Biodegradation of Volatile Hydrocarbons in Estuarine Environments

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A thesis submitted for the degree of Doctor of Philosophy

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2019

Summary

Benzene, toluene, isoprene, ethane and propane are volatile organic compounds (VOCs) commonly found in estuarine environments. Benzene and toluene are toxic and carcinogenic at varying concentrations. Isoprene, ethane and propane are emitted from marine environments to the atmosphere where they can alter atmospheric chemistry and thus climate in different ways. The identity of microbial communities associated with VOC degradation in estuarine environments is not completely elucidated. Aerobic and anaerobic degradation of these VOCs in the Colne estuary, UK, was investigated using both cultivation-dependent and independent approaches, to understand the effect of VOC-type, concentration and estuarine location on degradation and microbial community composition. Aerobic biodegradation of all VOCs occurred in sediment slurries. More than 80% of the benzene, toluene and isoprene added was degraded within 10 days, while there was more than 40% degradation of ethane and propane within 37 days. Degradation of VOCs correlated positively with the concentration added. Principal component analysis (PCA) of MiSeq-generated 16S rRNA gene sequence data revealed that bacterial communities enriched with ethane, propane or toluene were similar, while those with benzene and isoprene were distinct. The main aerobic genera enriched were: *Pseudomonas* (benzene), *Rhodococcus* (isoprene), *Amphritea* (propane and toluene). In ethane enrichments, *Azoarcus* and *Cycloclasticus* dominated at the head and middle of the estuary, respectively. Metabolically versatile *Mycobacterium hodleri* strain B and *Rhodococcus wratislaviensis* strain I with putative propane monooxygenase genes were isolated from sediment slurries. Anaerobic degradation of benzene, toluene and propane was observed in sediment slurries but there was no evidence of ethane

degradation. Compared with aerobic, anaerobic biodegradation of isoprene was much more equivocal. The bacterial genus primarily enriched with benzene was *Pelobacter* (95% 16S rRNA identity) along with the archaeal genus *Methanomassiliicoccus* (83-86% identity) and other novel *Euryarchaeota*. The main bacterial OTUs enriched on propane had ~90% 16S rRNA identity to *Smithella, Rugosimonospora* and *Desulfatiglans.* This study has provided an insight into the bacterial and archaeal populations associated with aerobic and anaerobic degradation of VOCs. It shows that different types of VOCs are consumed by specific taxa, which working together as a community, contribute to carbon cycling in estuarine environments.

Keywords: Archaea, Bacteria, biodegradation, estuarine environments, microcosms, volatile organic compounds.

Acknowledgements

I wish to thank my supervisor, Prof Terry McGenity for the advice and mentoring he provided throughout the PhD programme. I would also like to thank my board member, Dr Corinne Whitby for the feedback she provided during meetings. I appreciate the technical support provided by Farid Benyahia, John Green and Russell Smart. I wish to thank members of the Molecular Microbiology Laboratories (5.28 and 5.32) especially Dr Boyd McKew for performing part of the bioinformatic analyses of the MiSeq data. I appreciate Dr Florin Musat for providing training on anaerobic cultivation techniques at the Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research –UFZ, Leipzig, Germany. I am thankful to Dr Andrew Crombie of the University of East Anglia, UK, for the support he provided with the identification of the putative propane monooxygenase gene in my bacterial isolates.

This work was funded by the Tertiary Education Trust Fund (TETFUND) through the University of Uyo (UniUyo), Nigeria. I wish to express my gratitude to them and Prof Comfort Ekpo, the past Vice Chancellor, for nominating me. I wish to thank the Head, Department of Microbiology UniUyo, Prof J.P. Essien, Prof A.Y. Itah and Dr Samuel Eduok for the career support advice they provided. I am grateful to several members of the University of Essex community who made positive contributions to my life and family. They include Rev Julia Murphy, the Anglican Chaplain, Prof Hugh Ward, Prof Glyn Stanway, Dr Louise Beard, Dr Victor Utti, Dr Claire A. Passarelli and partner Hector, etc.

I express profound gratitude to my wife, Jacqueline Nguavese, our children, Chivirnen and Wuesenen for the support and love. I wish to say a big thank you to my mom Martina Korinjo and my mother-in-law, Christina Adugh for the financial support and love. I am grateful to my siblings, cousins and in-laws for all the love they showed. I thank our family friends Prof Aniedi and Odot Ette, Mr and Mrs John E. Udo, Prof. A. A. Fasanmade, Dr Paul and Julie Rusiecki, Hilary and John Le Seve, Dr Chima and Mrs Isabella Egbukhole, Dr Paul Ihom, Dr Jacob Atser, Mr Asuquo Medekong, Engr Mfon Ukpabio etc. for the support and kindness.

Finally, I am grateful to Almighty God for provision, knowledge, wisdom, good health and faithfulness.

Contents

Tables

Figures

Abbreviations

bp	base pair
BLAST	basic local alignment search tool
BTEX	benzene, toluene, ethylbenzene and xylene
BVOC	biogenic volatile organic compound
cDNA	complementary DNA
Ct	cycle threshold (in Q-PCR)
Cq	quantitation cycle (in Q-PCR)
CTAB	cetyl trimethylammonium bromide
DGGE	denaturing gradient gel electrophoresis
DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
GC-FID	gas chromatograph with flame ionisation detector
GMBA	2-glutathionyl-2-methyl-3-butenoic acid
IPP	Isopentenyl pyrophosphate
isoA	isoprene monooxygenase, α-subunit

isoMO	isoprene monooxygenase
MEP	methyl D-erythritol 4-phosphate
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
NTC	no template control (in Q-PCR)
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PEG	polyethylene glycol
psbA	photosystem II protein D1
ppbv	parts per billion by volume
ppmv	parts per million by volume
PTFE	polytetrafluoroethylene
Q-PCR	quantitative-PCR
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcription-PCR
RT-qPCR	reverse transcription-quantitative PCR
TAE	tris-acetate-EDTA buffer

Tris	tris(hydroxylmethyl)aminomethane
v/v	volume to volume
w/v	weight to volume
VOCs	volatile organic compounds

Chapter 1. General Introduction

1.1 Brief introduction

Hydrocarbons occur naturally as part of fossil resources like crude oil or through biosynthesis by plants, microbes and other eukaryotes (Ladygina et al., 2006, Xie et al., 2013, Hinrichs et al., 2006). Some of them are volatile, thus, referred to as volatile hydrocarbons or organic compounds (VOCs). In general, VOCs are defined as compounds that have a vapour pressure at 20^{o}C of less than 101.3 kPa and greater than 0.13 kPa (Derwent, 1995). They are present in the environment as vapour. Vapour is the gaseous state of a substance which could be liquid under normal conditions of temperature and pressure, whereas a gas must be liquified in a cylinder under those conditions (Hill, 1966). Examples include benzene, toluene, isoprene, ethane, propane etc. Those produced by living things are called biogenic VOCs (BVOCs). Others are introduced in the environment by human activities (anthropogenic) such as industrial discharge of effluents.

Isoprene, toluene, benzene, ethane and propane have very important industrial applications. The polymerisation of isoprene yield polyisoprene, a synthetic rubber that is used to produce medical equipment, baby bottle teats/nipples, toys, shoe soles, tyres, elastic films, threads for golf balls or textiles, etc. (Shell Chemicals, 2012). Isoprene is also used as a co-polymer in styrene-isoprene-rubber to produce pressure sensitive adhesives (Shell Chemicals, 2012). Toluene is used for the production of paints, thinners, adhesives, inks, benzene, pharmaceutical products and addition to gasoline mixtures to increase its octane ratings (Beauregard, 1993). Benzene is used for making plastics, resins, synthetic fibres, rubber lubricants, dyes, detergents, drugs and pesticides. The ethane – propane mixture is used as a petrochemical feedstock to

produce ethylene, an important monomer for plastic production (Shennan, 2006). Moreover, propane is commonly used as fuel for cooking and heating.

These VOCs impact the environment in diverse ways. Isoprene is as abundant as methane with a global annual emission ≈ 500 Tg C $year^{-1}$ (Guenther et al., 2012). Global emission estimates for ethane and propane are 9.2 – 9.6 Tg $year^{-1}$ and 9.6 – 10.5 Tg $year^{-1}$, respectively (Musat, 2015, Pozzer et al., 2010, Etiope and Ciccioli, 2009). The large amount of isoprene, ethane and propane emitted from terrestrial and marine environments alter atmospheric chemistry with detrimental impacts on climate (section 1.3). Benzene and toluene have an impact on health because of their toxicity and carcinogenicity. Acute exposure to benzene cause leukaemia (McHale et al., 2012, Smith et al., 2011, Snyder, 2012).

1.2 Sources of volatile hydrocarbons

VOCs in marine environments are often from combined sources including cold seeps, accidental oil spillage, industrial discharge, biogenic production, etc. Biogenic production of isoprene, ethane, propane and toluene has been demonstrated (Ladygina et al., 2006, Formolo, 2010, McGenity et al., 2018). VOCs are produced by organisms as secondary metabolites for reasons that have not been fully understood but some are in response to stress (Sharkey et al., 2008). There are hints that isoprene is produced to serve as volatile repellent of soil predators or as a growth inhibitor of competing microbes (Ladygina et al., 2006).

1.2.1 Biosynthesis of isoprene

Isoprene is the most abundant biogenic hydrocarbon produced mainly by plants (Harley et al., 1999, Loreto and Velikova, 2001, Sharkey et al., 2005, Sharkey et al., 2008,

Loreto and Delfine, 2000). It is also produced by bacteria (Julsing et al., 2007, Kuzma et al., 1995, Wagner et al., 1999, Xue and Ahring, 2011, Yang et al., 2012) and microphytobenthic communities (Acuña Alvarez et al., 2009, Exton et al., 2012, Dani et al., 2017, Moore et al., 1994, Shaw et al., 2003). Terrestrial vegetation emit 400 – 600 Tg C $year^{-1}$ while 0.1 – 11.6 Tg C $year^{-1}$ is emitted from marine environments (Guenther, 2006, Arneth et al., 2008, Shaw et al., 2010, Arnold et al., 2009, Luo and Yu, 2010, McGenity et al., 2018, Palmer and Shaw, 2005). Photosynthetic microalgae are the main source of isoprene in marine environments (McGenity et al., 2018).

