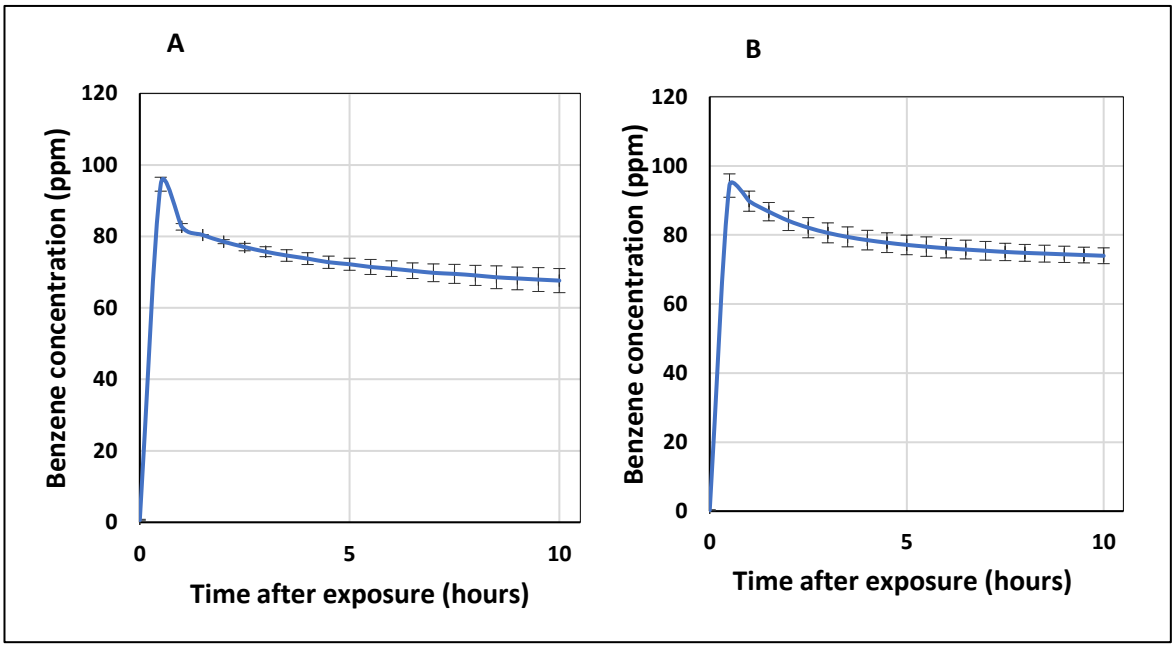


Fig. 3. 8 Benzene loss in sensor chamber (A) and plant chamber (B) in dual chamber system. Decline of initial benzene concentration (100 ppm) injected at 0 hour, expressed as the actual benzene concentration monitored. Data is presented as the mean ± SEM (n=2). The average rate of benzene loss rate was 2.84 and 2.13 ppm/hour in chamber A and B respectively.

3.1.6. Determination of VOC removal by plants in dual chamber system

Monitoring VOC removal by plants was carried out in the dual chamber system using Aeroqual sensors (detailed in section 2.12.1.6). According to the sample graphs from the first experiments "early" and last experiments "later" experimental results of benzene removal by *C. comosum* in plant propagating tray (pot) (Fig. 3. 9), it was observed that the sensor achieved the maximum concentration of benzene in early experiments but gradually declined when the number of experiments increased. This suggested that there must be an unexpected factor that was affecting sensor efficiency on monitoring VOC level inside the modified two-chamber system.

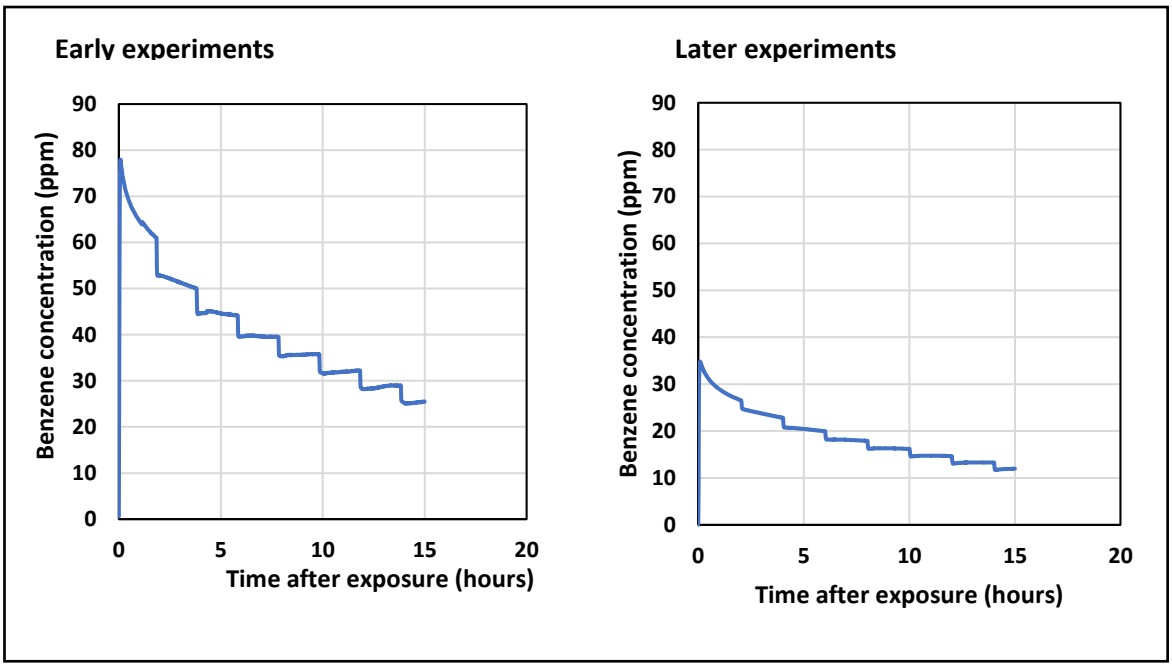


Fig. 3. 9 Benzene removal capacity of potted *C. comosum* measured in modified dual chamber system using Aeroqual digital sensors. Decline of initial benzene concentration (100 ppm) injected at 0 hour, expressed as the actual benzene concentration monitored. Sudden drop of benzene concentration in every two hours occurred after circulating air between two chambers. Maximum concentration ≈77.9 ppm measured in early experiment but had dropped to 34.8 ppm in the later experiment.

It was evident that over time, the sensitivity of the VOC sensor was declining, therefore the analysis data produced for the VOC removal by plants using Aroqual VOC sensors was less

reliable. Thus, it was essential to analyse the accuracy of sensors used in the experiment. Therefore, a sensor accuracy test was done with a new sensor (unused) and an old sensor.

3.1.7. Comparing sensitivity and accuracy of new and used Aeroqual sensors

The method to investigate Aeroqual sensor sensitivity is detailed in section 2.12.1.7. The new sensor (A) showed the maximum 94.70 ppm while the sensor (B) used in plant experiment (old sensor) achieved only 30.74 ppm concentration (Fig. 3. 10). This showed the new sensor had a higher sensitivity than the old sensor to detect 100 ppm benzene injected into the chambers, thus the new sensor measured the benzene level inside the chamber very close to the known initial value. Therefore, data from this experiment clearly showed that the used Aeroqual sensor had declined in its efficiency to measure the benzene level inside the chamber accurately over a period of time.

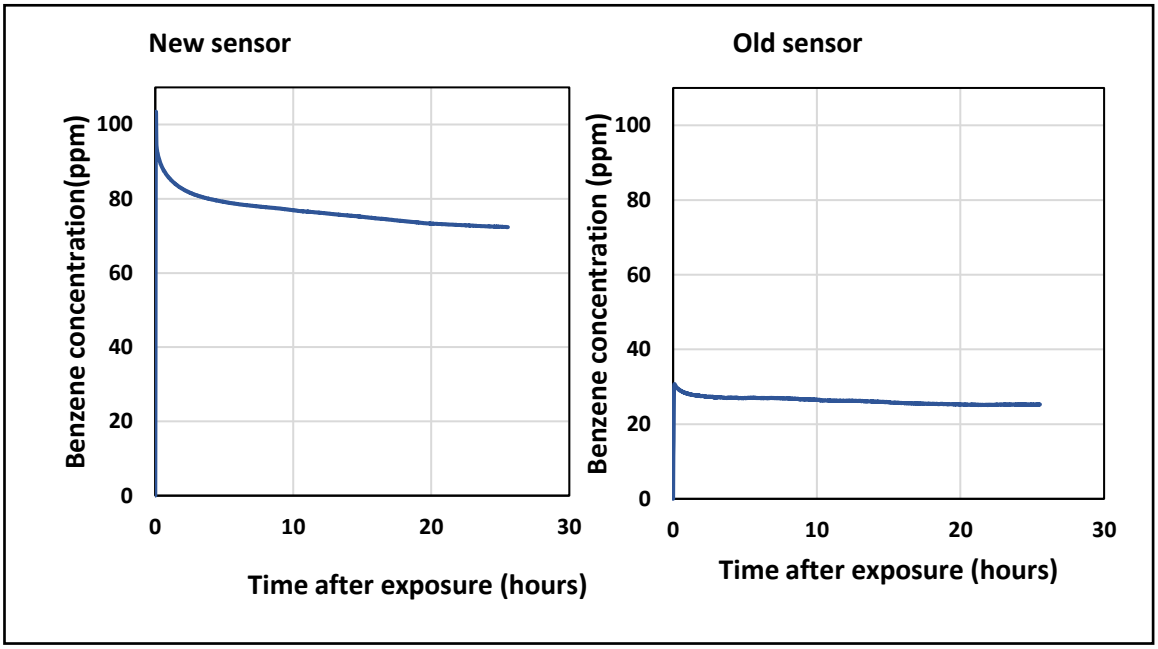


Fig. 3. 10 Sample graph to show maximum concentration of benzene detected by new and used sensors. Decline of initial benzene concentration (100 ppm) injected at 0 hour, expressed as the actual benzene concentration monitored. Initial benzene concentration (100 ppm) injected at 0 hours, maximum concentration 99.9 and 30.7 ppm measured by the new and old sensors respectively.

3.1.8. Conclusion

Aeroqual VOC monitors were used to optimise a method to analyse plant VOC removal efficacy in the sealed test chambers. Though initially, the sensor had shown a high sensitivity to VOC during the optimisation experiments performed in the empty chambers, according to the results obtained after inserting potted plants into the chambers, the sensitivity of the sensor had declined. This evidence clearly showed the unsuitability of the Aeroqual digital sensor in plant experiments. Therefore, it was decided to terminate using Aeroqual VOC monitors for VOC monitoring and no experiments were carried out for analysing the removal efficiency of toluene and n-hexane by plants. To fulfil the requirement of VOC monitoring in the study, a new VOC monitoring method was optimised using ATD-GC-FID techniques (detailed in sections 2.12.2).

3.2. Method development and investigation of VOC removal by plants using ATD-GC-FID method

Due to experiencing issues during VOC monitoring using Aeroqual digital monitors, a new analytical method was optimised (detailed in section 2.12.2). This section will present development of test chamber, optimisation of VOC monitoring method using a gas chromatography-flame ionisation detector. Also, the later section will discuss the benzene, toluene and m-xylene removal efficiency by plant monocultures, communities and compost.

3.2.1. Introduction

In the previous section, three VOCs including n-hexane was analysed using Aeroqual sensors to optimise a VOC monitoring method. The selection of n-hexane was based on because it is one of the common VOCs found in the indoor air. However, using the GC method, n-hexane gives a higher sensitivity *via* the solvent extraction method than thermal desorption and also it is used as a solvent during other polyaromatic hydrocarbon analysis. Therefore, to follow the same testing protocol to all VOC being tested, instead of n-hexane another common indoor VOCs: m-xylene was chosen to work in the plant experiments and analysed using GC/FID *via* thermal desorption method.

Analyte can be identified in the chromatogram based on their retention time. Therefore, once the GC column separates each VOC based on their polarity and size, they enter into the FID at different times through the ionisation process. Therefore, when studying different volatiles using GC/FID method, their retention times must be analysed as the first step.

3.2.2. Retention time analysis

FID is a non-selective detector which means it will detect all organic compounds in a sample. If the sample contains a mixture of unknown compounds, the chromatogram will show a peak for every single compound. Chromatographic retention time is the key to identify these compounds separately in the sample. Therefore, it was essential to inject and detect pure compounds (analytes) separately to get the correct retention times (detailed in section 2.12.2.2) for benzene, toluene and m-xylene (Fig. 3. 11).

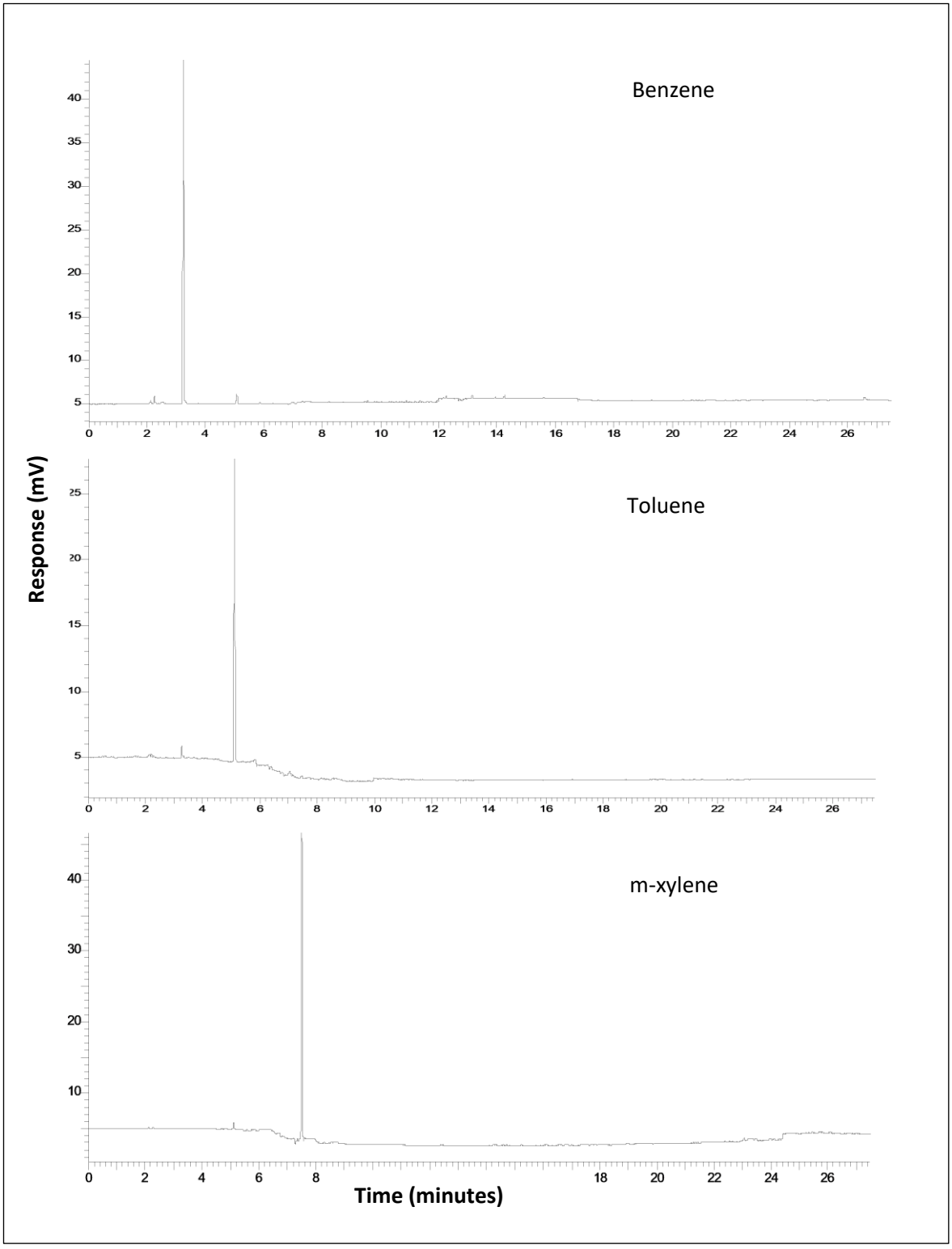


Fig. 3. 11 Retention times of benzene, toluene and m-xylene. Standard chromatogram produced by FID detector for pure compounds of benzene, toluene and m-xylene respectively. From these chromatograms, GC peak at the retention time of 3.2, 5.1 and 7.5 minutes for benzene, toluene and m-xylene were determined respectively.

After GC separation and FID detection of pure samples of benzene, toluene and m-xylene, their retention time, which occurred at 3.2, 5.1 and 7.5 minutes respectively, was recorded. Each VOC has a unique retention time during GC separation. Therefore, during sample analysis, these retention time peaks were used to identify each volatile compound. The peak area in the chromatogram is proportional to the concentration of VOC in the sample.

3.2.3. Chamber sealing material selection

Although experiments were conducted for the selection of chamber sealing materials using Aeroqual VOC sensors previously (detailed in sections 2.12.1 and 3.1.2), due to less reliability and low validity of the sensor that selection was not trustworthy to use in the plant VOC removal experiment. Therefore, the test chamber sealing material selection was re-conducted using GC/FID method to choose the material with minimum VOC loss during VOC monitoring (detailed in section 2.12.2.2). VOC loss can happen as results of leakage or chemical reaction inside the chamber. The best sealing material should maintain the maximum benzene concentration inside the chamber for a maximum period of time. All the glass chambers were the same size and purchased from the same supplier and there were no any visible gaps between the glass walls. Therefore, here we made an assumption that there is no VOC loss occurring other than VOC loss might be happening through the sealing material. Average GC peak area obtained for the 100 ppm benzene injected to the test chambers sealed using four different sealing materials were compared (Fig. 3. 12).

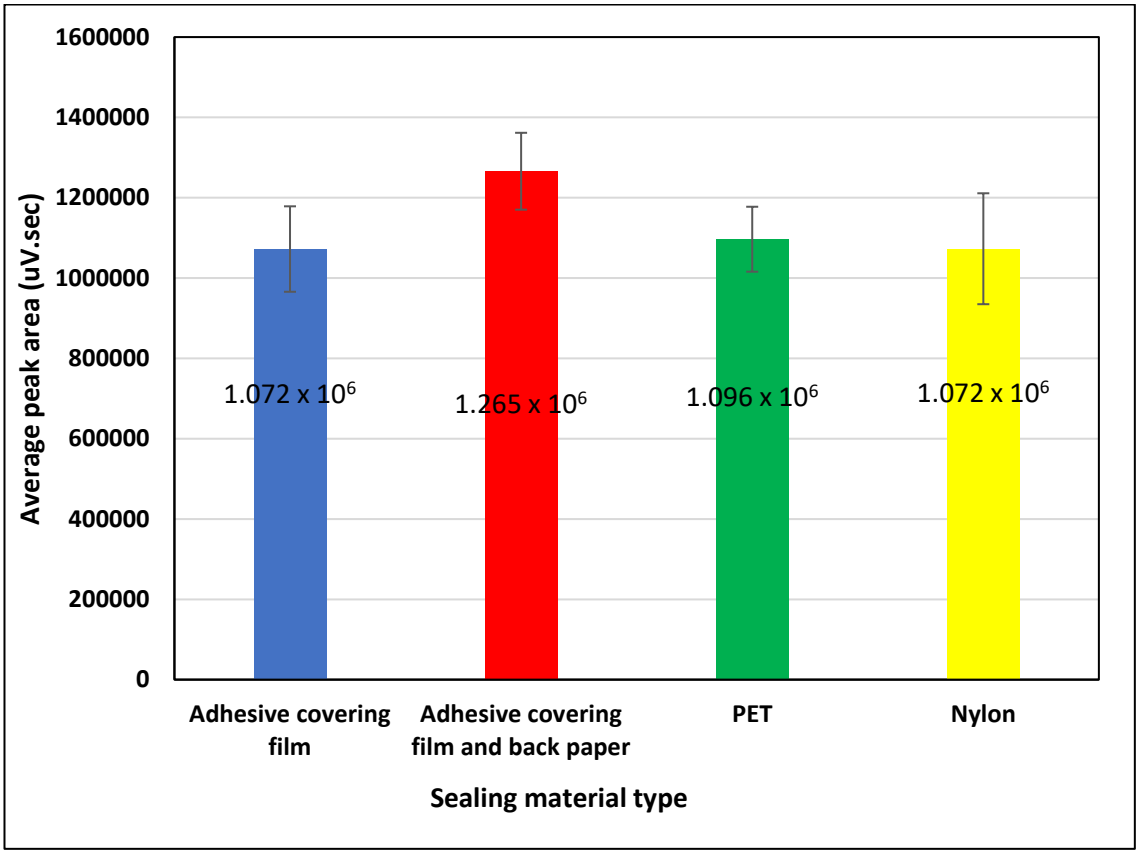


Fig. 3. 12 Average GC peak area obtained for the 100 ppm of benzene after the chamber was sealed using four different sealing materials; adhesive covering film, adhesive covering film with its back paper, PET and NYLON sheets respectively. Samples were analysed in each chamber separately for 48 hours. Vertical bars denote means ± 1SEM, (n=4), ($p>0.05$).

The maximum average peak area was obtained for the samples taken from the chamber sealed using adhesive covering film along with the paper left (back-paper) on the inside covering the area which was exposed to the inside of the tank (Fig. 3. 12). Though there was no significant difference ($p>0.05$) observed between four materials, based on the average peak area the minimum VOC loss had happened in the adhesive covering film with its back-paper method when compared to the other three methods. Thus, this sealing method showed a promising efficiency by reducing VOC loss than other methods, therefore, it was selected to use for sealing all the chambers in the proposed plant-VOC detoxification experiments.

3.2.4. Determination of standard calibration plots for benzene, toluene and m-xylene

Calibration of each analyte is essential during the GC analysis. Linearity of the calibration curve indicates the sensitivity of the system for the analytes within the low and high concentration range used (Fig. 3. 13). Following the retention time analysis and sealing material selection, the GC /FID system was calibrated for benzene, toluene and m-xylene concentration ranging between 2.5 ppm and 125 ppm (detailed in section 2.12.2.3). Concentration range was selected to cover the range corresponding to the concentrations to be established inside the test chambers. According to the calibrations (Fig. 3. 13), the linear relationship between the concentrations vs peak area for benzene, toluene and m-xylene were observed at R^2 0.9968, 0.9959 and 0.9981 respectively.

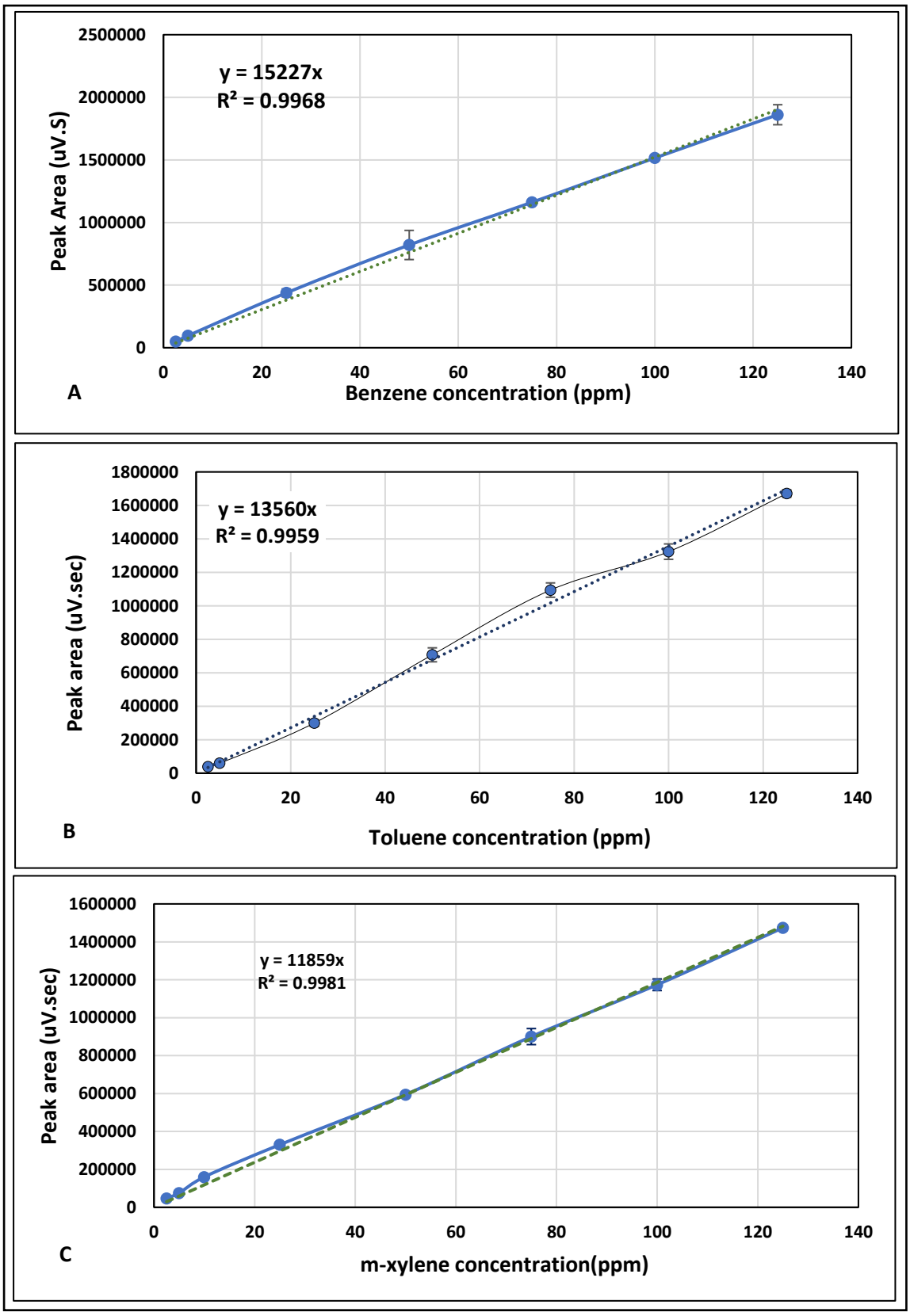


Fig. 3. 13 Calibration curves of benzene (A), toluene (B) and m-xylene (C). Seven-point calibration curves of each VOC ranging from 2.5 ppm to 125 ppm. Data is presented as the mean ± SEM (n=3). Dotted line shows theoretical coefficient of determination (R^2=1).

The high correlation coefficient ($R^2 \approx 1$) of benzene, toluene and m-xylene calibration curves demonstrated, there is a linear relationship between the VOC concentration (within 5-125 ppm concertation range) and GC peak area. Therefore, the GC/FID method was suitable for analysing air samples in the test chambers.

3.2.5. Quality control of the GC method

A Shewhart control chart (Fig. 3. 14) was produced to determine the accuracy of the GC calibration of benzene, toluene and m-xylene throughout the experimental period (detailed in section 2.12.2.3). Standard deviations obtained for the 25 ppm standard in the calibration curves were used in the Shewhart chart to set the upper control limit (UCL) and the lower control limit (LCL). UCL and LCL were three standard deviations above and below respectively from the mean concentration which is 25 ppm.

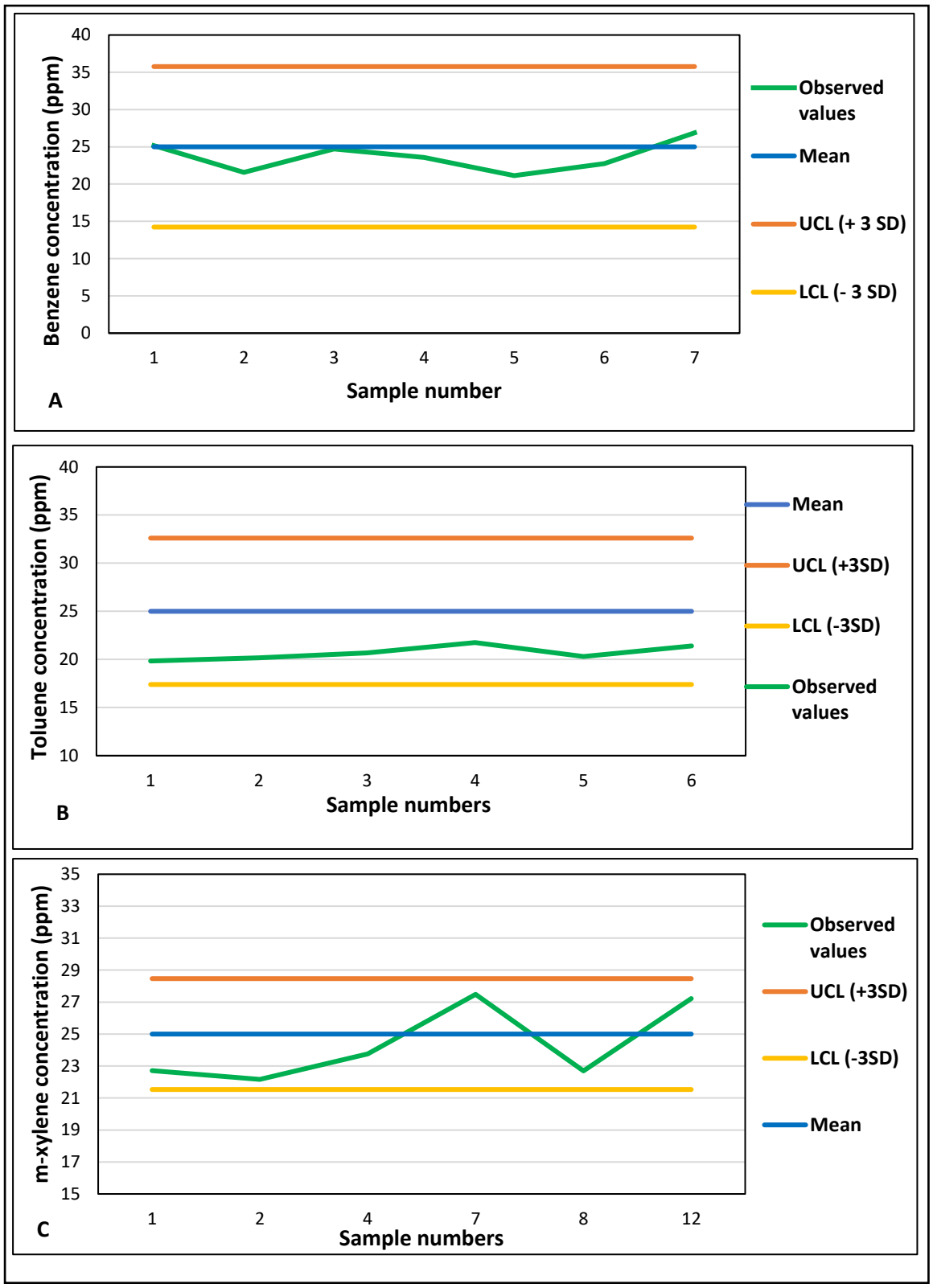


Fig. 3. 14 Shewhart chart of 25 ppm benzene (A), toluene (B) and m-xylene (C). UCL and the LCL are 3 Standard deviation (SD) above and below the mean concentration (25 ppm). 1 SD for the 25 ppm concentration in the benzene, toluene and m-xylene calibration curves equal to 3.58, 2.53 and 1.16 respectively.

According to the Shewhart charts, observed concentrations for the pure benzene, toluene and m-xylene liquid volumes which used to make 25 ppm concentration during calibration, did not exceed the UCL or LCL. Therefore, throughout the experimental period, GC calibration was accurate for all three VOCs under the used experimental conditions.

3.2.6. Possible VOC emission from the chamber materials and VOC production by plants and compost

The test chamber was made of glass, silicone sealant and adhesive covering film along with the paper left on the inside covering the area which was exposed to the inside of the tank. Except glass, the other two synthetic plastic materials were regarded as possible sources for different volatile emission which can cause interference with the monitoring VOC removal efficiency by plants in the chamber. Therefore, air samples from sealed empty chambers were analysed using ATD/GC/FID to observe possible VOC emissions (Fig. 3. 15).

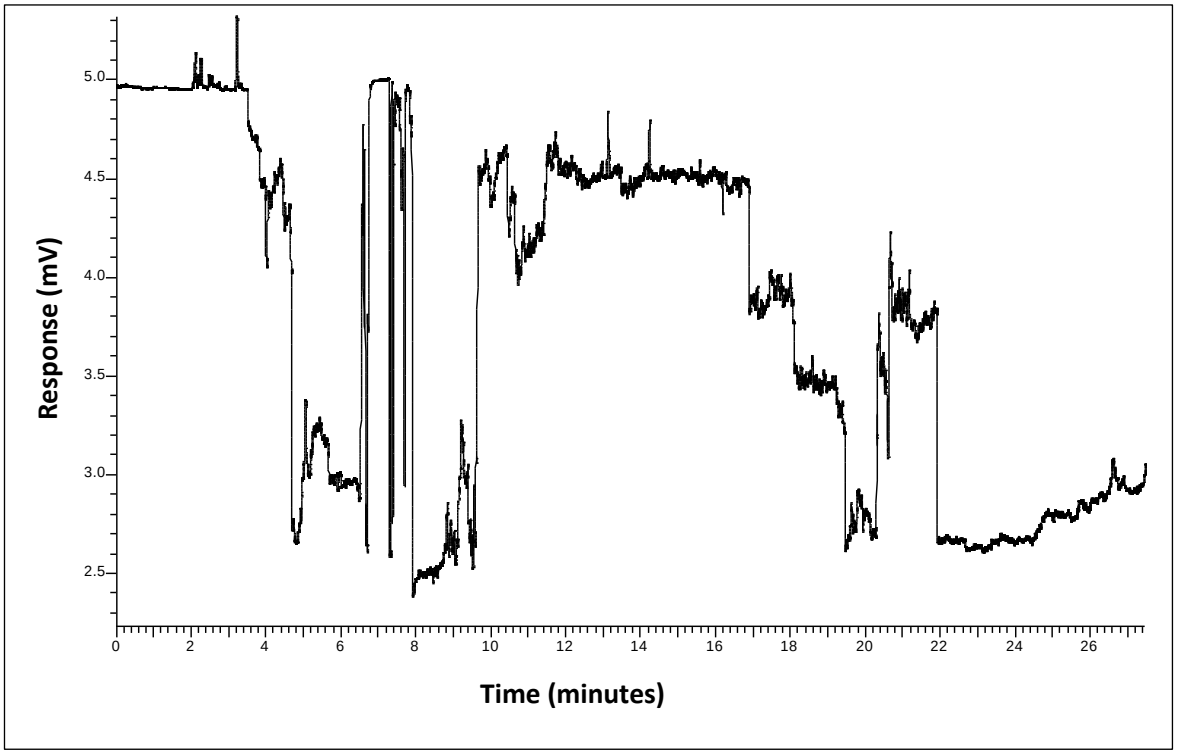


Fig. 3. 15 Gas chromatogram to show possible VOC emission by chamber materials.

According to the chromatogram (Fig. 3. 15), there was no detectable peak area for benzene, toluene, m-xylene or any unknown volatiles observed in the air samples taken from sealed empty

test chambers. Therefore, there was no measurable interference caused to VOC analysis due to chamber materials VOC emission.

Some plants and soil material (compost) produce VOCs which is known as biogenic VOC production. Therefore, plants and soil used in this study were investigated for their biogenic benzene, toluene and m-xylene production (detailed in section 2. 12.2.4) without VOC injection.

According to the chromatograms (Fig. 3. 16), there were peaks responsible for benzene, toluene and m-xylene emitted from plant monoculture with retention time 3.2, 5.1 and 7.5 minutes respectively. However, at the very low response level, the peak area which is the one proportional to the concentration of VOCs, were also very low and according to the calibration curve conducted previously for each VOCs, those peak areas represented less than 0.10 ppm concentrations. Therefore, the possible effect coming from plant biogenic benzene, toluene and m-xylene emission on the analysis of VOC removal by plants was considered as negligible.