Two major isoprene biosynthesis pathways have been determined. The first is the mevalonate (MVA) pathway, which is found in animals, fungi, archaea, some bacteria and the cytosol of higher plants, while the second is the non-mevalonate or 1-deoxy-D-xylulose-5-phosphate (DXP) found in bacteria, green algae and chloroplasts of higher plants (Figure 1.1) (Xue and Ahring, 2011, McGenity et al., 2018). The non-mevalonate pathway is more commonly known as the methylerythritol 4-phosphate (MEP) pathway (Yang et al., 2012, Julsing et al., 2007, Steinke et al., 2011). Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the universal precursors for isoprene and isoprenoid biosynthesis (Xue and Ahring, 2011). Isoprene synthase (IspS) catalyse the elimination of pyrophosphate from DMAPP to produce isoprene (Figure 1.1).

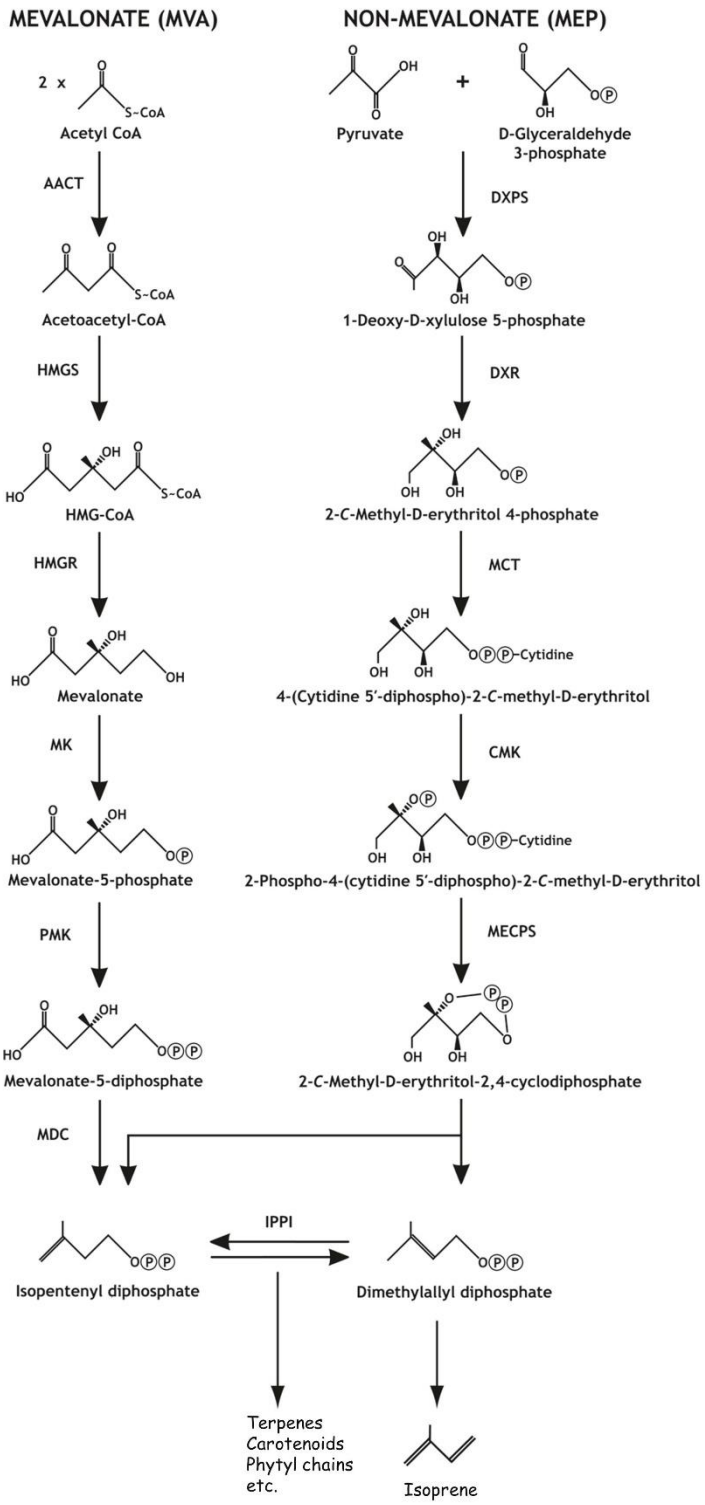


Figure 1.1. Mevalonate (MVA) and methylerythritol phosphate (MEP) biosynthetic pathways of isoprene and isoprenoids. Redrawn from McGenity et al. (2018). Enzymes for the MVA pathway shown: AACT acetoacetyl-CoA thiolase, HMGS 3-hydroxy-3-methylglutaryl-CoA synthase, HMGR 3-hydroxy-3-methylglutaryl-CoA reductase, MK mevalonate kinase, PMK phosphomevalonate kinase, MDC mevalonate 5-diphosphate decarboxylase. Note that an alternative route exists in Archaea for the conversion of mevalonate-5-phosphate to IPP (Vinokur et al., 2014). Enzymes for MEP pathway shown: DXPS 1-deoxyxylulose 5-phosphate synthase,

DXR 1-deoxyxylulose 5-phosphate reductoisomerase, MCT 2-Cmethyl-D-erythritol 4-phosphate cytidyl transferase, CMK 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase, MECPS 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase. The intermediate, (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP), from 2-Cmethyl-D-erythritol-2,4-cyclodiphosphate to IPP and DMAPP, is not shown. Enzymes common to both pathways are: IPPI isopentenyl diphosphate isomerase, ISPS isoprene synthase.

1.2.2 Biosynthesis of ethane and propane

Biogenic ethane or propane production has been known for decades (Oremland, 1981, Fukuda et al., 1984b, Fukuda et al., 1984a). Fukuda et al. (1984b) found 49 ethane-producing and 87 propane-producing strains belonging to fungi, yeasts, bacteria, and actinomycetes. A recent study in the SouthEastern Pacific detected ethane and propane production by microbial communities 380 m below seafloor (Hinrichs et al., 2006).

Methanogens produce ethane and propane in estuarine environments. Oremland (1981) shows ethane production by methanogenic cultures obtained from sediment slurries. Although the amount of ethane is little compared to methane ($1:10^4$), it explains why trace amount of ethane is found in estuarine sediments. The methanogenic enrichment produced ethane by the cleavage of ethylthioethanesulfonic acid (ethyl-S-CoM) (Oremland, 1981). More recently, ethane and propane production in anoxic sediments has been shown (Xie et al., 2013).

Ethanogenesis and propanogenesis are carried out by methanogens using ethylene and propanethiol respectively (Xie et al., 2013). Ethane is detectable in sediment slurries after 30 days of incubation with ethylene and 50 days incubation with ethanethiole (Xie et al., 2013).The biosynthetic pathways of ethane and propane were proposed earlier by Hinrichs et al. (2006) through ethanogenesis and propanogenesis (Figure 1.2). They proposed that ethane could be formed by an ethylated Co-enzyme-

M or, if present, ethanethiol could be a precursor for ethane production. Propanethiol could be the possible precursor for biogenic propane production (Formolo, 2010, Hinrichs et al., 2006).

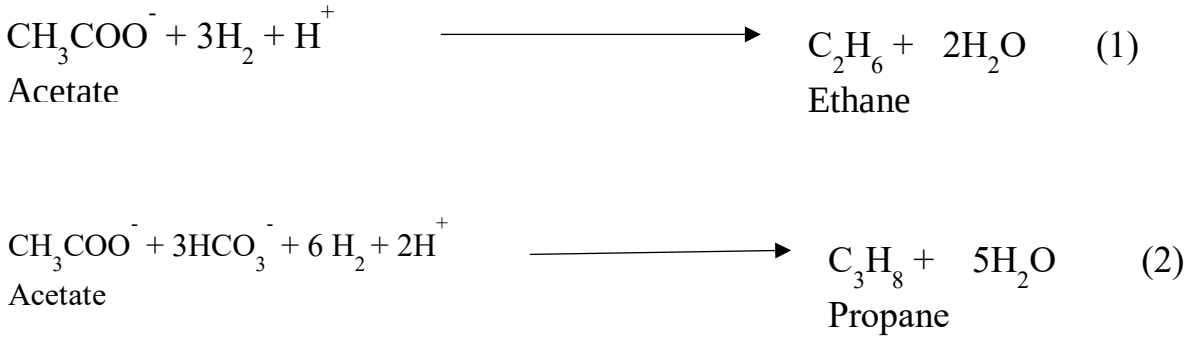


Figure 1.2. Proposed reactions for ethanogenesis (1) and propanogenesis (2). The reaction shows that ethanogenesis and propanogenesis are sinks for acetate and hydrogen in an anaerobic environment. Redrawn from Hinrichs et al. (2006).

1.2.3 Biosynthesis of benzene and toluene

Microbes have also been shown to produce toluene (Fischer-Romero et al., 1996) and benzene ring (Suzuki et al., 2006). The first bacterium shown to produce toluene is *Tolumonas auensis* isolated from anoxic sediments of a freshwater lake (Fischer-Romero et al., 1996). *Tolumonas auensis* transforms phenylalanine, phenyl-lactate, phenylpyruvate, and phenylacetate into toluene (Fischer-Romero et al., 1996). Its ability to produce toluene is dependent on the presence of any of these precursors in the medium. Monreal and Schnitzer (2013) detected a wide range of *n*-alkylbenzenes including methylbenzene (toluene) in the wheat rhizosphere at all the four growth stages of the wheat studied. They explained that the alkylbenzenes were produced by the depolymerisation and decomposition reactions of the plant tissues.

Although reports about the biosynthesis of benzene are scarce, Suzuki et al. (2006) demonstrated benzene ring biosynthesis from C_3 and C_4 primary metabolites by *Streptomyces griseus*. The objective of the study was to determine the pathway for the biosynthesis of grixazone. They were able to decipher the pathway for the biosynthesis of 3-amino-4-hydroxybenzoic acid, a benzene derivative and precursor to grixazone synthesis (Suzuki et al., 2006).

1.2.4 Concentration of volatile hydrocarbons encountered in estuarine environments

Estuaries are sites of high primary production. Millions of people around the world live near estuaries and depend on them for their food. They are used for fishing, shipping, recreation and aquaculture. By definition, estuaries are semi-enclosed bodies of coastal water having a free connection with the open sea and in which the seawater is measurably diluted with fresh water derived from a river or land drainage (O'Sullivan and Jacques, 1999). Most of the materials entering the oceans from land do so through estuaries.

Volatile hydrocarbons are emitted from estuarine environments. Biogenic isoprene, ethane, propane and toluene production has been observed in sediments (Xie et al., 2013, Acuña Alvarez et al., 2009, Exton et al., 2012, Jüttner, 1991, Mrowiec et al., 2005). Isoprene concentrations of water in the Colne estuary range from 0.7 – 451.8 pmol l^{-1} (Exton et al., 2012). Its gross production in sediments ranges from 0.15 – 0.71 pmol cm^{-2} h^{-1} which could result in an isoprene flux of 2.5 x 10^7 to 1.2 x 10^8 molecules cm^{-2} s^{-1} (Acuña Alvarez et al., 2009). Xie et al. (2013) reported ethane and propane production in anoxic estuary sediment as 0.079–2.252 µmol l^{-1} and 0.026–0.032 µmol l^{-1} slurry respectively.

Benzene and toluene are also emitted from sediments. Benzene and toluene were detected in sediment of Menai Strait,UK, using a stainless-steel funnel coupled to a small vacuum pump and solid-phase microextraction (SPME) (Bravo-Linares and Mudge, 2007). The benzene and toluene concentrations were 8.6 – 176.7 and <0.1 – 886.9 pg g^{-1} (w/w) respectively. Biogenic production is assumed to occur in the top 5 cm of sediments accounting for a benzene and toluene flux of 1108 and 8484 pg h^{-1} m^{-2} , respectively (Bravo-Linares and Mudge, 2007).

1.2.5 Nutrient input in the Colne estuary

Primary production along the Colne estuary is driven by microphytobenthos (MPB), an assemblage of photosynthetic microalgae, bacteria and archaea that inhabit surfaces of illuminated sediments (Underwood and Kromkamp, 1999, Kocum et al., 2002a, Kocum et al., 2002b). MPB are a source of organic carbon for benthic invertebrates and fish species (Nedwell et al., 2016) and form the base of the food chain in muddy and sandy estuaries (Underwood and Kromkamp, 1999).

The Colne is a nutrient-rich coastal estuary with seasonal variations in phosphate and nitrogen concentrations. The nutrient concentration is higher at the head of the estuary, but reduced towards the mouth (Brightlingsea) due to biological processes, increasing water volume and tidal activities etc. (Nedwell et al., 2016). Biogeochemical processes such as biodegradation of organic matter driven by microbial communities are key drivers of element cycling in the estuary (Nedwell et al., 2016).

Most of the nitrogen input into the estuary (≈60%) is contributed by nitrate and dissolved organic nitrogen (DON) while the other 40% is contributed by the Colchester sewage treatment work (STW) through a constant flow of ammonium-rich effluents into the estuary (Nedwell et al., 2016, Agedah et al., 2009). Inorganic nitrate and phosphate

concentrations as high as 1 mM and 50 µM are common at the head of the estuary where the STW is located (Nedwell et al., 2016).

1.3 Environmental impact of benzene, toluene, isoprene, ethane and propane.

1.3.1 Properties of isoprene and its environmental impact

Isoprene (2-methyl-1,3-butadiene) is volatile with a boiling point of 34^{o}C (Table 1.1). It is an unsaturated hydrocarbon with a carbon-carbon double bond which makes it highly reactive. It competitively removes free radicals of ·OH and NO_x thus delaying the oxidation of greenhouse gases like methane and carbon monoxide. The elongation of the residence of these gases in the atmosphere contributes to global warming (Collins et al., 2002).

Isoprene contributes to tropospheric ozone (O_3) formation and organic atmospheric nitrate oxidants when moderate to high levels of NOx are present. Tropospheric ozone formation by isoprene is possible only when the following conditions are met: the right UV wavelengths ($\lambda < 420$ nm) of sunlight and in the presence of nitrogen oxides (NO_x = NO + NO_2) (Harley et al., 1999). Isoprene reacts with OH radicals or O_3 to yield peroxy radicals (RO_2). In regions of high NO_x, RO_2 reacts with NO, to produce NO_2 (Figure 1.3). NO_2 is readily photolyzed ($\lambda < 420$ nm) to NO and ground-state oxygen atoms that rapidly combine with O_2 to form tropospheric ozone (Harley et al., 1999). Ozone is a greenhouse gas and has negative impact on the health of animals and plants (Ashworth et al., 2013).

$RH\ (e.g.\ isoprene) + OH + O_2 \rightarrow RO_2 + H_2O$

$RO_2 + NO \rightarrow RO + NO_2$

$RO + O_2 \rightarrow R'CHO + HO_2$

$HO_2 + NO \rightarrow OH + NO_2$

$2NO_2 + 2O_2 \rightarrow 2NO + 2O_3$

Net Reaction: $RH + 4O_2 \rightarrow R'HCO + 2O_3 + H_2O$

Figure 1.3. Transformation reaction of reduced hydrocarbons (e.g. isoprene) in the atmosphere. Redrawn from Sharkey et al. (2008).

Oxidation of isoprene produces products that contribute directly to the mass of secondary organic aerosols (SOA). SOA cause global cooling and promote cloud formation by absorbing or scattering solar radiation (Budisulistiorini et al., 2013, Chan et al., 2010, Lin et al., 2012, Shaw et al., 2010).

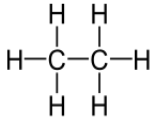
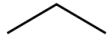
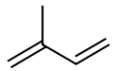
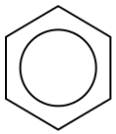
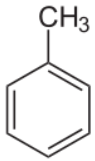
1.3.2 Properties of ethane and propane and their impact on the environment

Ethane (C_2H_6) and propane (C_3H_8) are saturated alkanes with -89^{o}C and -42.1^{o}C boiling points respectively (Table 1.1). They are not very reactive because of the non-polar σ-bonds (Singh et al., 2017). They are emitted in large amount from terrestrial and marine environments to the atmosphere where they contribute to the formation of ozone and organic aerosols (Etiope and Ciccioli, 2009, Pozzer et al., 2010). Ethane and propane levels in the atmosphere are believed to be rising again in the northern hemisphere largely due to oil and gas exploration (Helmig et al., 2016). Recent model studies reveal that the amount of petrogenic ethane and propane is 2 to 3 times higher than previously estimated (Dalsøren et al., 2018).

1.3.3 Properties of benzene and toluene and their impact on the environment

Benzene (C_6H_6) and toluene (C_7H_8) are monoaromatic hydrocarbons with 80.1^oC and 110.8^oC boiling points respectively. They migrate very rapidly in water because of their high solubility (Table 1.1). The main routes of exposure to these VOCs are air, drinking water, food and beverages. They are implicated with causing cancer and irritation effects at sub-lethal dose levels (Wallace, 1993). The United States Environmental Protection Agency (EPA) listed benzene and toluene among the priority pollutants and their risk level is 0.01 mg l^{-1} (Bianchi and Varney, 1989, World Health Organization, 2010) . Exposure to benzene at concentrations above 0.005 mg l^{-1} may result in anaemia or increased risk of cancer while exposure to toluene at concentrations ≥1 mg l^{-1} may damage the nervous system, kidney, and liver (Cao et al., 2009). The oral LD_{50} (lethal dose causing 50% mortality) in rats for benzene range from 3900 to 5970 mg kg^{-1} body weight (bw) while the LD_{50} of toluene ranges from 5000 – 7500 mg kg^{-1} bw (Speijers, 1993). In humans, high exposure to benzene (325 mg m^{-3}) or acute exposure (65 g m^{-3}) may result to leukaemia and death respectively (WHO, 2003) . According to the world health organization, the life cancer risk of benzene in drinking water is 10^{-5} mg l^{-1} but there is no specific guideline for air (World Health Organization, 2010).

Table 1.1. Physical properties of volatile hydrocarbons

VOC	Molecular Weight (g mol^{-1})	Chemical Structure	Source	Boiling point (°C)	Density gml^{-1}	Vapour Pressure @ 25°C (kPa)	Solubility in water @ 25°C (mg l^{-1})	Henry's Law Constant @ 25°C (KPa.m^3 mol^{-1})	Global Emission (TgC yr^{-1})	Biodegradation		Notes	References
										Aerobic	Anaerobic		
Ethane (C_2H_6)	30.07		Oil, Bacteria, fungi, yeast	-89.0	1.28	nd	60.4	nd	9.2–9.6	yes	yes	Anaerobic degradation poorly understood.	3,4,7,8,10, 11,12,13.
Propane (C_3H_8)	44.10		Oil, Bacteria, fungi, yeast	-42.1	1.55	nd	62.4	nd	9.6–10.5	yes	yes	Petroleum component	3,4,7,8,10
Isoprene (C5H8)	62.12		Plants, Bacteria, Actinomycetes, marine algae	34.0	0.681	nd	642	nd	412 - 601	yes	no	Not present in petroleum.	1,5,7,9
Benzene (C6H6)	78.11		Gasoline, Bacteria, yeast, fungi	80.1	0.8765	12.7	1780	0.55	nd	yes	yes	Degraded by few pure cultures. Trace amounts in petroleum	2,4 ,6
Toluene (C7H8)	92.14		Gasoline, Plants, bacteria, yeast, fungi	110.8	0.867	3.80	531	0.67	nd	yes	yes	Degraded readily in anaerobic environments	2,4,6

[1]Fall (1999), [2]Kotzias and Sparta (1993), [3]Shennan (2006), [4]Widdel et al. (2010), [5]Guenther et al. (2012), [6]Rabus et al. (2016b), [7]Ladygina et al. (2006), [8]Rojo (2009), [9]Acuña Alvarez et al. (2009), [10]Musat (2015), [11]Adams et al. (2013), [12]Bose et al. (2013), [13]Singh et al. (2017). VOCs arranged in ascending order by Molecular Weight. nd means no data.

1.4 Microbial ecology of benzene, toluene, isoprene, ethane and propane degraders

Microorganisms utilising benzene, toluene, isoprene, ethane, and propane live in a variety of ecosystems. Only a small proportion of these microbial communities have been studied and few pure strains obtained so far. This is because the physiology, nutrients and growth requirements for most microbial populations is poorly understood. Most microbial populations are interdependent on each other in the environment, functioning in a network or consortia, while sharing essential nutrients (or metabolites) required for growth. Such conditions are difficult to replicate in the laboratory following conventional microbiological techniques, making it impossible for them to grow. These barriers are gradually being overcome by recent advances in molecular biological approaches.