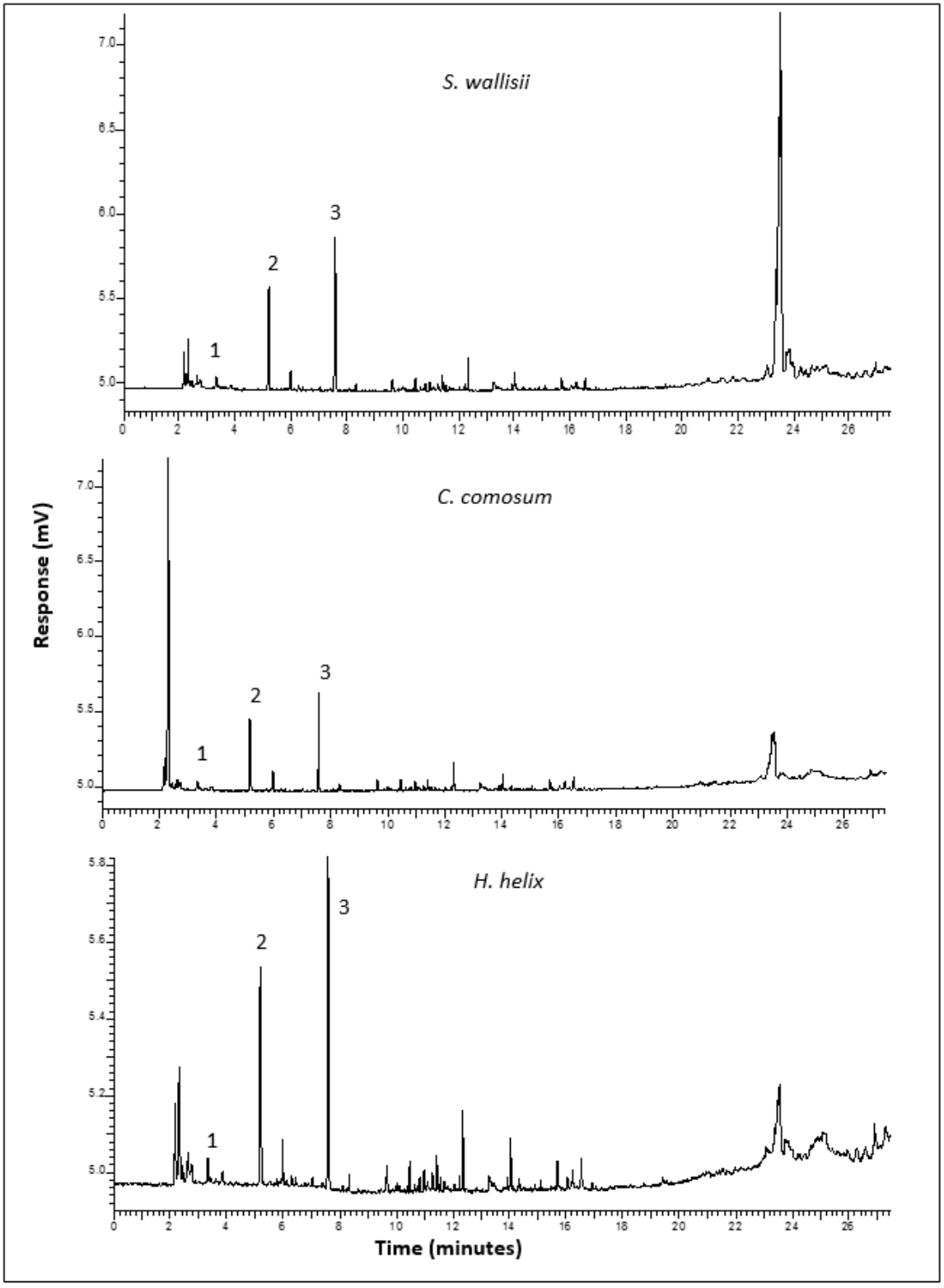


Fig. 3. 16 GC chromatographs to show possible VOC emissions from *S. wallisii*, *C. comosum* and *H. helix*; 1) benzene, 2) toluene and 3) m-xylene, peaks are marked at 3.2, 5.1 and 7.5 minutes respectively.

Air samples taken from the plant community and compost chambers also showed a very low-level emission of benzene, toluene and m-xylene (Fig. 3. 17).

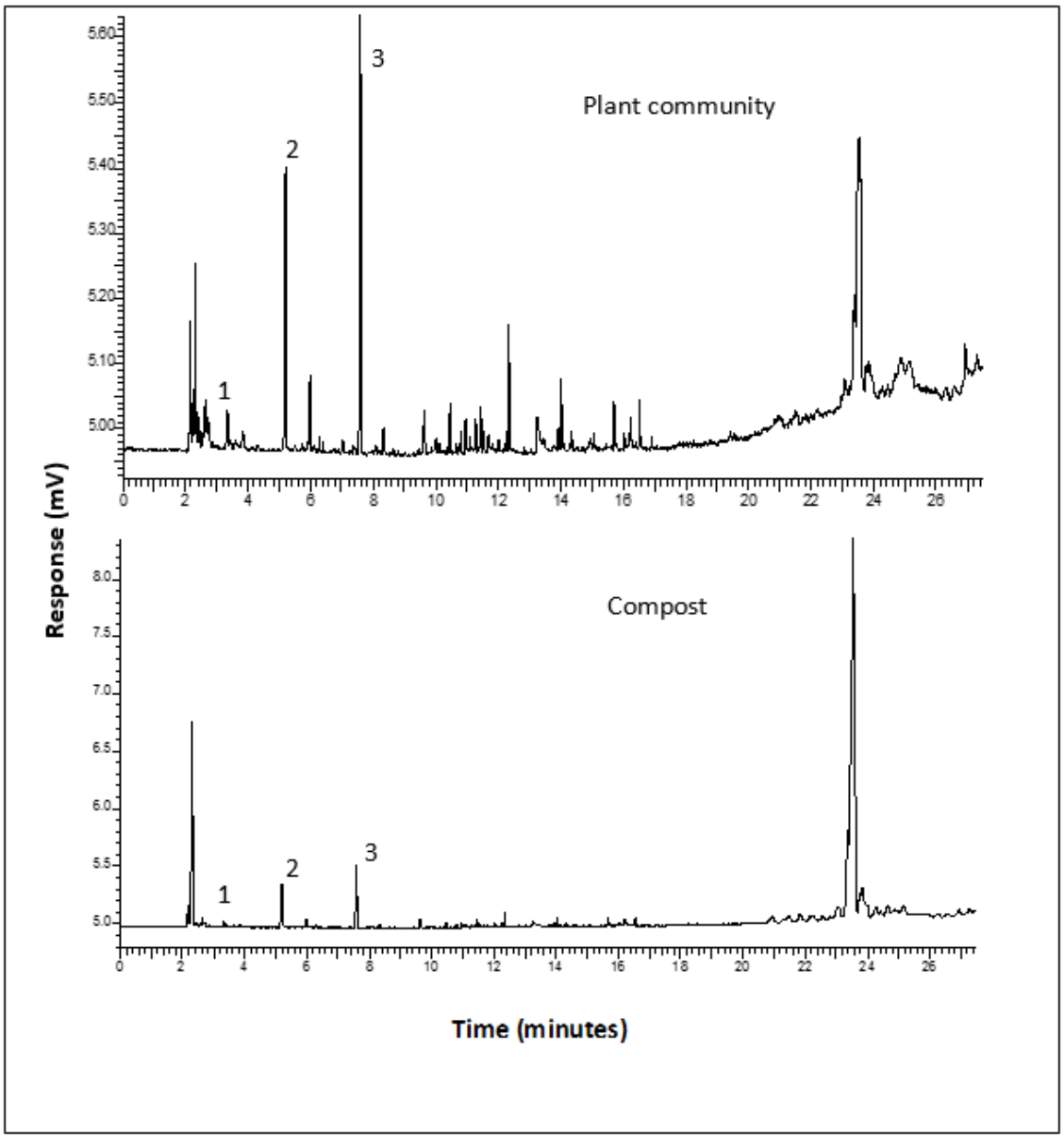


Fig. 3. 17 GC chromatograph to show possible VOC emissions from plant community and compost; benzene, 2) toluene and 3) m-xylene, peaks are marked at 3.2, 5.1 and 7.5 minutes respectively.

3.2.7. Analysis of benzene removal by plant monocultures, communities and compost

During studying 10 and 100 ppm VOC removal patterns, removal rates and time taken to the complete removal by plants and compost placed in the sealed test chambers, plants and plant growing medium in the propagating tray was considered as one unit. Therefore, the observations are based on per plant unit (i.e. per chamber). During the experiments, approximately the same size healthy plants were used and each plant and compost trays consisted equal volume of potting mixture. After the test period no visible foliar damage was observed at each of two concentrations used.

During 10 ppm benzene study, all the plants and compost removed benzene from the test chamber air. A quite similar patterns of benzene removal was observed by plant monocultures (single species), community (mixed species of *S. wallisii*, *C. comosum* and *H. helix*) and compost (Fig. 3. 18). In addition to the VOC removal by plants and compost, loss of benzene occurred due to leakage, adsorption or the possible chemical reactions inside the chamber air was analysed in the "empty chamber" /control (Fig. 3. 18). The amount of benzene loss in the control experiment (empty chamber) was deducted when calculating the rate of benzene removal by each plant system separately (Table 3. 3). The comparison of benzene removal rate was made based on the half-life time of VOC which is the time required to reduce 50% of the initial VOC concentration injected into the chamber air. In addition, the time taken to the complete removal of VOC from the test chamber air was compared (Table 3.8). Considering the benzene removal rate, no significant effect ($p=0.143$) was observed by the plant monocultures, community or compost (Table 3. 3).

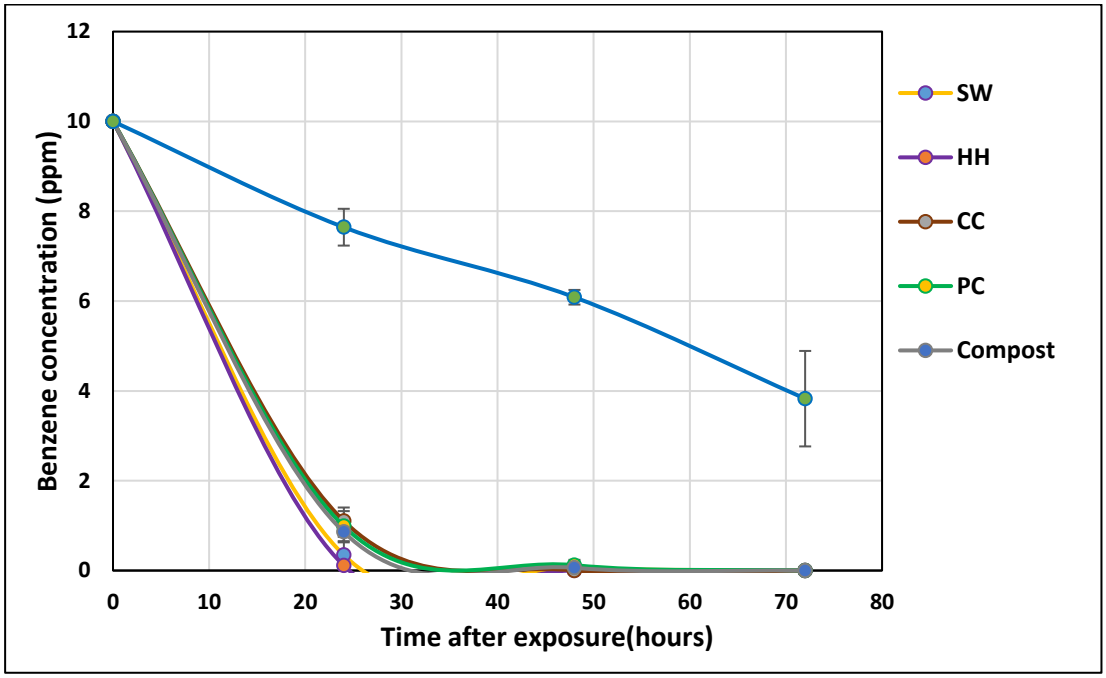


Fig. 3. 18 Benzene (10 ppm) removal by *S. wallisii* 'SW', *H. helix* 'HH', *C. comosum* 'CC', plant community 'PC' and compost in plant propagating trays (pots), relative to a plant free control 'empty chamber'. Data is presented as the mean ± SEM (n=2).

Table 3. 3 Average rate for 50% of initial 10 ppm benzene removal from test chamber air by potted plants and compost.

Plant type (n=2)	**Rate for 50% benzene removal (ppm pot^{-1} day^{-1})**
S. wallisii	8.01±0.40
H. helix	8.34±0.05
C. comosum	6.87±0.04
Plant community	7.25±0.34
Compost	6.88±0.40

All data were corrected for test chamber leakage, (Kruskal Wallis test, p=0.143).

The chamber containing *H. helix* and *S. wallisii* reached one-half ($T_{50\%}$) of the initial benzene concentration (10 ppm) within 11.05 and 11.28 hours respectively and both species were able to remove benzene from the test chamber air completely after 24.0 and 24.5 hours respectively (Fig. 3. 18). Taking into account the benzene removal rate, *H. helix* (8.34 ppm day^{-1} pot^{-1}) and *S. wallisii* (8.01 ppm day^{-1} pot^{-1}) showed the higher rates than other potted plants and compost (Table 3. 3)

at the time at which 50% of benzene had been removed from the test chambers. Potted *C. comosum*, plant community and compost removed one-half of the initial benzene concentration within 12 hours and the complete removal was performed after 30 hours from initial injection (Table 3.8). The plant community had removed benzene at the rate of 7.25 ppm day^{-1} pot^{-1} until the chamber air became one half of the initial benzene concentration. *C. comosum* and compost showed the lowest and similar rates for 50% benzene removal as 6.87 and 6.88 respectively. Therefore, based on the removal rate and time taken to the complete removal, *S. wallisii* and *H. helix* were more efficient than other potted plants in the phytoremediation of benzene at 10 ppm.

During 100 ppm benzene experiments, all the plants and compost removed benzene from the test chamber air. Different removal patterns were observed in the chambers (Fig. 3. 19), however no significant differences (p=0.078) were observed in the 100 ppm benzene removal rates by plant monocultures, community or compost (Table 3.3).

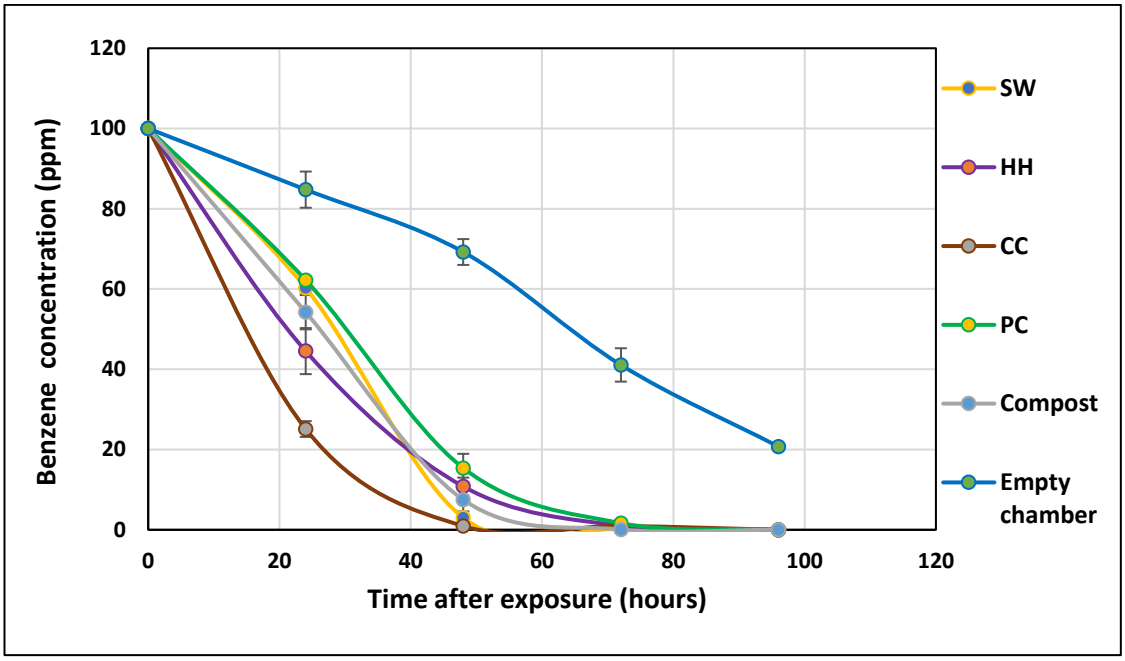


Fig. 3. 19 Benzene (100 ppm) removal capacity by *S. wallisii* 'SW', *H. helix* 'HH', *C. comosum* 'CC', plant community 'PC' and compost in plant propagating trays (pots), relative to a plant free control 'empty chamber'. Data is presented as the mean ± SEM (n=2).

Table 3. 4 Average rate for 50% of initial 100 ppm benzene removal from test chamber air by potted plants and compost.

Potted plant type (n=2)	**Rate for 50% benzene removal (ppm/pot^{-1} day^{-1})**
S. wallisii	26.98±0.05
H. helix	41.70±0.41
C. comosum	64.31±18
Plant community	25.12±0.01
Compost	31.18±0.21

All data were corrected for test chamber leakage, (Kruskal Wallis test, p=0.078).

The highest removal rate was 64.31 ppm day^{-1} pot^{-1} by *C. comosum* (Table 3.3) from the test chamber air during the half-life of benzene. Benzene concentration in the chamber air containing *C. comosum* reached its half-life within 15 hours (Fig. 3. 19) and complete removal occurred after 51 hours (Table 3.8). The plant community and *S. wallisii* removed initial 100 ppm benzene into its half-life at the fairly similar rates which were 25.12 and 26.98 ppm day^{-1} pot^{-1} respectively and it took 33 and 28.5 hours respectively to the reach half-life of benzene. After reaching the half-life, *S. wallisii* removed the benzene from the chamber completely within 52 hours and the plant community took 74 hours for complete removal (Table 3.8). *H. helix* showed comparatively a higher rate of removal at the beginning which was 41.7 ppm day^{-1} pot^{-1} rate and took only 22 hours to reach the half-life of benzene. However, the reduction rate declined at the later stage and it took 72 hours to remove the benzene from the chambers completely.

The half-life of 100 ppm benzene was 26 hours in the chamber containing compost and the benzene removal rate was 31.18 ppm day^{-1} pot^{-1} during that time. It was taken 62.5 hours for the complete removal of benzene from the test chamber air by compost. Therefore, based on the removal rate and the time taken for the complete removal of 100 ppm benzene from the chamber air, *C. comosum* and *S. wallisii* showed a higher phytoremediation efficiency than *H. helix*, plant community and compost.

The rate of benzene removal observed by each plant monoculture, community and the compost during half-life of 100 ppm benzene study were higher than the rates observed for 10 ppm benzene removal. Though in the 10 ppm experiment, compost had a relatively slower performance to remove benzene from the chamber air than other potted plants used (except *C. comosum*), in the 100 ppm experiment compost showed a relatively a higher removal rate during half-life of benzene. This indicated VOC removal capacity by the potting mixture can be different based on the concentration of VOC in the environment. Since the plant trays contained equal volume of potting mixture as well as the compost tray, these results suggested the majority of benzene were removed by the plant growing medium (potting mixture) during high concentration of benzene removal.

Considering the complete removal of benzene from chamber air*, S. wallisii* was able to remove 10 and 100 ppm benzene from chamber air completely within 24.5 and 52 hours respectively. Therefore, compared to the time duration taken for the complete removal by other potted plants and compost*, S. wallisii* showed a higher phytoremediation efficiency for high and low levels of benzene.

3.2.8. Analysis of toluene removal by plant monocultures, communities and compost

All the plants and compost reported the toluene removal from the test chamber air during 10 ppm toluene study (Fig. 3. 20). The half-life times of toluene removed by *S. wallisii*, plant community and *H. helix* were 10.7, 11.25 and 12.4 hours respectively. During this half-life removal stage, 10.6, 10.27 and 9.25 ppm day^{-1} pot^{-1} toluene removal rates were observed by each chamber respectively (Table 3. 5). *H. helix* declined its toluene removal efficiency after reaching less than 1 ppm concentration in the chamber air (Fig. 3. 20) and required another 50 hours for complete removal (Table 3. 8). *S. wallisii* and the plant community achieved the complete removal after 23.0 and 23.5 hours respectively which were earlier than other potted plants. Overall this indicated that *S. wallisii* and the plant community were more efficient than other potted plants and compost in the phytoremediation of toluene. *C. comosum* showed a lower rate (5.16 ppm pot^{-1} day^{-1}) during one half of toluene removal which performed at 21 hours. Within 48 hours,

chamber air containing *C. comosum* became less than 1.5 ppm toluene level. Similar to the toluene removal by *H. helix* at the later stage (after reaching half-life of toluene) as explained above, *C. comosum* showed a very slow removal rate of toluene for another ≈40 hours until removed completely. This showed, at lower concentrations of toluene, *C. comosum* and *H. helix* reduced their phytoremediation efficiency.

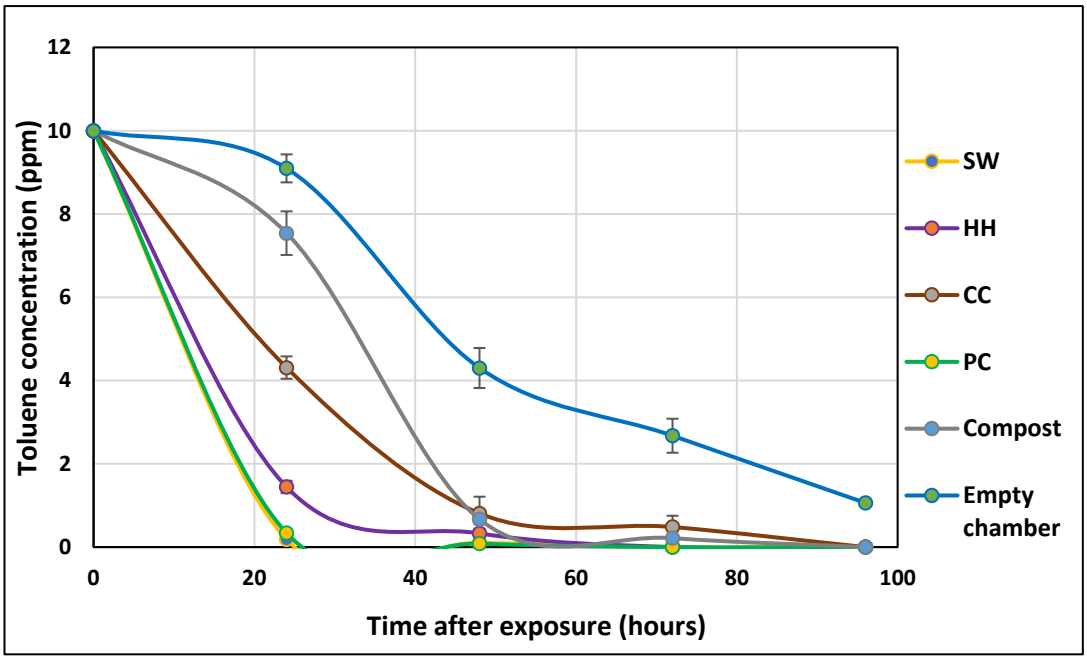


Fig. 3. 20 Toluene (10 ppm) removal by *S. wallisii* 'SW', *H. helix* 'HH', *C. comosum* 'CC', plant community 'PC' and compost in plant propagating trays (pots), relative to a plant free control 'Empty chamber'. Data is presented as the mean ± SEM (n=2).

Table 3. 5 Average rate for 50% of initial 10 ppm toluene removal from test chamber air by potted plants and compost

Potted plant type (n=2)	**Rate for 50% toluene removal (ppm pot^{-1} day^{-1})**
S. wallisii	10.60±0.06
H. helix	9.25±0.25
C. comosum	5.16±0.37
Plant community	10.27±0.12
Compost	1.77±0.21

All data were corrected for test chamber leakage, (Kruskal Wallis test, p=0.068).

The potting mix; compost maintained the slowest rate which was 1.77 ppm pot^{-1} day^{-1} during one half of toluene (10 ppm) removal. By performing a few times higher toluene removal rates by all plants than compost during half-life of toluene indicated that the presence of plants inside the chambers maximised the rate of removal. Thus, this observation clearly evidenced that the plants contributed to accelerating the rate of toluene phytoremediation. Compost removed the toluene level by 50% after 34 hours and the complete removal after 54 hours. (Table 3. 8) Compared to 10 ppm benzene removal by compost from the test chamber air, 10 ppm toluene removal rate by compost was lower than one-third of benzene removal rate. Also, similar to 10 ppm benzene removal, no significant differences (p=0.068) was observed between 10 ppm toluene removal rates performed by plant monocultures, community or compost (Table 3. 5).

During 100 ppm toluene study, all the plants and compost removed toluene from the test chamber air (Fig. 3. 21) and showed a higher removal rate during half-life time of initial 100 ppm toluene (Table 3. 6). No significant differences (p=0.254) were observed in the toluene removal rates performed by plant monoculture, community or compost. Removal rates declined from higher to lower as follows 78. 01 (*S. wallisii*), 76.05 (*C. comosum*), 70.91 (compost), 70.11 (*H. helix*) and 55.67 (plant community) ppm pot^{-1} day^{-1} respectively. This indicates the majority of toluene was declined by the plant growing medium. During one-half toluene removal, *S. wallisii,*

C. comosum, *H. helix* and compost performed it in less than 15 hours and only the plant community took 18.5 hours, thus the plant community performed at the slowest removal rate.

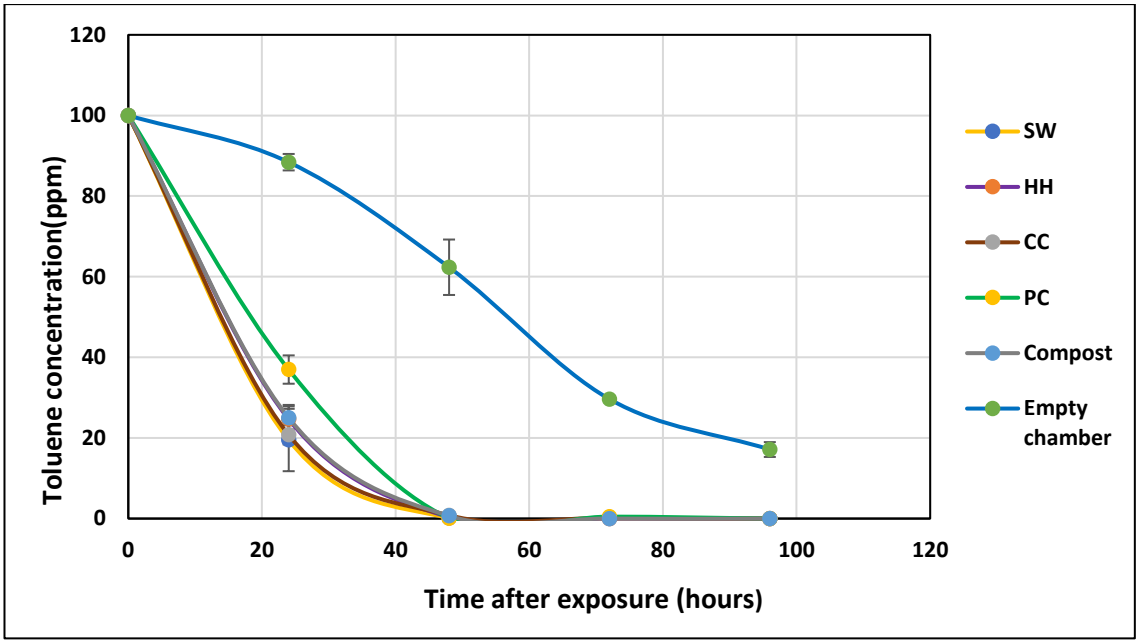


Fig. 3. 21 Toluene (100 ppm) removal by *S. wallisii* 'SW', *H. helix* 'HH', *C. comosum* 'CC', plant community 'PC' and compost in plant propagating trays (pots), relative to a plant free control 'Empty chamber'. Data is presented as the mean ± SEM (n=2).

Table 3. 6 Average rate for 50% of initial 100 ppm toluene removal from test chamber air by potted plants and compost

Potted plant type (n=2)	**Rate for 50% toluene removal (ppm/pot^{-1} day^{-1})**
S. wallisii	78.01±11.05
H. helix	70.11±3.53
C. comosum	76.05±2.20
Plant community	55.67±5.02
Compost	70.91±4.83

All data were corrected for test chamber leakage, (Kruskal Wallis test, p=0.254).

Comparatively to the phytoremediation of 100 ppm benzene from the chamber air, all plants and compost showed a higher removal rate and complete removal of toluene occurred around 49

hours by all pots (Table 3. 8). These results indicated the phytoremediation efficiency of VOC by plants may depend on the type of VOC.

During 10 ppm toluene study, compost conducted the slowest removal rate though, compost performed relatively a higher removal rate in the 100 ppm toluene study. Similar observations were found during benzene removal by compost as well. Therefore, this observation suggests the capability of potting mix to remove VOC from the air is increased when the initial concentration of VOC increased.

3.2.9. Analysis of m-xylene removal by plant monocultures, communities and compost

All the plants and compost removed m-xylene from the test chamber air as explained below. According to the 10 ppm m-xylene removal experiment (Fig. 3. 22), all plant chambers and the compost chambers removed 50% of m-xylene within 13-14 hours. Similar rates were observed for m-xylene removal by all plants and compost fluctuating around 3 ppm pot^{-1} day^{-1} (Table 3. 7) and no significant difference ($p=0.953$) was observed between m-xylene removal rates. m-xylene level in all chambers reached less than 1 ppm after 27 hours of initial injection, however, after that, there was a very slow removal rate of m- xylene in all chambers. The slow removal rate at the lower level (later stage) was also observed for phytoremediation of toluene and benzene. Decline of the removal rate at the lower concentrations of VOC indicated the progressive reduction of VOC concentration inside the chamber air to be removed by plants and compost. Relatively, *H. helix* showed the slightly higher removal rate, 3.47 ppm pot^{-1} day^{-1} than other plants during half-life of m-xylene removal in 10 ppm study. Based on the complete removal, the chamber containing *S. wallisii* removed m-xylene completely after 68 hours which indicated slightly higher phytoremediation efficiency than all other potted plants and compost which required approximately 92 hours to remove 10 ppm m-xylene (Table 3. 8). However, all plants conducted relatively lower removal rates during 10 ppm m-xylene removal than the removal rates observed

for benzene and toluene. Therefore, these results suggest the selected plants may not be better selection for phytoremediation of low concentration of m-xylene from air.

During 10 ppm benzene and toluene studies, removal rates by compost were less than or similar to the removal rate observed by plants. However, observations from the m-xylene study indicated removal rate performed by compost were slightly higher than all plants (except *H. helix*). This suggested exposing to m-xylene may cause declining of remediation efficiency in plants.

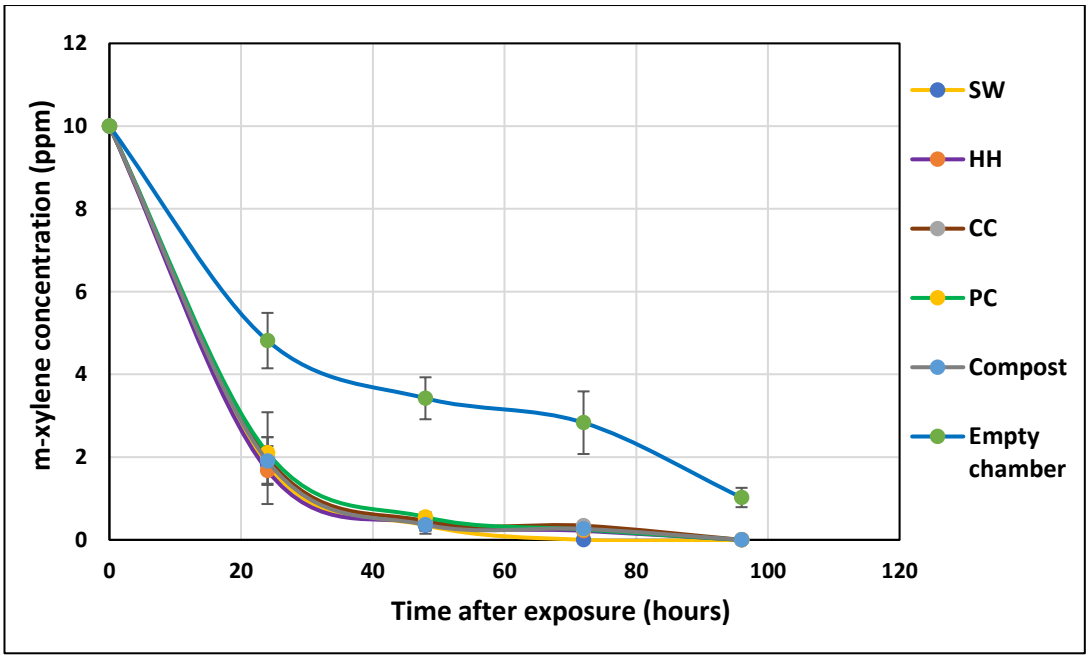


Fig. 3. 22 m-xylene (10 ppm) removal by *S. wallisii* 'SW', *H. helix* 'HH', *C. comosum* 'CC', plant community 'PC' and compost in plant propagating trays (pots), relative to a plant free control 'Empty chamber'. Data is presented as the mean ± SEM (n=2).

Table 3. 7 Average rate for 50% of initial 10 ppm m-xylene removal from test chamber air by potted plants and compost

Potted plant type (n=2)	**Rate for 50% m-xylene removal (ppm/pot^{-1} day^{-1})**
S. wallisii	3.33±0.41
H. helix	3.47±0.22
C. comosum	3.13±1.03
Plant community	3.09±0.38
Compost	3.40±0.34

All data were corrected for test chamber leakage, (Kruskal Wallis test, p=0.953).

Plant monoculture and community showed almost similar removal rate for the initial 10 ppm m-xylene injection (Table 3. 7). According to phytoremediation of 100 ppm m-xylene (Fig. 3. 23), all plants and control chambers removed m-xylene from the chamber air. Slightly similar removal pattern and the very close removal rates observed during for 50% of m-xylene removal (Table 3. 8). Likewise, 100 ppm benzene removal, *C. comosum* showed the highest removal rate (76.25 ppm pot^{-1} day^{-1}) of m-xylene. The lowest rate observed during half-life of m-xylene was by the plant community which was 63.2 ppm pot^{-1} day^{-1}. However, no significant differences (p=0.238) were observed among the removal rates performed by different chambers (Table 3. 8). A very close half-life of m-xylene was observed in the chambers containing plants and compost varying between 14- 16 hours. Similar to the results obtained for 10 ppm m-xylene removal, removal rate was declined at the lower concentration (later stage) and the pots took nearly 100 hours to remove m-xylene completely from the chamber air (Table 3. 8).

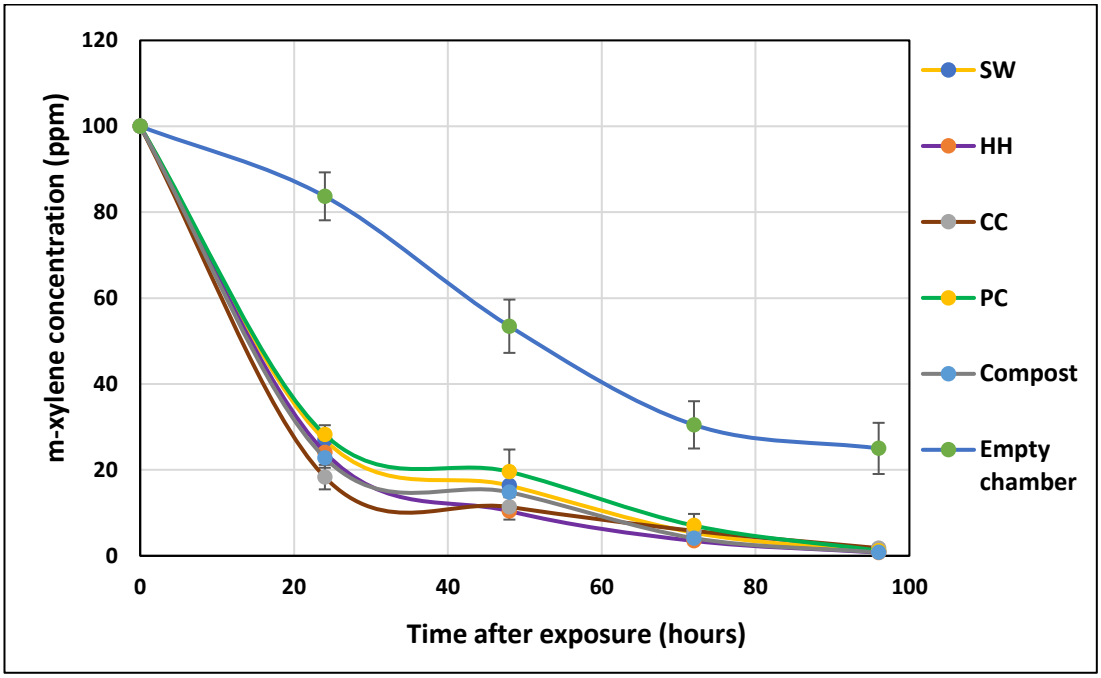


Fig. 3. 23 m-xylene (100 ppm) removal by plant *S. wallisii* 'SW', *H. helix* 'HH', *C. comosum* 'CC', plant community 'PC' and compost in plant propagating trays (pots), relative to a plant free control 'Empty chamber'. Data is presented as the mean ± SEM (n=2).

Table 3. 8 Average rate for 50% of initial 100 ppm m-xylene removal from test chamber air by potted plants and compost

Potted plant type (n=2)	**Rate for 50% m-xylene removal (ppm/pot^{-1} day^{-1})**
S. wallisii	65.45±1.02
H. helix	67.52±1.04
C. comosum	76.25±3.74
Plant community	63.20±2.83
Compost	69.65±4.05

All data were corrected for test chamber leakage, (Kruskal Wallis test, p=0.238).

As a comparison of 10 ppm and 100 ppm m-xylene removal rate, plant community showed the lowest rates in both experiments as 3.09 and 63.2 ppm pot^{-1} day^{-1} respectively. It was noticed that at the lower concentration of m-xylene (later stage), the removal rate declined in all chambers. This was also observed during benzene and toluene removal. This is probably because at the

beginning, all plant and control chambers achieved a higher removal rate by stimulating the system due to initial exposure to VOC. However, at the lower concentration in the later stage level, plant and soil may decline the rate of removal because there was no additional amount of VOC to stimulate the system. In addition, any adsorbed VOC by soil (compost) at the initial stage may be desorbed into the chamber air in the later stage which may cause low removal rates by plants.