Molecular biology techniques have improved our understanding of the structure and functioning of microbial communities which is the theme of microbial ecology. They are designed to detect nucleic acids or gene products in samples or the environment (*in situ*) (Morgan, 1991). In general, they involve extracting total community DNA (or RNA) from the natural sample (e.g. sediment); amplifying a marker gene (e.g. *16S rRNA* gene) by the polymerase chain reaction (PCR); sequencing gene(s) of interest; comparing the "marker" sequences to those available on databases, identifying them and carrying out phylogenetic analyses to determine evolutionary relationship with other organisms (Tunlid, 1999). Approaches aimed at DNA sequencing can be used to understand the taxonomic and functional diversity of extant, dormant, metabolically active or extinct populations in an environment while mRNA sequence analysis provide insight into the metabolically active populations (Clark et al., 2018). Recent advance in sequencing technology (next generation sequencing) and

computing power have revolutionized the field of microbial ecology providing tones of data in relatively short time. Using these tools, ecologists can identify microbial populations, described how they live and engineer them to carryout vital services such as bioremediation of polluted environments (Tunlid, 1999).

1.4.1 Ecology of benzene and toluene-degrading microbial communities

Benzene and toluene (BT)-utilising microbial communities inhabit ecosystems with a benzene or toluene source such as gasoline-polluted environments. These environments become polluted due to accidental discharge of gasoline during transport, leakage of underground storage tanks, etc. It is this pollution of diverse environments (soils, groundwater, sediments etc) and BT toxicity that exacerbated interest in research to understand their biodegradation (Widdel et al., 2010). A few BT- degrading bacterial taxa have been identified in oxic or anoxic environments and some isolates obtained. These bacterial populations play a key role of removing benzene or toluene from polluted environments.

Aerobic degradation of aromatic hydrocarbons is performed by species of the genus *Alcaligenes*, *Acinetobacter*, *Pseudomonas*, *Rhodococcus*, and *Nocardia* (Cao et al., 2009). *Pseudomonas* is the best known aerobic aromatic hydrocarbon-degrading genus because of its ability to degrade a wide range of aromatic compounds, from benzene to benzo(pyrene) (Cao et al., 2009). Several hydrocarbon utilising *Pseudomonas* strains exist in pure culture (Hildebrand et al., 1994, Lang et al., 2010, Lin et al., 2013, Ramirez-Bahena et al., 2014, Romanenko et al., 2008, Vanparys et al., 2006). However, benzene and toluene degrading capability is not restricted to those genera. Fries et al. (1994) isolated eight *Azoarcus* strains degrading toluene

under nitrate reducing conditions from a variety of contaminated and noncontaminated environments of the world. All isolates were able to utilise toluene aerobically and anaerobically (Fries et al., 1994). Three of those isolates also degrade benzene aerobically (Fries et al., 1994). Toluene consumption is observed in enrichments with samples from diverse environments including: a pristine rain forest soil from Cameroon; industrial waste or sludge from two states of Brazil; a big island of forest soil and Kauai sugar cane soil from Hawaii, USA; Huntington beach in California,USA; a deep sandy aquifer (24 to 26-m deep) in Michigan; sediment from a wood pulp treatment lagoon in Ontario, Canada; and a compost pile in Michigan, USA (Fries et al., 1994).

Anaerobic benzene and toluene-degrading populations under sulfate-reducing conditions have been identified. The main toluene-degrading populations (or phylotypes) include *Desulfobacula toluolica* Tol2 (Rabus et al., 1993), Strain oXyS1 and Strain mXyS1 (Harms et al., 1999). The dominant phylotype identified in benzene degrading enrichments include *Pelobacter*-related *Thermotogales, Deltaproteobacterium, Peptococcaceae, Desulfobacteraceae, Pelotomaculum-related* and *Epsilonproteobacteria* (Vogt et al., 2011).

Syntrophy plays a key role in anaerobic benzene and toluene degradation. McInerney et al. (2009) defined anaerobic syntrophy as “a thermodynamically interdependent lifestyle where metabolically distinct microorganisms are tightly linked by the need to maintain the exchange of metabolites (usually hydrogen, formate, and acetate) at very low concentrations, in order to degrade an organic compound leading to methane production”. Benzene and toluene are converted to benzoyl-CoA then consumed by a syntrophic consortia (McInerney et al., 2008). Syntrophy occurs in sulfate-reducing environments as well (Vogt et al., 2011, Nauhaus et al., 2007).

1.4.2 Ecology of isoprene-degrading microbial communities

Isoprene production and degradation has been studied extensively in terrestrial and marine environments since its discovery almost 60 years ago (McGenity et al., 2018, Sharkey and Monson, 2017). In terrestrial environments, rapid isoprene degradation has been reported in a variety of soils including tropical and boreal forest soils. Isoprene consumption was demonstrated in temperate soil chambers 20 years ago (Cleveland and Yavitt, 1997). Soil is a very important isoprene sink due to its ability to consume isoprene at rates as high as 62 pmol g^{-1} h^{-1} (Gray et al., 2015).

There are just a few studies on isoprene degradation in terrestrial aquatic environments, however, the most well characterized isoprene degrading *Rhodococcus* strain AD45 was isolated from a freshwater lake sediment (Van Hylckama Vlieg et al., 1998, Van Hylckama Vlieg et al., 2000). To date, the main isoprene enrichments or culture reported in terrestrial environments are either Actinobacteria or Proteobacteria from the genera *Rhodococcus*, *Pseudomonas*, *Klebsiella* and *Alcaligenes* (McGenity et al., 2018).

Reports of isoprene degradation in marine environments is relatively new compared to terrestrial environments. Nevertheless, isoprene degradation has been demonstrated in marine environments in the UK and beyond (Johnston et al., 2017, Acuña Alvarez et al., 2009). Isoprene-degrading bacterial communities were enriched in samples from the Colne estuary, UK; a brackish lagoon (Étang de Berre, France); in marine waters (between the islands of Hoga and Kaledupa, Indonesia) and other coastal marine environments in the UK (Penarth, South Wales, and the Western Channel Observatory L4 sampling station) (Acuña Alvarez et al., 2009, Johnston et al., 2017).

Isoprene-degrading communities in marine environments are phylogenetically diverse but the main isolates or phylotypes commonly identified in marine environments are of the phylum Actinobacteria or Proteobacteria, often dominated by *Rhodococcus, Mycobacterium* or *Stappia* (Johnston et al., 2017, Acuña Alvarez et al., 2009, McGenity et al., 2018).

1.4.3 Ecology of ethane and propane-degrading microbial communities

Ethane- and propane-degrading bacterial communities inhabit both terrestrial and marine environments (Shennan, 2006, Kulikova, 1995, Musat, 2015, Redmond et al., 2010, Valentine et al., 2010, Davis et al., 1956). They live in soil, coastal sediments, oilfields, sub-surface sediments, deep waters, oceans and natural gas seeps (Musat, 2015) where they contribute to carbon cycling.

Soil-dwelling ethane and propane degraders are non-spore formers adapted to living under starvation conditions (Shennan, 2006). They are typical soil dwellers which are metabolically versatile due to the low amount of ethane or propane available at any given point in time (Shennan, 2006). They are predominantly Gram-positive bacteria including *Arthrobacter*, *Corynebacterium*, *Mycobacterium* and *Rhodococcus* (Musat, 2015). Marine propane and ethane degrading bacterial communities are phylogenetically more diverse including *Proteobacteria*, *Firmicutes, Chloroflexi* (Musat, 2015, Redmond et al., 2010, Savage et al., 2010).

Table 1.2. Biodegradation of VOCs and the concentrations used in microcosms

VOC	Volume of VOC added/ How	Size of Vial (bottle)	Final Conc in Microcosm	Sample	Notes	References
Benzene	np	25 ml	0.25 mM (250 µM)	Sediments cores	Anaerobic benzene oxidation	1
Benzene	np	np	2 mM	np	Anaerobic benzene oxidation	2
Benzene	np	100 ml	1.02 mM (80 mg l^{-1})	Ground water	Aerobic benzene degradation	3
Benzene	np	160 ml	0.05 mM (50 µM)	Sediment Slurries	Anaerobic benzene degradation	4
Benzene	np	110 ml	0.81mM (64 mg l^{-1})	Ground water	Aerobic degradation	5
Benzene	Neat	120 ml	0.64 mM (50 mg l^{-1})	Slurries from sandy aquifers	Aerobic degradation. 10ml of oxygen was injected into the headspace	6
Toluene	np	50 ml	0.5 mM	np	Aerobic and anaerobic degradation	7
Toluene	Neat	np	0.3 mM (300 µM)	np	Anaerobic degradation	8
Toluene	np	250 ml	0.05 mM (50 µM)	Sediment	Anaerobic degradation	9
Toluene	Neat	120 ml	0.64 mM (50 mg l^{-1})	Slurries from sandy aquifers	Aerobic degradation	6
Isoprene	Neat	125 ml	4.2 mM	sediment	Aerobic degradation	10
Ethane and Propane	4.0 ml (0.16 mmol)	40 ml	np	Sediment slurry	Anaerobic degradation of propane	11

[1]Zhang *et al.* (2012), [2]Holmes et al. (2011), [3]Fahy *et al.* (2006), [4]Kazumi *et al.* (1997), [5]Aburto et al. (2009), [6]Alvarez and Vogel (1991), [7]Shinoda and Sakai (2004), [8]Evans et al. (1991a), [9]Edwards and Wills (1992), [10]Acuña Alvarez *et al.*, (2009), [11]Savage *et al.*, (2010). np means not provided in source article.

1.5 Aerobic degradation of volatile hydrocarbons

Aerobic degradation of benzene, toluene, ethane, propane and isoprene has been demonstrated in some environments (Tables 1.1 & 1.2). Oxygen is required both as a terminal electron acceptor and for activation of the hydrocarbon. In general,

aerobic degradation of volatile hydrocarbons is initiated by the incorporation of an atom of molecular oxygen into the hydrocarbon with the help of monooxygenases (Shennan, 2006). Soluble di-iron monooxygenases (SDIMO) are the group of enzymes commonly used by hydrocarbon degraders (Leahy et al., 2003a, Coleman et al., 2006).

1.5.1 Aerobic degradation of isoprene

Aerobic isoprene degradation has been demonstrated in soils and coastal sediment environments (Cleveland and Yavitt, 1998, Acuña Alvarez et al., 2009, McGenity et al., 2018) with key Isoprene degraders isolated and characterized (Johnston et al., 2017, Khawand et al., 2016, Acuña Alvarez et al., 2009, Van Hylckama Vlieg et al., 2000).