The removal rate of m-xylene by compost during the half-life was slightly higher than the plants in 100 ppm (except *C. comosum*) experiment. This probably happened due to exposure to a higher concentration of m-xylene, that may cause high abiotic stress to plants and thus the initial removal rate (phytoremediation efficiency) would be slower in the plant containing chambers. In addition, these results demonstrated the capability of potting mixture to remove high concentration of m-xylene from the chamber air. Similar observations were also found during 100 ppm benzene and toluene studies.

3.2.10. Comparison of results and Conclusions

At the low (10 ppm) and high (100ppm) concentrations of benzene, toluene and m-xylene experiments, all plant monocultures, community and compost removed gaseous benzene, toluene and m-xylene from the test chamber air. However, no significantly different removal rates were observed during BTX removal experiments. Based on the removal rates and the time taken for the complete removal of VOC from the test chamber air, study showed some plants performed better removal efficiencies while some plants showed a relatively lower removal performance for the three VOCs tested. In addition, study showed that plants performed VOC removal efficiency differentially for the low and high concentrations of the same VOC.

At 10 ppm benzene study, *S. wallisii* and *H. helix* performed higher removal rates while *S. wallisii* followed by the plant community achieved the best removal rates for 10 ppm toluene removal. Therefore, those plants are more suitable for removing lower concentrations (≈10 ppm) of benzene and toluene from indoor air. In 10 ppm benzene removal, all plants except *C. comosum*

conducted higher removal rates than the compost. The rate observed for the removal of toluene by *C. comosum* was equal to the rate conducted by compost. Also, all plants performed better than compost during 10 ppm toluene study. Therefore, this study showed that plants enhanced the phytoremediation of low-level benzene and toluene (except *C. comosum*) from air.

Interestingly, in 10 ppm m-xylene experiment, compost performed a considerably higher removing rates than plants performed (except *H. helix*). Also, plants removed m-xylene from the chamber air at a lower rate than the rate they performed during 10 ppm benzene and toluene phytoremediation. Therefore, the selected plant species; *S. wallisii*, *H. helix and C. comosum* may not be a good selection for efficient removal of lower concentrations of m-xylene from the air.

The best removal rate of 100 ppm benzene and m-xylene from chamber air was observed by *C. comosum* while the best removal rate of 100 ppm toluene was performed by *S. wallisii* followed by *C. comosum*. Interestingly, at the higher concentration (100 ppm) of benzene, toluene and m-xylene, plant community reported the lowest removal rates than the plant monocultures. Probably this may happen due to a few reasons. Exposure to a high concentration of VOC, plants get a high abiotic stress which reduce the plant and roots growth (Gerhardt *et al.*, 2009). In the mixed culture system, plants acquired different abiotic stress levels based on the plant species which may probably higher than the plant stress in a monoculture. Also, in a plant community, mixed plant species conducting interspecies competition for the nutrients is higher than the intraspecific competition in the monocultures (Hortal *et al.*, 2017). In addition, the combination of selected three plant species may not be the best selection for removing high concentration of tested VOCs. Thus, due to single or a combination of these factors, the overall VOC removal rate by the plant community was reduced compared to the monocultures.

The time taken to reach the half-lives and the complete removal of VOC from chamber air were compared (Table 3. 9). At the 10 ppm VOCs concentration all the plants removed the 50% of VOCs within 10.7 to 13.7 hours except 10 ppm toluene removal by *C. comosum* and compost which took 21 and 34 hours respectively (Table 3. 9). In the same experiment, the maximum time taken to reach less than 1 ppm concentration inside the chamber was 46 hours observed for the toluene

removal by compost. In all other 10 ppm experiments, plants and compost removed VOC concentration completely or into less than 1 ppm concentration within less than 46 hours. Comparatively, it was observed that a long time was required for the complete removal of 10 ppm m-xylene by plants and compost than the time taken for the complete removal of 10 ppm benzene and toluene.

Table 3. 9 Half-life of VOC and the time required for the complete removal from the test chamber

VOC type	VOC dose (ppm)	Type of potted plants system									
		S. wallisii		*C. comosum*		*H. helix*		Plant community		Compost	
		Half-life (hours)	Complete removal (hours)	Half-life (hours)	Complete removal (hours)	Half-life (hours)	Complete removal (hours)	Half-life (hours)	Complete removal (hours)	Half-life (hours)	Complete removal (hours)
Benzene	**10**	**11.28±0.1**	**24.5±1.5**	**12.0±0.5**	**30±2.9**	**11.05±0.2**	**24±1**	**12.0±0.10**	**34±1.5**	**12.0±0.0**	**31.5±1.1**
	100	**28.5±0.4**	**52±0.5**	**15±2.1**	**51±1.8**	**22±0.3**	**72±1.1**	**33±2.1**	**74±3.3**	**26±1.1**	**62.5±2.2**
Toluene	**10**	**10.7±0.3**	**23±1.5**	**21±2.2**	**96±3.5**[2]	**12.4±0.5**	**65±2.0**[1]	**11.25±0.25**	**23.5±1.0**	**34±1.5**	**54±3.1**[3]
	100	**13.5±0.4**	**48±0.8**	**14.1±0.6**	**51±1.1**	**14.8±0.1.5**	**50±0.8**	**18.5±2.1**	**48±0.9**	**14.3±0.8**	**49±0.1**
m-xylene	**10**	**13.4±0.9**	**68±1.2**[4]	**13.7±0.5**	**92±0.1**[4]	**13.1±0.8**	**91±0.8**[4]	**13.6±0.8**	**92±0.4**[4]	**13.3±0.2**	**92±0.4**[4]
	100	**16±0.6**	**≈100**	**14±1.1**	**≈100**	**15±0.8**	**≈100**	**16.8±1.5**	**≈100**	**14±1.5**	**≈100**

[1] Toluene level declined less than 1 ppm after 26 hours of initial induction, however it took 65 hours for the complete removal

[2] Toluene level declined less than 1 ppm after 45 hours of initial induction, however it took 96 hours for the complete removal

[3] Toluene level declined less than 1 ppm after 46 hours of initial induction, however it took 54 hours for the complete removal

[4] m-xylene level declined less than 1 ppm after 23-35 hours of initial induction, however it took ≈92 hours for the complete removal (except *S. wallisii*)

According to the complete removal of 100 ppm VOC from the test chambers, all the plants and compost took a minimum of 48 hours or more than 48 hours. At the 100 ppm VOC study, *C. comosum* commonly performed a better half-life removal rate for all three VOCs by removing 50% of all single VOC within 14-15 hours (Table 3.8). Therefore, *C. comosum* grown in the potting mixtures is suitable choice for remediate high concentrations of VOC from air.

Similar to the observations in complete removal of 10 ppm m-xylene, complete removal of 100 ppm m-xylene by plants and compost took relatively more time than the time required for the removal of 100 ppm benzene and toluene (except 100 ppm toluene removal by *C. comosum*). Therefore, based on the time taken for the complete removal of 10 ppm and 100 ppm m-xylene, this study showed that the selected three plant species and plant mixed culture (community) are not a better option to remove m-xylene from indoor air.

As mentioned previously, the plant community showed a lower phytoremediation efficiency at 100 ppm VOC concentrations, by maintaining longer half-lives than plant monocultures. Therefore, the findings suggested plant monocultures are better option to remediate high concentrations of VOCs than communities.

Results from this study showed that higher removal rates performance by most of plant chambers than compost chamber during 10 ppm benzene and toluene removal experiments. Probably this occurred because at the lower concentrations, plant faced a lower stress, therefore a higher plant-soil microorganism interaction conducted. Thus, the presence of plants accelerates phytoremediation of lower concentration of benzene and toluene from the chamber air. Opposite to this observation, at the higher concentration of VOC, compost also showed high removal rates. Each plant and compost trays contained a similar volume of potting mixture. Therefore, at 100 ppm concentration, potting medium (compost) performed as the main contributor to remove VOC at the higher concentration.

Compost/ soil has the ability to adsorb the high concentrations of VOC from the air and act as a VOC sink (Ramirez *et al.*, 2010). But due to exposing to a higher concentration of VOC, plants may

reduce their tolerance against the VOC stress, therefore phytoremediation efficiency by plants can be reduced. Therefore, the findings suggest that the phytoremediation efficiency depended on several factors such as the type of VOC, the concentration of VOC, type of plant species used and the plant growing medium.

As a summary, in each experiment, the highest remediation rate was performed by a plant monoculture: *S. wallisii, H. helix* or *C. comosum.* In overall, all plant monocultures conducted higher removal rates of benzene, toluene and m-xylene than the plant community, except 10 ppm toluene and benzene removal. Therefore, these findings indicated that the plant monocultures have higher efficiency to remediate VOC from the air than the plant community. Also, our findings showed that plants are the main contributor of VOC removal at the lower concentration, however at the higher concentration of VOC, potting mix was the main contributor of VOC removal.

Chapter 4. Isolation and Identification of VOC degrading bacteria from rhizosphere and compost

4.1. Introduction

Bacterial identifications based on the traditional culture methods still play an important role in research because culture-based identification reflects the composition of unknown microbiomes to some extent. In addition, it facilitates the direct isolation of bacteria from the samples. In this study, unknown bacteria were isolated from the rhizosphere of VOC exposed and non-exposed (fresh/control plants) *S. wallisii, C. comosum* and *H. helix,* and fresh compost (VOC unexposed). Exposing plants to VOC, rhizosphere soil extraction and isolating VOC degrading bacteria from soil samples are detailed in sections 2.3, 2.4 and 2.11 respectively. To identify them at the genus level, morphological, biochemical and physiological tests were conducted on each bacterium (detailed in section 2.13.1 and Appendix 02). Based on the culture characteristics of each bacterium, their taxonomy was predicted using Bergey's manual of determinative bacteriology, 9th edition (Bergey *et al.*, 1994). Then the 16s rRNA gene from these bacteria was sequenced and mapped against the Gene Bank database in NCBI to identify them at the species level (detailed in section 2.13.2). The collaborative approach with both culture-dependent and independent identifications enhanced the understanding of VOC degrading bacteria present in the rhizosphere and compost samples.

4.2. Results

4.2.1. Isolation and identification of VOC degrading bacteria based on culture methods

Bacteria in soil possess hydrocarbon-degrading enzymes such as monooxygenase, dioxygenase and dehydrogenase (Djokic *et al.*, 2011; Ferrera-Rodríguez *et al.*, 2013; Gianfreda, 2015). Research showed that the different taxonomical and functional bacterial compositions between contaminated and the uncontaminated land, and these differences occurred mainly because a higher bioremediation activity takes place in the contaminated sites than noncontaminated soil (Barragán *et al.*, 2008; Djokic *et al.*, 2011; Ferrera-Rodríguez *et al.*, 2013; Gianfreda, 2015). A higher proportion of hydrocarbon-degrading bacteria inhabit in the rhizosphere of plants grown in the oil-contaminated lands than the plants grown in the non-contaminated area (Ferrera-

Rodríguez *et al.*, 2013; Seyedi *et al.*, 2013; Yergeau *et al.*, 2014). This suggests, there can be differences between the rhizosphere bacterial composition in the plants exposed and non-exposed to gaseous VOC. Following the incubation period, the presence of bacterial growth was observedFig. 4. 1a) after incubating the rhizosphere soil inoculated MSA under the gaseous VOC while no bacterial growth occurred (Fig. 4. 1b) in the negative-control experiments, where the same soil suspension used from the above experiments were inoculated on MSA and incubated under the same conditions without VOC in the air (Table 4. 1).

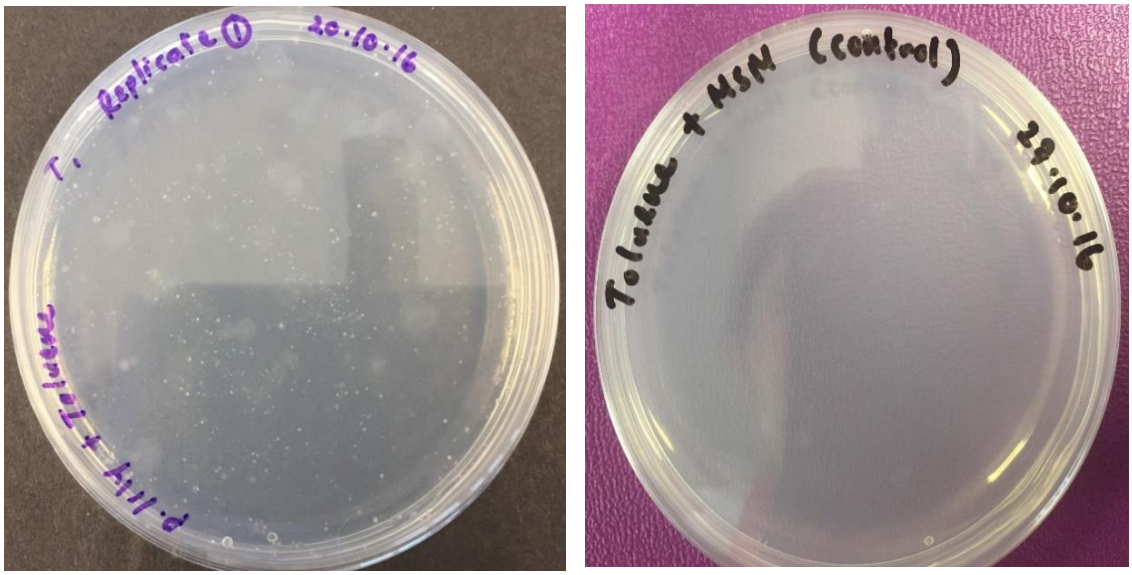


Fig. 4. 1a and **Fig. 4.** 1b Presence of bacterial colonies and no (absence) bacterial colonies on the MSA inoculated *S. wallisii* rhizosphere soil suspension, incubated under gaseous toluene.

Table 4. 1 Total number of bacterial colonies observed in the MSA plates following incubation under the gaseous VOC conditions

VOC treatment on soil inoculated MSA	**Plant species used in the batch experiment**	**Total number of colonies observed on MSA[1]**		
		VOC treated[2]	**VOC untreated[3]**	**Fresh compost**
Benzene	*S. wallisii*	0	8	4
	C. comosum	10	0	
	H. helix	3	4	

VOC treatment on soil inoculated MSA	Plant species used in the batch experiment	Total number of colonies observed on MSA[1]		
		VOC treated[2]	VOC untreated[3]	Fresh compost
Toluene	*S. wallisii*	4	11	3
	C. comosum	6	9	
	H. helix	5	9	
m-xylene	*S. wallisii*	5	0	0
	C. comosum	7	6	
	H. helix	5	0	

[1]-This is the total number of colonies (99 colonies) counted on MSA after incubation period, during identification them through the culture and molecular methods, only ≈50 colonies were selected for the tests

[2]- Plants were exposed to gaseous VOC for four weeks during batch experiment

[3]- Plants were non-exposed to VOC for four weeks during batch experiment

To reduce the time and cost required to test bacteria, only a limited number of colonies were selected. In total, 24 bacteria were picked from the rhizospheres where the plants were exposed to 100 ppm of VOCs (Table 4. 2). In addition, 29 bacteria were picked from the control plants rhizosphere where the plants had not been exposed to VOC (Table 4. 3). Based on the culture methods, these bacteria were classified up to the genus level.

The MSA medium did not contain a carbon or energy source for the bacteria. The plates inoculated with the same soil suspensions but incubated under no VOC supplement (negative control) showed no bacterial growth. This showed absence of bacterial growth was resulted by the absence of a carbon and energy sources in the medium. Presence of bacterial growth on the MSA when the incubating environment contained VOC concluded the capability of rhizosphere bacteria to utilize benzene, toluene and m-xylene as their sole carbon and energy source.

Therefore, isolated bacteria can phytoremediate gaseous benzene, toluene and m-xylene from the air. In detail, four bacteria from *S. wallisii,* fourteen from *C. comosum* and six from *H. helix* were isolated and analysed with the classical microbiology culture tests. Based on these culture tests observations, the taxonomy of each unknown bacterium was predicted (Table 4. 2).

Table 4. 2 Culture-based identifications of VOC degrading bacteria isolated from VOC treated plants' rhizosphere (Bergey *et al*. 1994).

	Isolate tag	Shape	Gram staining	Spores	Oxidase	Catalase	Motility	O/F test	Glucose acid	Anaerobic growth	Acid fast	Predicted genus
Bacteria isolated from *S. wallisii*	SWT1	R	+	-	-	+	-	-	+	-	-	*Agromyces*
	SWT3	R	+	-	-	+	-	O	+	-	-	*Microbacterium*
	SWX1	R	-	ND	+	+	+	O	+	-	ND	*Pseudomonas*
	SWX2	R	-	ND	+	+	+	O	+	-	ND	*Pseudomonas*
Bacterial isolated from *C. comosum*	CCB1	R/S	+	-	-	+	-	-	+	-	-	*Agromyces*
	CCB2	R	+	+	-	+	+	F	+	-	-	*Bacillus*
	CCB3	R/S	+	-	+	+	-	O	+	-	-	*Micrococcus*
	CCB4	R/S	+	-	-	+	-	F	+	-	-	*Microbacterium*
	CCB5	R	-	ND	-	+	-	-	-	-	ND	*Brucella*
	CCB6	R	+	-	-	+	-	O	-	-	-	*Arthrobacter*

	Isolate tag	Shape	Gram staining	Spores	Oxidase	Catalase	Motility	O/F test	Glucose acid	Anaerobic growth	Acid fast	Predicted genus
	CCT1	R	+	-	-	+	-	O	-	-	-	*Arthrobacter*
	CCT2	R/S	+	-	-	+	-	O	+	-	-	*Microbacterium*
Bacteria isolated from *C. comosum*	CCT3	R/S	+	-	-	+	+	O	+	-	-	*Microbacterium*
	CCT4	R/S	+	-	-	+	-	F	+	-	-	*Microbacterium*
	CCX1	R	-	ND	+	+	+	O	+	-	ND	*Pseudomonas*
	CCX2	R	-	ND	+	+	+	O	+	-	ND	*Pseudomonas*
	CCX3	R	-	ND	-	+	+	O	+	-	ND	*Pantoea*
	CCX4	R	-	ND	+	+	+	O	+	-	ND	*Pseudomonas*
Bacteria isolated from *H. helix*	HHB1	R	+	-	-	+	-	O	+	-	-	*Rhodococcus*
	HHT1	R	+	-	-	+	-	O	+	-	-	*Microbacterium*
	HHT2	R	+	-	-	+	-	F	+	-	-	*Microbacterium*
	HHT3	R	+	-	-	+	+	O	+	-	-	*Microbacterium*

	Isolate tag	Shape	Gram staining	Spores	Oxidase	Catalase	Motility	O/F test	Glucose acid	Anaerobic growth	Acid fast	Predicted genus
	HHX1	R	-	ND	+	+	+	O	+	-	ND	*Pseudomonas*
	HHX2	R	-	ND	+	+	+	O	+	-	ND	*Pseudomonas*

Abbreviations are SW *= S. wallisii,* CC*, C. comosum,* HH*= H. helix,* B= benzene, T=toluene, X= m-xylene, R=rod shaped (bacillus), S= spherical /coccus, O= oxidation, F=fermentation, + = strains are positive, - = strains are negative, S = sphere (coccus), ND= not detected.

Following the incubation period and sub-culturing process, four VOC degrading bacteria from *S. wallisii* were isolated. In detail, there were two toluene-degrading and two m-xylene degrading bacteria. Using culture identification tests, toluene-degrading bacteria were identified belonging to the genus *Agromyces* (SWT1) and *Microbacterium* (SWT3) while m-xylene degrading bacteria belonged to the genus *Pseudomonas* (SWX1 and SWX2). However, no benzene degrading bacteria were isolated from *S. wallisii*.

During the isolation of bacteria from VOC treated plant rhizosphere, the majority of bacteria were isolated from *C. comosum*. There were six benzene-degrading, four toluene-degrading and four m-xylene-degrading bacteria isolated from *C. comosum* rhizosphere. According to the culture analysis, one bacterium from each of the genera *Agromyces* (CCB1), *Bacillus* (CCB2), *Micrococcus* (CCB3), *Microbacterium* (CCB4), *Brucella* (CCB5) and *Arthrobacter* (CCB6) was identified as benzene degraders. Toluene-degrading bacteria found in *C. comosum* were classified to the genus *Arthrobacter* (CCT1) and *Microbacterium (*CCT2, CCT3 and CCT4*).* Culture identifications revealed that m-xylene degrading bacteria found in *C. comosum* belonged to the genus *Pseudomonas* (CCX1, CCX2 and CCX4) and *Pantoea* (CCX3).

In total, six bacteria were isolated from *H. helix*: one benzene-degrading, three toluene-degrading and two m-xylene-degrading bacteria. According to the culture tests, *Rhodococcus* (HHB1) for the benzene degradation, *Microbacterium* (HHT1, HHT2 and HHT3) for toluene degradation and *Pseudomonas* (HHX1 and HHX2) for the m-xylene degradation were identified in *H. helix*.

In summary, culture identification revealed that most of the VOC degrading bacteria in the rhizosphere of VOC treated plants belonged to the genera *Microbacterium* and *Pseudomonas.* Also, the majority of m-xylene degrading bacteria belonged to the genus *Pseudomonas*.

There were 29 bacteria isolated from the rhizosphere of control plants and compost which were not exposed to benzene, toluene and m-xylene, but the soil suspensions were inoculated on the MSA and incubated in the closed jar supplied with VOC. In detail, there were eleven bacteria in the rhizosphere of *S. wallisii,* eight bacteria from *H. helix*, seven bacteria from *C. comosum* and

three bacteria from compost were isolated and identified based on their culture characteristics (Table 4. 3).

Table 4. 3 Culture-based identifications of VOC degrading bacteria isolated from control plants' rhizosphere (Bergey *et al.*, 1994).

	Isolate tag	Shape	Gram staining	Spores	Oxidase	Catalase	Motility	O/F test	Glucose acid	Anaerobic growth	Acid fast	Predicted genus
Bacteria isolated from *S. wallisii*	SWBC1	R	+	-	-	+	-	F	+	-	-	*Microbacterium*
	SWBC3	R	+	-	-	+	+	F	+	-	-	*Microbacterium*
	SWBC4	R	+	-	-	+	-	F	+	-	-	*Microbacterium*
	SWBC6	R	+	+	-	+	+	F	+	-	-	*Bacillus*
	SWTC1	R/S	+	-	-	+	-	-	+	-	+	*Rhodococcus*
	SWTC5	R	+	-	-	+	-	-	+	-	+	*Rhodococcus*
	SWTC7	R/S	+	-	-	+	-	-	-	-	+	*Rhodococcus*
	SWTC8	R	+	-	-	+	-	F	+	-	-	*Microbacterium*
	SWTC10	R/S	+	-	-	+	-	F	+	-	+	*Rhodococcus*
	SWTC11	R	+	-	-	+	-	F	+	-	-	*Microbacterium*

	Isolate tag	Shape	Gram staining	Spores	Oxidase	Catalase	Motility	O/F test	Glucose acid	Anaerobic growth	Acid fast	Predicted genus
	SWTC12	R	+	-	-	+	-	-	-	-	-	*Aeromicrobium*
Bacteria isolated from *C. comosum*	CCTC2	R	+	-	-	+	+	O	+	-	-	*Microbacterium*
	CCTC3	R	+	-	-	+	+	F	+	-	-	*Microbacterium*
	CCTC5	R	+	-	-	+	-	F	+	-	-	*Microbacterium*
	CCTC7	R	+	+	-	+	+	F	+	-	-	*Microbacterium*
	CCTC11	R	+	+	-	+	+	O	+	-	-	*Microbacterium*
	CCXC1	R	+	+	-	+	+	O	-	-	-	*Bacillus*
	CCXC2	R	-	ND	+	+	+	-	-	-	ND	*Pseudomonas*
	HHBC1	R	+	-	+	+	+	O	-	-	-	*Arthrobacter*
Bacteria isolated from *H. helix*	HHBC6	R	-	ND	+	+	+	F	+	-	ND	*Rhizobium*
	HHTC2	R	+	-	-	+	+	F	+	-	-	*Cellulomonas*

	Isolate tag	Shape	Gram staining	Spores	Oxidase	Catalase	Motility	O/F test	Glucose acid	Anaerobic growth	Acid fast	Predicted genus
	HHTC3	R	-	ND	+	+	+	-	+	-	ND	*Agrobacterium*
	HHTC4	R	+	-	-	+	-	O	+	-	-	*Rhodococcus*
	HHTC6	R	+	-	-	+	-	-	-	-	-	*Aeromicrobium*
	HHTC7	R	+	-	-	+	+	F	+	-	-	*Microbacterium*
	HHTC9	R	-	ND	+	+	+	F	+	-	ND	*Agrobacterium*
Bacteria isolated from Compost	JIBC1	R/S	+	-	+	+	-	-	-	-	-	*Micrococcus*
	JIBC2	R	-	ND	-	+	-	-	-	-	ND	*Brucella*
	JITC1	R	-	ND	-	+	-	-	-	-	ND	*Brucella*

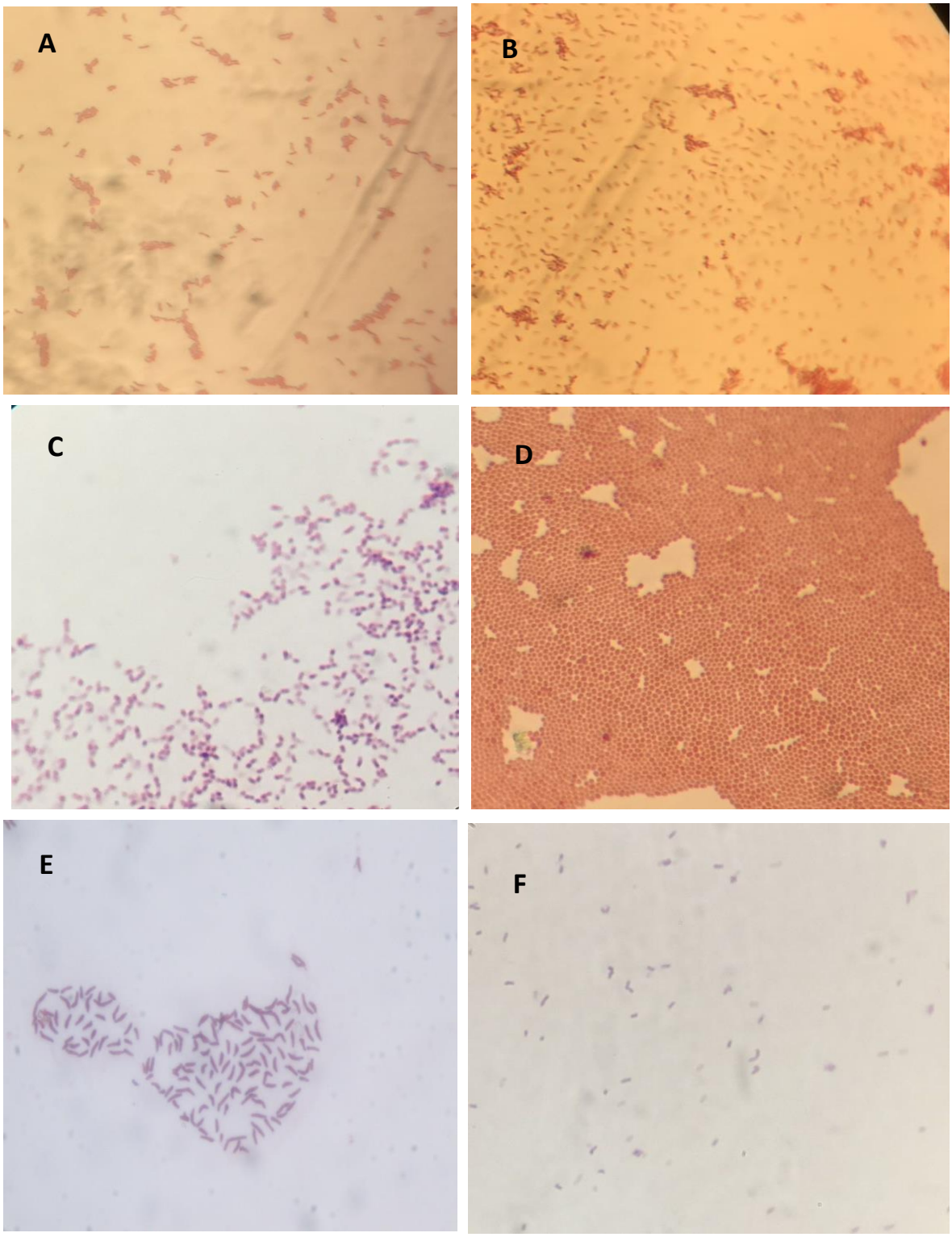
Abbreviations are SW = *S. wallisii*, CC*, C. comosum*, HH= *H. helix*, JI= compost, B= benzene, T=toluene, X= m-xylene, C=control (VOC untreated during batch experiment), R=rod shaped (bacillus), S= spherical /coccus, O= oxidation, F=fermentation, + = strains are positive, - = strains are negative, S = sphere (coccus), ND= not detected.

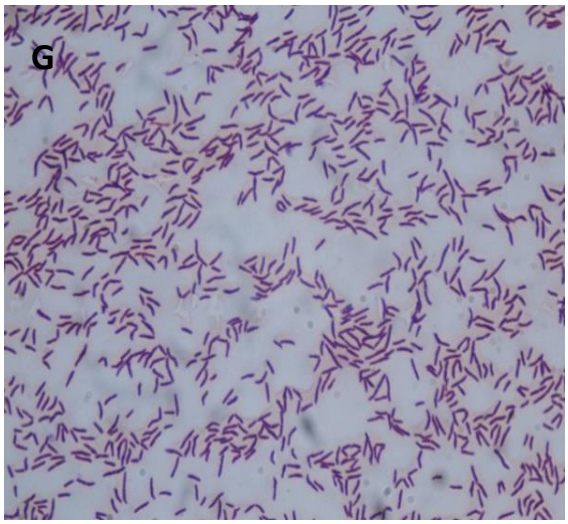
More VOC degrading bacteria were isolated from the control plants (where the plants were not exposed to 100 ppm VOC at the initial four weeks period, but the rhizosphere soil inoculated plates were incubated in the jar containing VOC vapours) rhizosphere of *S. wallisii* than *H. helix* and *C. comosum.* In detail, there were four benzene-degrading and seven toluene-degrading bacteria isolated from *S. wallisii* control plants. However, no bacteria could be isolated from the *S. wallisii* control plants rhizosphere following the m-xylene treatment. According to the culture identifications, *Microbacterium* in the rhizosphere of *S. wallisii* control plants were able to degrade both benzene (SWBC1, SWBC3 and SWBC4) and toluene (SWTC8 and SWTC11) from the atmosphere. In addition, benzene degrading *Bacillus* (SWBC6) and toluene degrading *Rhodococcus* (SWTC1, SWTC5, SWTC7 and SWTC10) and *Aeromicrobium* (SWTC12) were identified in the rhizosphere of *S. wallisii* control plants.

Five toluene-degrading bacteria and two m-xylene-degrading bacteria were found in the *C. comosum* control plants rhizosphere however, no bacteria could be isolated following the benzene treatment. All toluene-degrading bacteria belonged to the genus *Microbacterium* (CCTC2, CCTC3, CCTC5, CCTC7, CCTC11) while *Bacillus* (CCXC1) and *Pseudomonas* (CCXC2) were identified for their capability to degrade m-xylene. In *H. helix* control plants' rhizosphere, one bacterium from each of the genera *Arthrobacter* (HHBC1) and *Rhizobium* (HHBC6) were found as benzene degraders while six bacteria identified as toluene degraders. They were *Cellulomonas* (HHTC2), *Agrobacterium* (HHTC3 and HHTC9), *Aeromicrobium* (HHTC6), *Rhodococcus (*HHTC4*)* and *Microbacterium* (HHTC7). However, no m-xylene-degrading bacteria were isolated from the rhizosphere of *H. helix* control plants. According to the biochemical test observations, the majority of the bacteria in control plants belonged to the genus *Microbacterium* followed by *Rhodococcus*.

In addition to isolating bacteria from rhizosphere, VOC degrading bacteria in potting mix (compost) were isolated. Two benzene-degrading (JIBC1 and JIBC2) and one toluene-degrading (JITC1) bacterium identified from compost samples and they were classified to the genus *Micrococcus* (JIBC1) and *Brucella* (JIBC2 and JITC1). No m-xylene degrading bacteria were isolated

from the compost samples. Fig. 4. 2 shows representative Gram staining images of each bacteria isolated from soil samples.

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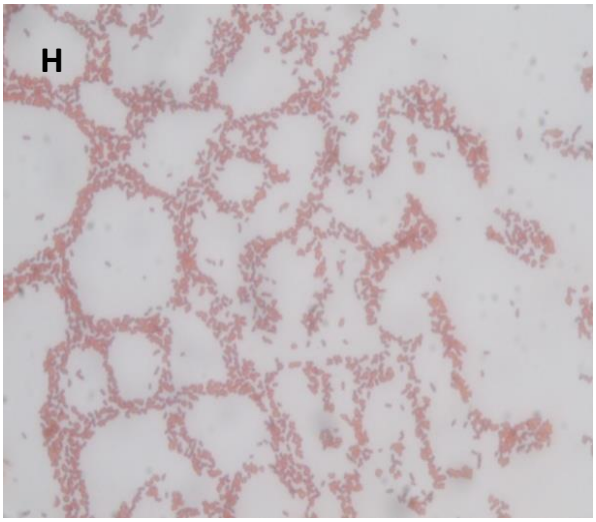
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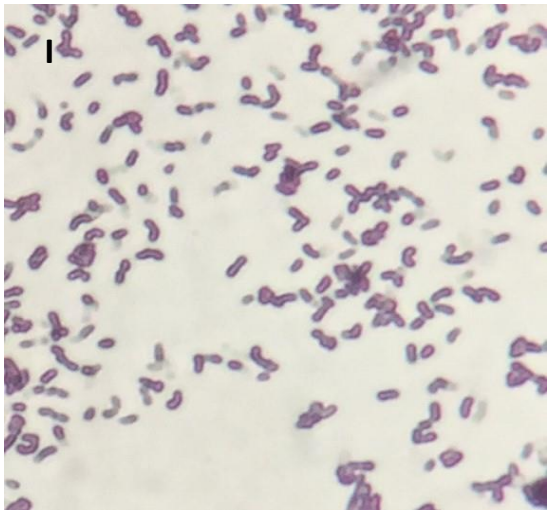
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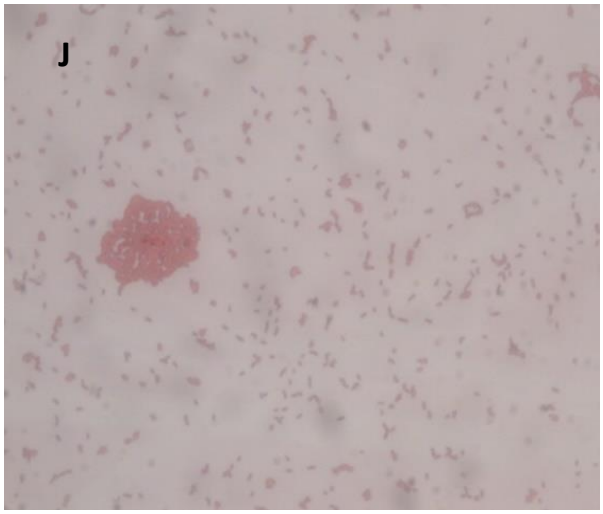
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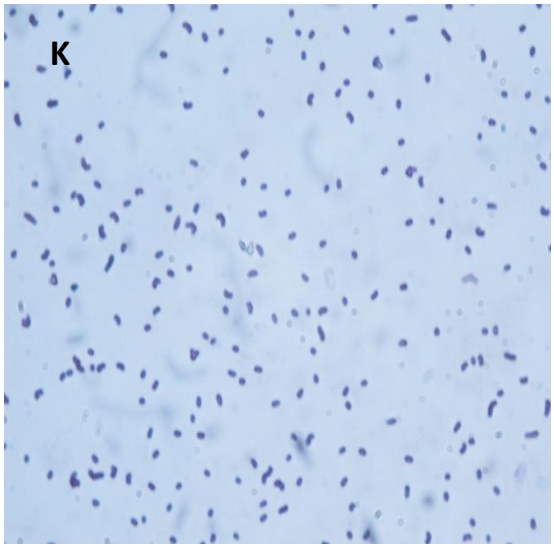
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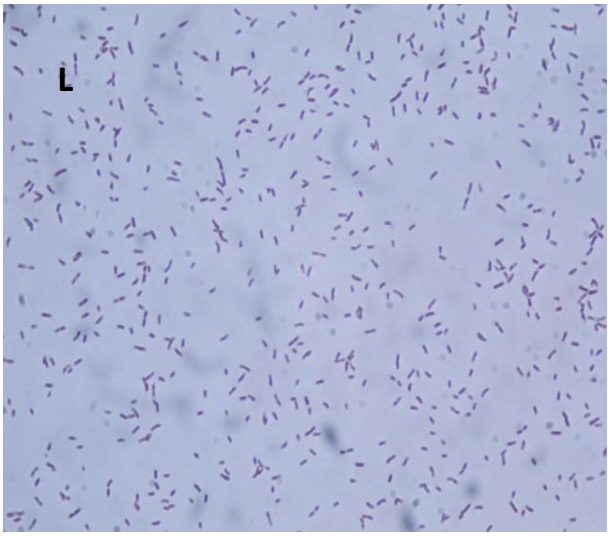
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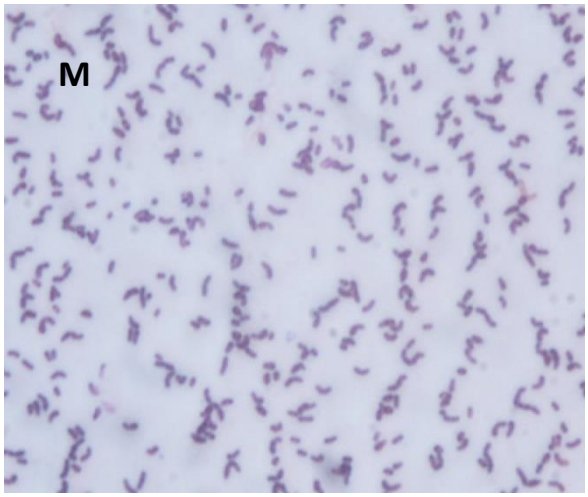

Fig. 4. 2 Representative microscopic image of Gram stained VOC degrading bacteria isolated from soil samples, under magnification x 1000. Abbreviations are A=SWX1 (*Pseudomonas*), B= CCX3 (*Pantoea*), C =JIBC1 (*Micrococcus*), D= JIBC2 (*Brucella*), E =CCB2 (*Bacillus*), F =SWT1 (*Agromyces*), G=HHTC7 (*Microbacterium*), H=HHBC6 (*Rhizobium*), I= CCB6 (*Arthrobacter*), J= HHTC3 (*Agrobacterium*), K= HHB1 (*Rhodococcus*), L= HHTC2 (*Cellulomonas*), M= HHTC2 (*Aeromicrobium*).

Common culture characteristics of bacteria were as follows (Table 4. 2 and Table 4. 3). *Microbacterium* was Gram-positive, rod-shaped, non-spore forming, oxidase negative, catalase positive, aerobic, non-acid fast, glucose oxidative or fermentative, motile or non-motile and positive for the acid production from glucose. *Rhodococcus* was identified as Gram-positive, rod-shaped, non-spore forming, oxidase negative, catalase positive, aerobic, non-motile, glucose oxidative or fermentative, positive or negative for the acid production from glucose and acid-fast or non-acid fast bacteria. *Arthrobacter* showed rod shape, Gram-positive, non-spore forming, oxidase negative, catalase positive, non-motile, aerobic, negative for the acid production from glucose and non-acid fast. *Pseudomonas* was Gram-negative, rod-shaped, motile, oxidase and catalase positive bacteria. *Agromyces* showed Gram-positive, non-motile, oxidase negative, catalase positive, non-spore forming, aerobic and positive for the acid production from glucose.

Micrococcus was Gram-positive, coccus or very short rod shape, non-spore forming, oxidase and catalase positive, non-motile and either carbohydrate oxidative or no reaction observed on carbohydrate. Some bacteria from the genus *Micrococcus* were positive for acid production from glucose. Genus *Brucella* was identified as rod-shaped, Gram-negative, catalase positive, oxidase negative or positive, non-motile, carbohydrate oxidative or observed no reaction on carbohydrate. *Bacillus* was Gram-positive, rod-shaped, spore-forming, oxidase negative, carbohydrate fermentative, and catalase positive, motile and positive for the acid production from glucose. *Pantoea* was Gram-negative, rod-shaped, oxidase negative, catalase positive, motile, carbohydrate oxidative and positive for the acid production from glucose.

Aeromicrobium showed Gram-positive, non-spore forming, oxidase negative, catalase positive, non-motile, negative for the acid production from glucose, aerobic and non-acid fast. *Rhizobium* was rod shape, Gram-negative, oxidase and catalase positive, motile, glucose fermentative and aerobic. *Cellulomonas* was rod shape, Gram-positive, non-spore forming, oxidase negative, catalase positive, carbohydrate fermentative, aerobic and positive for the acid production from glucose. *Agrobacterium* was Gram-negative, rod-shaped, oxidase and catalase positive, motile, positive for the acid production from glucose and found fermentative or no reaction on carbohydrate.

By performing isolation and identification of VOC degrading bacteria, it was expected to observe the compositional differences between the rhizosphere of VOC treated and control plants. In the control plants experiment, though the plants were not exposed to VOCs during the initial four-week period, some bacteria in the rhizosphere samples of these plants were able to utilise VOCs as their sole carbon source. During isolating VOC degrading bacteria, totals of 24 and 29 bacteria from VOC treated and untreated (control) plants rhizosphere were isolated respectively and then predicted their genus level based on culture test observations. Thus, culture-dependent method enabled an understanding of physiology and metabolism of unknown microorganisms.

Studying unknown bacteria based on the culture-independent method can be carried out based on their DNA or RNA analysis without cultivation. Mapping sequencing data to a reference

database enables identifying the closest species or genus level of unknown bacteria, thus this is less labour consuming and faster approach compared to culture methods. The Percentage of identity, based on the sequencing data, expresses the level of similarity between the unknown bacterium and reference bacteria in the database. When the percentage of identity is low, this indicates the unknown bacterium can be a new bacterium strain or species. Also, bacterial genomic information enables comparing phylogenetic and functional similarities and differences between different bacteria. Sequencing data can be used for further downstream applications such as designing species-specific primers. Therefore, to get a closer look at the isolated bacteria from plant rhizosphere, their 16 rRNA gene sequencing identification was conducted.

4.2.2. Molecular identification of VOC degrading bacteria

The same set of bacteria studied in the culture method was investigated in this section. Though the culture-based analysis identified benzene, toluene and m-xylene degrading bacteria, that identification was limited to the genus level. Also, there were often more than one bacterium identified belonging to the same genus, therefore it was necessary to classify them to sub-genus or species level. Such classifications were carried out by using molecular methods and each bacterium was identified up to the species level with a 97% or more than 97 % identity (except JIBC1 and CCX3). During molecular identification, bacterial genomic DNA extraction and amplification of the 16S rRNA gene using 27F/1492 primer pair was conducted (detailed in section 2.13.2.1). There was a total of 53 bacteria isolated using culture methods and DNA from each single bacterium was amplified and run on the agarose gel separately to confirm the presence of expected amplicons (Fig. 4. 3).

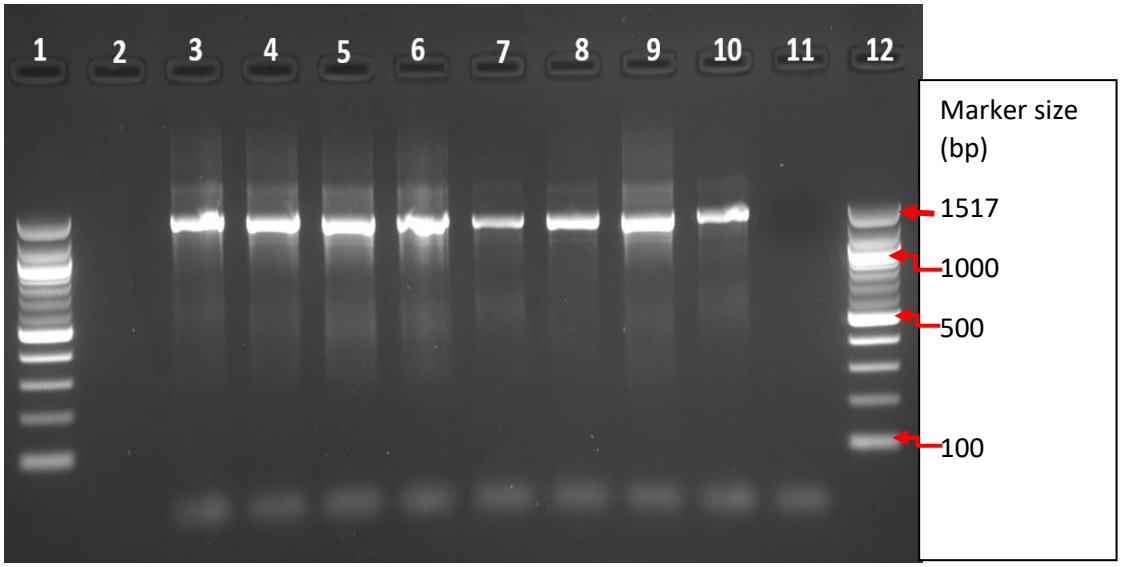

Fig. 4. 3 Representative Agarose gel image to show PCR amplicons (1485 bp) of 16S rRNA gene in bacterial genomic DNA extracted from rhizosphere soil samples. Lanes are: 100 bp DNA ladder (1), empty well (2), SWX1 (3), CCX1 (4), JIBC1 (5), CCB1 (6), CCT1 (7), HHB1 (8), HHT1 (9), SWT1 (10), negative control (11) and 100 bp DNA ladder (12).

Cloning PCR amplicons into vectors following the colony PCR was performed for the selected (only for the bacteria mentioned in the Fig. 4. 5) unknown bacteria to certify the successful amplification of the 16S rRNA gene during PCR. Therefore, PCR amplicons from the selected bacteria were purified and cloned into pGEM ® -T Easy vector and plated on LB agar plates containing ampicillin, X-Gal and IPTG (Fig. 4. 4). White colour colonies on the LB medium indicates successful transformations while no (unsuccessful) transformations appeared blue.

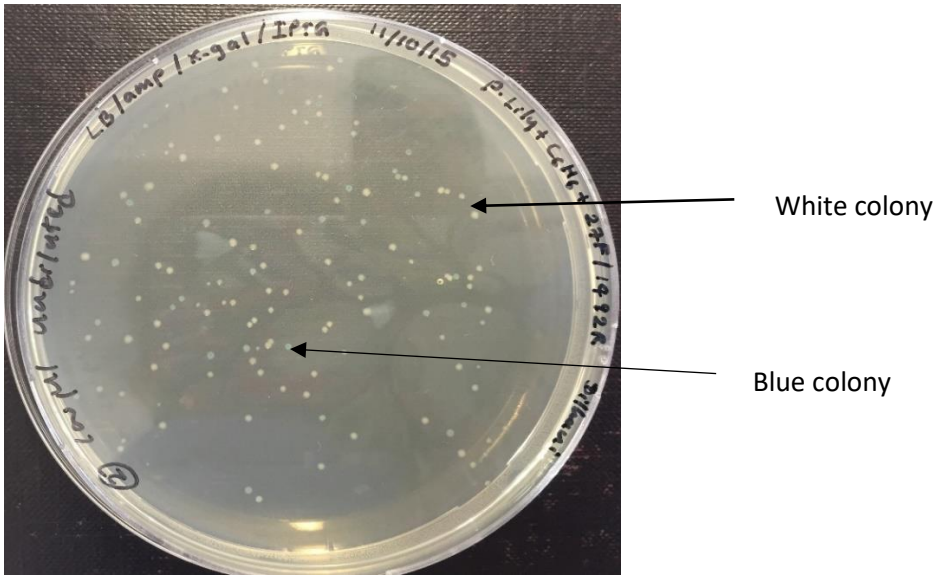

Fig. 4. 4 Example of successful cloning of 16S rRNA gene amplicons from SWBC1 into *Escherichia coli* JM109 high-efficiency competent cells. White and blue colonies are recombinant vector containing cells and non-recombinant vector containing cells respectively.

Transformant colonies were used for colony PCR (detailed in section 2.13.2.2). Agarose gel image (Fig. 4. 5) shows the presence of 1485 amplicon in the colony PCR products obtained from the selected isolates.

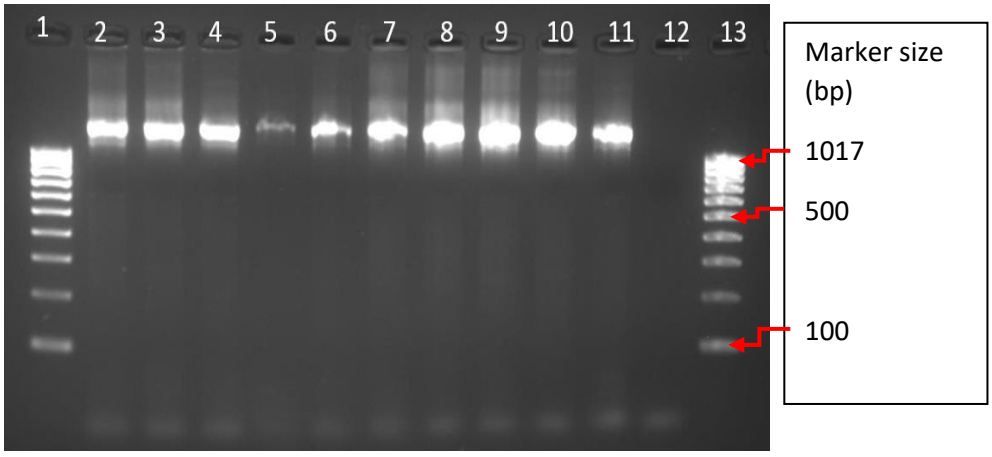

Fig. 4. 5 Agarose gel image to show colony PCR product amplicons (1485 bp) obtained for the bacteria listed. Lanes are: 100 bp DNA Hyper ladder (1), SWX1 (2), CCX1 (3), JIBC1 (4), CCB1 (5), CCT1 (6), HHB1 (7), HHT1 (8), SWT1 (9), SWBC1 (10), SWBC3 (11), negative control (12) and 100 bp DNA Hyper ladder (13).

Then, the 16S rRNA gene amplicons obtained from the PCR were sequenced by the Sanger sequencing approach. Only the forward strand was sequenced using the 27F primer as the sequencing primer in the reaction. The successful sequencing result of SWBC1 is shown below as an example and the full sequencing list for the all bacterial can be found in Appendix 3.

>SWBC1_27F

TTACCATGCAGTCGAACGGTGAAGCCAAGCTTGCTTGGTGGATCAGTGGCGAACGGGTGA

GTAACACGTGAGCAACCTGCCCTGGACTCTGGGATAAGCGCTGGAAACGGCGTCTAATAC

TGGATATGAGCTTTCACCGCATGGTGGGGGTTGGAAAGATTTTTCGGTCTGGGATGGGCT

CGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGC

CTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG

CAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGAGGGATGA

CGGCCTTCGGGTTGTAAACCTCTTTTAGCAAGGAAGAAGCGTGAGTGACGGTACTTGCAG

AAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTAT

CCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAATCCCG

AGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGG

AATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAG

ATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAGATA

CCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCTTTCCACGGATTCC

GTGACGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC

AAAGGAATTGACGGGGACCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGC

GAAGAACCTTACCAAGGCTTGACTACACG

After mapping 16S rRNA gene sequencing reads of bacteria isolated from VOC treated plant rhizosphere into the nucleotide BLAST database, all the strains were identified up to the species level with the identity percentage ranging from 97% to 100% (Table 4. 4). In most cases, query nucleotide sequence aligned with more than one reference sequence in the database with the same or very close highest identities, therefore in a phylogenetic tree, those species/strains were closely branched (Fig. 4. 6). For example, bacteria SWTC10 showed 99.90% identity with the 16S

rRNA sequence of *Rhodococcus qingshengii* strain djl-6-2 and *Rhodococcus degradans* strain CCM 4446. Also, bacteria CCT3 shared 99.90% identity with *Microbacterium barkeri* strain DSM while sharing 98.97% identity with *Microbacterium oryzae* strain MB10 (Fig. 4. 6).

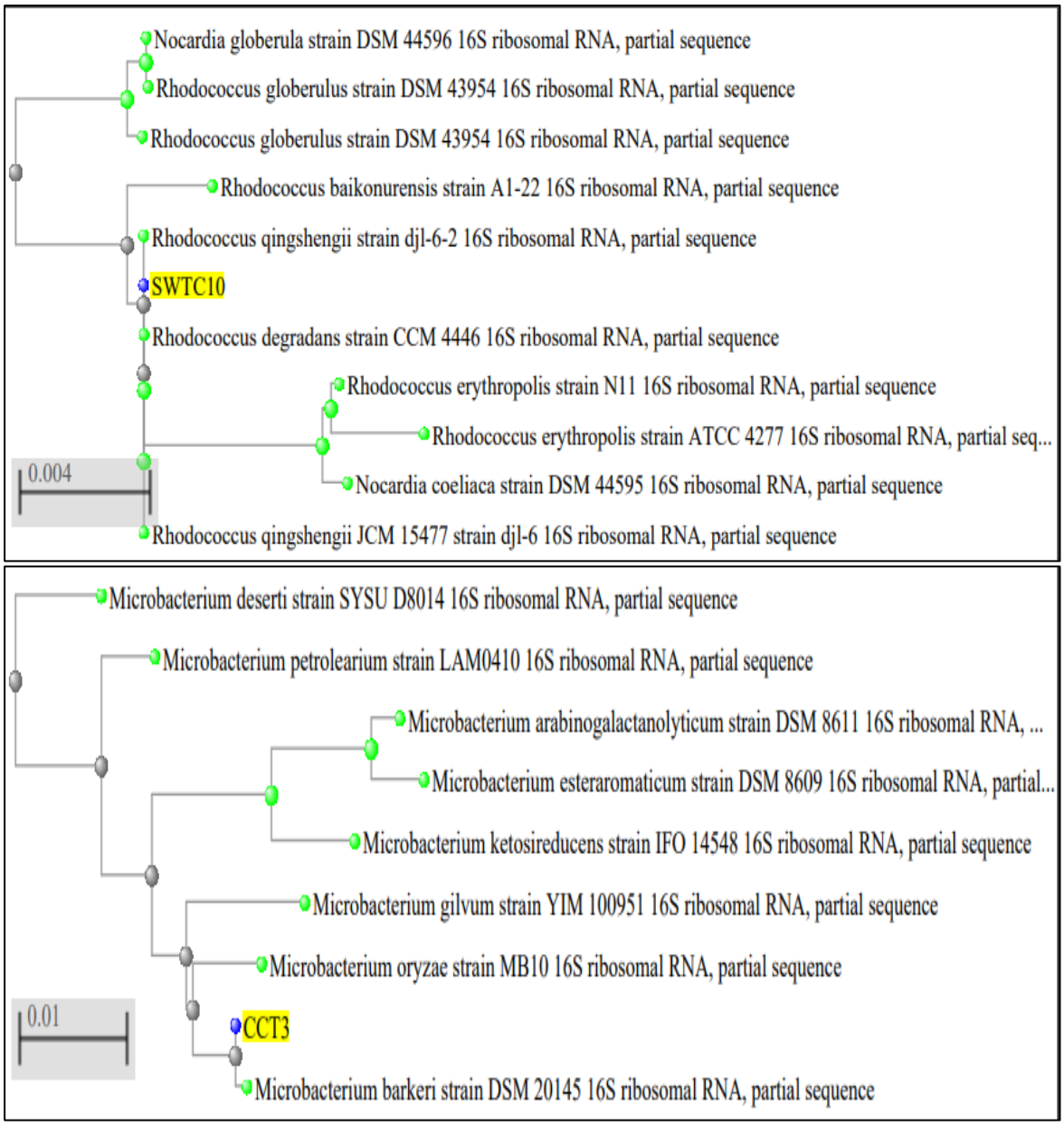

Fig. 4. 6 phylogenetic tree example of the bacteria SWTC10 and CCT3

During sequencing, the query reads were produced using one primer, therefore only partial sequences of the 16S rRNA gene for the test bacteria were obtained ranging the read length from 700-1000bp in most of the test bacteria. However, it was sufficient to identify closely related species (nearest species) and also the evolutionary relationships between the test bacterium and reference bacteria (Fig. 4. 9).

Table 4. 4 Identification of VOC degrading bacteria isolated from VOC treated plants by analysing bacterial 16S rRNA gene sequences.

	Isolate Tag	Gene Bank Accession no	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
Bacteria isolated from *S. wallisii*	**SWT1**	NR_116743.1	*Agromyces atrinae*	1477	965	1777	99.90
		NR_026165.1	*Agromyces ramosus*	1482	932	1580	96.18
		NR_029092.1	*Agromyces cerinus*	1465	934	1572	95.89
	SWT3	NR_116483.1	*Microbacterium ginsengiterrae*	1427	982	1759	98.59
		NR_025405.1	*Microbacterium phyllosphaerae*	1478	981	1751	98.49
		NR_042983.1	*Microbacterium natoriense*	1429	981	1748	98.40
	SWX1	NR_156987.1	*Pseudomonas paralactis*	1428	239	442	100
		NR_114225.1	*Pseudomonas mucidolens*	1462	239	442	100
		NR_113583.1	*Pseudomonas synxantha*	1527	239	442	100
		NR_024928.1	*Pseudomonas gessardii*	1516	239	442	100
		NR_024901.1	*Pseudomonas libanensis*	1516	239	442	100

	Isolate Tag	Gene Bank Accession no	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
	SWX2	NR_114226.1	*Pseudomonas plecoglossicida*	1462	941	1725	99.68
		NR_114224.1	*Pseudomonas monteilii*	1462	940	1720	99.58
		NR_102854.1	*Pseudomonas entomophila*	1526	939	1714	99.47
Bacteria isolated from *C. comosum*	**CCB1**	NR_116743.1	*Agromyces atrinae*	1477	977	1799	99.90
		NR_026165.1	*Agromyces ramosus*	1482	942	1587	95.93
		NR_029092.1	*Agromyces cerinus*	1465	946	1799	95.85
	CCB2	NR_044524.1	*Paenibacillus xylanexedens*	1514	954	1735	99.27
		NR_040853.1	*Paenibacillus pabuli*	1500	952	1725	99.06
		NR_112728.1	*Paenibacillus amylolyticus*	1478	949	1716	98.85
	CCB3	NR_112644.1	*Leifsonia lichenia*	1465	994	1827	99.80
		NR_116501.1	*Leifsonia soli strain TG-S248*	1343	965	1755	99.28
		NR_043663.1	*Leifsonia shinshuensis*	1500	983	1766	98.69

	Isolate Tag	Gene Bank Accession no	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
	CCB4	NR_028944.1	*Microbacterium aerolatum*	1345	903	1642	99.23
		NR_025368.1	*Microbacterium foliorum*	1480	945	1700	98.75
		NR_116483.1	*Microbacterium ginsengiterrae*	1427	942	1683	98.43
	CCB5	NR_104702.1	*Arthrobacter psychrochitiniphilus*	1446	961	1690	97.76
		NR_117355.1	*Arthrobacter livingstonensis*	1379	936	1639	97.50
		NR_042258.1	*Arthrobacter stackebrandtii*	1517	959	1666	97.36
	CCB6	NR_104702.1	*Arthrobacter psychrochitiniphilu*	1446	759	1339	97.94
		NR_117355.1	*Arthrobacter livingstonensis*	1379	736	1299	97.87
		NR_042258.1	*Arthrobacter stackebrandtii*	1517	758	1323	97.55
	CCT1	NR_041546.1	*Arthrobacter humicola*	1463	968	1784	99.90
		NR_041545.1	*Arthrobacter oryzae*	1465	963	1755	99.38
		NR_026236.1	*Pseudarthrobacter oxydans*	1486	923	1674	99.14

	Isolate Tag	Gene Bank Accession no	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
	CCT2	NR_026164.1	*Microbacterium barkeri*	1468	813	1498	99.88
		NR_117527.1	*Microbacterium oryzae*	1419	805	1454	98.89
		NR_159264.1	*Microbacterium album*	1470	795	1397	97.67
	CCT3	NR_026164.1	*Microbacterium barkeri*	1468	968	1784	99.90
		NR_117527.1	*Microbacterium oryzae*	1419	959	1735	98.97
		NR_146699.1	*Microbacterium gilvum*	1515	916	1640	98.49
	CCT4	NR_026164.1	*Microbacterium barkeri*	1468	972	1788	99.79
		NR_117527.1	*Microbacterium oryzae*	1419	963	1738	98.87
		NR_146699.1	*Microbacterium gilvum*	1515	918	1640	98.39
	CCX1	NR_112073.1	*Pseudomonas monteilii*	1462	782	1408	99.11
		NR_102854.1	*Pseudomonas entomophila*	1526	781	1408	98.86
		NR_114226.1	*Pseudomonas plecoglossicida*	1462	781	1408	98.86

	Isolate Tag	Gene Bank Accession no	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
	CCX2	NR_117826.1	*Pseudomonas corrugata*	1442	932	1681	98.94
		NR_116299.1	*Pseudomonas brassicacearum*	1468	937	1683	98.84
		NR_028929.1	*Pseudomonas kilonensis*	1528	933	1679	98.83
	CCX3	NR_116247.1	*Pantoea conspicua*	1344	121	206	96.03
		NR_104940.1	*Atlantibacter hermannii*	1478	121	206	96.03
		NR_116115.1	*Pantoea vagans*	1429	121	206	96.03
	CCX4	NR_114224.1	*Pseudomonas monteilii*	1462	951	1740	99.58
		NR_102854.1	*Pseudomonas entomophila*	1526	950	1740	99.48
		NR_114226.1	*Pseudomonas plecoglossicida*	1462	950	1735	99.48
Bacteria isolated from *H. helix*	**HHB1**	NR_115708.1	*Rhodococcus qingshengii*	1489	791	1461	100
		NR_145886.1	*Rhodococcus degradans*	1473	791	1461	100
		NR_104776.1	*Nocardia coeliaca*	1507	781	1443	100

	Isolate Tag	Gene Bank Accession no	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
		NR_037024.1	*Rhodococcus erythropolis*	1476	781	1443	100
	HHT1	NR_028944.1	*Microbacterium aerolatum*	1345	868	1583	99.31
		NR_025368.1	*Microbacterium foliorum*	1480	909	1633	98.70
		NR_116502.1	*Microbacterium azadirachtae*	1373	881	1576	98.44
	HHT2	NR_114984.1	*Microbacterium chocolatum*	1509	772	1415	99.61
		NR_126283.1	*Microbacterium mangrovi*	1484	774	1415	99.49
		NR_042263.1	*Microbacterium hydrocarbonoxydans*	1495	774	1415	99.49
	HHT3	NR_026164.1	*Microbacterium barkeri*	1468	968	1779	99.79
		NR_117527.1	*Microbacterium oryzae*	1419	959	1729	98.87
		NR_146699.1	*Microbacterium gilvum*	1515	917	1637	98.39
	HHX1	NR_114477.1	*Pseudomonas mendocina*	1457	561	1002	98.42
		NR_125523.1	*Pseudomonas chengduensis*	1529	560	994	98.25

	Isolate Tag	Gene Bank Accession no	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
		NR_114072.1	*Pseudomonas alcaliphila*	1462	560	994	98.25
	HHX2	NR_114224.1	*Pseudomonas monteilii*	1462	701	1273	99.15
		NR_102854.1	*Pseudomonas entomophila*	1526	700	1267	99.01
		NR_114226.1	*Pseudomonas plecoglossicida*	1462	700	1267	99.01

Identity: the highest percent identity of all query-subject alignments, Max score: the highest alignment score from that database sequence, length of 16S rRNA: the number of nucleotides in the subject sequence, Length of query aligned: number of nucleotides in query aligned with the subject, Abbreviations are SW = *S. wallisii*, CC*, C. comosum*, HH= *H. helix*, B= benzene, T=toluene, X= m-xylene.

Following mapping 16S rRNA gene sequences of isolated bacteria from the rhizosphere samples, most of them were identified up to the nearest species level. In some cases, bacterial species from different genera were identified for the query read sequence with the same highest identity (e.g. CCX3 and HHB1). 16S rRNA gene sequence of bacterium CCX3 resulted in nearest identifications from two bacterial genera: *Pantoea* or *Atlantibacter*. Similar to the CCX3, blastn analysis classified bacteria HHB1 as a *Rhodococcus* or *Nocardia* species with the same identities (Fig. 4. 7).

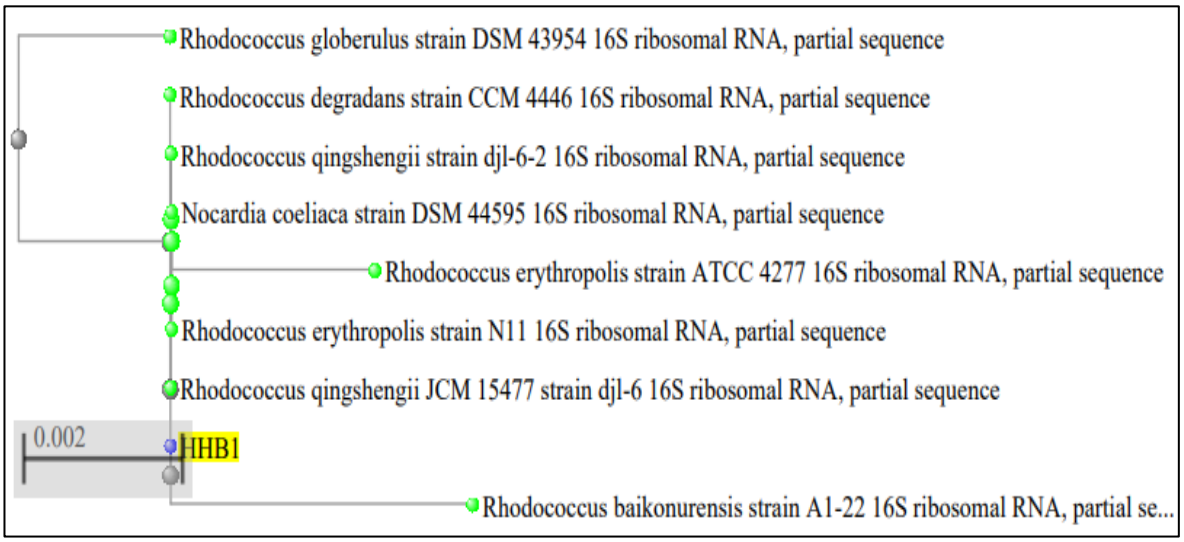

Fig. 4. 7 Phylogenetic tree to show reference species from different genera related with bacterium HHB1

According to the 16S rRNA gene sequence analysis for the 24 cultures isolated from VOC exposed plants rhizospheres, they were classified to 9 genera as *Pseudomonas, Pantoea, Arthrobacter, Agromyces, Paenibacillus, Leifsonia, Microbacterium, Nocardia* and *Rhodococcus.* Except for the genus *Pseudomonas* all other seven genera belonged to the phylum Actinobacteria, so the majority of isolates (71%) were Gram-positive soil bacteria. Most of the identifications obtained using two methods; culture test and sequence analysis were similar at the genus level (Table 4. 5).

Table 4. 5 Genus level classification of VOC degrading bacteria isolated from VOC treated plants rhizosphere and compost.

Isolate	**Genus identification by culture method**	**Nearest species identification by sequence analysis**
SWT1	*Agromyces*	*Agromyces atrinae*
SWT3	*Microbacterium*	*Microbacterium ginsengiterrae*
SWX1	*Pseudomonas*	*Pseudomonas paralactis*
SWX2	*Pseudomonas*	*Pseudomonas plecoglossicida*
CCB1	*Agromyces*	*Agromyces atrinae*
CCB2	*Bacillus**	*Paenibacillus xylanexedens*
CCB3	*Micrococcus**	*Leifsonia lichenia*
CCB4	*Microbacterium*	*Microbacterium aerolatum*
CCB5	*Brucella**	*Arthrobacter psychrochitiniphilus*
CCB6	*Arthrobacter*	*Arthrobacter psychrochitiniphilu*
CCT1	*Arthrobacter*	*Arthrobacter humicola*
CCT2	*Microbacterium*	*Microbacterium barkeri*
CCT3	*Microbacterium*	*Microbacterium barkeri*
CCT4	*Microbacterium*	*Microbacterium barkeri*
CCX1	*Pseudomonas*	*Pseudomonas monteilii*
CCX2	*Pseudomonas*	*Pseudomonas corrugata*
CCX3	*Pantoea*	*Pantoea conspicua*
CCX4	*Pseudomonas*	*Pseudomonas monteilii*
HHB1	*Rhodococcus*	*Rhodococcus qingshengii*
HHT1	*Microbacterium*	*Microbacterium aerolatum*
HHT2	*Microbacterium*	*Microbacterium chocolatum*
HHT3	*Microbacterium*	*Microbacterium barkeri*

Isolate	Genus identification by culture method	Nearest species identification by sequence analysis
HHX1	*Pseudomonas*	*Pseudomonas mendocina*
HHX2	*Pseudomonas*	*Pseudomonas monteilii*

Abbreviations are SW = *S. wallisii*, CC, *C. comosum*, HH= *H. helix*, B= benzene, T=toluene, X= m-xylene, * -different identifications based on culture and molecular method

Compared to the sequence analysis, most of the bacterial identifications carried out in the culture methods were agreed with only a few exceptions (*). Those exceptions were observed for the bacteria CCB2, CCB3 and CCB5. Bacterium CCB2 was identified as *Paenibacillus* based on the sequence analysis, but it was classified as *Bacillus* in culture methods. Both genera; *Bacillus* and *Paenibacillus* show similar reactions to the biochemical tests used in the culture method. Also, the genus *Paenibacillus* belonged to the genus *Bacillus* previously and was reclassified as a separate genus later (Ash *et al.*, 1993). Bacterium CCB3 was classified as belonging to the genus *Micrococcus* in the culture tests, however, it was identified as a bacterium from the genus *Leifsonia* according to the sequence analysis. Bacterial genus *Leifsonia* was not mentioned in the manual used to classify them, thus based on the culture test responses it was classified to the genus *Micrococcus* since the bacterium CCB3 showed most of biochemical test characteristics similar to the genus *Micrococcus.* Bacteria from the genera *Micrococcus* and *Leifsonia* can show similar biochemical characteristics: both can be Gram-positive, aerobic, non-spore forming, oxidase positive, catalase positive, non-motile and positive or negative for the acid production from glucose (Kocur *et al.*, 2006; Madhaiyan *et al.*, 2010). Thus, in the absence of such genus information in a manual, it can be easily classified into a different genus. Bacterium CCB5 were observed as Gram-negative bacteria during culture analysis, thus they were classified to Gram-negative genus *Brucella*. But according to their 16S rRNA sequence analysis, they were identified as Gram-positive *Arthrobacter.* Genus *Arthrobacter* shows Gram variables, thus young cultures can be stained as Gram-negative while mature cultures stained as Gram-positive bacteria. However, genetically they are classified as Gram-positive bacteria (Mulllakhanbhai and Bhat,

1965; Mongodin *et al.*, 2006). Except the Gram difference, *Brucella* and *Arthrobacter* share most of common biochemical characteristics: both genera show rod or short rod shape, catalase positive, oxidase negative, non-motile and non-spore forming bacteria (Hayashi *et al.*, 1993; Kyebambe, 2005; Mongodin *et al.*, 2006).

Also, common bacteria from different soil samples were identified. For example, bacteria HHT1 and CCB4 (*Microbacterium aerolatum*), CCT4 and CCT3 (*Microbacterium barkeri*), CCB1 and SWT1 (*Agromyces atrinae*), CCX1 and CCX4 (*Pseudomonas monteilii*) and CCB5 and CCB6 (*Arthrobacter psychrochitiniphilus*) were branched closely in the phylogenetic tree (Fig. 4. 9) based on their sequence similarities.

During mapping 16S rRNA gene sequencing reads of bacteria isolated from control plant rhizosphere, all the strains were identified up to the genus level ranging identity percentage from 99% to 100% (Table 4. 6). For a query sequence, multiple hits were selected from the blast results since more than one reference species shared similar or very close highest sequence identities with the query according to the 16S rRNA gene sequence analysis.

Table 4. 6 Identification of VOC degrading bacteria isolated from control plants by analysing bacterial 16S rRNA gene sequences Identity.