The first elaborated report on the aerobic degradation of isoprene and the enzyme involved in the process was carried out by Van Hylckama Vlieg et al. (2000) . In their study, they characterized the gene cluster involved in isoprene metabolism in *Rhodococcus* strain AD45. Ten open reading frames (ORFs), *isoABCDEFGHIJ,* were discovered that encoded putative proteins important for the degradation of isoprene. Crombie et al. (2015), using *Rhodococcus* strain AD45, demonstrated that all the genes in the *iso* gene cluster are essential to isoprene metabolism. Isoprene-degrading bacterial strains use the soluble di-iron monooxygenase enzyme, isoprene monooxygenase for substrate activation (Crombie et al., 2015, McGenity et al., 2018). This enzyme is encoded by *isoABCDEFGHIJ* were recently characterized in detail by Crombie et al. (2015).

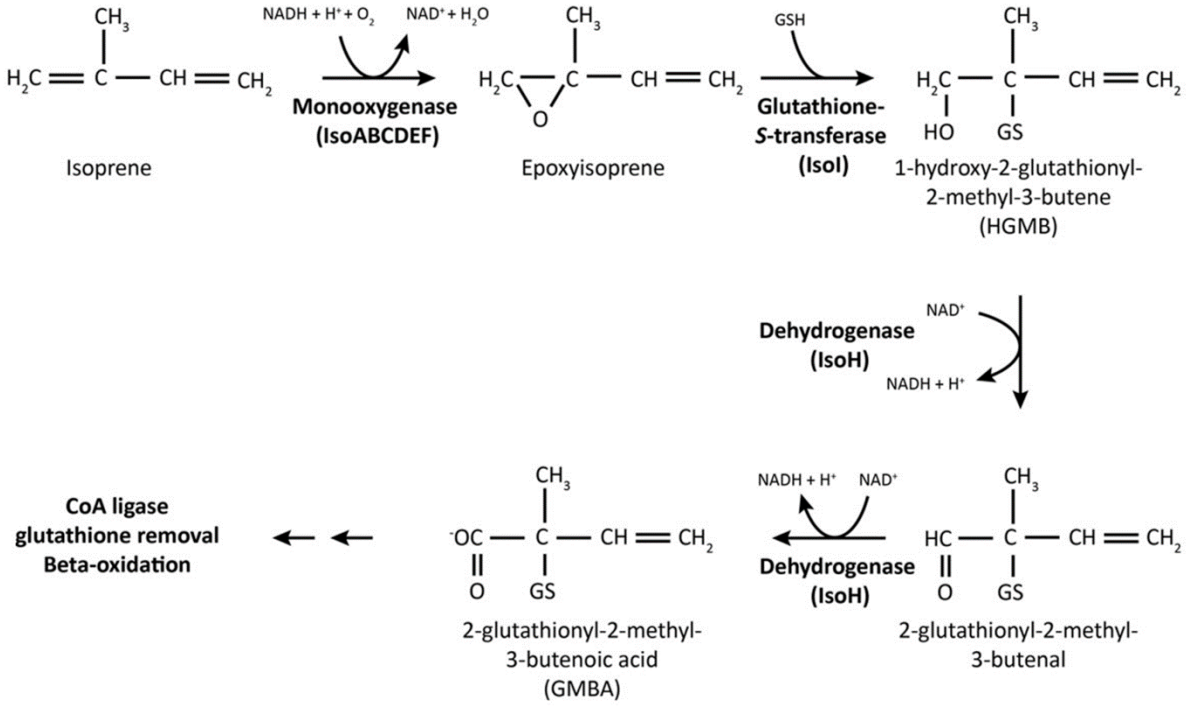


Figure 1.4. The pathway of isoprene catabolism. Redrawn from Crombie et al. (2015).

Van Hylckama Vlieg et al. (2000) proposed the pathway shown in Figure 1.4, which has been confirmed by Crombie et al. (2015). The pathway starts with the oxidation of the methyl-substituted double bond by a monooxygenase, encoded by *isoABCDEF*. This reaction yields 1,2-epoxy-2-methyl-3- butene, which is a substrate for *IsoI*. This enzyme catalyses the glutathione-dependent opening of the epoxide ring, yielding the stable glutathione conjugate HGMB. Two consecutive oxidation steps by *IsoH* in which the hydroxyl is oxidized to a carboxylate then result in the formation of GMBA (2-glutathionyl-2-methyl-3-butenoic acid). Anaerobic degradation of isoprene, however, has not yet been reported.

1.5.2 Aerobic degradation of ethane and propane

Alkanes are saturated hydrocarbons that are stable and unreactive. Aerobic ethane- or propane-degrading bacteria break the apolar C-H bond of alkanes using

molecular oxygen and monooxygenases (Figure 1.5) (Jaekel, 2011, Shennan, 2006, Coleman et al., 2006). Aerobic degradation of C_2 - C_4 alkanes is initiated either by the oxidation of a terminal or sub-terminal methyl group (Figure 1.6) to render the corresponding primary and/or secondary alcohols which are further oxidized to aldehydes by alcohol dehydrogenases (ADH).

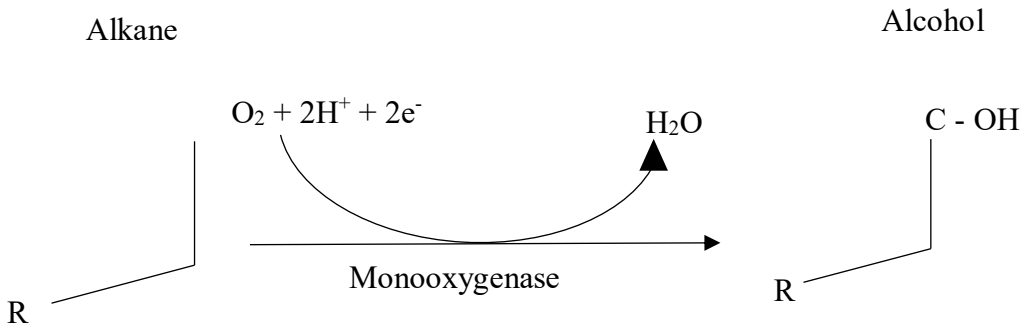


Figure 1.5. Aerobic activation of alkanes. A reaction showing oxidation of alkanes to alcohols. Redrawn from Jaekel (2011).

Some microorganisms, such as *Pseudonocardia* sp. TY-7, can oxidize propane via terminal and/or sub-terminal oxidation (Kotani et al., 2006, Shennan, 2006). Butane monooxyganase produced by *Thauera butanivorans* (Dubbels et al., 2009) oxidizes C2 – C4 alkanes through terminal oxidation of the methyl groups only (Arp, 1999). Ethane, propane and *n*-butane are utilised predominately by Gram-positive bacteria belonging to the actinnobacterial genera *Corynebacterium, Mycobacterium, Nocardia* and *Rhodococcus* (CMNR) and a few Gram-negative bacteria (Shennan, 2006).

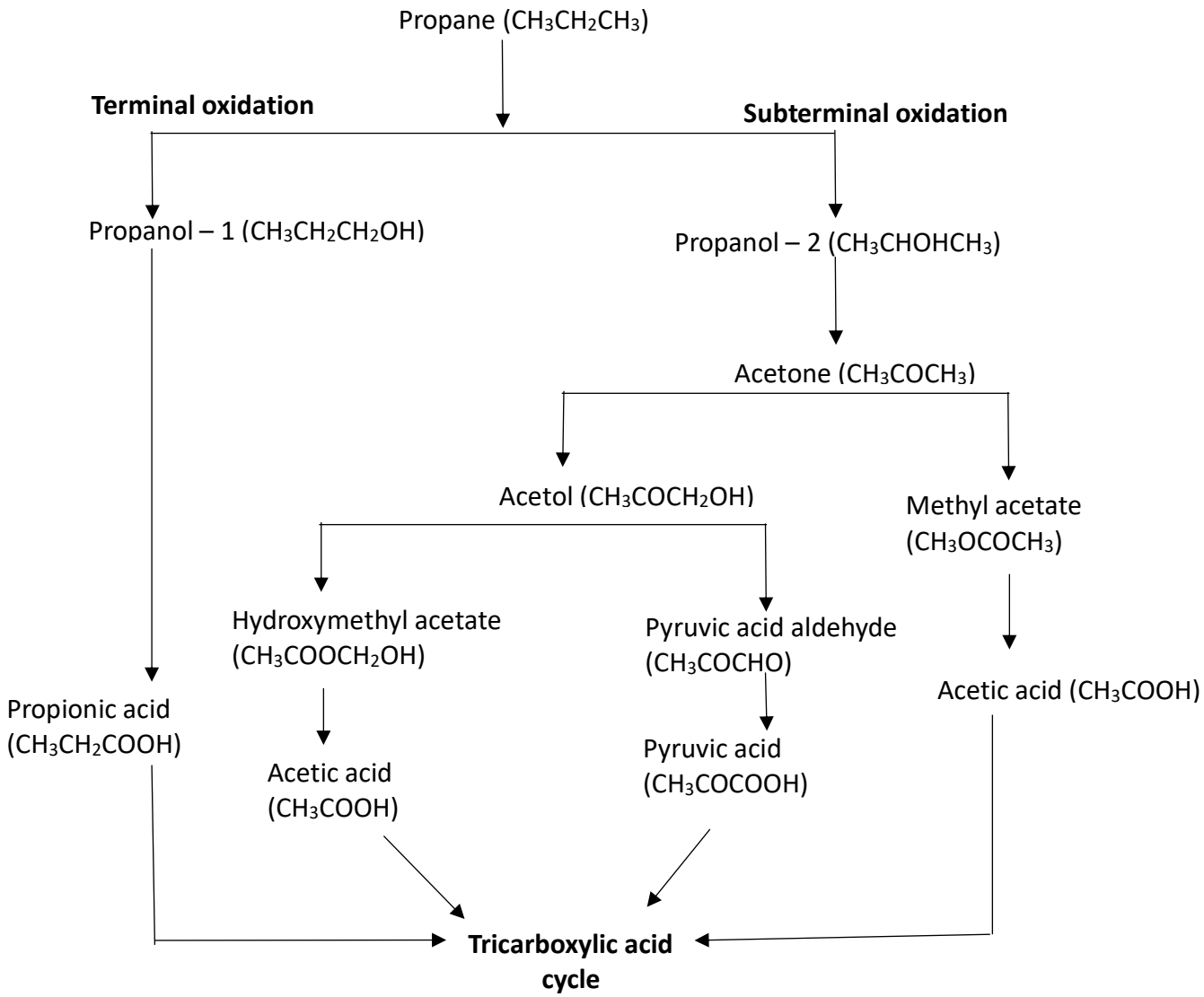


Figure 1.6. Propane assimilation pathway. Redrawn from Kulikova (1995).