	Isolate Tag	Gene Bank Accession number	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
Bacteria isolated from *S. wallisii*	**SWBC1**	NR_044937.1	*Microbacterium trichothecenolyticum*	1461	913	1657	99.13
		NR_134085.1	*Microbacterium jejuense*	1441	913	1633	98.49
		NR_134086.1	*Microbacterium kyungheense*	1443	910	1620	98.27
	SWBC3	NR_114986.1	*Microbacterium maritypicum*	1345	951	1751	99.89
		NR_044931.1	*Microbacterium oxydans*	1466	950	1748	99.79
		NR_026162.1	*Microbacterium liquefaciens*	1474	947	1729	99.47
	SWBC4	NR_114986.1	*Microbacterium maritypicum*	1345	923	1700	99.89
		NR_044931.1	*Microbacterium oxydans*	1466	922	1696	99.78
		NR_026162.1	*Microbacterium liquefaciens*	1474	919	1677	99.46
	SWBC6	NR_044524.1	*Paenibacillus xylanexedens*	1514	895	1628	99.33
		NR_040853.1	*Paenibacillus pabuli*	1500	890	1602	98.78

	Isolate Tag	Gene Bank Accession number	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
		NR_112728.1	*Paenibacillus amylolyticus*	1478	888	1589	98.45
	SWTC1	NR_149270.1	*Rhodococcus pedocola*	1472	998	1829	99.70
		NR_109454.2	*Rhodococcus canchipurensis*	1519	988	1773	98.70
		NR_114500.1	*Rhodococcus koreensis*	1473	986	1760	98.50
	SWTC5	NR_149270.1	*Rhodococcus pedocola*	1472	1000	1832	99.70
		NR_109454.2	*Rhodococcus canchipurensis*	1519	990	1777	98.70
		NR_114500.1	*Rhodococcus koreensis*	1473	988	1764	98.50
	SWTC7	NR_149270.1	*Rhodococcus pedocola*	1472	966	1770	99.69
		NR_109454.2	*Rhodococcus canchipurensis*	1519	956	1714	98.66
		NR_114500.1	*Rhodococcus koreensis*	1473	954	1701	98.45
	SWTC8	NR_042263.1	*Microbacterium hydrocarbonoxydans*	1495	203	375	100
		NR_117603.1	*Microbacterium murale*	1356	164	300	99.39

	Isolate Tag	Gene Bank Accession number	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
		NR_116483.1	*Microbacterium ginsengiterrae*	1427	193	315	94.61
	SWTC10	NR_115708.1	*Rhodococcus qingshengii*	1489	964	1775	99.90
		NR_145886.1	*Rhodococcus degradans*	1473	964	1775	99.90
		NR_024784.1	*Rhodococcus baikonurensis*	1348	961	1759	99.59
	SWTC11	NR_114986.1	*Microbacterium maritypicum*	1345	954	1720	98.86
		NR_044931.1	*Microbacterium oxydans*	1466	953	1716	98.76
		NR_025548.1	*Microbacterium paraoxydans*	1490	953	1711	98.65
	SWTC12	NR_042659.1	*Aeromicrobium ponti*	1396	937	1690	98.94
		NR_024846.1	*Aeromicrobium erythreum*	1474	924	1635	97.99
		NR_104488.1	*Aeromicrobium halocynthiae*	1358	909	1574	97.01
Bacteria isolated from C.	**CCTC2**	NR_028944.1	*Microbacterium aerolatum*	1345	880	1605	99.32
		NR_025368.1	*Microbacterium foliorum*	1480	922	1664	98.93

	Isolate Tag	Gene Bank Accession number	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
		NR_116483.1	*Microbacterium ginsengiterrae*	1427	919	1648	98.61
	CCTC3	NR_028944.1	*Microbacterium aerolatum*	1345	893	1629	99.39
		NR_025368.1	*Microbacterium foliorum*	1480	935	1688	98.94
		NR_116483.1	*Microbacterium ginsengiterrae*	1427	932	1672	98.62
	CCTC5	NR_028944.1	*Microbacterium aerolatum*	1345	914	1663	99.24
		NR_025368.1	*Microbacterium foliorum*	1480	959	1724	98.76
		NR_116483.1	*Microbacterium ginsengiterrae*	1427	956	1707	98.46
	CCTC7	NR_028944.1	*Microbacterium aerolatum*	1345	940	1707	99.16
		NR_025368.1	*Microbacterium foliorum*	1480	983	1773	98.89
		NR_116483.1	*Microbacterium ginsengiterrae*	1427	980	1757	98.59
	CCTC11	NR_028944.1	*Microbacterium aerolatum*	1345	914	1668	99.35
		NR_025368.1	*Microbacterium foliorum*	1480	956	1727	98.96

	Isolate Tag	Gene Bank Accession number	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
		NR_116483.1	*Microbacterium ginsengiterrae*	1427	953	1711	98.65
	CCXC1	NR_044524.1	*Paenibacillus xylanexedens*	1514	620	1114	98.73
		NR_040853.1	*Paenibacillus pabuli*	1500	618	1105	98.41
		NR_112728.1	*Paenibacillus amylolyticus*	1478	616	1098	98.09
	CCXC2	NR_116299.1	*Pseudomonas plecoglossicida*	1462	951	1740	99.58
		NR_024910.1	*Pseudomonas monteilii*	1517	950	1735	99.48
		NR_102854.1	*Pseudomonas entomophila*	1526	949	1729	99.37
Bacteria isolated from *H. helix*	**HHBC1**	NR_027199.1	*Paenarthrobacter nitroguajacolicus*	1488	890	1635	99.78
		NR_026233.1	*Paenarthrobacter aurescens*	1480	886	1607	99.22
		NR_026235.1	*Paenarthrobacter ilicis*	1486	884	1592	98.88
	HHBC6	NR_117203.1	*Rhizobium nepotum*	1371	921	1685	99.57
		NR_041396.1	*Agrobacterium tumefaciens*	1457	914	1646	98.81

	Isolate Tag	Gene Bank Accession number	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
		NR_118035.1	*Rhizobium skierniewicense*	1400	913	1635	98.60
	HHTC2	NR_125452.1	*Cellulomonas pakistanensis*	1421	809	1465	99.02
		NR_029288.1	*Cellulomonas hominis*	1394	807	1454	98.78
		NR_042936.1	*Cellulomonas denverensis*	1422	806	1448	98.65
	HHTC3	NR_136460.1	*Youhaiella tibetensis*	1447	959	1746	99.38
		NR_147729.1	*Methyloterrigena soli*	1420	957	1738	99.27
		NR_136441.1	*Paradevosia shaoguanensis*	1407	956	1729	99.07
	HHTC4	NR_037021.1	*Rhodococcus fascians*	1424	925	1659	98.61
		NR_156055.1	*Rhodococcus sovatensis*	1414	921	1650	98.61
		NR_116275.1	*Rhodococcus cercidiphylli*	1480	923	1652	98.40
	HHTC6	NR_041384.1	*Aeromicrobium ginsengisoli*	1483	982	1762	98.69
		NR_044983.2	*Aeromicrobium fastidiosum*	1472	981	1762	98.69

	Isolate Tag	Gene Bank Accession number	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
		NR_043207.1	*Aeromicrobium alkaliterrae*	1473	977	1731	98.09
	HHTC7	NR_114986.1	*Microbacterium maritypicum*	1345	914	1648	98.92
		NR_044931.1	*Microbacterium oxydans*	1466	913	1644	98.81
		NR_025405.1	*Microbacterium phyllosphaerae*	1478	909	1626	98.48
	HHTC9	NR_136460.1	*Youhaiella tibetensis*	1447	899	1635	99.34
		NR_147729.1	*Methyloterrigena soli*	1420	897	1628	99.23
		NR_136441.1	*Paradevosia shaoguanensis*	1407	896	1618	99.01
Bacteria isolated from compost	**JIBC1**	NR_041545.1	*Arthrobacter oryzae*	1465	172	294	96.63
		NR_026191.1	*Arthrobacter pascens*	1474	171	283	95.53
		NR_026194.1	*Paenarthrobacter nicotinovorans*	1468	169	279	95.48
	JIBC2	NR_041546.1	*Arthrobacter humicola*	1463	939	1725	99.79
		NR_041545.1	*Arthrobacter oryzae*	1465	945	1733	99.68

	Isolate Tag	Gene Bank Accession number	Nearest species	Length of 16S rRNA	Length of query aligned	Max score	Identity %
		NR_026236.1	*Pseudarthrobacter oxydans*	1486	892	1624	99.33
		NR_025084.1	*Pseudarthrobacter sulfonivorans*	1457	918	1679	99.57
	JITC1	NR_026191.1	*Arthrobacter pascens*	1474	917	1668	99.35
		NR_041545.1	*Arthrobacter oryzae*	1465	916	1663	99.24

The highest percent identity of all query-subject alignments, Identity: the highest percent identity of all query-subject alignments, length of 16S rRNA: the number of nucleotide in the subject sequence, Length of query aligned: number of nucleotides in query aligned with the subject, Abbreviations are SW = *S. wallisii*, CC, *C. comosum*, HH= *H. helix*, JI= compost, B= benzene, T=toluene, X= m-xylene, C= C=control (VOC untreated during batch experiment)

Based on the 16S rRNA gene sequencing analysis, VOC degrading bacteria isolated from control plants' rhizosphere samples were classified among 10 genera as *Microbacterium, Paenibacillus, Rhodococcus, Aeromicrobium, Paenarthrobacter, Rhizobium, Cellulomonas, Pseudomonas, Arthrobacter, Youhaiella* and *Pseudarthrobacter.* Top significant species from different genera were observed for the bacteria HHBC6 (Rhizobium and *Agrobacterium*), HHTC3 (Fig. 4. 8) and HHTC9 (*Youhaiella, Methyloterrigena* and *Paradevosia*) based on the 16S rRNA gene sequence analysis.

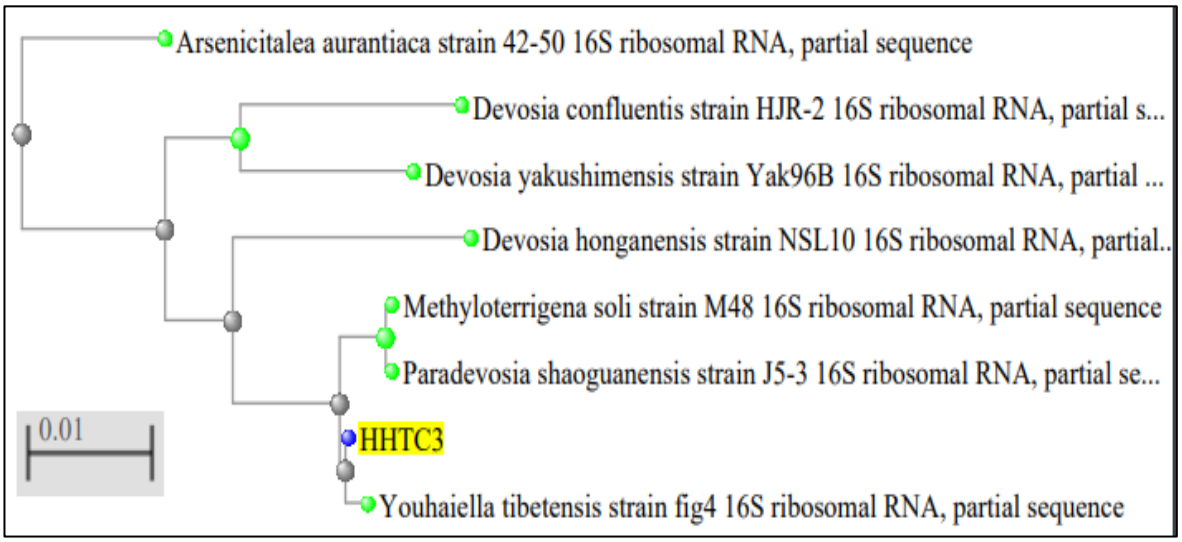

Fig. 4. 8 Phylogenetic tree to show reference species from different genera related with bacterium HHTC3

Similar to the observation taken from VOC treated plants, the majority of bacteria (93%) were Gram-positive in the control plants experiment. Only two Gram-negative bacterial genera: *Pseudomonas* and *Rhizobium* were observed. As mentioned in the previous section, most of the bacterial identifications were similar between the culture test results and sequence analysis. However, there were a few discrepancies (*) between the culture and molecular-based results obtained for bacteria isolated from control plant rhizosphere. The full list of culture method predicted bacterial genera names and 16S rRNA sequence identification of bacteria isolated from VOC treated plants are listed in Table 4. 7.

Table 4. 7 Genus level classification of VOC degrading bacteria isolated from VOC treated plants rhizosphere and compost.

Isolate	**Genus identification by culture method**	**Species identification by sequence analysis**
SWBC1	*Microbacterium*	*Microbacterium trichothecenolyticum*
SWBC3	*Microbacterium*	*Microbacterium maritypicum*
SWBC4	*Microbacterium*	*Microbacterium maritypicum*
SWBC6	*Bacillus**	*Paenibacillus xylanexedens*
SWTC1	*Rhodococcus*	*Rhodococcus pedocola*
SWTC5	*Rhodococcus*	*Rhodococcus pedocola*
SWTC7	*Rhodococcus*	*Rhodococcus pedocola*
SWTC8	*Microbacterium*	*Microbacterium hydrocarbonoxydans*
SWTC10	*Rhodococcus*	*Rhodococcus qingshengii*
SWTC11	*Microbacterium*	*Microbacterium maritypicum*
SWTC12	*Aeromicrobium*	*Aeromicrobium ponti*
CCTC2	*Microbacterium*	*Microbacterium aerolatum*
CCTC3	*Microbacterium*	*Microbacterium aerolatum*
CCTC5	*Microbacterium*	*Microbacterium aerolatum*
CCTC7	*Microbacterium*	*Microbacterium aerolatum*
CCTC11	*Microbacterium*	*Microbacterium aerolatum*
CCXC1	*Bacillus**	*Paenibacillus xylanexedens*
CCXC2	*Pseudomonas*	*Pseudomonas plecoglossicida*
HHBC1	*Arthrobacter**	*Paenarthrobacter nitroguajacolicus*
HHBC6	*Rhizobium*	*Rhizobium nepotum*
HHTC2	*Cellulomonas*	*Cellulomonas pakistanensis*
HHTC3	*Agrobacterium**	*Youhaiella tibetensis*

Isolate	Genus identification by culture method	Species identification by sequence analysis
HHTC4	*Rhodococcus*	*Rhodococcus fascians*
HHTC6	*Aeromicrobium*	*Aeromicrobium ginsengisoli*
HHTC7	*Microbacterium*	*Microbacterium maritypicum*
HHTC9	*Agrobacterium**	*Youhaiella tibetensis*
JIBC1	*Micrococcus**	*Arthrobacter oryzae*
JIBC2	*Brucella**	*Arthrobacter humicola*
JITC1	*Brucella**	*Pseudarthrobacter sulfonivorans*

Abbreviations are SW = *S. wallisii*, CC, *C. comosum*, HH= *H. helix*, JI= compost, B= benzene, T=toluene, X= m-xylene, C= control (VOC untreated during batch experiment), * -different identifications based on culture and molecular method

SWBC6 and CCXC1 were identified as Gram-positive *Bacillus* according to culture-dependent method, but sequence analysis revealed them as *Paenibacillus.* Though culture results classified bacteria HHTC3 and HHTC9 to *Agrobacterium*, their sequence analysis revealed them as *Youhaiella tibetensis*. Bacteria HHBC1 was *Arthrobacter* in culture method, but it was identified as *Paenarthrobacter*. Bacteria from the genus *Paenarthrobacter* previously belonged to the genus *Arthrobacter* and later they were classified as a new genus, however, this new genus was not mentioned in the reference manual used in this study (Busse, 2016). Both genera: *Arthrobacter* and *Paenarthrobacter* shared similar characters for the general biochemical tests, however they are considered as separate genera based on the different chemotaxonomic characteristics (compositions of the cell wall, lipid profile and cell proteins) (Busse, 2016; Schumann and Busse, 2017). Also, in the phylogenetic tree, bacteria from genera *Arthrobacter* and *Paenarthrobacter* branched very closely indicating their close evolutionary relationships (Fig. 4. 9).

Bacteria: JIBC2 and JITC1 were observed as Gram-negative bacteria during culture analysis, thus they were classified to Gram-negative genus *Brucella*. But according to their 16S rRNA sequence analysis, they were identified as Gram-positive *Arthrobacter* and *Pseudarthrobacter* respectively.

Genus *Arthrobacter* shows Gram variables, thus young cultures can be stained as Gram-negative while mature cultures stained as Gram-positive bacteria. However, genetically they are classified as Gram-positive bacteria (Mulllakhanbhai and Bhat, 1965; Mongodin *et al.*, 2006). *Pseudarthrobacter* is a novel genus originated from *Arthrobacter* (Busse, 2016; Schumann and Busse, 2017). Bacteria from the genus *Pseudarthrobacter* exhibits biochemical characters similar to the genus *Arthrobacter*, however based on different chemotaxonomic characteristics between them, they are considered as different genera (Busse, 2016; Schumann and Busse, 2017). Bacterium JIBC1 was classified as Micrococcus based on the culture characteristics, however, it was identified as *Arthrobacter oxydans.* Some bacteria from genera *Micrococcus* and *Arthrobacter* share similar biochemical and morphological characteristics, however they can be differentiated based on their 16S rRNA gene sequences analysis (Crocker *et al.*, 2000).

In the phylogenetic tree (Fig. 4. 9), close similarities between the test bacteria and reference bacterial sequences and also the similarities among the test bacteria from different soil samples can be identified. For example, test bacteria CCTC3 and CCTC7 (*M. aerolatum*) and CCXC1 and SWBC6 (*Paenibacillus xylanexedens*) were clustered closely (sister groups) based on their 16S rRNA gene sequences. Also, benzene (CCB4) and toluene (CCTC2, CCTC3, CCTC5, CCTC7 and CCTC11) degrading *M. aerolatum* species clustered closely in the phylogenetic tree.

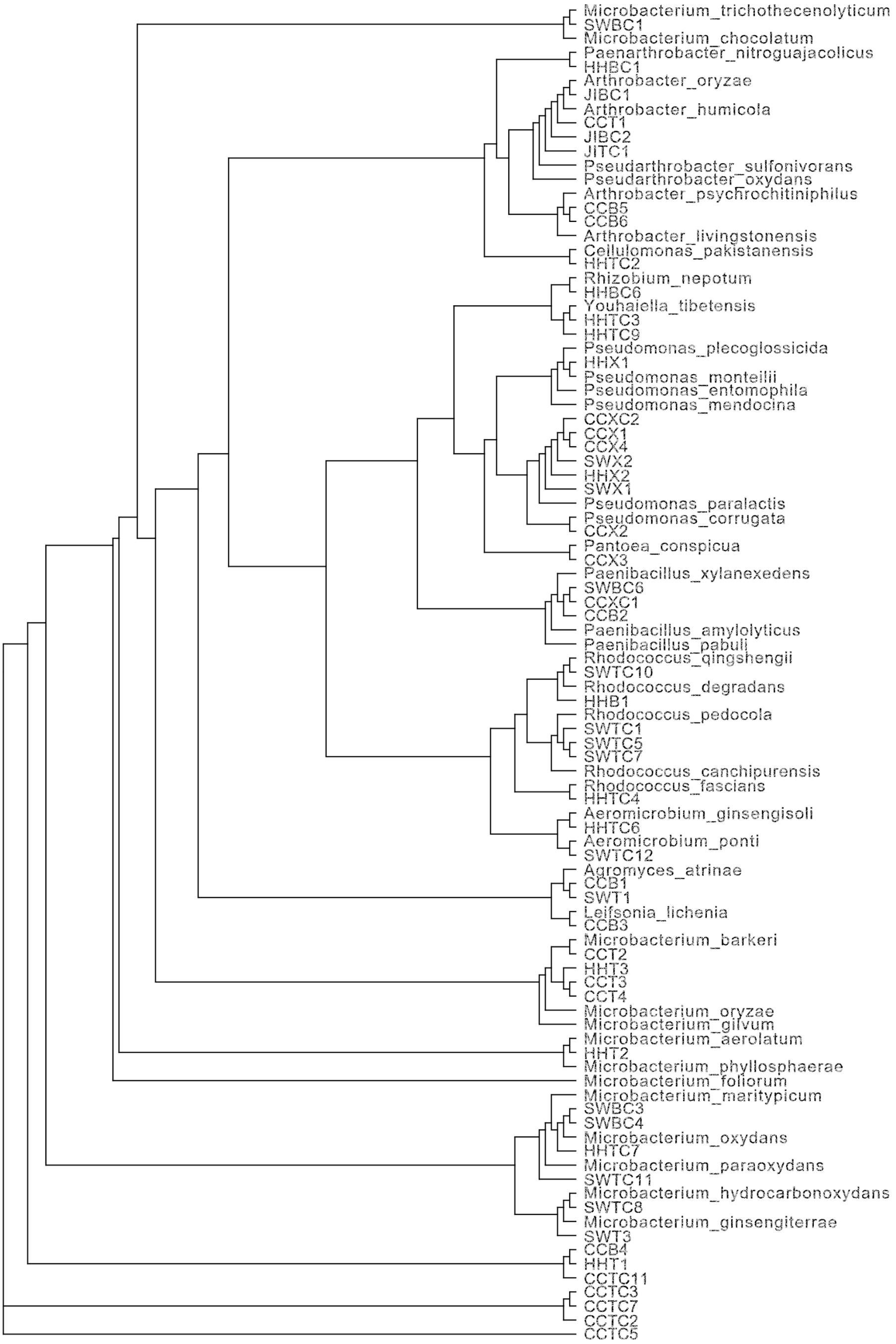

Fig. 4. 9 Phylogenetic tree indicating all test bacteria and their nearest species identifications based on the 16S rRNA sequence analysis

4.2.3. Summary of results and conclusion

We isolated and identified gaseous benzene, toluene and m-xylene-utilizing bacteria in the rhizosphere of plants exposed and non-exposed to VOC vapour for four weeks. According to identifications, the majority of bacteria were Gram-positive. Also, more bacteria were isolated from soil samples in the presence of toluene than any other VOC. No bacterial growth was observed on the MSA plates inoculated with the same soil suspension with no VOC present (negative control experiment). MSA did not contain a carbon and energy source, therefore the isolated bacteria in the presence of VOC vapour, were able to use VOC as their sole carbon and energy source. Therefore, these results concluded that the isolated bacteria from the rhizosphere of *S. wallisii*, *C. comosum*, *H. helix* utilize gaseous benzene, toluene and m-xylene.

Most of the bacteria isolated from VOC treated and control plants belonged to the genus *Microbacterium*. Bacteria from genera *Microbacterium, Rhodococcus*, *Paenibacillus, Arthrobacter* and *Pseudomonas* were identified in both VOC treated and control plants' rhizosphere (Fig. 4. 10). This indicated they might be acting as key bacteria with the VOC degradation capability. *Agromyces, Micrococcus*, *Brucella, Leifsonia* and *Pantoea* were observed only in the VOC treated plants while *Aeromicrobium*, *Rhizobium, Cellulomonas, Paenarthrobacter, Pseudarthrobacter,* and *Youhaiella* were observed only in the control plants rhizosphere. The common key bacteria found in both experiments were able to utilise VOC vapour as the carbon and energy source for their growth faster than the slow growing bacteria, thus they colonise quickly by outcompeting the growth of slow growers (bacteria observed only in the VOC treated plants or control plants). Therefore, when picking a bacterial colony from MSA plate, there is a high possibility of picking fast growers than slow growers. Another possible suggestion is, during the four weeks VOC treatment period (detailed in section 2.3), through the root exudates and quorum signals possibly plants and root microbes respectively altered the rhizosphere microbiome to some extent to degrade VOC from the atmosphere. As a result, the abundance of some bacteria who utilize VOC can be higher in the VOC exposed plants' rhizosphere than VOC non-exposed plants. Therefore, the bacteria isolated from VOC exposed plants: plants grown under the VOC contaminated

condition, may represent some of the bacteria involved during phytoremediation of gaseous VOC. However, the control plants were not exposed to VOCs. Therefore, the alteration of rhizosphere microbiome in control plants, grown under the non-contaminated conditions, for phytoremediation process was possibly less than the VOC treated plants. Following the rhizosphere soil extraction from control plants, the plant-soil interaction was terminated. Therefore, the bacteria isolated from control plants can be represented by the VOC degrading bacteria available in the plants when the plants were not influenced by atmospheric VOC. Compared to number of isolates picked from the rhizosphere, there were only a few bacteria (totally three bacteria: JIBC1, JIBC2 and JITC1) isolated from compost samples and all of them belonged to the genera *Arthrobacter* or *Pseudarthrobacter. Pseudarthrobacter* is a novel genus originated from *Arthrobacter* (Busse, 2016; Schumann and Busse, 2017). Therefore, it was concluded *Arthrobacter* is an abundant bacterium in compost samples with the capability of utilizing VOC vapours from air. Only three bacteria could be isolated, probably because other VOC degrading bacteria in compost samples are slow growers in the MSA medium or most of them are unculturable.

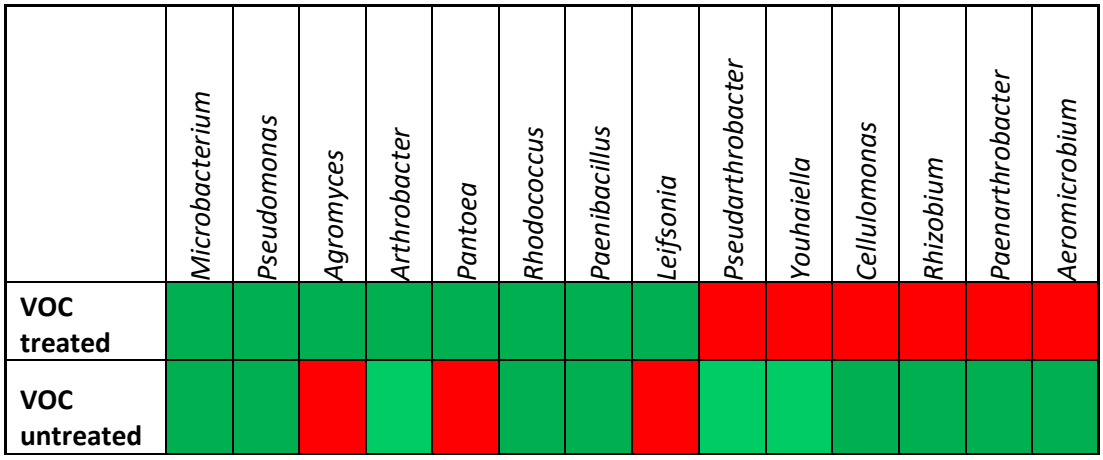

Fig. 4. 10 Comparison of bacterial groups found in rhizosphere where the plants exposed and non-exposed to VOC during the four weeks treatment period. Green colour indicates presence of bacteria while absence of bacteria represented by red colour.

In this chapter, we isolated and identified a list of gaseous VOC utilizing bacteria in three plant species and compost. Though the soil contains millions of bacteria, only a limited number was isolated following incubating the soil inoculated MSA supplied with VOC (BTX) vapours. Probably, this could have happened due to the unculturability of most of VOC degrading soil bacteria and also, they can be slow growers on the supplied medium. Therefore, molecular-independent microbiome profiling approach can be used to enhance the understanding of VOC degrading bacteria in the rhizosphere and compost samples.

Chapter 5. The impact of benzene exposure on the rhizosphere microbiome of S. wallisii, C. comosum and H. helix

5.1. The impact of benzene exposure on the taxonomical profiles of the rhizosphere microbiome

This section reports the impact of benzene exposure on the rhizosphere microbiome of three plant species. Taxonomic profiles of benzene exposed plants were compared to unexposed plants to identify differences in the rhizosphere bacterial community.

5.1.1. Introduction

Culture-dependent methods allow us to explore the diversity of the plant rhizosphere microbiome to some extent, however, the capacity of bacterial identification is limited because using this traditional approach only a small percentage of (between 0.1-1%) total environmental microorganisms can be cultured (Pham and Kim, 2012; Mendes *et al.*, 2013; Sun *et al.*, 2014). Culture-independent techniques have a greater capacity to identify unknown bacteria from soil samples than the culture methods because, through metagenomic DNA extraction, DNA from both culturable and un-culturable microbes in environmental samples can be extracted (Schloss and Handelsman, 2008). High-throughput sequencing studies based upon the 16S rRNA gene facilitate the identification of complex bacterial communities, such as plant rhizospheres.

In this study, bacterial community diversity was investigated following the exposure of three plant species: *S. wallisii*, *C. comosum* and *H. helix* to benzene. Microbiome analysis was conducted through amplification and sequencing of the bacterial 16S rRNA variable region 4.

5.1.2. Evaluation of commercial DNA extraction kits for total DNA extraction from rhizosphere

The concentration of the DNA extracted using commercial DNA extraction Kits were evaluated through fluorometric analysis (detailed in section 2.5) (Table 5. 1).

Table 5. 1 DNA concentrations assessed by fluorometric determinations

DNA extraction method	**Replicate number**	**DNA quantification after extraction (ng/µL)**
QIAamp fast DNA stool mini kit	1 2 3	2.25 4.20 3.11
MO BIO PowerSoil® DNA isolation kit.	1 2 3	11.16 13.56 11.80

DNA extracted from the rhizosphere of benzene untreated *S. wallisii* using MO BIO PowerSoil® DNA isolation kit yielded more than double the concentration of DNA as the DNA resulted from the QIAamp fast DNA stool mini kit (Table 5. 1). Therefore, MO BIO PowerSoil® DNA isolation kit was more suitable to use as the DNA extraction kit in this study. The performance of the MO BIO PowerSoil® DNA isolation kit to extract total community DNA was further evaluated to validate this section. Successful amplifications (Fig. 5. 1 and Fig. 5. 2) were obtained from bacterial 16S rRNA gene and fungi ITS regions in the DNA extracted from rhizosphere of benzene untreated *S. wallisii* (detailed in section 2.6).

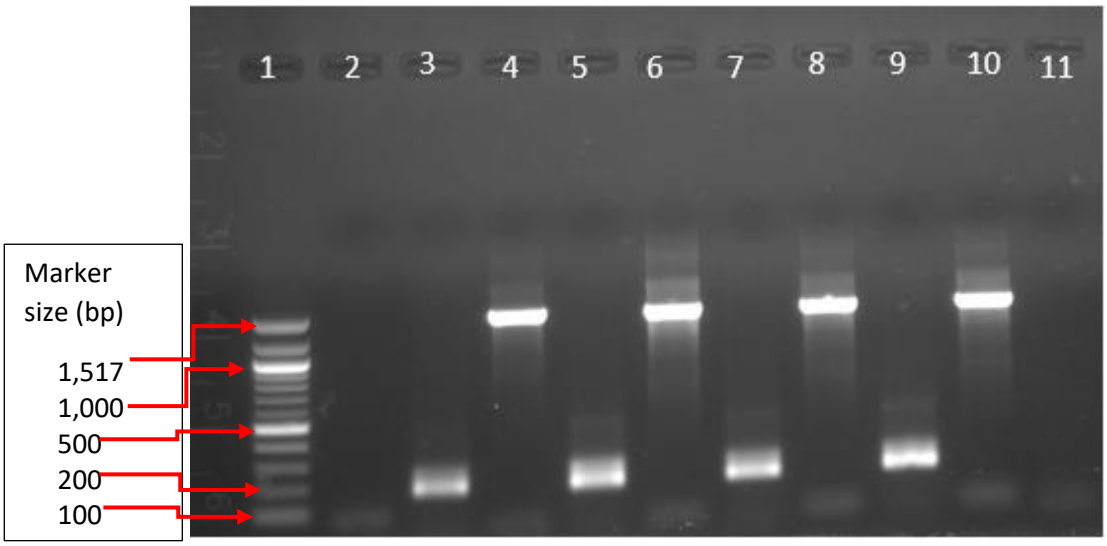

Fig. 5. 1 Agarose gel image to show bacterial 16S rRNA gene amplicons obtained from benzene untreated *S. wallisii* rhizosphere bacterial community following DNA extraction using MO BIO PowerSoil® DNA isolation kit. Lanes are: 100bp DNA ladder (1), PCR control without DNA (2,11), V3/V4 region amplicons (200 bp) obtained using EUB 518/338 primers (3, 5, 7 and 9), V1/V9 amplicons (1485 bp) obtained using 1492R/27F primers (4, 6, 8 and 10).

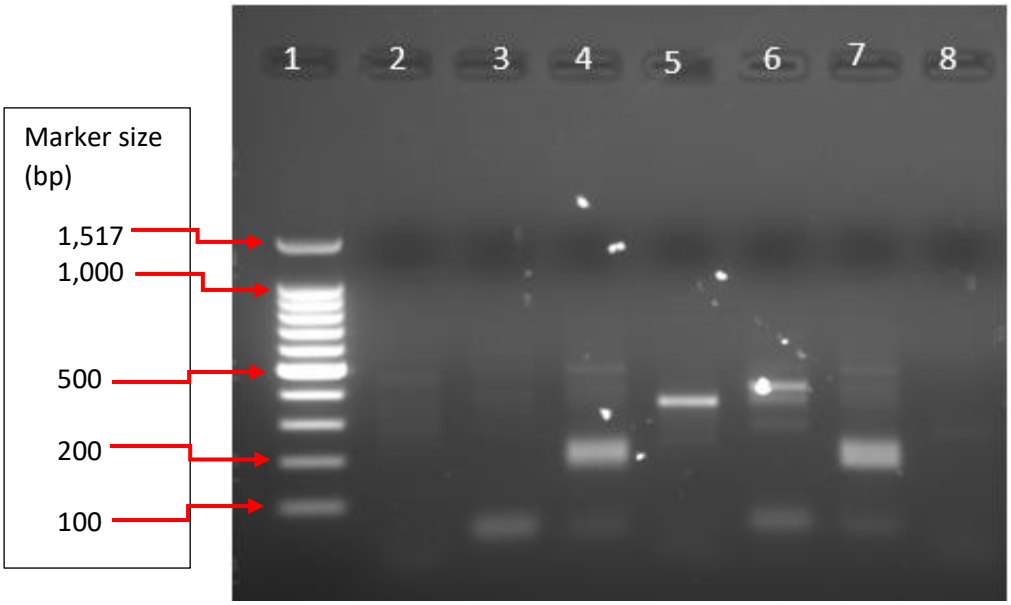

Fig. 5. 2 Agarose gel image to show bacterial 16S rRNA gene and fungi ITS region amplicons obtained from benzene untreated *S. wallisii* rhizosphere bacterial community following DNA extraction using MO BIO PowerSoil® DNA isolation kit. Lanes are 100bp DNA ladder (1), blank well (2 and 8), PCR control without DNA (3), ITS1 region amplicons (≈350 bp) obtained using

ITS1F/ITS2R primers (5), ITS2 region amplicons (≈400 bp) obtained using ITS3F/ITS4R primers (6) and V3/V4 region amplicons (194 bp) obtained using probio uni/R primers (4 and 7).

This trial verified, successful DNA extraction from two types of soil organisms (bacteria and fungi) using MO BIO PowerSoil® DNA isolation kit, thus it was suitable for the soil community DNA extraction. Therefore, all the total DNA extractions in this study were conducted using MO BIO PowerSoil® DNA isolation kit. In addition, based on the above results, primer pair Probio uni/R was selected to quality check all DNA samples prior to sequencing V3/V4 regions of bacterial 16S rRNA gene through Illumina Miseq sequence and the primer pair 1492R/27F was selected to amplify entire 16S rRNA gene through Sanger sequencing detailed in section 2.6 and 2.11.2.1 respectively.

5.1.3. PCR amplification of bacterial DNA from benzene exposed plant rhizosphere and Illumina MIseq sequencing summary

All the soil samples, selected following the exposure of plants to 10 ppm benzene for four weeks (detailed in section 2.3), contained the expected bacterial 16S rRNA gene (refer Appendix 4 for the concentration of DNA). Therefore, they were suitable for sequencing through Illumina MiSeq sequence method. PCR followed by the agarose gel verified the presence of the expected hypervariable region (V3/V4) of the 16S rRNA gene in DNA extracted from the rhizosphere of both VOC exposed and non-exposed *S. wallisii*, *C. comosum* and *H. helix* as well as VOC non-exposed compost samples (Fig. 5. 3).

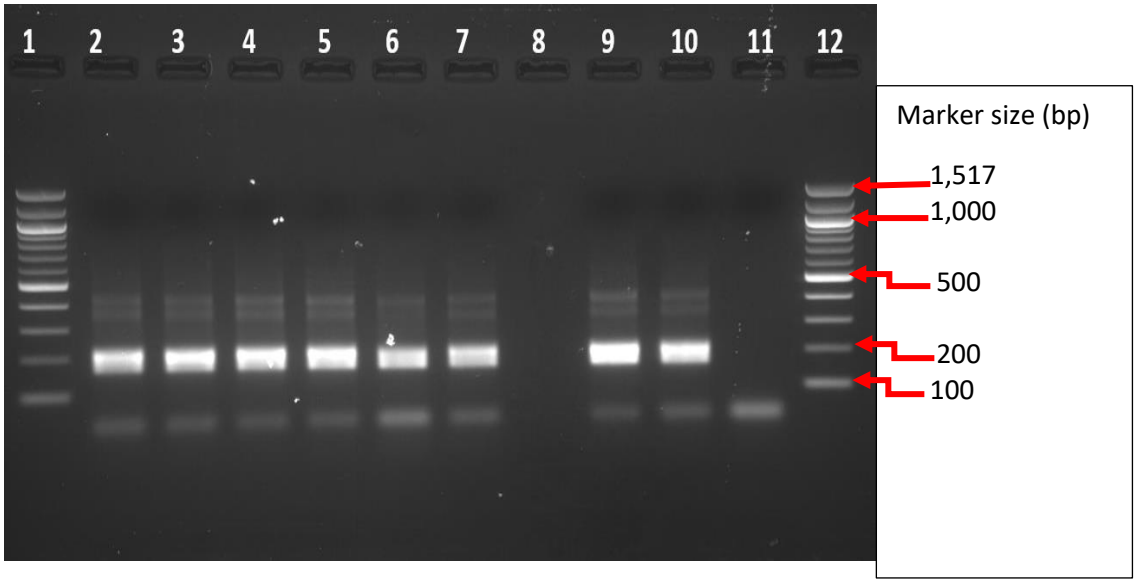

Fig. 5. 3 Agarose gel image to show V3/V4 amplicons (194 bp) obtained from rhizosphere bacterial community DNA. Lanes are: 100bp DNA ladder (1, 12), benzene treated *C. comosum* (2), benzene untreated *C. comosum* (3), benzene treated *H. helix* (4), benzene untreated *H. helix* (5), and benzene treated *S. wallisii* (6), benzene untreated *S. wallisii* (7), blank well (8), compost (9, 10) and PCR control without DNA (11).

During sequencing (detailed section 2.9.1), DNA samples were amplified using V3V4_F/R primer pair, amplicons were barcoded, sequencing libraries prepared (service provided by MWG Eurofins), and sequenced using the Illumina MiSeq sequencing platform (Eurofins Genomics, Germany) with a 430 bp paired-end read matrix conducted. A total 6,551,516 paired-end reads were obtained for the whole study (comprising 14 soil DNA samples) (Fig. 5. 4). Based upon the number of raw reads passing initial quality control, the largest and the smallest bacterial communities were benzene treated *H. helix* (1,839,801 reads) and compost (133,106 reads) samples respectively.

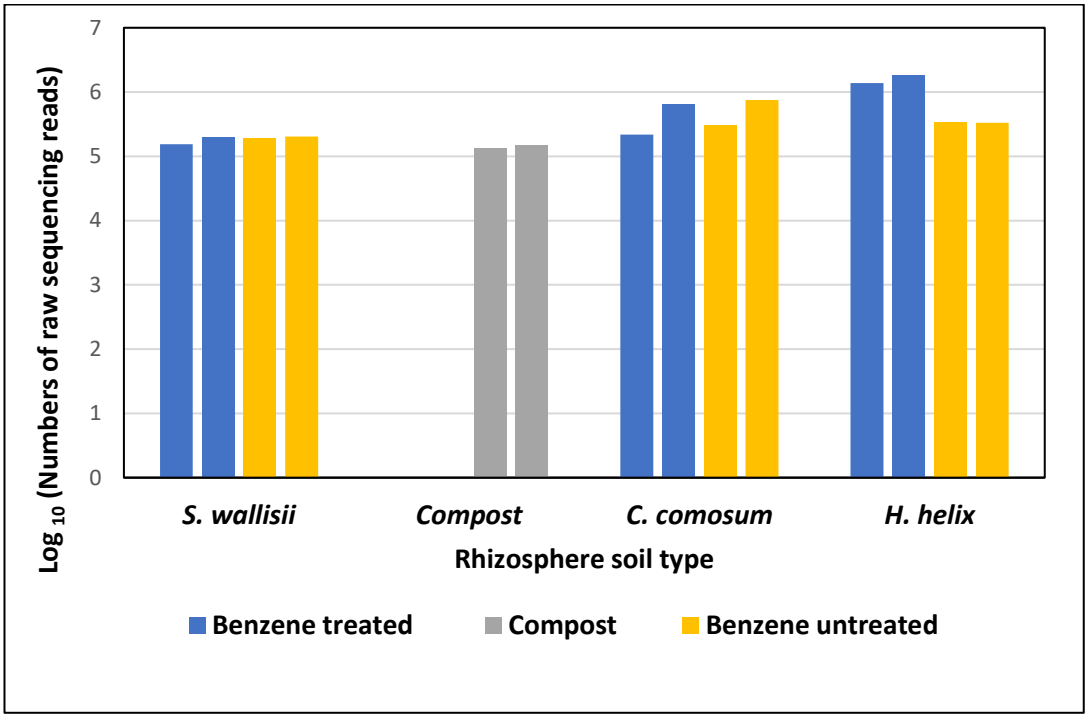

Fig. 5. 4 The number of raw sequencing reads generated from benzene treated and untreated *S. wallisii, C. comosum* and *H. helix* rhizosphere samples and compost following amplification and sequencing of the V3-V4 region of the 16S rRNA gene in bacteria

The V4 regions in the sequencing reads were filtered through trimming out the V3 region from long V3/V4 reads. This reduced the distance matrix in the data set, thus it reduced computational memory usage during the analysis. Following subsampling the sequencing libraries to the smallest number of sequences (detailed in section 2.9.1) there was a total of 1,863,484 reads for the analysis.

5.1.4. Alpha diversity analysis

According to the paired-end Illumina MiSeq reads analysis, performed in the QIIME implemented microbiome analysis platform NEPHELE (detailed in section 2.11), there was a total of 530,745 quality passing reads from all samples. Also, a total 56,573 unique reads (OTUs) were found from the sequencing pool. Alpha diversity was (number of taxonomic units and their abundance in each sample) calculated based on four metrics; observed species richness, phylogenetic diversity (PD), Shannon index and Chao1 estimator (Table 5. 2.) In detail, observed species richness estimated the number of unique sequences (OTUs) in each sample, Chao1 estimator and phylogenetic

diversity (PD) estimated rhizosphere community richness and the Shannon index was used for community diversity analysis.

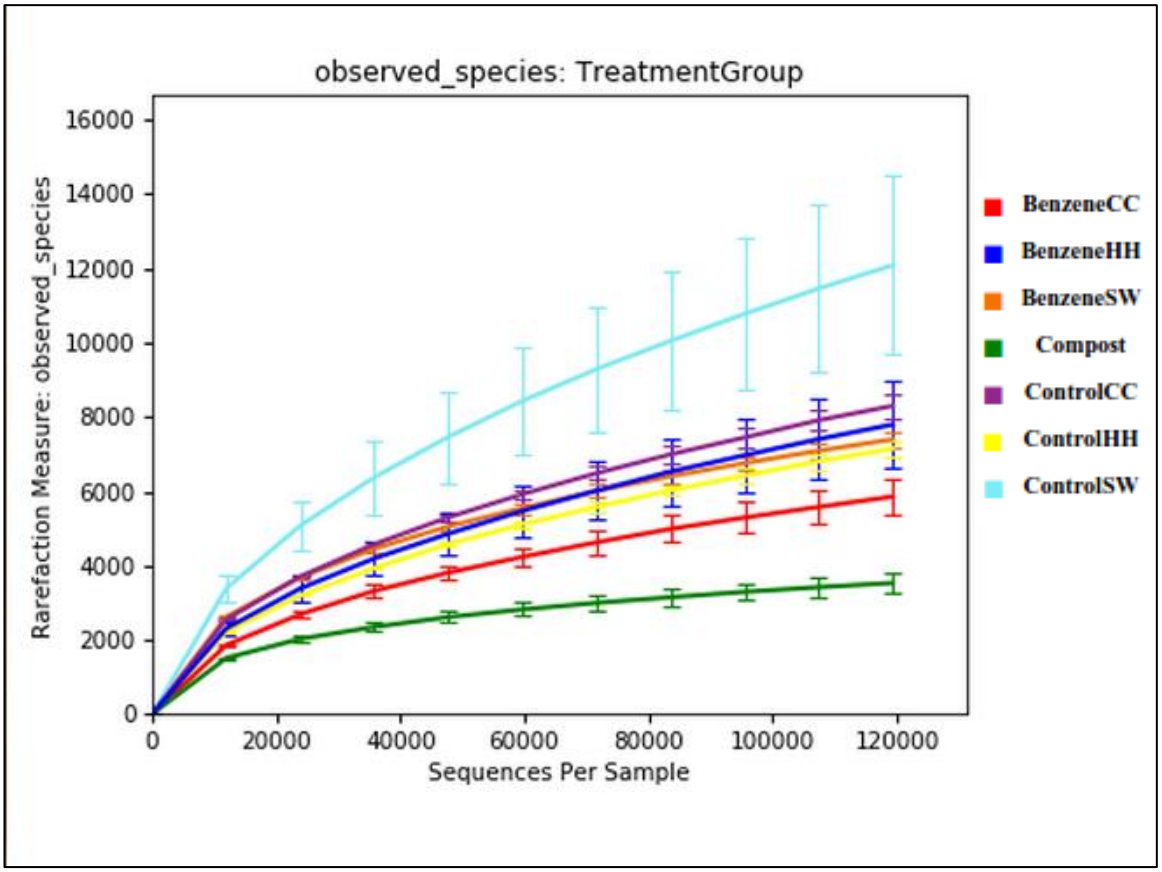

Fig. 5. 5 Alpha diversity rarefaction curves of each rhizosphere community to show the estimated number of OTUs with 97% similarity. Sample key indicates (Benzene) benzene treated, (Control) benzene untreated, (CC) *C. comosum*, (HH) *H. helix* and (SW) *S. wallisii*.

During sampling raw reads, 120,000 raw sequences from each sample were rarefied to see the number of OTUs (species) in the samples (Fig. 5. 5). Each individual curve represents the estimated species richness in each rhizosphere sample. Benzene untreated *S. wallisii* (ControlSW) showed the highest species richness while bacterial community in compost demonstrates the least species richness. The steep slope of the curves (except compost) indicate more species richness might be examined by further sampling, however, all the curves become less steep as they move from left to right, this indicates that additional sampling probably will only result in a few new species. Therefore, the rarefaction curves conclude that all the samples considered had a sufficient number of sequences (sampling depth) for community diversity analysis.

Table 5. 2 Alpha diversity metrics for rhizosphere samples extracted from benzene treated and untreated *S. wallisii*, *C. comosum*, *H. helix* and compost.

Sample (n=2)	**Observed species richness (number of unique OTUs)**	**Phylogenetic diversity (PD)**	**Shannon index**	**Chao1**
BenzeneSW[1]	7,398±212	338±13	10.21±0.11	11,648±570
ControlSW[2]	12,083±2,399	510±81	10.84±0.24	21,682±4,510
Compost	3,532±266	168±11	8.53±0.01	5,176±431
BenzeneCC[3]	5,857±503	265±26	8.18±0.29	10,088±1485
ControlCC[4]	8,299±338	356±14	9.89±0.12	14,685±1,024
BenzeneHH[5]	7,797±1,180	344±58	9.66±0.15	14,953±3,137
ControlHH[6]	7,150±224	316±7	9.57±0.16	13,277±802

[1]- benzene treated *S. wallisii,* [2]- benzene untreated *S. wallisii,* [3]- benzene treated *C. comosum,* [4]- benzene untreated *C. comosum,* [5]-benzene treated *H. helix*, [6]- benzene treated *H. helix,* The data is presented as the mean ± SEM.

According to the Shannon index and Chao1 estimator in control plant samples (benzene untreated), *S. wallisii* showed the greatest diversity and species richness followed by *C. comosum* and *H. helix*. Following benzene treatment, community diversity and species richness in the rhizosphere of *S. wallisii* and *C. comosum* decreased more than their control rhizosphere communities. Also, the community diversity in *C. comosum* was less than the all sample communities considered in the experiment. However, in contrast to *S. wallisii* and *C. comosum*, Shannon diversity and Chao1 in *H. helix* increased following benzene treatment. Considering all soil communities, the lowest species richness was observed in the compost community.

5.1.5. Beta diversity analysis

Beta diversity analysis shows the compositional differences of soil microbial communities between samples. Beta diversity in the rhizosphere and compost bacterial communities were calculated based on the phylogenetic estimator: weighted UniFrac metric and visualised through PCoA plot.

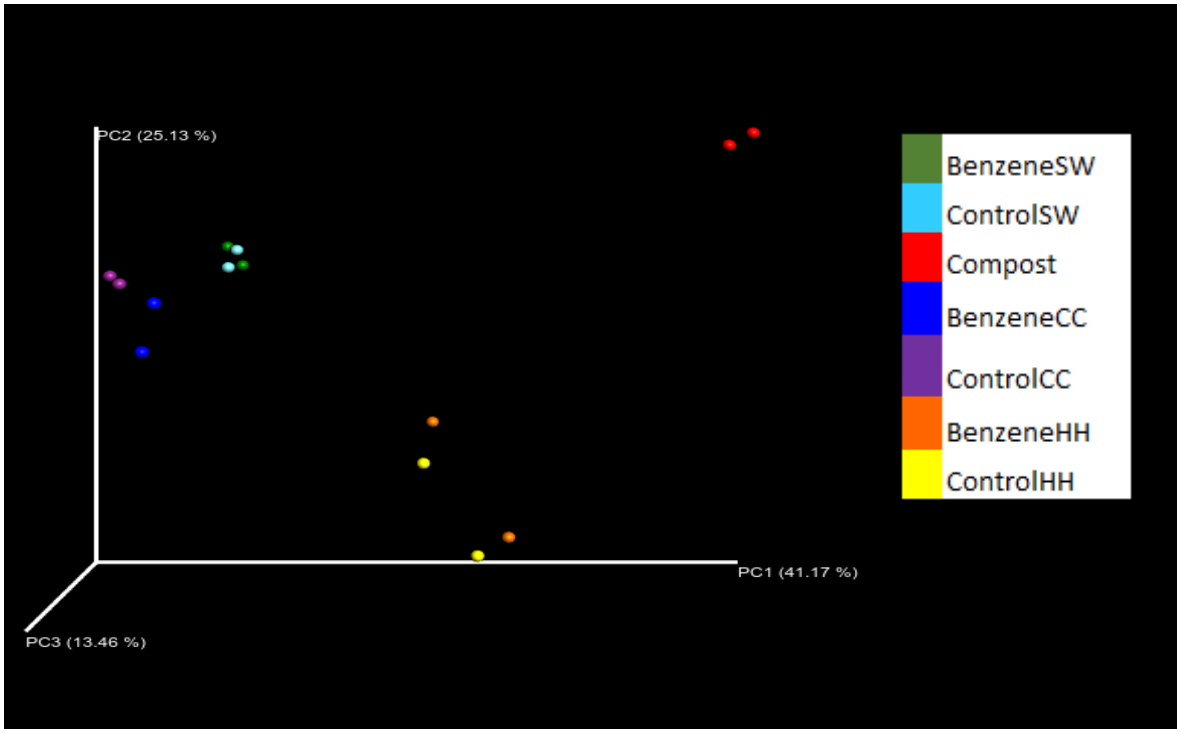

Fig. 5. 6 PCoA plot on weighted UniFrac distances for rhizosphere and compost bacterial communities. Sample key indicates (Benzene) benzene treated, (Control) benzene untreated, (CC) *C. comosum*, (HH) *H. helix* and (SW) *S. wallisii.*

In the PCoA plot (Fig. 5. 6), each point indicates a single sample and the distance between two points shows the amount of compositional difference between them. The replicates of compost samples are positioned furthest away from the rhizosphere samples and this major separation indicated different bacterial compositions between compost and rhizosphere. Benzene treated and untreated same plant species rhizosphere samples clustered very closely, indicating that their microbiome community compositions are very similar. Therefore, the results suggest that whilst benzene treatment appeared to impact upon the rhizosphere (changes of alpha diversity), this difference in community diversity structure was less than between the different plant species.

5.1.6. Rhizosphere and compost bacterial community composition

During the community analysis of 14 soil samples, 82.7% unique reads were classified to the phylum level, 82.1% to the class level, 80.9% to the order level, 79.2% to the family level, 72.4% at the genus level and 4.3% to the species level. Relative abundance as a percentage of the total community reported in the taxonomy summary were used for the multiple group comparison.

Descriptive statistics (i.e. mean relative abundance %) were used to compare the abundance of bacteria at phylum, class and the genus levels since the sample size was low (n=2). For all soil samples, Proteobacteria was the most abundant phylum with a percentage abundance ranging between 51.6 % and 75.7% (Fig. 5. 7 and Table 5. 3). Next, the bacterial phylum abundance in all rhizosphere communities was dominated by Bacteroidetes (12.5%- 18.8%), followed by Actinobacteria (4.3%- 9.4%), Acidobacteria (0.8%- 11.2%) and Gemmatimonadetes (1.3%-1.8%). Actinobacteria (21%) was the second dominant phylum in the compost samples followed by Bacteroidetes (14.9%), Saccharibacteria (5.1%) and Acidobacteria (2.7%).

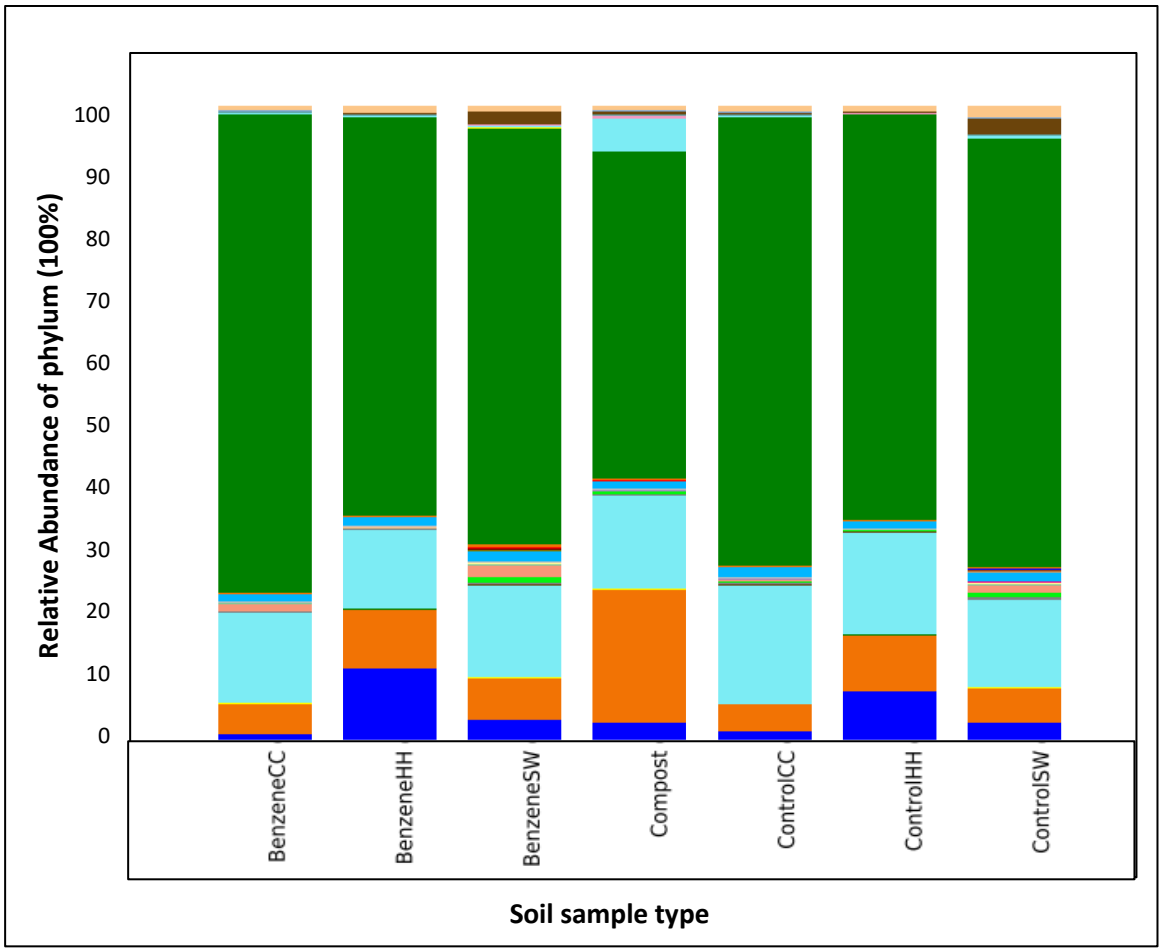

Fig. 5. 7 Distribution of dominant bacterial phyla taxonomic assignments for all soil samples. For clarity only the most abundant phyla are named. Sample key indicates (Benzene) benzene treated, (Control) benzene untreated, (CC) *C. comosum,* (HH) *H. helix* and (SW) *S. wallisii.*

Table 5. 3 Relative abundance (%) of dominant bacterial phyla in each soil samples

Legend	Phylum name	Benzene treated *C. comosum* (BenzeneCC) %	Benzene treated *H. helix* (BenzeneHH) %	Benzene treated *S. wallisii* (BenzeneSW) %	Compost %	Benzene untreated *C. comosum* (ControlCC) %	Benzene untreated *H. helix* (ControlHH) %	Benzene untreated *S. wallisii* (Control SW) %
	Proteobacteria	75.7	63.1	65.8	51.6	70.9	64.2	67.8
	Bacteroidetes	14.4	12.5	14.5	14.9	18.8	16.3	13.8
	Actinobacteria	5.0	9.4	6.5	21.0	4.3	8.9	5.5
	Acidobacteria	0.8	11.2	3.2	2.7	1.3	7.6	2.6
	Gemmatimonadetes	1.3	1.4	1.8	1.2	1.6	1.3	1.6
	Saccharibacteria	0.1	0.2	0.4	5.1	0.1	0.2	0.4
	Verrucomicrobia	0.2	0.3	2.0	0.7	0.4	0.2	2.7
	Cyanobacteria	1.2	0.1	1.8	0.1	0.3	0.1	1.3
	Chlorobi	0.1	0.1	0.3	0.0	0.2	0.1	0.3
	Unassigned	0.8	1.0	0.8	0.7	1.0	0.8	1.9

At the class level (Fig. 5. 8 and Table 5.4), Alphaproteobacteria (32.6% - 40.1%) were dominant in all rhizosphere communities followed by Gammaproteobacteria (8.3% - 32.5%), Sphingobacteriia (8.9% - 14.5%) and Betaproteobacteria (4.0% - 14.8%). Sphingobacteriia (14.1%) and Actinobacteria (11.3%) were the second and third dominant bacterial classes found in the compost samples respectively. In addition, the abundance of Thermoleophilia (8.2%) and an uncultured bacterial class from the phylum Saccharibacteria (4.9%) were higher in the compost samples than in all rhizosphere communities. Also, the relative abundance of the class Acidobacteria was higher (8.7% and 6.0% in benzene treated and untreated) in *H. helix* rhizosphere than other samples.

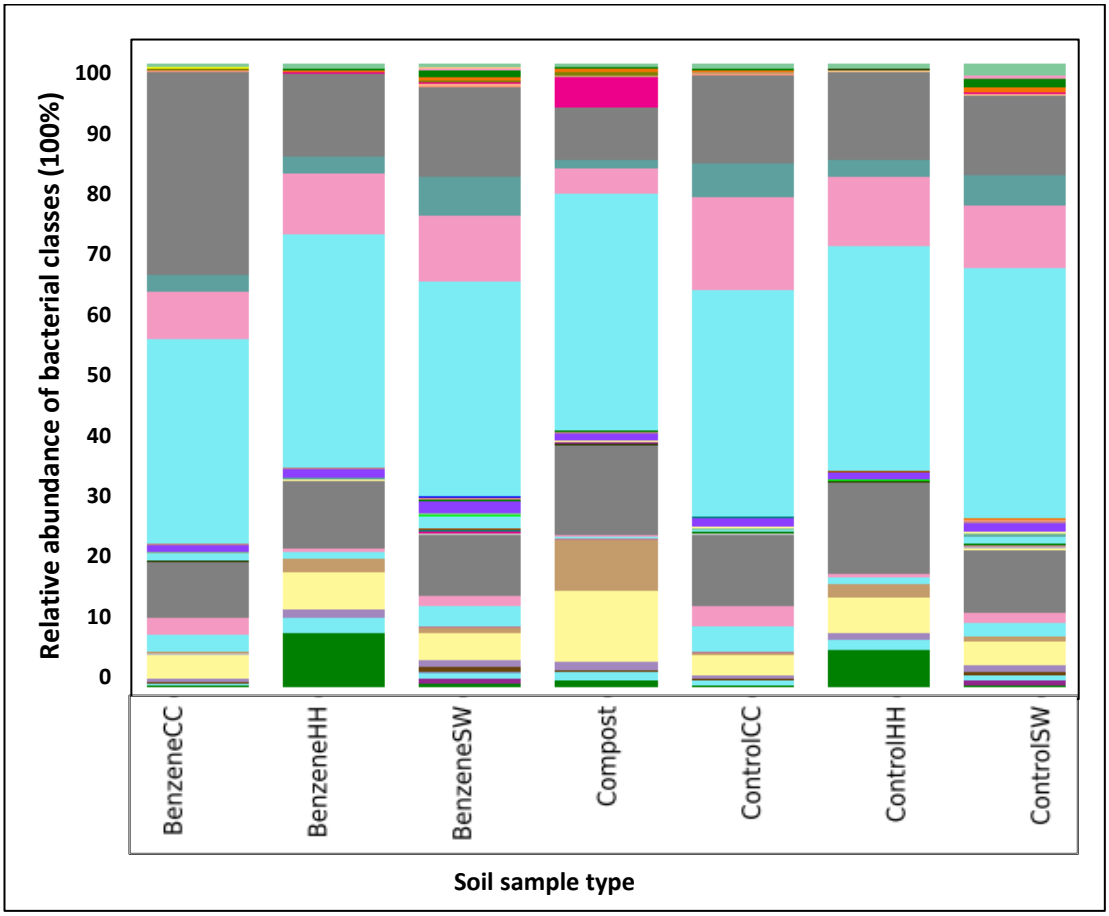

Fig. 5. 8 Distribution of dominant bacterial classes taxonomic assignments for all soil samples. For clarity only the most abundant are named. Sample key indicates (Benzene) benzene treated, (Control) benzene untreated, (CC) *C. comosum*, (HH) *H. helix* and (SW) *S. wallisii*. Description of legends with the percentage of relative abundance are shown in the Table 5.4.

Table 5. 4 Relative abundance (%) of dominant bacterial classes in each soil sample.

Legend	Class name	Benzene treated *C. comosum* (BenzeneCC) %	Benzene treated *H. helix* (BenzeneHH) %	Benzene treated *S. wallisii* (BenzeneSW) %	Compost %	Benzene untreated *C. comosum* (ControlCC) %	Benzene untreated *H. helix* (ControlHH) %	Benzene untreated *S. wallisii* (Control SW) %
	Alphaproteobacteria	32.6	37.4	34.2	37.9	36.3	35.9	40.1
	Gammaproteobacteria	32.5	13.2	14.5	8.3	14.1	14.3	12.6
	Sphingobacteriia	8.9	11.1	9.6	14.1	11.3	14.5	10.0
	Betaproteobacteria	7.6	9.6	10.8	4.0	14.8	11.3	10.0
	Deltaproteobacteria	2.9	2.9	6.0	1.4	5.4	2.5	4.8
	Actinobacteria	3.9	6.1	4.5	11.3	3.3	5.8	3.7
	Cytophagia	2.6	0.9	3.3	0.3	4.0	1.0	2.2
	Flavobacteria	2.8	0.5	1.5	0.4	3.4	0.7	1.5
	Gemmatimonadetes	1.2	1.4	1.7	1.0	1.4	1.2	1.5
	Thermoleophilia	0.4	2.1	0.9	8.2	0.4	2.1	0.8
	Uncultured bacterium from Saccharibacteria	0.1	0.1	0.4	4.9	0.1	0.1	0.3
	Acidobacteria	0.2	8.7	0.6	1.0	0.3	6.0	0.4

Mean relative abundance of the most abundant genera was compared between different soil communities and the full list of most abundant genera with their mean relative abundances is listed in the Appendix 5. At the genus level, the most abundant genera were uncultured bacterial genus from the family Chitinophagaceae (7.3% pooled mean), genus *Rhizomicrobium* (5.0% pooled mean), *Pseudomonas* (3.9% pooled mean), *Rhodanobacter* (3.9% pooled mean), *Rhizobium* (2.6% pooled mean), *Paraburkholderia* (2.6% pooled mean), *Sphingomonas* (2.1%

pooled mean), *Sphingobium* (1.9% pooled mean), *Pseudolabrys*(1.7% pooled mean), *Devosia* (1.5% pooled mean), *Flavobacterium* (1.0% pooled mean), *Novosphingobium,* (1.0% pooled mean) *Dongia* (1.6% pooled mean) and *Acidibacter* (0.6% pooled mean) in all soil samples. The most abundant genera in compost were *Pseudolabrys* (8.0%), *Rhizomicrobium* (7.7%) and *Rhodanobacter* (6.7%).

In the rhizosphere of *S. wallisii*, following benzene treatment, genus *Pseudolabrys* decreased in relative abundance (from 3.0% to 2.4%) and it was the most abundant genus observed. However, the second dominant genus in *S. wallisii, Acidibacter* increased relative abundance (from 0.7% to 2.3%) following benzene treatment. For *S. wallisii*, there was also an increase in the average relative abundance of the genus *Cellvibrio* (from 1.4% to 1.5%), *Haliangium* (from 0.5% to 1.0%), *Hyphomicrobium* (from 0.7% to 1.1%), *Woodsholea* (from 1.4% to 2.1%) and *Nicotiana otophora* species (from 1.0% to 1.6%) following benzene treatment. Conversely, there was a decrease in the relative abundance of *Mesorhizobium* (from 1.2% to 0.9%), *Flavobacterium* (from 1.0% to 0.7%) and *Luteimonas* (from 1.2% to 1.0%) due to benzene treatment into *S. wallisii.*

The response of *Rhodanobacter* to benzene treatment varied with plant species. In *S. wallisii* (from 2.4% to 1.7%) and *H. helix* (from 6.2% to 4.7%), *Rhodanobacter* relative abundance decreased following the benzene treatment, but in *C. comosum* (from 1.1% to 3.3%) relative abundance of *Rhodanobacter* increased. Similar results were observed for the genus *Rhizobium* following benzene treatment. In both *S. wallisii* (from 2.4% to 1.7%) and *H. helix* (from 1.9% to 1.4%) there were decreases in the relative abundance of *Rhizobium*, but in *C. comosum* the abundance of the genus increased (from 3.2% to 7.9%) following benzene treatment. The relative abundance of genus *Methylotenera* also decreased following benzene treatment from 1.9% to 1.3% in *S. wallisii* and from 3.0% to 1.6% in *C. comosum*. Interestingly, the relative abundance of *Sphingomonas* decreased for all three plant rhizosphere communities following benzene treatment. In *S. wallisii* and *C. comosum*, the relative abundance of *Devosia* and *Rhizomicrobium* decreased, but in *H. helix* the relative abundance of both genera was increased following the benzene treatment. The relative abundance of *Flavobacterium* in *S. wallisii* (from 1.0% to 0.7%)

and *C. comosum* (from 2.9% to 2.6%) decreased following benzene treatment. While relative abundance of *Hirschia* increased in *S. wallisii* from 0.8% to 1.0%, in *C. comosum* it was decreased from 1.0% to 0.3%. Conversely, the relative abundance of *Sphingobium* decreased in *S. wallisii* (from 2.5% to 1.8%) while increased in *C. comosum* (from 4.0% to 6.3%). Following benzene treatment, *Pseudomonas* decreased in relative abundance from 1.5% to 1.1 in *S. wallisii*. Therefore, these results show that the impact of benzene was to cause compositional changes in the relative abundance of some bacterial genera in *S. wallisii*.

In *C. comosum*, the two most abundant genera: *Pseudomonas* (from 2.1% to 22.9%) and *Rhizobium* (from 3.2% to 7.9%) increased their relative abundance following benzene treatment. Genus *Enterobacter* (from 1.3% to 0.1%), *Novosphingobium* (from 3.6% to 0.8%), *Caulobacter* (from 1.1% to 0.6%), *Dyadobacter* (from 1.0% to 0.6%), *Arthrobacter* (from 1.2% to 0.9%), *Acidibacter* (from 1.0% to 0.5%) *Varlovorax* (from 1.4% to 0.5%) in *C. comosum* decreased following the benzene treatment. Conversely, following benzene treatment, the relative abundance of *Pedobacter* (from 1.3% to 1.7%), *Brevundimonas* (from 0.6% to 1.0%) and *Stenotrophomonas* (from 0.1% to 1.3%) increased in *C. comosum*. Relative abundance of *Pseudolabrys* decreased in *C. comosum* from 1.4% to 1.1% while *H. helix* showed an increase from 1.2% to 1.3%.

In *H. helix*, *Rhizomicrobium* and *Rhodanobacter* were observed as the most abundant genera. *Rhizomicrobium* (from 6.2% to 7.3%) relative abundance increased following benzene treatment, but the relative abundance of *Rhodanobacter* (from 6.2% to 4.7%) decreased. The relative abundance of *Granullcella* (from 3.0% to 3.3%), *Bryobacter* (from 1.1% to 1.7%), *Acidothermus* (from 0.5% to 1.3%), *Dongia* (from 1.8% to 2.5%) and *Dokdonella* (from 0.7% to 1.2%) in *H. helix* increased following benzene treatment. Conversely, the relative abundance of *Mucilaginibacter* (from 4.8% to 2.4%) *Paraburkholderia* (from 6.5% to 4.1%) decreased following benzene treatment.

Overall, there were increases and decreases of the relative abundance of dominant bacteria in rhizosphere due to exposure to benzene. Presence or absence of different key bacteria between rhizosphere and compost indicated that the plant species adjust the soil community structure.

5.1.7. Summary of results and conclusions

The results of this study showed that changes in the composition of rhizosphere bacterial communities occurred following 10 ppm benzene treatments to the plants. Such changes occurred as a decrease or increase of the relative abundance following benzene treatment. At the genus level, the largest alteration in relative abundance in *S. wallisii* was identified for the genus *Acidibacter* by increasing relative abundance from 0.8% to 2.6%, following benzene treatment. In *C. comosum*, the largest shift in relative abundance at the genus level was observed for *Pseudomonas* increasing from 2.1% to 22.9%. In *H. helix*, the major shifts at the genus level occurred for genera *Mucilaginibacter* decreasing from 4.8% to 2.4% and *Paraburkholderia* by decreasing relative abundance from 6.5% to 4.1% following benzene treatment. The above observations show that due to exposing to benzene, there is an increase or decrease of the relative abundance of specific bacteria in the rhizosphere.

Investigation of the *S. wallisii, C. comosum* and *H. helix* rhizosphere bacterial community composition showed the dominance of the bacterial phyla; Proteobacteria, Bacteroidetes, Actinobacteria and Acidobacteria. However, the compost bacterial community dominated by the members of the phylum Proteobacteria followed by Actinobacteria and Bacteroidetes. The different compositions between compost and plant rhizospheres bacterial communities at the phylum stage revealed the major role of adjusting the rhizosphere microbiome composition (apart from the compost microbiome composition) is conducted by the presence of plants.

The most abundant genera were *Pseudolabrys* and *Rhizomicrobium* in compost, *Pseudolabrys* and *Acidibacter* in *S. wallisii, Pseudomonas* and *Rhizobium* in *C. comosum* and *Rhizomicrobium* and *Rhodanobacter* in *H. helix* respectively. Considering both rhizosphere and compost samples, the dominant key bacteria, major phyla, classes and genera were commonly observed in both soil

types. However, different relative abundances of those key bacterial groups among soil samples indicated the presence of plants and type of plant have altered the compositional constituent of its bacterial community.

Rhizosphere bacterial community diversity was analysed based on alpha and beta diversity analysis. Alpha diversity revealed that the three-plant species rhizosphere had a higher bacterial species richness than the compost samples. This evidence suggested the presence of plants had enriched its rhizosphere bacterial composition. Beta diversity PCoA plot showed a major separation of compost from rhizosphere, this indicated that overall compositional differences in the bacterial communities between the compost and rhizosphere. This is because, benzene treatment impacted upon the rhizosphere microbiome of all plant species by increasing or decreasing the relative abundance of bacterial taxa, however, it is less than the impact of plant species on the microbiome. Thus, as a final output, same plant species' benzene treated, and control samples clustered closely in the PCoA plot.

By comparing the above results obtained from sequencing whole bacterial community in benzene treated and untreated plant rhizosphere with the culture test results obtained in chapter 4, there were two bacterial groups identified commonly by both approaches. In the culture method, bacterial strains from genera *Arthrobacter* and *Rhizobium* were isolated after soil suspensions treated with benzene and also those genera were identified among the most abundant bacterial genera list in the sequencing pool. The majority of culture method identified species belonged to the phylum Actinobacteria and the high-throughput sequence analysis also revealed Actinobacterial as one of the dominant bacterial phyla in rhizosphere and compost samples.

The bacterial genera *Pseudomonas* and *Devosia* were dominant in the rhizosphere of plants according to the whole community analysis, however, strains from those genera could not be isolated in culture method following the benzene treatment. But there were isolated strains from the genus *Pseudomonas* in culture methods following m-xylene treatment. The rest of the bacterial strains isolated using culture method following benzene treatment belonged mostly to the bacterial genera *Rhodococcus*, *Microbacterium*, *Paenarthrobacter and Paenibacillus.* They

were identified in the community sequencing analysis with a low abundance (less than 0.01% pool mean). Conversely, most of the high abundant bacterial groups identified in the benzene treated and untreated plant rhizosphere and compost using the high-throughput method could not be isolated using culture tests. This is because only a tiny fraction of bacteria can be isolated in microbiological media, but the whole community sequence analysis reveals almost all the bacterial groups in a community.

5.2. Prediction of functional profiles of rhizosphere bacterial communities using 16S rRNA gene sequences

The aim of this section is to study the functional changes of the plant rhizosphere following exposure to 10 ppm benzene. In addition, the functional profiles of rhizosphere and compost will be compared based on the average relative abundance of functions.

5.2.1. Introduction

Functional characteristic in a microbiome can be studied using metatranscriptomics or metaproteomics studies. However, these approaches are expensive, so only a limited number of samples can be sequenced. PICRUST is a software which can predict functional profiles of an unknown microbiome using their 16S rRNA taxonomical sequences. Therefore, to predict bacterial functional annotation of benzene treated and untreated plant rhizosphere and compost, bacterial community DNA sequences used in the previous section were re-analysed (detailed in section 2.9.3). Functional predictions were assigned to three KEGG levels and based on the abundance of functions statistical analysis was performed and graphs were made using STAMP software (detailed in section 2.9.3).

5.2.2. Comparison of functional predictions among soil samples

Based on the KEGG orthology database, PICRUST predicted functional analysis assignment using 16S rRNA taxonomical sequences for up to three KEGG levels. At the KEGG level 1, PICRUST inferred 6 functional categories as; “metabolism”, “genetic information processing”,

"environmental information processing", "cellular process", "human disease" and "organismal system" (Fig. 5. 9).