1.5.3 Aerobic degradation of benzene and toluene

The aerobic degradation of toluene and benzene requires the activation of the benzene ring by oxygenases to overcome the resonance stability of the ring. Microorganisms that degrade toluene and benzene oxidize these compounds using dioxygenases or monooxygenases, which are iron-containing and oxygen-activating enzymes (Wackett and Hershberger, 2000). The various aerobic degradation pathways of toluene and benzene have catechol as a common intermediate.
The benzene ring is activated by the dioxygenase to *cis*-dihydrodiol and hydrogenated to produce catechol. The ring cleavage of catechol is achieved either by the *ortho*-cleavage pathway (intradiol) or by the *meta*-cleavage pathway (extradiol), Figure 1.7.

In the *ortho*-pathway, the aromatic ring is split between the two hydroxyl groups to yield *cis,cis*-muconic acid . The intermediates are further transformed to acetyl-CoA and succinate before entering the tricarboxylic acid cycle where it is mineralised to CO_2 (Figure 1.7). In the *meta*-cleavage pathway, the benzene ring is broken at a position adjacent to the two hydroxyl groups to yield hydroxymuconic semialdehyde (Figure 1.7). The intermediates are further transformed to pyruvate and acetaldehyde before entering the tricarboxylic acid cycle for final degradation to carbon dioxide (Rabus et al., 2016b).
Toluene is oxidized to catechol via two parallel pathways (Wackett and Hershberger, 2000). In the first pathway toluene is oxidized by dioxygenase to yield *cis*-1,2-dihydroxydihydro-3,5-cyclohexadiene, then the intermediate is oxidized by a dehydrogenase to 3- methylcatechol. In the parallel pathway, a monooxygenase oxidizes toluene to *o*-cresol (2-hydroxytoluene) then to 3- methylcatechol. The

subsequent steps from the metabolism of catechol through dioxygenolytic ring cleavage are similar for both pathways.

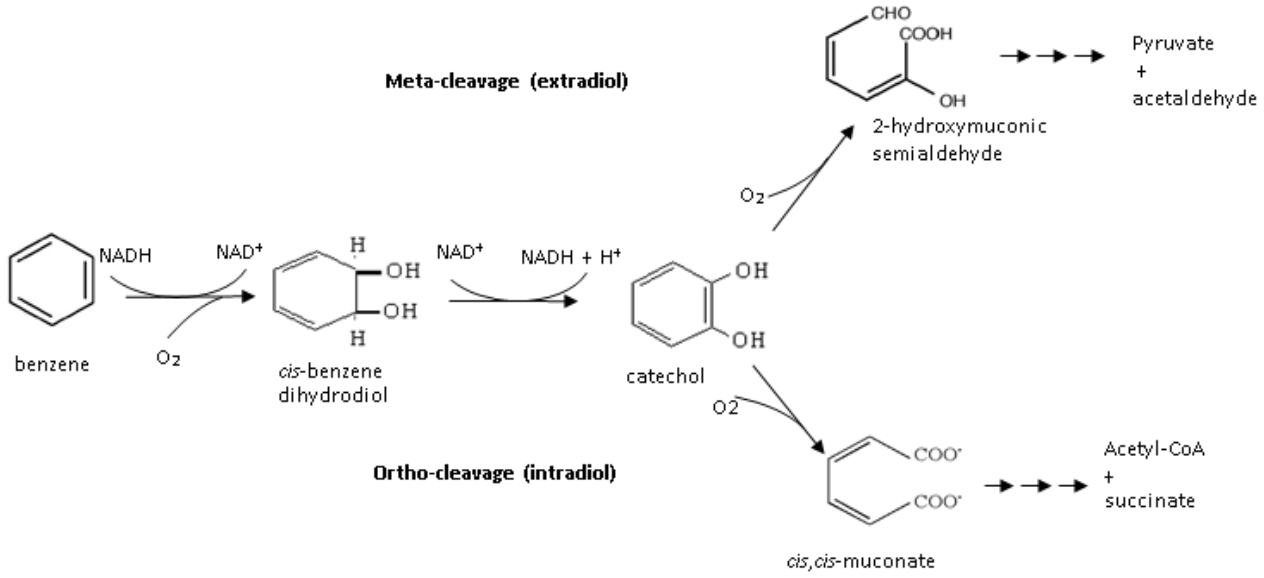


Figure 1.7. Aerobic degradation of benzene by dioxygenases. Redrawn from Weelink (2008).

1.6 Anaerobic degradation of volatile hydrocarbons

There is increasing interest in the study of anaerobic degradation of hydrocarbons because a vast number of hydrocarbons are found in anoxic environments. Anaerobes utilize hydrocarbons as a source of carbon and energy with nitrate, iron (III), or sulfate as electron acceptor, under conditions of methanogenesis, or by anoxygenic photosynthesis (Widdel et al., 2010).

1.6.1 Anaerobic degradation of ethane and propane

There are two major mechanisms involved in anaerobic alkane degradation. The first mechanism starts with the activation of the alkane at a sub-terminal position by the addition of a fumarate molecule to the alkane, yielding an alkylsuccinate derivative or an organic radical intermediate which is subsequently linked to CoA and converted to an acyl-CoA that can be further metabolized by β-oxidation (Rojo, 2009). The

second mechanism was reported by Kniemeyer et al. (2007) where the fumarate molecule is added to one of the terminal carbon atoms of propane. The second mechanism has so far been observed in no other alkane apart from propane. Anaerobic degradation of short chain alkanes under sulphate-reducing and methanogenic conditions has been reported (Kniemeyer et al., 2007, Savage et al., 2010). No degradation was observed under methanogenic condition in both studies. Nevertheless, *n*-propane and *n*-pentane were degraded under sulfate-reducing conditions (Savage et al., 2010). In the same study, ethane and butane were not degraded under sulfate-reducing conditions. Propane degradation started after 80 days of incubation and more than 90% of the propane added was metabolised after 467 days of incubation. The net sulfate reduction was 67% of the theoretical value expected (Savage et al., 2010).

Degraders of non-methane alkanes are mostly members of the *Proteobacteria*, *Betaproteobacteria* or *Deltaproteobacteria* and *Firmicutes* (Savage et al., 2010, Widdel et al., 2010). Kniemeyer et al. (2007) isolated a number of sulfur-reducing bacteria (SRB) that were were able to degrade propane. A pure culture named strain BuS5 that could degrade propane and butane anaerobically under sulfate-reducing conditions was also isolated. 16S rRNA gene analyses revealed that strain BuS5 isclosely related to SRBs, *Desulfosarcina/Desulfococcus*. *Desulfotomaculum*- like SRB also degrade propane at 60^{o}C (Kniemeyer et al., 2007).

1.6.2 Anaerobic degradation of benzene and toluene

Anaerobes degrading BT compounds under anoxic conditions have evolved mechanisms for overcoming the resonance stability of the benzene ring. There are three broad mechanism through which the benzene ring is activated in anoxic

environments: i) hydroxylation of benzene to phenol, ii) carboxylation of benzene to benzoate, and iii) methylation of benzene to toluene (Figure 1.8)(Zhang et al., 2012). The anaerobic degradation of toluene and benzene has benzoyl-CoA as a common intermediate (Figure 1.8) (Wackett and Hershberger, 2000). Most of the organisms involved in anaerobic degradation of BTEX lack the genes that encode monooxygenase production (Holmes et al., 2011, Zhang et al., 2012).

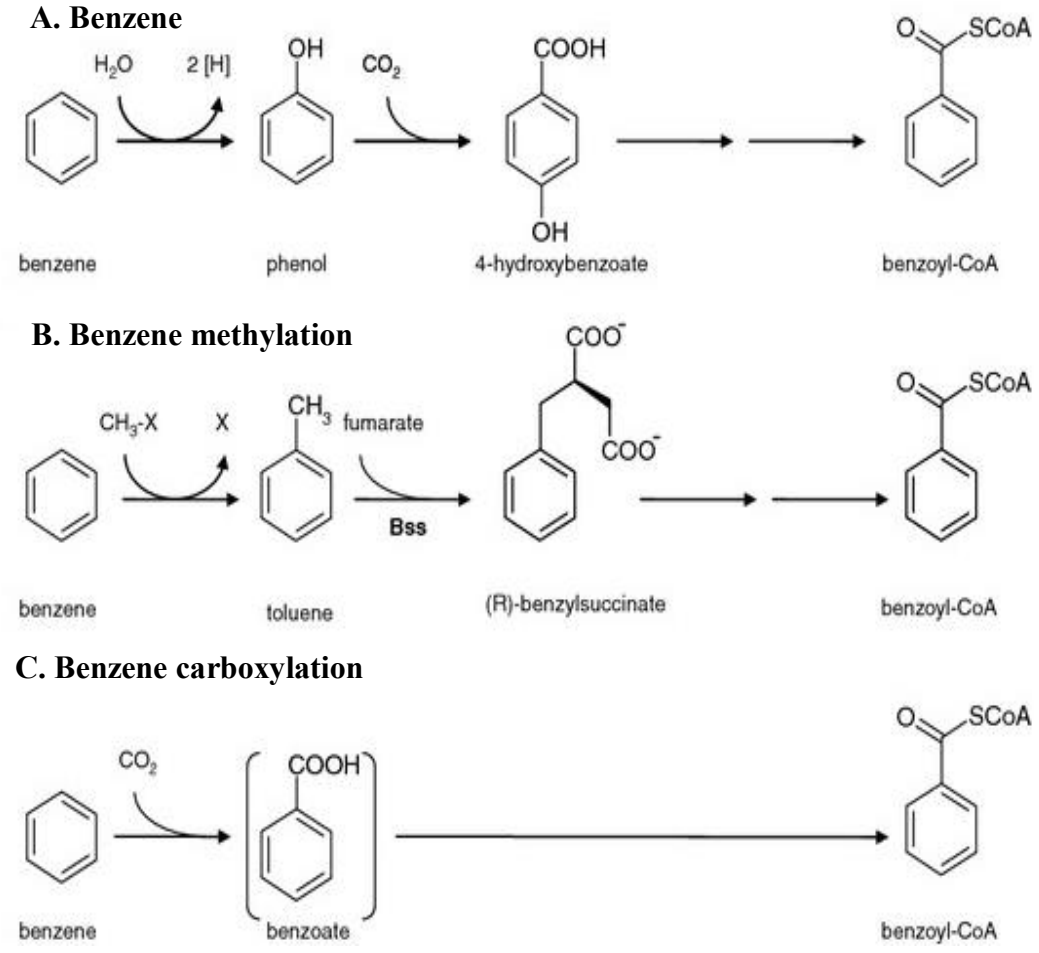


Figure 1.8. Mechanism of the initial steps of benzene degradation under anaerobic conditions. Redrawn from Weelink (2008).

i) Anaerobic degradation of benzene and toluene coupled with Fe(III) as electron acceptor.