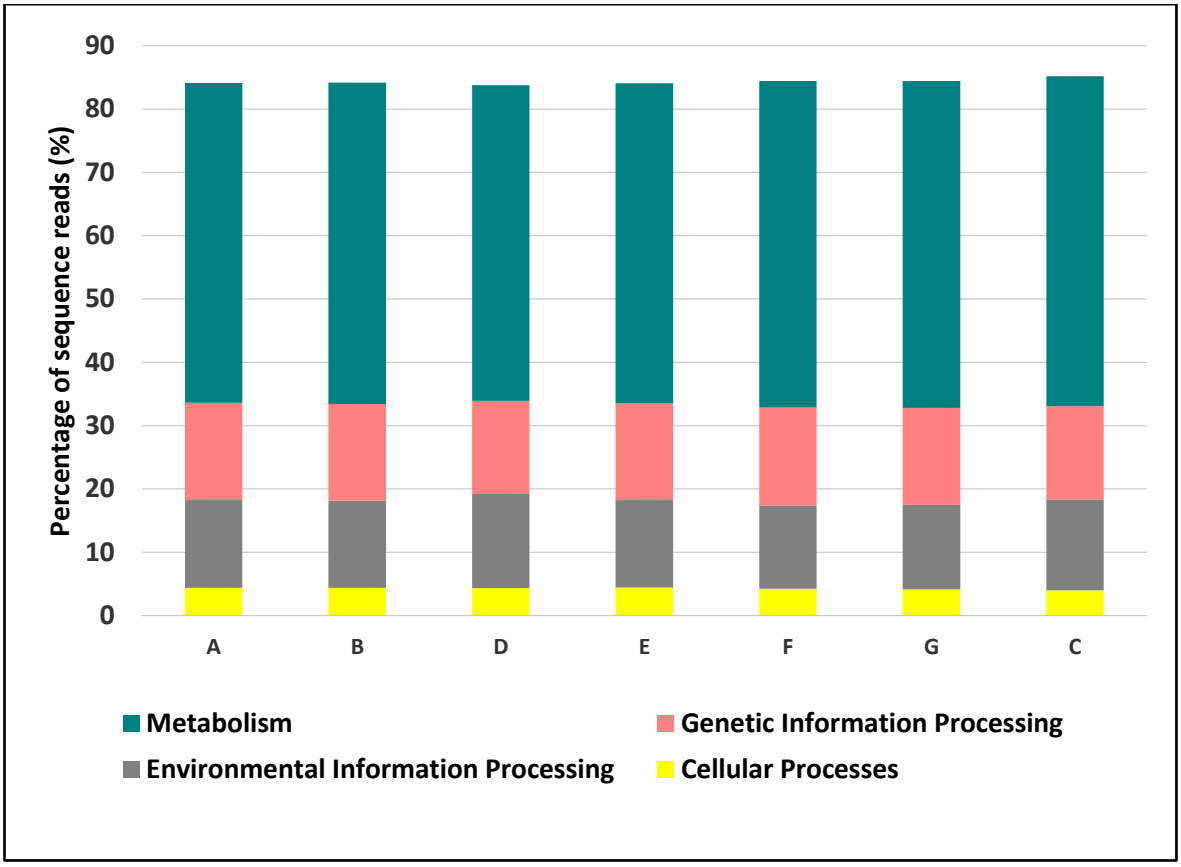

Fig. 5. 9 Percentage of sequence reads assigned to KEGG level 1 functions among all soil samples. Percentage of sequence reads assigned to KEGG level 1 functions among all soil samples. Functional profiles for human disease and organismal system are excluded. Sample key indicates A-(benzene treated *S. wallisii*), B-(control *S. wallisii*), C-(compost), D-(benzene treated *C. comosum*), E-(control *C. comosum*), F-(benzene treated *H. helix*) and G-(control *H. helix).*

More than 50% of sequences from each soil sample were assigned to the functional protein (Fig. 5. 9) involved in the metabolism followed by genetic information processing (> 14.7%), environmental information processing (>13.1%) and cellular processes (>4.0%). The other two KEGG level 1 functions; human disease and organismal systems accounted ≈2% sequences and approximately 13.5% sequences were unclassified into functional categories. At the KEGG level 2, there were 41 functional protein encoding gene families identified and the majority of KEGG level 2 functions belong into the functional group Metabolism (Table 5. 5). KEGG level 2 function,

membrane transport accounted for the greatest number of functional protein (pool mean >11.1%) followed by amino acid metabolism (pool mean=10.8%). The relative abundance of those functional proteins was higher in the compost community than all rhizosphere communities. In addition, the carbohydrate metabolism (pool mean=10.2%) was higher in the compost samples and *H. helix* rhizosphere than *C. comosum* and *S. wallisii*. Proteins involved in the function, xenobiotic biodegradation and metabolism showed (4.2% pool mean) a higher relative abundance in the compost samples than all rhizosphere samples. The functional abundance of membrane transport was greater in the compost (pool mean=12.5%) followed by rhizosphere of benzene treated *C. comosum* (12.2%) than all other soil samples. However, there are very little changes in the mean relative abundance of KEGG level 2 functions between the treatment and control of same plant species (Table 5. 5). Therefore, based on KEGG level 2 functional categories, our findings indicated that exposure to low concentration of benzene has little influence on rhizosphere bacterial functions.

Table 5. 5 Mean functional abundance (%) assigned to KEGG level 2 for all data set. Functions from human disease and organismal systems are excluded.

Function	Benzene treated *S. wallisii* (%)	Control *S. wallisii* (%)	Benzene treated *C. comosum* (%)	Control *C. comosum* (%)	Benzene treated *H. helix* (%)	Control *H. helix* (%)	Compost (%)
Cellular Processes; Cell Growth and Death	0.7	0.7	0.6	0.7	0.6	0.6	0.7
Cellular Processes; Cell Motility	3.3	3.3	3.4	3.3	3.2	3.1	2.9
Cellular Processes; Transport and Catabolism	0.4	0.4	0.3	0.4	0.4	0.4	0.4
Environmental Information Processing; Membrane Transport	11.3	11.2	12.2	11.2	10.7	10.9	12.0
Environmental Information Processing; Signal Transduction	2.3	2.3	2.4	2.3	2.2	2.2	2.1
Environmental Information Processing; Signalling Molecules and Interaction	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Genetic Information Processing; Folding, Sorting and Degradation	2.2	2.2	2.1	2.2	2.2	2.1	2.1
Genetic Information Processing; Replication and Repair	6.7	6.7	6.4	6.7	6.8	6.7	6.4
Genetic Information Processing; Transcription	2.3	2.4	2.4	2.4	2.5	2.5	2.4
Genetic Information Processing; Translation	4.1	4.0	3.7	3.9	3.9	3.9	3.8

Function	Benzene treated *S. wallisii* (%)	Control *S. wallisii* (%)	Benzene treated *C. comosum* (%)	Control *C. comosum* (%)	Benzene treated *H. helix* (%)	Control *H. helix* (%)	Compost (%)
Metabolism; Amino Acid Metabolism	10.8	10.8	10.8	10.8	10.9	11.0	11.2
Metabolism; Biosynthesis of Other Secondary Metabolites	1.0	1.0	1.0	0.9	1.1	1.1	1.0
Metabolism; Carbohydrate Metabolism	9.9	10.0	9.7	10.0	10.4	10.3	10.5
Metabolism; Energy Metabolism	5.7	5.6	5.4	5.5	5.4	5.5	5.5
Metabolism; Enzyme Families	1.9	1.9	1.8	1.8	1.9	1.8	1.7
Metabolism; Glycan Biosynthesis and Metabolism	1.9	1.9	1.8	1.9	2.1	2.1	1.8
Metabolism; Lipid Metabolism	3.9	3.9	3.9	3.9	4.1	4.1	4.2
Metabolism; Metabolism of Cofactors and Vitamins	4.1	4.1	4.0	4.1	4.0	4.0	4.0
Metabolism; Metabolism of Other Amino Acids	2.1	2.1	2.1	2.1	2.1	2.1	2.2
Metabolism; Metabolism of Terpenoids and Polyketides	2.2	2.2	2.1	2.2	2.2	2.2	2.4
Metabolism; Nucleotide Metabolism	3.1	3.1	2.9	3.0	3.0	3.0	3.0
Metabolism; Xenobiotics Biodegradation and Metabolism	3.9	4.1	4.1	4.2	4.1	4.2	4.6

Functional protein-coding gene family in the soil samples were clustered in a PCoA plot according to the PICRUST inference at KEGG level 3 (Fig. 5. 10), there is a clear distinction of overall functions in compost samples from all other plant rhizosphere samples. The deviation of compost from the plant rhizosphere indicated that the presence of plant is the key factor to regulate its soil community functional diversity. Also, clustering samples in the same plant rhizosphere together (except *S. wallisii* samples) based on their functional predictions, indicated that the type of plant species is one of the main factors to determine its rhizosphere functions. Also, the close clustering pattern of same plant species samples suggests that the benzene treatment has had little influence on plant rhizosphere functional diversity.

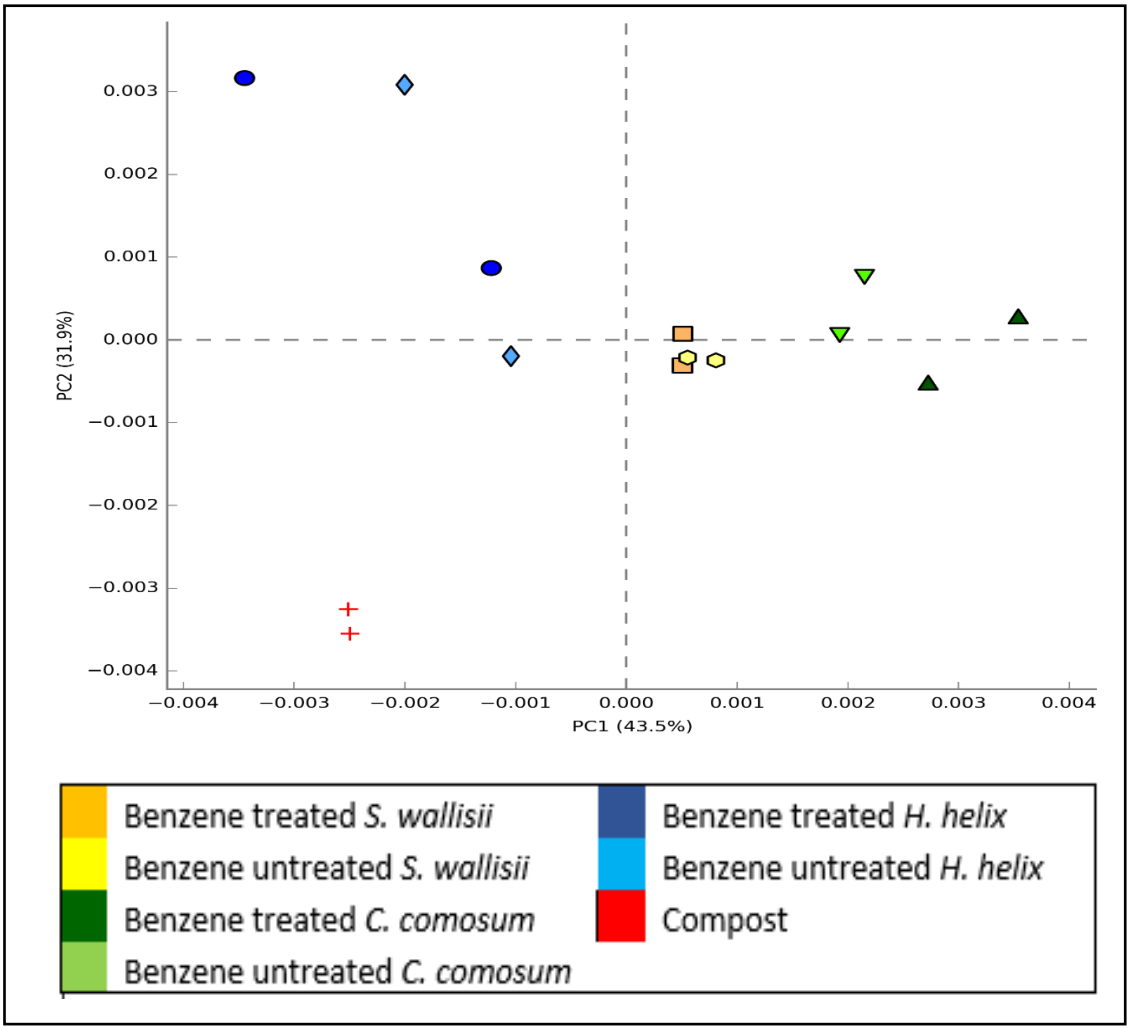

Fig. 5. 10 Overall functional differences among soil samples. PICRUST inferred PCoA to compare KEGG level 3 functional differences in soil samples

There was a total of 207 active functional features found among soil communities, according to the PICRUST predicted KEGG level 3 functional traits. The most abundant functions were ABC transporters (ATP-binding cassette) followed by the two-component system, bacterial motility protein and bacterial secretion system (Fig. 5. 11, Fig. 5. 12, Fig. 5. 13 and Fig. 5. 14 respectively) in all soil samples. ABC transporters are one of the predominant protein families in bacteria (Higgins, 1992; Fath and Kolter, 1993; Minz *et al.*, 2013; Feng *et al.*, 2018). Interestingly, the abundance of ABC transporter proteins in compost samples was higher than all rhizosphere samples. Proteins involved in the two-component system enables rhizosphere bacteria to respond to the changes in surrounding environments (Berg and Smalla, 2009). PICRUST predictions showed that the abundance of proteins in the two-component system was higher in all rhizosphere samples than compost. The majority of functional proteins in the bacterial secretion system are produced by Gram-negative bacteria (Gauthier and Brett Finlay, 2001; Green and Mecsas, 2016). PICRUST analysis inferred that the abundance of bacterial secretion system proteins is higher in all rhizosphere samples than compost. There was an increase of bacterial secretion system proteins in *C. comosum* following the benzene treatment compared to the control *C. comosum*. Bacterial motility proteins are the components required during motility through flagellum (Rajagopala *et al.*, 2007). PICRUST analysis showed that all rhizosphere samples contained a higher abundance of motility proteins than compost.

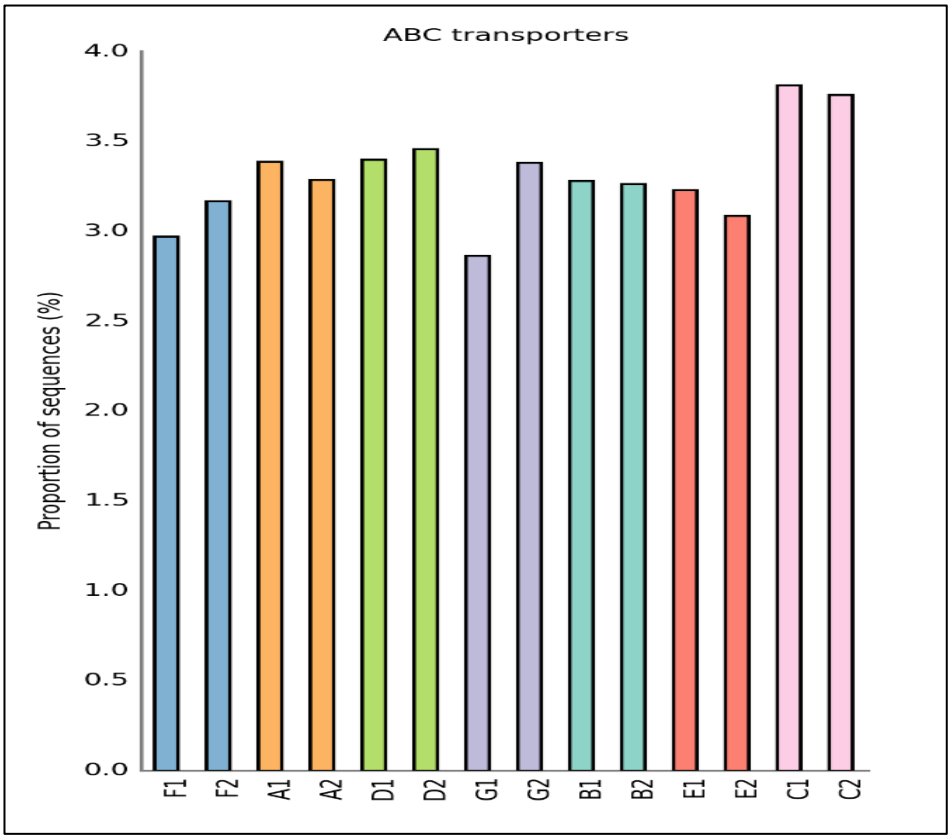

Fig. 5. 11 Relative abundance of selected KEGG level 3 function "ABC transporters" in soil samples. Sample key indicates A1/A2-(benzene treated *S. wallisii*), B1/B2-(control *S. wallisii*), C1/C2-(compost), D1/D2-(benzene treated *C. comosum*), E1/E2-(control *C. comosum*), F1/F2-(benzene treated *H. helix*) and G1/G2 (control *H. helix*).

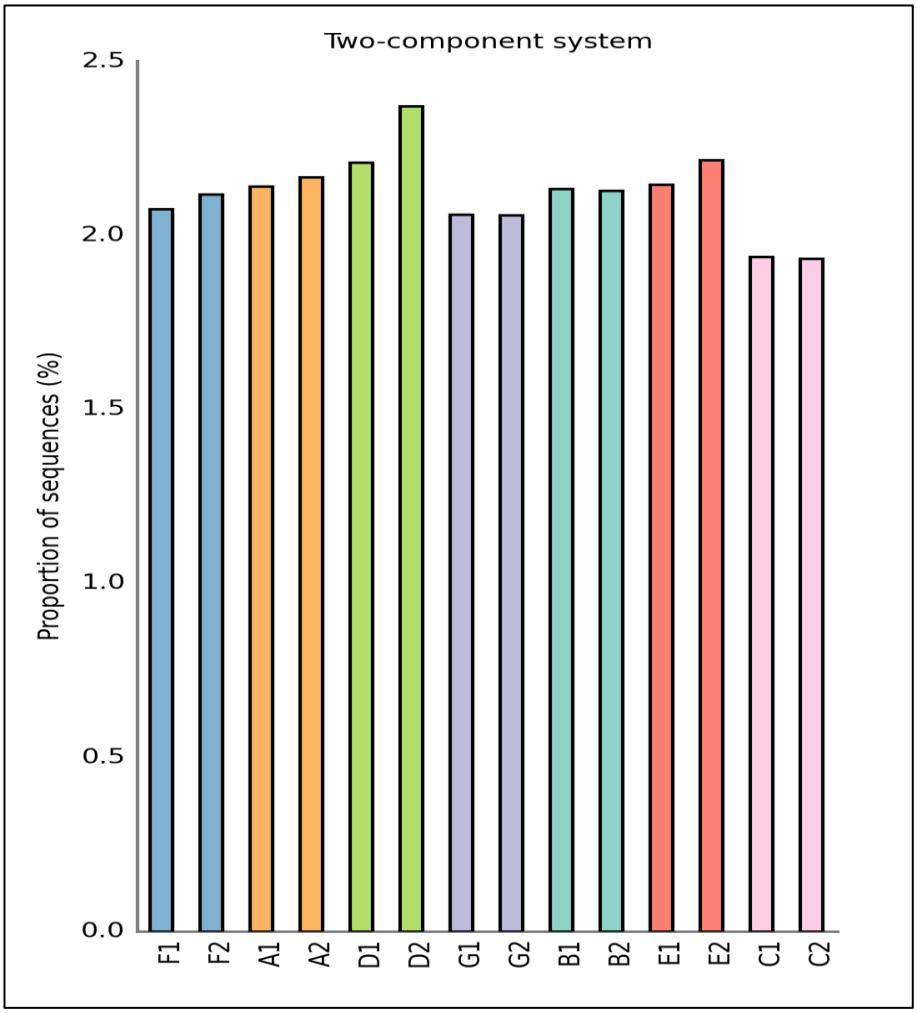

Fig. 5. 12 Relative abundance of selected KEGG level 3 function "two-component system" in soil samples. Sample key indicates A1/A2-(benzene treated *S. wallisii*), B1/B2-(control *S. wallisii*), C1/C2-(compost), D1/D2-(benzene treated *C. comosum*), E1/E2-(control *C. comosum*), F1/F2-(benzene treated *H. helix*) and G1/G2 (control *H. helix*).

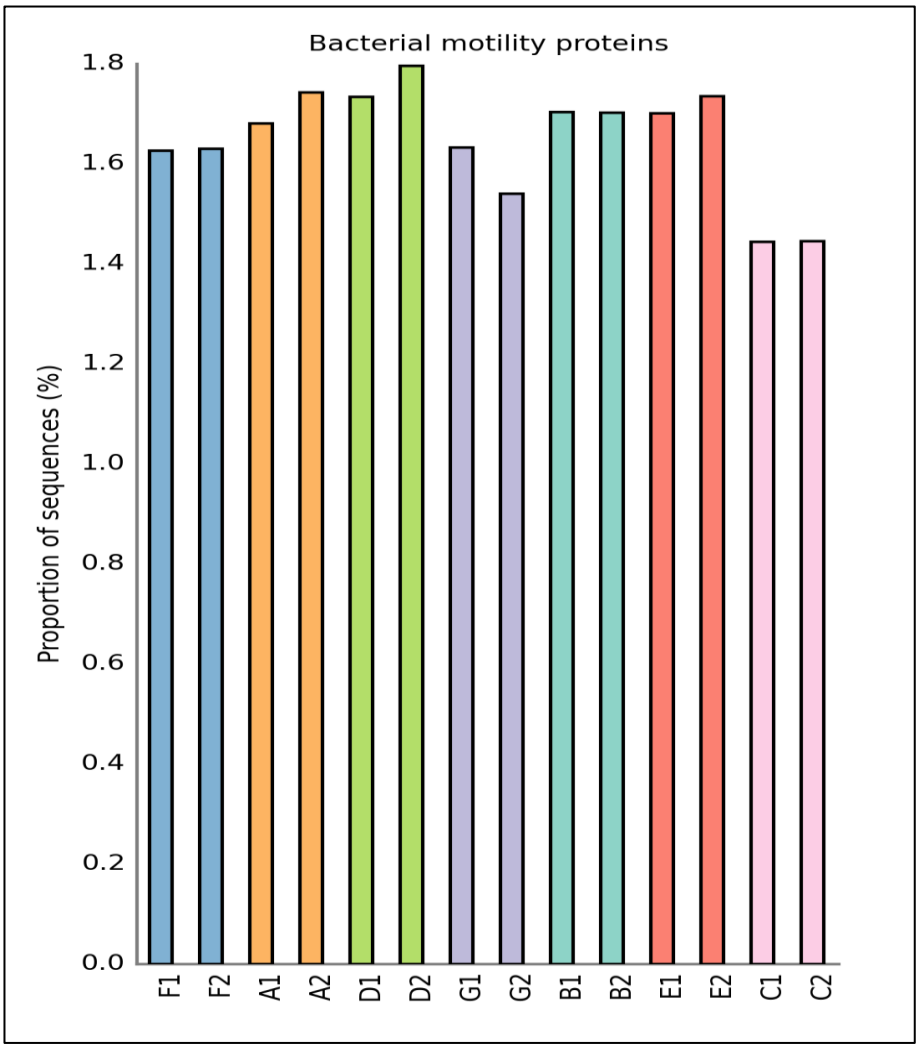

Fig. 5. 13 Relative abundance of selected KEGG level 3 function "bacterial motility protein" in soil samples. Sample key indicates A1/A2-(benzene treated *S. wallisii*), B1/B2-(control *S. wallisii*), C1/C2-(compost), D1/D2-(benzene treated *C. comosum*), E1/E2-(control *C. comosum*), F1/F2-(benzene treated *H. helix*) and G1/G2 (control *H. helix*).

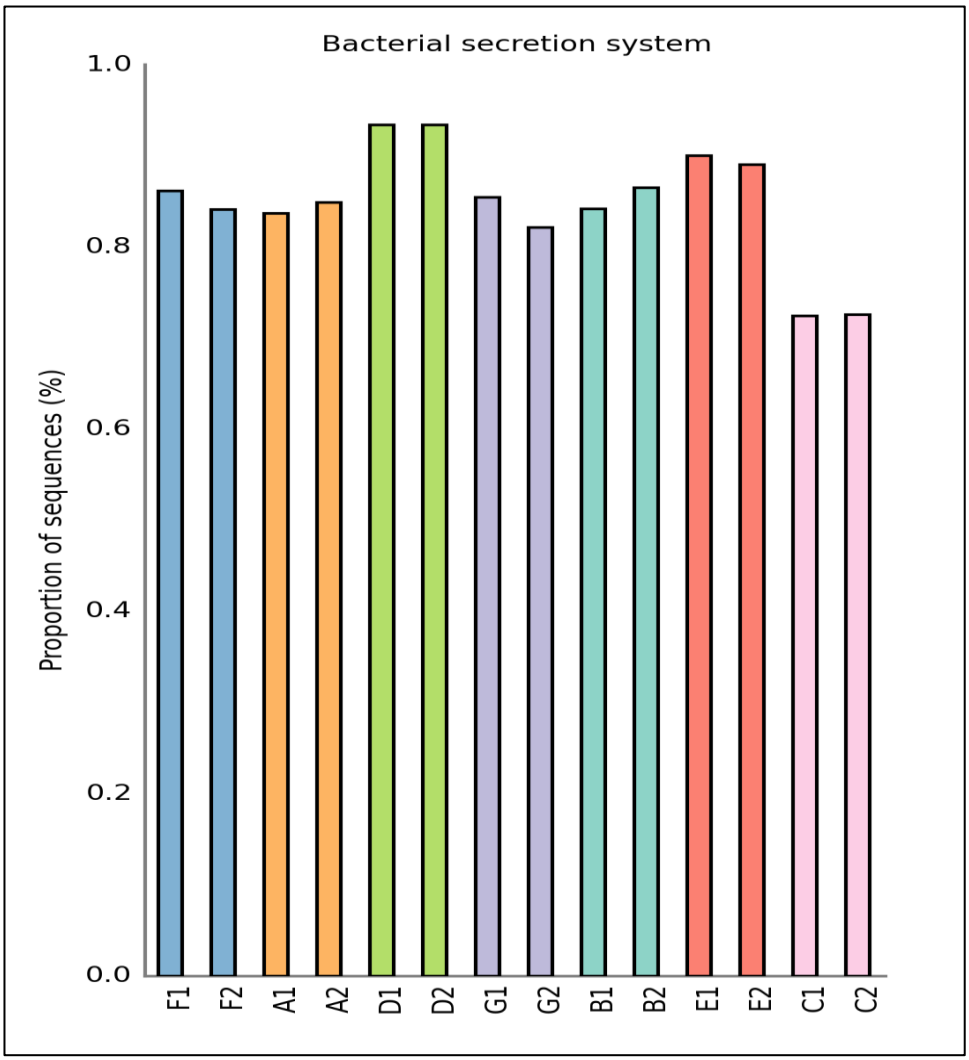

Fig. 5. 14 Relative abundance of selected KEGG level 3 function "bacterial secretion system" in soil samples. Sample key indicates A1/A2-(benzene treated *S. wallisii*), B1/B2-(control *S. wallisii*), C1/C2-(compost), D1/D2-(benzene treated *C. comosum*), E1/E2-(control *C. comosum*), F1/F2-(benzene treated *H. helix*) and G1/G2 (control *H. helix*).

However, little differences were observed between benzene treated and untreated samples at the KEGG level 3.

Individual genes are represented by "K" numbers in the KEGG database. By taking a closer look at the functional profile of benzene treated and untreated (control) plant rhizosphere communities, three main KEGG pathway enzymes (reductases group enzymes) coding genes: K00446, K07104 and K03268 involved during benzene degradation were identified. PICRUST predicted gene K00446 (*dmpB* and *xylE* :encoding catechol 2,3-dioxygenase) represents monooxygenase enzyme

which catalysis oxidation and reduction reactions during electron transferring from one donor to another (Margesin *et al.*, 2003). K00446 showed a greater abundance in the benzene untreated *C. comosum* samples than all soil samples except benzene untreated *S. wallisii*. K07104 (*catE*: encoding catechol 2,3-dioxygenase); one of the internal enzymes from the group oxidoreductases was higher in the benzene treated and untreated *H. helix* and compost samples than other soil samples. K03268 (*todC1*, *bedC1* and *tcbAa*: encoding benzene/ toluene and chlorobenzene dioxygenase respectively) catalysis oxidation and reduction reactions during electron transferring from two donors (Masai *et al.*, 1995). *H. helix* samples showed a higher abundance of K03268 than all other soil samples.

5.2.3. Conclusions

Functional predictions for the benzene treated and untreated plant rhizosphere and compost were conducted for three KEGG levels based on PICRUST predictions. At the KEGG level 1, the majority of the functions belonged to the function metabolism followed by genetic information processing. At the KEGG level 2, there were 29 different functions observed and most of the differences occurred between compost and different plant rhizospheres or among rhizosphere of different plant species. Therefore, this observation indicated the presence of plants and type of plant species influenced the different composition of functional diversity in a plant rhizosphere or soil microbiome. However, at the KEGG level 2, very little differences were observed between the same plant species due to benzene treatment. Our observation, close clustering pattern of same plant species benzene treated and control samples also indicated, 10 ppm benzene exposure has a little influence on the rhizosphere microbiome while the type of plant species was the main factor for affecting on the functional composition of rhizosphere.

Likewise in KEGG level 1 and 2, principal coordinate analysis of KEGG level 3 functional traits revealed overall, there were major differences between compost and plant rhizosphere samples. Also, despite the benzene treatment, by clustering same plant species samples closely indicted the similar functional diversity between them.

Based on PICRUST, the predicted four individual gene functions: ABC transporters, two-component system, bacterial secretion system and bacterial motility protein, there were little changes observed between the same plant species treatment and the control. Most of the differences occurred among different plant species. For example, a higher abundance of K03268 (benzene dioxygenase) found in the *H. helix* samples while all other communities indicated the lower abundance. Therefore, this suggested *H. helix* adjusts the abundance of enzyme K03268 in its rhizosphere through plant-microbes interactions. Considering the changes occurred between the same plant rhizosphere due to benzene treatment, the functional enzyme K0446 in *C. comosum* showed a decrease of abundance following benzene treatment.

As a summary, functional predictions revealed differences were mainly observed between compost and plant rhizosphere. Similar to the taxonomical composition between benzene treated and untreated plant rhizosphere, overall, there were no drastic changes that occurred in the relative abundance of functional traits in rhizosphere due to exposure to 10 ppm benzene.

Chapter 6. Discussion, conclusions and future work

6.1. Overview

This chapter will explain the key findings in this research reflecting upon the extent to which the specific objectives of this study were met. Also, the chapter will place findings from Chapters 3, 4 and 5 into context with the existing literature. In chapter 3, the potential of plant monocultures and plant mix culture to phytoremediate VOC from test chamber air was addressed based on their VOC removing rates and complete removal. In addition to the plants, as an experimental control, the capability of plant growing medium (compost) to remove VOC from air was studied. Two different VOC concentrations, 10 ppm and 100 ppm, were tested during the study. During presenting of the data, VOC removal rates were expressed as reduction of the concentrations per plant unit grown in the propagating tray (i.e. per chamber).

Secondly, the work specifically focused on the isolation and identification of gaseous VOC degrading bacteria from plant rhizosphere and compost are detailed in Chapter 4. Identification of unknown bacteria was carried out based on both culture-dependent and independent approaches. Finally, in Chapter 5, rhizosphere microbiome of three plants species were studied to understand possible taxonomical and functional changes in the bacterial community due to exposure to benzene. In addition to the plants, bacterial community composition in the fresh compost was studied. Each experiment conducted in this work achieved one or more of the specific objectives defined in Chapter 1.

6.2. Selection of three plant species to make plant community and overall VOC removal ability by plant monoculture and community

The research presented in this thesis revealed the ability of plant monocultures and communities to remove different indoor VOCs at two different concentrations. The efficiency of plant communities to phytoremediate indoor VOCs had not been studied prior to this study, and understanding this phenomenon is important in the use of mix cultures to reduce indoor volatiles. The few studies that studied this aspect, only focused on the VOC removal conducted by plants grown in the green wall systems (Irga *et al.*, 2018; Torpy and Zavattaro, 2018; Torpy *et al.*, 2018).

Though the green wall can be compromised of mix plants composition, during the manipulation of a green wall, separately grown plants in small pots are fixed into the wall, thus the roots in the mix plants species are not well integrated with each other compared to the plants grown together in a propagating tray (Su and Lin, 2015; Mikkonen *et al.*, 2018). Therefore, different plant species grown in a big pot or a propagating tray represent a physically well cooperated community.

During selection of plant species to design a plant community (detailed in section 2.2), plant species: *S. wallisii, C. comosum* and *H. helix* were chosen since those species had shown high phytoremediation ability according to the literature. According to Yoo *et al.*, (2006), aerial parts of *S. wallisii* and *H. helix* showed a higher removal efficiency of benzene and toluene in the daytime than night. In another study, *H. helix* removed benzene and toluene from the test chambers efficiently in the daytime (Yang *et al.*, 2009). Previously, *C. comosum* had reported high removal efficiency of benzene from the atmosphere (Sriprapat and Thiravetyan, 2016; Setsungnern *et al.*, 2017). In addition, these three plants species are extensively used as indoor ornamental plants, are easy to grow and the average size of mature plants was suitable to perform experiments inside the test chambers.

Though the indoor vertical green wall system is popular due to being less spacious, maintenance costs (for the irrigation system, initial fixation on the wall and repairs) and labour cost are higher than a potted plant/ plants in propagating tray systems. Compared to these drawbacks of the green wall system, plant community manipulated in the propagating trays are suitable to use inside any indoor environment. The plant community was manipulated using three plant species in the plant propagating trays, they were small in size, well arranged, easily portable, needed little indoor space and required low maintenance cost. Therefore, compared to the vertical green wall system, plant community in a suitable propagating tray is a better choice for indoor environment.

This study has added to the understanding of the indoor VOC removal ability by plant communities. Our findings showed the capability of plant communities to remove gaseous VOC from air. Especially, during 10 ppm toluene removal, the plant community showed a higher removal efficiency than benzene and m-xylene removals. However, overall, plant monocultures

conducted higher removal rates than the plant community during the rest of the experiments. Therefore, our findings suggest that use of single plant species in pots and green wall systems can be the best option for the efficient removal of indoor VOCs.

6.3. 10 ppm VOC removal by plant monocultures, communities and compost

The findings of this research showed that the potential of the three plant monocultures: *S. wallisii*, *C. comosum* and *H. helix*, and the plant community, and compost (plant growing medium) in plant propagating trays to remove 10 ppm concentration of gaseous VOC (benzene, toluene and m-xylene) from the test chamber air. However, no significant differences were observed for the VOC removal rate of chambers. At the low concentration of benzene (10 ppm), *H. helix* followed by *S. wallisii* demonstrated the highest removal rates and showed complete benzene removal at around 24 hours. This concluded that those two species have higher phytoremediation efficiencies during low concentration benzene detoxification. Similar to our finding, Yoo *et al.*, (2006) showed the high removal rate of benzene by *H. helix* and *S. wallisii* in a test chamber study based on removal rate per plant leaf area. However, contradictory results were observed by Sriprapat and Thiravetyan, (2016). According to their study, the maximum benzene removal rate was performed by *C. comosum* compared to all other species including *H. helix* in a hydroponic condition.

During 10 ppm toluene study our findings revealed that the highest removal rates were by *S. wallisii* followed by the plant community. Both plant units conducted complete toluene removal within 24 hours, thus they have better phytoremediation capabilities for toluene than the other plants tested. In addition, our findings revealed that the rates of benzene and toluene removal by three plant monocultures and plant community were higher than the m-xylene removal rates. Therefore, the selected plant models were identified as less efficient plants during phytoremediation of low concentration of gaseous m-xylene. Our observation of low phytoremediation rate of m-xylene is supported by studies using different plant species. Kim *et al.*, 2014 showed higher removal rates (per pot) of toluene than xylene by potted *D. fragrans, F. japonica, Schefflera actinophylla* and *Ficus benghalensis*. Also, similar findings were reported by

Orwell *et al.*, (2006) during 10 ppm toluene and m-xylene removal by potted *Spathiphyllum* "Sweet chico" and *D. deremensis*. With these previous caveats, our findings suggest gaseous xylene removal efficiency by plants can be relatively lower than the removal efficiency of other dominant VOC (e.g. benzene and toluene). However, this suggestion is contradicted by the findings from Mosaddegh *et al.*, (2014). According to their study, xylene removal rate (per leaf area) by *D. deremensis* and *Opuntia microdasy* were higher than benzene and toluene removal rates.

During 10 ppm toluene removal, we observed *S. wallisii* and *H. helix* exhibited higher removal rates than the rate observed for benzene removal. In previous studies, Yang *et al.*, (2009) and Yoo *et al.*, (2006) showed higher removal rates of toluene than benzene by *S. wallisii, C. comosum* and *H. helix.* However, their findings were based on the per leaf area. By comparing our observations with the previous literature, it suggests that phytoremediation rate by a plant species can provide different results based on whether the rate is calculated per propagating tray unit, per leaf area or per other parts of the plants such as roots. Most houseplants are propagated in pots or propagating trays, thus the VOC removal capacity per propagating tray provided direct measurement of VOC removal by plant and potting mix together which is important during indoor plant selection and designing plant community or green walls.