The first axenic culture shown to degrade benzene in anoxic environments was grown on benzene with iron(III) as the electron acceptor. The hyperthermophilic archaeon *Ferroglobus placidus* degrades benzene to carbon dioxide with Fe(III) as the sole electron acceptor (Holmes et al., 2011). In the study, the putative carboxylase activates benzene. *F. placidus* converts benzene directly to benzoate via the class I benzoyl coenzyme A (benzoyl-COA) reductase pathway. Fe(III) is reduced to Fe(II) as shown in Figure 1.9. BT degradation with Fe(III) as electron acceptor is shown by a decrease in the concentration of BT compound, an increase in the number of degraders and the reduction of Fe(III) to Fe(II).

$$C_6H_6 + 30\ Fe^{3+} + 12H_20 \longrightarrow 6CO_2 + 30Fe^{2+} + 30H^+$$

Figure 1.9. Biodegradation of benzene coupled with reduction of Fe(III) to Fe(II).

Members of the family Geobacteriaceae are the main species involved in the degradation of BT with iron(III) as the electron acceptor. *Geobacter* strain Ben**,** isolated from Fe(III)-reduction zone of a petroleum-contaminated aquifer in Bemidji, degrades both benzene and toluene (Zhang et al., 2012), but the benzene - dependent growth is slow.

A wide range of Fe(III) compounds or Fe(III) chelators are used as electron acceptors. Those often used include Fe(III) oxide, Fe(III) nitrilotriacetate, Fe(III)

pyrophosphate, Fe(III) citrate, or anthraquinone-2,6-disulfonate (AQDS) (Winderl et al., 2007, Zhang et al., 2012, Holmes et al., 2011).

ii) Anaerobic degradation of benzene and toluene coupled with sulfate reduction.

Microorganisms degrading BT under sulfate reduction are phylogenetically diverse including members of the *Desulfobacteriaceae* family of *Deltaproteobacteria* (Vogt et al., 2011). The BT compounds are degraded with sulfate as the electron acceptor. In their study of the degradation of benzene under sulfate-reducing conditions, the loss of sulfate is shown to be consistent with the stoichiometric degradation of benzene (Kazumi et al., 1997). A pioneering study of the anaerobic degradation of BT with sulfate as the electron acceptor observed that toluene degradation ceased whenever sulfate was used up in the microcosm (Edwards et al., 1992).

The loss of sulfate gives rise to sulfide production. Benzene consumption and sulfide production rates showed a correlation of about 1.25 to 2.8 mole sulfide per mol benzene degraded (Taubert et al., 2012). This accumulation of sulfide during degradation might inhibit or slow further degradation of BT (Edwards et al., 1991).

iii) Anaerobic degradation of benzene and toluene coupled with nitrate reduction and denitrification.

The degradation of toluene coupled with nitrate reduction and by denitrifying bacteria has been widely reported (Evans et al., 1991b, Evans et al., 1991a, Shinoda et al., 2004). A denitrifying bacterium, *Thauera sp.* strain DNT-1, cultivated under oxygen-limiting conditions with nitrate, first degraded toluene as oxygen was consumed and

lateras nitrate was reduced (Shinoda et al., 2004). The degradation is achieved through a dioxygenase-mediated pathway and the benzylsuccinate pathway respectively. Therefore, the organism has both the toluene dioxygenase (*tod*) and benzylsuccinate synthase (*bss*) genes (Shinoda and Sakai, 2004) .

iv) Anaerobic degradation of benzene and toluene coupled with methanogenesis

Degradation of benzene or toluene under conditions of methanogensis is accompanied by the concomitant production of methane. Microbial utilization of benzene results in the accumulation of methane and the release of CO_2 (Kazumi *et al.*, 1997). Kazumi *et al.* (1997) observed that over 80% of benzene added to MI aquifer sediments is recovered as carbon dioxide and methane. They were 113% ($^{14}CO_2$) and 64% ($^{14}CH_4$) of the expected theoretical values (Kazumi et al., 1997).

1.7 The rationale for study, hypothesis, aims and specific objectives of the thesis

1.7.1 Justification for study

Estuaries are important ecosystems, because they provide safe breeding ground for many aquatic species, support rare plant communities, create jobs and revenue through commercial activities like fishing, recreation, tourism etc. (Nedwell et al., 2016, New Jersey Sea Grant Consortium, 2010). In the Niger Delta region of Nigeria, coastal communities depend on them for their livelihood. Food, timber, traditional medicines, raw materials etc. are obtained there (Zabbey et al., 2010). Fishing is the main occupation of inhabitants of these riverine communities. Important oil-producing cities like Port Harcourt, Uyo, Owerri, Calabar etc. are also located near estuaries.

Prominent amongst them are Qua Iboe river, Imo river, Cross river and Bonny estuaries.

Environmental pollution caused by anthropogenic activities such as accidental oil spillage has a huge impact on estuarine environments. VOCs, petrogenic or biogenic, emitted from estuarine environments also impact atmospheric chemistry as highlighted in section 1.3. Consequences include loss of biodiversity, agricultural lands and jobs; restiveness and migration from fishing communities in the Niger Delta (Adekola et al., 2017, Akanni et al., 2018, Albert et al., 2018, Chinedu and Chukwuemeka, 2018, Linden and Palsson, 2013, Osuagwu and Olaifa, 2018).

Biodegradation of pollutants by microbial communities plays an ecological role of removing substances from the environment. However, only a few studies have looked at the biodegradation of VOCs in estuarine environments. For instance, aerobic isoprene degradation was demonstrated in temperate soils about 20 years ago (Cleveland and Yavitt, 1997), whereas marine isoprene degradation was demonstrated recently (Acuña Alvarez et al., 2009), which illustrates that cycling of volatile hydrocarbons in estuarine environment is poorly understood. Furthermore, the identity of bacterial communities or populations involved with the biodegradation process has not been fully described.

Microbial communities with hydrocarbon-degrading capability are ubiquitous and diverse. Consequently, it is expected that bacterial and archaeal species with the ability to aerobically or anaerobically degrade volatile hydrocarbons will be found in estuarine environments. The findings will provide an insight into the fate of volatile hydrocarbons in estuarine environments. They could be of biotechnological significance with potential applications in bioremediations.

1.7.2 Aim

The cycling of volatile hydrocarbons in estuarine environment is not fully understood. The aim of this project is to investigate the aerobic and anaerobic degradation of ethane, propane, benzene, toluene and isoprene in the Colne estuary, UK, particularly to identify key bacterial and archaeal communities involved with the biodegradation of these volatile hydrocarbons using both cultivation-dependent and independent approaches.

1.7.3 Specific objectives

Chapter 3

3.1 To measure the effect of three different concentrations of benzene, toluene, isoprene, ethane and propane on the rate of aerobic degradation in single-substrate sediment slurry microcosms using GC-FID.

3.2 To enrich and identify the main bacterial populations degrading benzene, toluene, isoprene, ethane using cultivation-independent techniques.

Chapter 4

4.1 To measure the rates of anaerobic degradation of benzene, toluene, isoprene, ethane and propane in sediment slurry microcosms supplemented singly with these hydrocarbons using samples from three different locations along the Colne Estuary, using GC-FID.

4.2 To identify the bacterial and archaeal (if any) populations enriched in the presence of benzene, toluene, isoprene, ethane and propane using cultivation-

independent techniques, DGGE and Illumina MiSeq analysis of PCR-amplified 16S rRNA genes.

Chapter 5

5.1 To isolate and characterize aerobic ethane and propane-degrading bacteria.

5.2 To sequence the genomes of bacterial strains isolated and identify the ethane or propane metabolic gene cluster.

Chapter 6

6.1 To measure the diel variation in the expression of isoprene monooxygenase (*isoA*) gene *in-situ* in the Colne estuary sediments using reverse transcription quantitative PCR.

1.7.4 Hypotheses

a. Rates of aerobic degradation of volatile hydrocarbons will be faster at lower concentrations. This was tested by setting up microcosms with varying concentration of each VOC and monitoring degradation over time by GC-FID (chapter 3).

b. The type of VOC degraded will determine the composition of the VOC-degrading microbial communities. To test the hypothesis, samples from the Colne estuary were amended with individual VOCs in microcosms. OTUs (97% similarity threshold) identified in those enrichments were analysed by PCA to understand the effect of VOC-degraded on the microbial community composition (chapter 3 and 4).

c. Due to salinity and nutrient differences along the Colne estuary, the sample location will influence the composition of the VOC-degrading microbial

community. To test this hypothesis, samples were collected at three locations in the Colne estuary: the head (the Hythe), middle (Wivenhoe) and mouth (Brightlingsea). They were collected from the Hythe (salinity ≤ 5) and Wivenhoe (salinity, 3 - 31) for the aerobic (Chapter 3); and an additional site, Brightlingsea (salinity > 30), for the anaerobic experiment (Chapter 4). An individual VOC was added in microcosms. OTUs identified in enrichments were analysed by PCA to understand the effect of sampling location on microbial community composition.

d. Anaerobic degradation of hydrocarbons will be slower and more stochastic than aerobic degradation. Anaerobic degradation of VOCs is poorly understood. The stochastic nature is due to the difficulty of hydrocarbon activation in anoxic environments, which may take days to years. That is why VOCs like benzene which are degraded readily in aerobic environments tend to persist in anoxic ones (Vogt et al., 2011). This hypothesis was tested in anaerobic microcosms amended with each VOC; and a side-by-side aerobic and anaerobic experiment to understand degradation of isoprene.

e. Ethane and propane-degrading bacterial isolates will possess a soluble diiron centre monooxygenase (SDIMO) enzyme for substrate activation, since this is the most typical mechanism for alkane activation. Bacterial isolates obtained from first enrichments (Chapter 3) were genome sequenced and annotated in Rapid Annotation using Subsystem Technology (RAST) (Chapter 5). The genomes were analysed for ethane and propane degrading enzymes.

f. Isoprene monooxygenase genes will be more highly expressed during the day, when more isoprene is being produced by phototrophic microalgae,

compared with the night, in an estuarine intertidal sediment. This was tested in chapter 6 with sediment samples collected in Wivenhoe.