Based only on the phytoremediation efficiency performed by the plant community, these research findings showed that the plant community removed toluene from the chamber air faster than benzene followed by m-xylene. Therefore, the combination of three species can be a better solution for the removal of low concentrations (≈10 ppm) of toluene but not benzene or m-xylene. There is no published research evidence based on the potted plant community VOC detoxification efficacy, however, Torpy *et al.*, (2018) reported the MEK (methyl ethyl ketone) removal by a green wall composed of eighteen plant species grown as a mixed species culture. They showed a single pass of MEK through the green wall removed 56.6 ± 0.86% of the VOC. This suggests, changing the composition of a plant community by including different plant species may result in different kinetics for VOC removal than we observed in this study.

During 10 ppm VOC experiments, our findings showed that unused potting mixture (compost) also removed gaseous VOC efficiently. Very early studies based on phytoremediation efficiency of potted plants carried out by Bill Wolverton showed that potting mix in the two plants species: *Dieffenbachia maculate* and *Nephrolepis exaltata* removed more than 50% of xylene (53% and 50.5% respectively) in a test chamber experiment (Wolverton & Wolverton, 1993). Also, Orwell *et al*. (2006) reported a higher removal rate of 10 ppm toluene and m-xylene by the potting mix used with *Spathiphyllum* "sweet Chico" and *D. deremensis.* Therefore, during phytoremediation, plants and the plant growing medium together contribute for the removal of VOC from air.

Based on the complete removal of 10 ppm VOC, it was noticed that the removal rate declined in the later stage. Especially, when the chamber air declined less than 1 ppm VOC, this lower VOC concentration remained for a long time. This might have occurred as a result of the biochemical and physiological responses of plants, plant associated microbes and microbes in compost. Plants can be stimulated for the phytoremediation process through exposure to an initial high concentration of VOC, but when the exposure concentration declines with time, the removal stimulation is also reduced, thus, as a result phytoremediation efficiency can decline to some extent (Lee *et al.*, 2002; Tarran *et al.*, 2007). Also, the low-level constant VOC concentration can be attributed by biogenic VOC emission by plants and soil microorganisms and or through the VOC desorption by soil. Previous studies showed the capability of soil to adsorb and desorb atmospheric VOC (Neu, 1996; Insam and Seewald, 2010). Desorption of VOC from soil is based on the adsorption-desorption kinetics of each VOC type (Rogers *et al.*, 1980; Lin *et al.*, 1994; Neu, 1996; Ruiz *et al.*, 1998; Insam and Seewald, 2010). In some instances, soil absorbed VOC can be taken up by plant roots and transported into the leaves followed by emission through stomata (Helmig *et al.*, 1999; Abhilash *et al.*, 2009; Nwoko, 2010; Soleimani *et al.*, 2011). In addition, soil bacteria produce VOC known as microbial VOC (MVOC) which is involved in maintaining microbe-microbe and plant-microbe interactions (Raza *et al.*, 2016; Wang *et al.*, 2016; Schulz-Bohm *et al.*, 2017). We reported possible VOC emission by unused plants and compost (VOC non-exposed plants and compost) in section 3.2.6 and according to that there were very low level (less than 1

ppm) BTX emission inside the test chamber. Therefore, the constant low-level BTX concentration observed during the experiments probably occurred as one or combination of few phenomenon explained above.

6.4. 100 ppm VOC removal by plant monocultures, communities and compost

Our study reported the ability of three plant monocultures, plant communities and compost in plant propagating trays to remediate 100 ppm concentrations of gaseous VOC from the test chamber air. According to the VOC removal rate, there were no significant differences found among different plant monocultures, communities or compost. In the 100 ppm benzene and m-xylene experiments, *C. comosum* showed the highest removal rates and also during the 100 ppm toluene study, the removal rate by *C. comosum* was only less than *S. wallisii*. These observations indicated the higher capacity of *C. comosum* to remove high concentrations of VOC from air. Similar to our observations, a previous study reported the removal of high concentrations of gaseous benzene (500 ppm) by *C. comosum* with 68.77% removal efficiency (Setsungnern *et al.*, 2017). Therefore, our findings and previous literature indicate that the VOC removal efficacy of plants could be affected by plant species, type of growing medium, type of VOC and the concentration of VOC in the air.

According to our findings, virgin potting mixture (compost) exhibited a greater capacity to remove high concentrations (100 ppm) of VOC from air. Soil has a high potential to accumulate volatile hydrocarbons including BTX vapour from air, through adsorption and absorption(Jaynes and Vance, 1999). Following sorption, soil moisture dissolves VOC by enhancing the soil microorganisms respiration and biomass production (Smolander *et al.*, 2006; Ramirez, Lauber and Fierer, 2010; Greenberg *et al.*, 2012). When the concentration of volatiles in the air increases, soil acts as a VOC sink by increasing the VOC adsorption rate (Asensio *et al.*, 2007; Peñuelas *et al.*, 2014). VOC adsorbed by soil can be desorbed as volatiles into the air through volatilation and a part of volatiles go through biodegradation (Manuela *et al.*, 2015; Osagie and Owabor, 2015). According to Lin *et al*. (1996), soil has shown its ability to adsorb different concentrations of benzene ranging between 10-1000 ppm. We observed a higher VOC removal rate by all plant

monocultures, communities and compost for the initial 100 ppm concentration than 10 ppm concentration. Supporting our observations, Orwell *et al.*, (2006) reported that during 100 ppm toluene and m-xylene removals, potting mix VOC removal efficiency increased by a few orders of magnitude higher than the 10 ppm removal rates. Therefore, our findings and the previous literature indicated when the plant unit composed with a high phytoremediation efficiency plant species, plant-compost unit together can perform a great removal of high concentration of VOC from air.

According to our findings in the 100 ppm study, the plant communities achieved the lowest VOC removal rates which were less than the removal rates observed by all plant monocultures. This is a novel finding and it indicates that the combination of different plant species in a mixed culture reduces its overall phytoremediation efficiency than single species (plant monocultures) at the presence of high concentration of VOC in air. Hortal *et al.*, (2017) showed that in a plant monoculture, there is less intraspecific competition for getting the nutrients, water and sunlight than the interspecific competition in mixed plant community for the above requirements. They also showed that lower root growth under the interspecific than intraspecific conditions. Intraspecific or interspecific competition is influenced by the rhizosphere microbiome community (Kanchiswamy, 2015). In the intraspecific condition, same species plants develop a rhizosphere microbiome with similar bacterial composition, however under the interspecific condition each plant species in the community develops plant species specific communities, abundance and functions (Bakker *et al.*, 2014; Hortal *et al.*, 2017). Hortal *et al.*, (2017) also showed the reduction of soil rhizosphere enzyme activity in the interspecific condition compared to intraspecific. Therefore, our findings of lower phytoremediation efficiency by plant communities can be attributed to high interspecific competition.

Also, we observed at the higher concentration level, some plant monocultures performed lower VOC removal rates than compost. This observation indicated that following exposure to high concentration of VOC, plants reduce their phytoremediation efficiency. Orwell *et al*. (2006) suggested that high concentrations of VOC such as 100 ppm or repeated induction of VOC into the

test chamber, may cause toxicity to plants or plant-associated microorganisms thus, as a result, VOC removal efficiency can be reduced. Exposure to high concentrations of VOC causes high abiotic stress to the plant and also volatiles can react with atmospheric ozone by increasing oxidative stress which influences the physiological and biochemical processes of the plants (Blande *et al.*, 2014). Also, this can alter the colonisation of microorganisms in the rhizosphere resulting in changes of microbial enzyme activity (van Overbeek and Saikkonen, 2016; Hartikainen *et al.*, 2009).

6.5. Isolation and Identification of gaseous benzene, toluene, m-xylene degrading bacteria in soil samples

Our study reported a list of gaseous VOC degrading bacteria in plant rhizosphere and compost. Bacteria were isolated from the rhizosphere of VOC exposed and non-exposed *S. wallisii*, *C. comosum, H. helix* and VOC non-exposed compost samples respectively. Those isolated species had the ability to use BTX as their sole carbon and energy source for the growth. The majority of them were Gram-positive soil bacteria belonging to the phylum Actinibacteria. Singh and Celin, (2010) reported most of BTEX (Benzene, toluene, ethylbenzene, and m-xylene) degrading bacteria isolated from soil samples polluted by hydrocarbons were also Gram-positive. The findings from Surendra *et al*. (2017) further corroborated our observations. They isolated five bacterial strains from petroleum-contaminated soil on the minimal salt medium under the supplement of BTEX and reported that all the isolates were Gram-positive bacteria from genera *Bacillus, Alcaligenes* or *Ochrobactrum*. Smalla *et al.*, (2001) also suggest Gram-positive bacteria can be more dominant in rhizosphere samples than previously thought. Also, Chun *et al.*, (2010) reported that total rhizosphere bacteria isolated from nine plant species including *S. wallisii* were able to remove gaseous benzene, toluene and m,p,o xylene respectively. However, they did not carry out the bacterial identifications (Chun *et al.*, 2010).

We identified bacteria from genera *Microbacterium, Rhodococcus*, *Paenibacillus, Arthrobacter* and *Pseudomonas* commonly in both VOC exposed and non-exposed plant rhizosphere, while

Agromyces, Micrococcus, *Brucella, Leifsonia* and *Pantoea* were observed only in the VOC treated plants. Ferrera-Rodríguez *et al.*, (2013) reported that the hydrocarbon degrading *Arthrobacter, Rhodococcus, Pseudomonas* and *Leifsonia* in the rhizosphere of plants in the diesel contaminated soil. In the same study they demonstrated the presence of hydrocarbon degrading genes: alkB (encode enzyme alkane hydroxylase), ndoB (encode dioxygenase) and xylE (encode 2-3-catechol dioxygenase) in those bacteria.

We reported the VOC degrading ability of bacteria isolated from the genera *Aeromicrobium*, *Rhizobium, Cellulomonas, Paenarthrobacter, Pseudarthrobacter,* and *Youhaiella* in the control plants rhizospheres and *Arthrobacter* and *Pseudoarthrobacter* in fresh soil (compost) samples. According to previous studies, several bacteria were identified with VOC degrading capability though, most of them were isolated from contaminated soil, the rhizosphere of plants grown in the contaminated soil, air purifying bioreactors, or rhizosphere of plants maintained under the VOC vapour supplied in chambers (Hori *et al.*, 2001; Lee *et al.*, 2002; Mathur *et al.*, 2007; Mikkonen *et al.*, 2018). However, there is little evidence of VOC degrading bacteria isolated from non-VOC contaminated soil or plant rhizosphere grown in the fresh air (non-exposed to VOC). Previous research reported the capability of bacteria from four genera including *Micrococcus* and *Bacillus* isolated from rhizosphere of *Populus deltoids* grown in the non-contaminated environment to remove naphthalene, anthracene, benzene, toluene and xylene (Bisht *et al.*, 2010, 2014, 2015).

Our findings showed that the bacteria from genera *Microbacterium*, *Arthrobacter*, *Agromyces* and *Rhodococcus* utilized both benzene and toluene as their sole carbon and energy source while *Paenibacillus* utilized gaseous benzene and m-xylene. Hori *et al.*, (2001) reported the isolation of gaseous toluene degrading *Rhodococcus* species from a trickle bed air-biofilter filled with sludge from industrial wastewater. Also, later they showed the capability of the same strains to remove twelve different VOCs including benzene and xylene. We reported that bacteria from the genera *Leifsonia*, *Rhizobium* and *Paenarthrobacter* utilized gaseous benzene while *Aeromicrobium*, *Pseudarthrobacter, Cellulomonas* and *Youhaiella* utilized toluene. *Pantoea* and *Pseudomonas*

were observed to be m-xylene utilizers. Supporting our findings, Otenio *et al*. (2005) reported the capability of *Pseudomonas putida* to degrade gaseous m-xylene. Also, they reported gaseous toluene removal by *Pseudomonas putida.* In another study, PAH-degrading *Paenibacillus* species were isolated from petroleum-contaminated soil and the rhizosphere of salt marsh plants which utilized naphthalene and phenanthrene as their sole carbon source (Daane *et al.*, 2002). Ridgway *et al*. (1990) reported the isolation of about 300 gaseous VOC degrading bacteria from well-water and petroleum-contaminated aquifer cores belonging to the genera *Pseudomonas*, *Micrococcus*, *Nocardia* and *Alcaligenes*. Later they showed the capability of those bacteria to use VOC as the sole carbon source. In another study, bacteria from the genera *Bacillus*, *Pseudomonas* and *Streptomyces* were isolated from the rhizosphere of *Ramonda serbica* and *Ramonda nathaliae* by inoculating soil samples on TSA medium. The isolated bacteria were able to degrade BTX by using it as their sole carbon source (Djokic *et al.*, 2011). Previous findings from (Bell *et al.*, 2011) showed the abundance of *Aeromicrobium* in petroleum contaminating soil.

Though one gram of soil is estimated to contain millions of bacteria (Fierer *et al.*, 2007), we only isolated a total of 53 bacteria through culturing, because only a small fraction (0.1-1%) of environmental bacteria are culturable using traditional culturing methods (Amann *et al.*, 1995). We noticed that the presence and absence of some bacterial genera under the different VOC supplements may be caused due to their growth rate, capability of using BTX as their sole carbon and energy source or that fast-growing bacteria outcompeted the slow growers. At the initial colony picking step, there was a higher chance to pick fast growers from the MSA plate than slow growers. Common bacteria isolated from VOC exposed and non-exposed plants: *Microbacterium, Rhodococcus*, *Paenibacillus, Arthrobacter* and *Pseudomonas* could be the fast growers while the bacteria observed only in the one type of plant: *Agromyces, Micrococcus*, *Brucella, Leifsonia, Pantoea, Aeromicrobium*, *Rhizobium, Cellulomonas, Paenarthrobacter, Pseudarthrobacter,* and *Youhaiella* may represent the slow growers. During the experimental time, soil suspension inoculated MSA plates were incubated 7-10 days by supplying gaseous VOC. By increasing the

incubation time, we will probably be able to isolate more slow growing bacteria. Such changes remain for future studies.

Our methodology was based on the isolation of bacteria utilizing a single VOC, however, the observation may be different if the soil samples are exposed to a mixture of BTX. There is some evidence from previous studies that the degradation of a mixture of VOC by bacteria. Lee *et al*. (2002) reported that *Stenotrophomonas maltophilia* isolated from a bioreactor did not utilize xylene, but the same bacteria utilized xylene from the binary mixture of xylene and toluene. Therefore, as a future direction, benzene, toluene and m-xylene degrading bacteria isolated from this study could be used to test their capability to utilize a mixture of three VOC.

In our study, we did a direct isolation of VOC utilizing bacteria from rhizosphere samples by inoculating rhizosphere soil on MSA following exposing them to gaseous VOC. However, in some studies, bacteria were isolated first on a nutrient-rich medium such as TSA or NA followed by studying their capability to degrade VOC (Singh and Celin, 2010; Djokic *et al.*, 2011). Similar to our method, another study showed the isolation of seventeen bacteria belonging to the genera *Pseudomonas* and *Bacillus* on the MSA medium from the rhizosphere of *Solanum melongena* utilizing toluene as their sole carbon source (Kundan *et al.*, 2017). Seyedi *et al*. (2013) reported the isolation of twenty-three petroleum-degrading bacteria from the rhizosphere of wild oat (*Avena fatua L*.) collected from the petroleum-contaminated soil. They belonged to the genera *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Bacillus*, *Achromobacter*, *Ochrobactrum*, *Paenib*acillus, *Microbacterium*, *Curtobacterium* and *Sphingobacterium* and were able to utilize petroleum as the sole carbon source.

In summary, we showed the capability of some rhizosphere bacteria to utilize BTX as their sole carbon and energy sources under the laboratory conditions. In the *in situ* phytoremediation (rhizormediation), those identified bacteria may also involve to the degradation of different VOCs from the environment. Plants maintained in the presence and absence of BTX and also compost samples contained BTX utilizing bacteria. Therefore, the VOC degradation can be identified as one of the common and major biochemical pathways in soil bacteria.

6.6. Taxonomical and functional classification of plant rhizosphere exposed, non-exposed to 10 ppm benzene and compost

In this study, we compared the bacterial composition in the three plants species exposed and non-exposed to 10 ppm benzene and fresh soil (compost). We have observed Proteobacteria, Bacteroidetes, Actinobacteria and Acidobacteria, Gemmatimonadetes as the most dominant phyla in all soil samples. According to the literature, these are the dominant bacterial phyla in the rhizosphere of most plants and bulk soil (Pisa *et al*. 2011; Minz *et al*. 2013; Donn *et al.*, 2015; Newman *et al*. 2016). Bacteria from phylum Proteobacteria and Actinobacteria in the rhizosphere of willows grown in the hydrocarbon contaminated sited had shown high remediation activities, however, those phyla are also ubiquitous in the plant grown in non-contaminated sites (Yergeau *et al.*, 2014; Correa-garc *et al.*, 2018). Therefore, based on our findings and the previous literature, these bacterial phyla in the soil can be identified as common bacterial taxonomies in the soil.

At the class level, we observed the dominance of Alphaproteobacteria, Gammaproteobacteria, Sphingobacteriia, Betaproteobacteria, Deltaproteobacteria, Actinobacteria, Cytophagia and Flavobacteria in all rhizosphere samples. Supporting our findings, previous studies have reported that high abundance of these bacterial classes in rhizosphere of different plants (Pisa *et al.*, 2011; Bell *et al.*, 2014). Bacteria from Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Acidobacteria had reported dominancy in the rhizosphere of willow plants grown in contaminated sites than non-contaminated sites plants (Yergeau *et al.*, 2014).

At the genus level, *Rhizomicrobium, Pseudomonas, Rhodanobacter, Rhizobium, Paraburkholderia, Sphingomonas, Sphingobium, Pseudolabrys, Devosia, Flavobacterium, Novosphingobium* and *Dongia* were dominant in all soil samples. The most abundant genera in compost were *Pseudolabrys*, *Rhizomicrobium* and *Rhodanobacter*. In Support of our findings, previous studies reported the high abundance of these genera in soil samples (Ahmad, Ahmad and Khan, 2008; Kämpfer *et al.*, 2006; Peterson *et al.*, 2006; Young *et al.*, 2007, 2008; Liu *et al.*, 2010; Ueki *et al.*,

2010; Panwar, *et al.*, 2012; Won *et al.*, 2015; Xue *et al.*, 2015; Cipriano *et al.*, 2016; Cho, Lee and Whang, 2017; Wang *et al.*, 2018). Some of the bactera identified in this study had previously shown their hydrocarbon degradation ability, symbiotic relationships with plants and plant growth promoting activity. *Sphingobium* associated with plant rhizosphere, is capable of degrading PAH from oil-contaminated soil (Young *et al.*, 2007, 2008). *Rhizobium* bacteria is well-known for its symbiotic relationships between plants (Panwar, *et al.*, 2012; Correa-garc *et al.*, 2018)**.** They are abundant in root nodules especially in legumes, and fix nitrogen from the atmosphere (Hirsch *et al.*, 2003; Vanparys *et al.*, 2005; Badri *et al.*, 2009; Wolińska *et al.*, 2017). Previous studies reported that bacteria from the genus *Devosia* in the legumes also fix nitrogen from the atmosphere (Vanparys *et al.*, 2005; Wolińska *et al.*, 2017). Root exudates such as flavonoids attracts rhizobium bacteria through plant-microbe interactions (Faure, Vereecke and Leveau, 2009; Bibi *et al.*, 2012; van Overbeek and Saikkonen, 2016). Research based on *Arabidopsis thaliana* showed that bacteria from the genus *Paraburkholderia* in rhizosphere promote plant growth through volatile emissions (phytostimulation), thus considered as a plant growth promoting rhizobacteria (Compant *et al.*, 2008; Suárez-Moreno *et al.*, 2012; Ledger *et al.*, 2016). High abundance bacterial genera: *Agrobacterium*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Novosphingobium* and *Sphingomonas* found in our study, reported their benzene and other PAH degrading ability in previous studies conducted in the polluted soil (Aitken *et al.*, 1998; Daugulis and McCracken, 2003; Liu *et al.*, 2005; Luo *et al.*, 2012).

According to the functional analysis, we observed the majority of sequences belonged to KEGG level 1 functions, metabolism and genetic information processing. Literature also reported that the majority of functional genes in soil microbiome belonged to these two functions (Chaparro *et al.*, 2014; Paul *et al.*, 2016). We observed rhizosphere functional diversity has little influence on exposure 10 ppm benzene treatment to the plants since same plant species' benzene treated and control samples clustered closely. In the PCoA, major separation occurred between bulk soil and rhizosphere functional diversities, indicating that the functional diversity in a plant is highly affected by the plant and differ from the diversity from compost (Chaudhary *et al.*, 2012). It also

indicated the benzene exposure has made little influence on the functional diversity of the plant rhizosphere.

During functional analysis we reported that the high abundance of three main KEGG orthology genes: K00446 (*dmpB* and *xylE),* K07104 (catE) and K03268 (*todC1, bedC1* and *tcbAa*) in all soil samples. previous studies reported that enzymes encoded by these genes involved in degradation of different hydrocarbons. (Masai *et al.*, 1995; Suyama *et al.*, 1996; Daly *et al.*, 1997). Rochman *et al.*, (2017) demonstrated that the presence of genes dmpB, xylE and *todC1, bedC1* in the bacteria isolated from oil sands tailings pond. They belonged to bacterial orders Gammaproteobacteria, xanthomonadales, Actinomysetales, Rhodocyclales, Burkholderiales, Rhizobiales, Pseudomonadales and Rhodobacterales. Another study demonstrated the high abundance of the genes *dmpB, todC1* and *xylE* in *Pseudomonas* and *Rhodococcus* species were isolated from petroleum-contaminated soil (Masai *et al.*, 1995; Suyama *et al.*, 1996; Daly *et al.*, 1997; Mukherjee *et al.*, 2017). In addition to *Pseudomonas*, the same genes (*dmpB, todC1* and *xylE*) were reported in the taxa Oxalobacteraceae, *Cupriavidus*, Brucellaceae, and *Ochrobactrum* in petroleum contaminated soil (Mukherjee *et al.*, 2017; Correa-garc *et al.*, 2018).

Higher expression of hydrocarbon degrading genes was previously reported in the rhizosphere of poplar, pea, alfalfa and sugar beet grown in the contaminated sites than the non-contaminated sites. Also, rhizobacteria in these plants showed higher utilisation of hydrocarbon as their main source of carbon than simple sugars (Jones, 1998; Lugtenberg *et al.*, 2001; Naik *et al.*, 2009; Ramachandran *et al.*, 2011).

During rhizoremediation, interactions between the plant and microbes occurred though plant root exudates and plant growth promoting rhizobacterial activities. Root exudates promote the growth of hydrocarbon-degrading bacteria and also improve desorption of hydrocarbon molecules from soil particles facilitating them available to rhizobacteria. Rhizobacterial activities promote plant growth by enhancing plant hormone production. Also, plant growth promoting rhizobacteria secrete indoleacetic acid, cytokinin and gibberellic acid that enhance plant root development (Correa-garc *et al.*, 2018).

In this study, the taxonomical differences between the benzene treated and control rhizosphere was compared based on the descriptive statistics (mean relative abundance) since our sample number was small (n=2) (due to budgetary constraint in the study). Though there were great changes in the abundance of some bacterial taxonomies (e.g. genus *Pseudomonas* in *C. comosum*) following the benzene treatment, due to low number samples and the presence of high variations in the relative abundance among the same group replicates, the statistical significance lowered. Therefore, we did not present any significant differences. However, by increasing the number of samples during the sequencing step, it will allow achieving a normal distribution in the same group samples and this will facilitate to observe statistical significances of bacterial taxonomies between control and VOC treated samples.

6.7. Suitability of Aeroqual digital monitors to analyse VOC level in plant experiment

In this study, our first choice was Aeroqual digital monitors to analyse VOC concentrations inside the test chambers. The selection of Aeroqual monitors was based on its automated function to monitor VOC levels in indoor and outdoor environments continuously for a long time. Thus, at the beginning of the research, it was a better choice for the study than the GC/FID method which allows analysis of a limited number of samples per day. There were no or few reports of the use of Aeroqual VOC sensors in plant test chamber experiments to measure VOC levels, however, it is extensively used for the indoor and outdoor VOC measurements (Wijewickrama *et al.*, 2016; Berry *et al.*, 2017; Spinelle *et al.*, 2017a, 2017b). Therefore, the use of Aeroqual monitors in the plant experiments was challenging and novel. Our findings showed the declining efficiency in the Aeroqual sensor during the study. This could be the first study using Aeroqual sensors in plant experiments and our findings showed the unsuitability of the Aeroqual sensor for the plant test chamber-based experiments.

6.8. Recommendations for future research

Further exploration of several aspects of this study would be an advantage in enhancing indoor phytoremediation systems. Use of different concentrations of BTX and other VOC which are identified as dominant in indoor air could be useful in future experiments. Since this work was the first study of the investigation of phytoremediation efficiency of plant communities (in propagating trays), using different compositions of plant communities to monitor their VOC removal rate would improve further understanding in this field. Future research can also be expanded by changing the number of plant species in a community. Also, the mixture of BTX or other VOC can be used to analyse the phytoremediation efficiency by the plant communities.

Also, future research can be extended by exposing rhizosphere soil into a mixture of BTX or different indoor VOCs to isolate and identify bacterial species with VOC degrading ability. In addition, the isolated bacteria in this study can be introduced to enhance the *in situ* bioremediation processes in contaminated sites. As such there remains a number of gaps within our knowledge that are obvious areas for future research. In addition, by studying taxonomical and functional changes in the plant rhizosphere following exposure to high concentrations (≈100 ppm) of BTX, will enhance the understanding of lower phytoremediation efficacy of plants we observed for the high concentration of BTX.

6.9. Conclusions of the findings

Findings from this work concluded that plant monocultures have a high capacity to remove low concentrations of BTX from indoor air compared to the communities. Therefore, use of monocultures for the indoor phytoremediation system is more suitable than the mix culture system. Use of multiple monocultures (monoculture of different plant species) would also enhance the indoor air quality since that provides the best features of each monoculture separately.

Our findings also showed the great capacity of compost to adsorb high concentration of BTX from air, thus compost can be applied to remove high VOC concentrations from atmosphere during

accidental spillages. Based on the VOC removal by monoculture, we observed the higher ability of *C. comosum* than other plants to remove high concentrations of VOC, therefore it is a better option to use in the presence of high concentrations of VOC in newly constructed buildings. Also, we showed higher phytoremediation ability by *S. wallisii*, *C. comosum* and *H. helix* monocultures than the plant communities at low concentration of VOC, therefore use of plant monocultures rather than mix species culture will enhance indoor air quality. We identified a list of gaseous BTX utilizing rhizosphere bacteria in rhizosphere, thus this observation supports previous suggestions of the capability of rhizosphere bacteria to detoxify gaseous VOC during phytoremediation. BTX degrading bacteria identified in this study can be used to enhance the activity in the bioreactors which is used for the phytoremediation process. Our observation of the presence of common BTX degrading bacteria in the BTX exposed and non-exposed plants indicated that the phytoremediation ability of bacteria in the rhizosphere are influenced by the type of plant more than the influenced of exposure to VOC. We reported there was little changes in the taxonomical and functional diversity in the rhizosphere of VOC exposed and non-exposed plants indicated that the major impact for organising rhizosphere community was type of plant species other than VOC exposure. Therefore, these plants are suitable to use in any indoors to enhance the indoor air quality. Findings from this study can be used to maximise the phytoremediation of VOC from indoor air in terms of enhancing human wellbeing.

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Appendix 1: Characteristics of potted plants unit used during VOC monitoring

Plant characteristics (per tray) (n=8)	**Leaf area (m^2)**	**Dry weight of unit (weight of root, shoot and compost) (g)**
S. wallisii	0.535±0.095	2292±296.6
C. comosum	0.730±0.105	2420±184.9
H. helix	0.175±0.045	1987±188.1
Plant community	0.565±0.094	2116±100.1
Compost	N/A	1605±126.0

Appendix 2: Biochemical identification tests for gaseous BTX degrading bacterial strains

Gram staining procedure

Fresh cultures of isolated bacteria were grown on NA for 36-48 hours incubation at 25 °C. Using a sterilised metal loop, a drop of distilled water was placed on a clean glass slide. A small amount of fresh bacterial culture was transferred using sterilised loop and mixed with water on the slide to make a smear, allowed to dry and heat fixed by passing the slide 2-3 times above a Bunsen flame. The slide was flooded with crystal violet, incubated for 2 minutes, rinsed in distilled water and stained for 30 seconds with iodine solution. Excess stain was removed with 95% ethanol for approximately 5-10 seconds. The slide was then rinsed in distilled water, added few drops of safranin, incubated for 1 min, gently rinsed in distilled water, blotted dry gently and allowed to air dry prior to examination under oil immersion microscopy at x 1000 magnification. Gram-positive bacteria appeared purple while Gram-negatives were pink.

Catalase Test

Fresh cultures of isolated bacteria were grown on NA for 36-48 hours incubation at 25 °C. A small sample of a fresh culture was transferred onto a coverslip using a disposable plastic loop. A drop of hydrogen peroxide (3% v/v) was added on the coverslip. An immediate production of gas bubbles was indicative of a positive reaction.

Spore Staining

Fresh cultures of isolated bacteria were grown on NA for 36-48 hours incubation at 25 °C. A heat fixed smear of each bacterium was prepared as described for Gram stain above. Malachite green was applied and heated to steaming for 5 min. The smear was washed for about 30 s with sterilised DW and counterstained with 1% safranin. The slide was then rinsed in distilled water, blotted dry gently and allowed to air dry prior to examination under oil immersion microscopy at x 1000 magnification. Vegetative cells appeared pink/red while spores ellipse inside the cells and appeared green.

Oxidase Test

Fresh cultures of isolated bacteria were grown on NA for 36-48 hours incubation at 25 °C. A fresh bacterial colony was removed aseptically using a plastic loop and rubbed on an oxidase strip containing N-N-N'-N'-tetramethyl-p-phenylennediamine dihydrochloride. Formation of a purple-blue colour within 10 to 30 seconds was indicative of a positive result.

Motility test

Fresh cultures of isolated bacteria were grown on NB for 24 hours incubation at 25 °C. using a tooth pick, Vaseline was applied in the four edges of a coverslip. Using a sterilised metal loop, a drop of culture broth was placed in the middle of a coverslip. A microscopic cavity slide was depressed on the drop of the culture, turned over the slide quickly so the drop of the culture was hanging into the centre of the cavity. The edge of the drop was observed for the movements of bacteria under the low power objective.

Glucose oxidase/fermentation test

All the Constituents (except glucose) in Hugh-Leifson glucose broth (HLGB) (Table 2.1.) were mixed in 100 ml distilled water, heated to 95 °C, added 1 % glucose to the medium. The solution mixture was dispensed to test tubes (8-9 ml). The tubes were capped, autoclaved and allowed to cool down to room temperature. Fresh bacterial culture grown in NB for 24 hours at 25 °C was inoculated in duplicate tubes by stabbing the HLGB medium with a straight nichrome wire. A layer of melted sterilised mineral oil of 1 cm depth was added to one of the duplicates to create an anaerobic environment. The tubes were incubated at 25 °C for 48 hours or up to 14 days. Yellow colour in both open and sealed tubes indicated glucose fermentative bacteria. Yellow colour in only the open tube while no colour change in the sealed tube indicated glucose oxidative bacteria. No colour changes in either tubes indicated that bacteria did not metabolise glucose.

Glucose acid/ gas production from glucose

Glucose acid test broth was prepared using constituents detailed in the Table 2.1. The solution was dispensed (6-8 ml) in test tube containing inverted Durham tube and autoclaved. The tubes were inoculated with fresh culture of bacteria, incubated at 25 °C, for 24-48 hours. Acid production (fermentation of medium) from glucose was specified by turning pink colour medium into yellow colour and gas production appeared by the presence of small bubbles in the inverted Durham tubes.

Anaerobic growth

Fresh bacterial culture grown on NB was inoculated on NA plates, placed in the anaerobic jar. Anaerobic gas generator pack (Oxoid, UK) was opened, placed immediately inside the jar, incubated for 24-48 hours at 25 °C. Presence of bacterial growth indicated Anaerobic growth.

Acid fast test

Fresh cultures of isolated bacteria were grown on NA for 36-48 hours incubation at 25 °C. A heat fixed smear of each bacterium was prepared as described for Gram stain above. Carbol fuchsin was applied, heated until steaming started and allowed cool for 5 minutes. The slide was washed

with distilled water, covered with 3% (v/v) acid alcohol for 5 minutes. The slide was then rinsed in distilled water, dried in air prior to examination under oil immersion microscopy at x 100 magnification. Acid fast bacteria appeared as bright red to purple colour while non-acid fast bacteria appeared blue colour.

Appendix 3: 16S rRNA gene sequences of BTX degrading bacteria

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Appendix 4. Concentrations of rhizosphere DNA extracted from 10 ppm benzene treated and untreated plants and benzene untreated compost

Rhizosphere sample	10 ppm benzene treatment	Replicate	DNA concentrations (ng/µl)
S. wallisii	Treated	1	15.3
		2	13.5
	Untreated	1	16.3
		2	15.4
C. comosum	Treated	1	19.4
		2	21.4
	Untreated	1	31.6
		2	24.0
H, helix	Treated	1	23.6
		2	34.8
	Untreated	1	38.2
		2	27.2
Compost	Untreated	1	10.6
		2	9.2

Appendix 5. List of most abundant bacteria genera with their percentage of mean relative abundance

Genus name	Benzene treated *C. comosum* (BenzeneCC) %	Benzene treated *H. helix* (BenzeneHH) %	Benzene treated *S. wallisii* (BenzeneSW) %	Compost %	Benzene untreated *C. comosum* (ControlCC) %	Benzene untreated *H. helix* (ControlHH) %	Benzene untreated *S. wallisii* (Control SW) %
Genus from family Chitinophagaceae	6.0	7.4	7.1	13.7	6.3	7.9	7.1
Rhizomicrobium	1.3	7.3	1.8	7.7	1.6	6.2	2.6
Pseudomonas	22.9	0.9	1.1	0.0	2.1	0.9	1.5
Rhodanobacter	3.3	4.7	1.7	6.7	1.1	6.2	2.4
Rhizobium	7.9	1.4	1.7	0.3	3.2	1.9	2.4
Paraburkholderia	0.1	4.1	0.1	0.6	0.0	6.5	0.1
Sphingomonas	1.8	2.0	1.1	0.6	2.2	2.7	3.6
Sphingobium	6.3	0.3	1.8	0.0	4.0	0.5	2.5
Pseudolabrys	1.1	1.3	2.4	8.0	1.4	1.2	3.0
Devosia	1.7	1.2	1.9	2.7	2.1	1.1	2.0
Flavobacterium	2.6	0.2	0.7	0.0	2.9	0.5	1.0
Novosphingobium	0.8	0.5	0.1	0.1	3.6	0.7	0.3
Dongia	0.3	2.5	0.6	1.7	0.4	1.8	0.8
Acidibacter	0.5	0.3	2.6	0.1	1.0	0.2	0.8
Cellvibrio	0.3	0.2	1.5	0.0	0.4	0.0	1.4
Haliangium	0.4	0.9	1.0	0.5	0.8	0.9	0.5
Hyphomicrobium	0.2	0.2	1.1	0.2	0.3	0.3	0.7
Woodsholea	0.1	0.9	2.1	0.0	0.7	0.4	1.4
Nicotiana otophora	0.0	0.0	1.6	0.0	0.0	0.0	1.0
Mesorhizobium	0.9	0.8	0.9	2.3	0.6	1.1	1.2
Luteimonas	0.8	0.1	1.0	0.1	0.4	0.3	1.2
Methylotenera	1.6	0.1	1.3	0.0	3.0	0.1	1.9
Hirschia	0.3	0.3	1.0	0.0	1.0	0.2	0.8
Enterobacter	0.1	0.0	0.0	0.0	1.3	0.0	0.0
Caulobacter	0.6	0.4	0.2	0.1	1.1	0.4	0.4

Genus name	Benzene treated *C. comosum* (BenzeneCC) %	Benzene treated *H. helix* (BenzeneHH) %	Benzene treated *S. wallisii* (BenzeneSW) %	Compost %	Benzene untreated *C. comosum* (ControlCC) %	Benzene untreated *H. helix* (ControlHH) %	Benzene untreated *S. wallisii* (Control SW) %
Dyadobacter	0.6	0.1	0.4	0.0	1.0	0.1	0.3
Arthrobacter	0.9	0.1	0.1	0.1	1.2	0.0	0.1
Varlovorax	0.5	0.3	0.4	0.0	1.4	0.6	0.2
Pedobacter	1.7	0.6	0.7	0.0	1.3	1.5	0.7
Brevundimonas	1.0	0.2	0.4	0.3	0.6	0.3	0.7
Stenotrophomonas	1.3	0.2	0.1	0.0	0.1	0.8	0.2
Granullcella	0.1	3.3	0.1	0.2	0.1	3.0	0.1
Bryobacter	0.1	1.7	0.6	1.2	0.76	1.1	0.5
Acidothermus	0.1	1.3	0.2	1.2	0.1	0.5	0.3
Dokdonella	0.2	1.2	1.0	0.1	0.2	0.7	0.7
Mucilaginibacter	0.4	2.4	0.2	0.3	0.4	4.8	0.2