Chapter 2. Materials and Methods

2.1 Sample site description

The Colne estuary is in Essex, South East England, United Kingdom. It is a nutrient-rich estuary which covers an area of 14.5 km^2 and it is surrounded by 900 ha of saltmarsh (Nedwell et al., 2016). The estuary is about 16 km long from East Mill Weir to the mouth of the estuary at Brightlingsea where it enters the North Sea with a corresponding longitudinal gradient of salinity and solute concentrations (Nedwell et al., 2016). It is mesotidal with tidal amplitude > 4 m (Nedwell et al., 2016).

Three sampling sites were chosen along the Colne estuary across three salinity zones (Figure 2.1). The first site, located at the head of the estuary has low salt concentration, the Hythe, 51º52′47.3″N; 0º55′43.1″E, Salinity = 0.5 - 5, (Acuña Alvarez et al., 2009, Exton et al., 2012, Nedwell et al., 2016); the second site is mid–estuary located at Wivenhoe , 51º52′8.7″N; 0º56′32.8″E, Salinity = 3 - 31, (Acuña Alvarez et al., 2009, Exton et al., 2012) and the third site is at the mouth of the estuary at Brightlingsea 51º48′19.67″N, 1º00′06.53″E, Salinity > 30 (Nedwell et al., 2016).

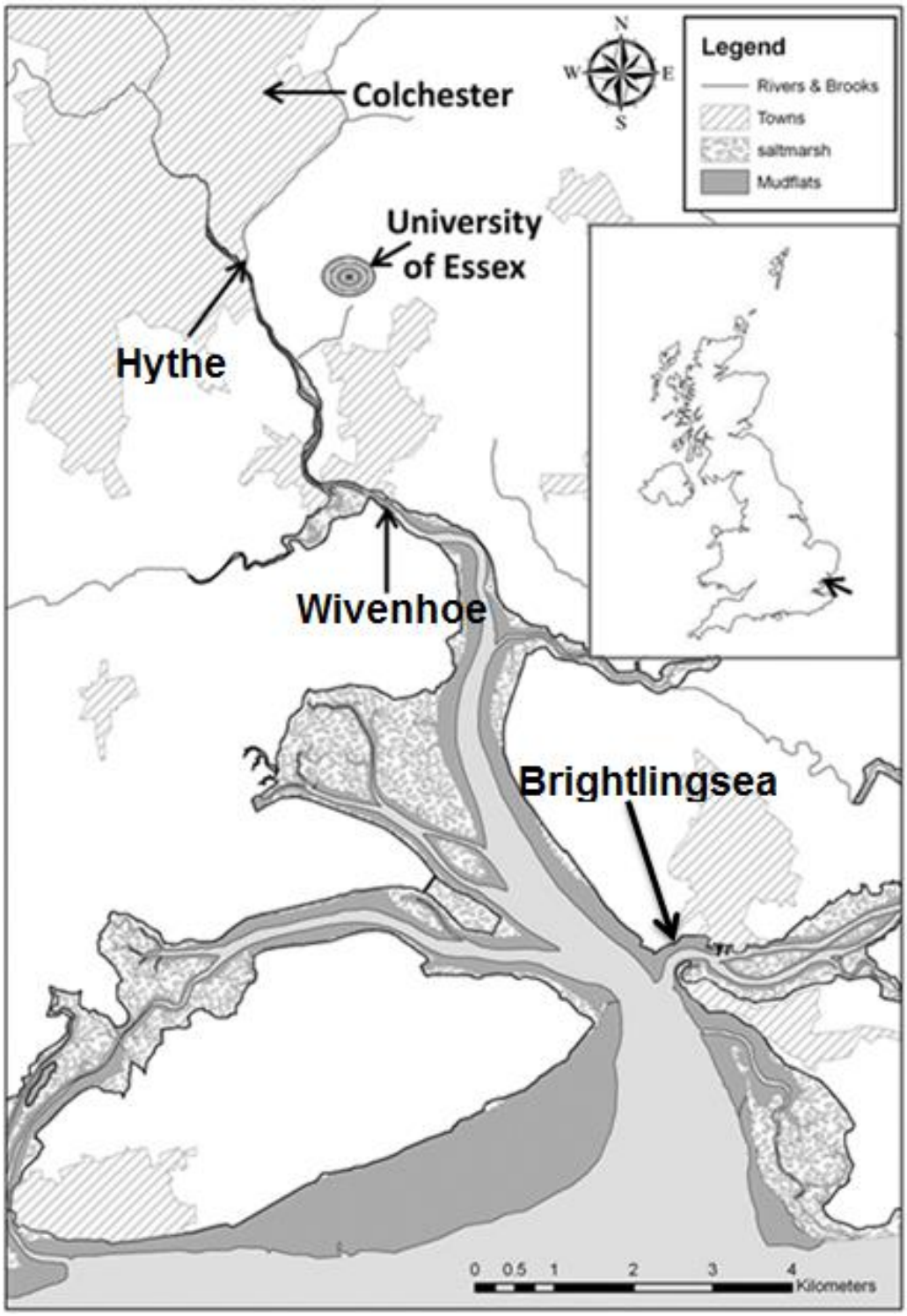


Figure 2.1. Map showing the Colne Estuary, Essex, United Kingdom. Samples used for microcosms were collected at the Hythe (51º52′47.3″N; 0º55′43.1″E), Wivenhoe (51º52′8.7″N; 0º56′32.8″E) and Brightlingsea (51º48′19.67″N; 1º00′06.53″E). Map provided by Dr Steven McMellor, University of Essex, UK.

2.2 Microcosm design for biodegradation studies

The biodegradation of benzene, toluene, isoprene, ethane and propane at the Colne estuary was studied in single substrate microcosms. These microcosms were made from sediment and/or estuarine water samples collected at the Colne estuary and established in 125-ml serum bottles, aluminium crimp seals with polytetraflouroethene (PTFE)-lined septa. Microcosms were designed to answer different research questions throughout the project. Therefore, their design and set up varied slightly with each experiment. In general, three sets of microcosms were set up for all experiments: test microcosms (live samples) to study biodegradation (aerobic or anaerobic); killed controls or sterile controls to check for abiotic loss of hydrocarbon; and a No-VOC control to ensure the difference in microbial community was due to hydrocarbon addition. All microcosms or enrichments were in triplicate. Microcosm design for each experiment is described in the respective chapters (Chapter 3-5) of this thesis.

2.3 Gas Chromatography Flame Ionization Detector (GC-FID) analysis

Benzene, toluene, isoprene, ethane and propane concentration in microcosms were monitored using a UNICAM 610 Series Gas chromatograph fitted with 1.5 m packed column (10% apiezon L on Carbo Wax 60 - 80 mesh) and a flame ionization detector (FID). The injector and detector temperatures were set at 160^{o}C while the column temperature was 120^{o}C. Nitrogen was used as the carrier gas at a flow rate of 40 ml min^{-1}. The detector was set at medium sensitivity for the analyses of benzene, toluene and isoprene or at low sensitivity for the analyses of ethane and propane. An aliquot (100 µl) of the headspace gas was collected using a gas tight SGE syringe

with a steel side-port needle and injected manually into the sample port of the GC-FID.

The GC-FID was calibrated by analysing standards. Benzene standards were made by diluting the stock solution (1.6 g l^{-1}) to concentrations ranging from 5 – 40 mg l^{-1}. Toluene standards were diluted from a stock solution (0.5 g l^{-1}) to concentrations ranging from 10 – 50 mg l^{-1}. The concentration of ethane and propane standards ranged from 1 – 20 % v/v. They were transferred into serum bottles from a latex bladder as described in section 2.4. Isoprene standards were diluted from a stock solution (section 2.4) to concentrations ranging 1.0×10^5 – 8.6×10^6 ppbv. A linear relationship between response (average peak area) and concentration was plotted for each VOC. Representative calibration curves for benzene, toluene, isoprene, ethane and propane are shown in Appendix 1.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated using the signal-to-noise method as described by Shrivastava and Gupta (2011). The peak-to-peak noise around the analyte retention time was 10243 mV and obtained from the chromatogram printout of the GC-FID. LOD and LOQ were set to 3 times and 10 times the noise value respectively.

2.4 Chemicals and stock solutions

Pure Grade Propane (N2.0) 99% and compensated pass Grade Ethane (N2.0) 99% were supplied by BOC, UK. AnalaR Benzene (99.7%) and AnalaR Toluene (99.5%) were supplied by BDH Laboratories, England. Isoprene (99%), containing <1000 ppm *p-tert*-butylcatechol as inhibitor was supplied by Sigma-Aldrich, USA.

Benzene stock solution (1.6 g l^{-1}) was prepared by adding 109.5 µl of benzene (99.7%) to 60 ml sterile distilled water in a 125-ml serum bottle. The toluene stock solution (0.5 g l^{-1}) was prepared by adding 34.59 µl of toluene (99.5%) to 60 ml sterile distilled water in a 125-ml serum bottle. The isoprene stock solution was prepared as described by Murphy (2017) and Acuña Alvarez et al. (2009) by adding 10 ml of isoprene (Sigma >99%, 464953) to 125-ml serum bottle. The isoprene stock was warmed at 30^{o}C for 30 min, then a gastight syringe was used to transfer isoprene from the headspace to 125-ml serum bottles. Ethane or propane was transferred from a cylinder into a latex bladder before transferring into microcosms as described in the respective chapters (Chapter 3 - 5).

2.5 Agarose gel electrophoresis

Agarose (1% or 1.5% w/v) was dissolved in 1xTAE buffer and melted in a microwave. Molten gels were cast in UV transparent trays. DNA samples were run using 1xTAE at a voltage ranging from 90 – 120 V for 30 minutes or longer. Gels were stained in 0.5 µg/ml ethidium bromide for a minimum of 30 minutes; visualized on a UV 60 transilluminator (Alpha Innotech MultiImage™ Light Cabinet) and photographed using the embedded AlphaImager EP software. Sizes of DNA fragments were estimated using GeneRuler™ DNA Ladder Mix (Thermo Scientific™).

2.6 Preparation of artificial seawater minimal media, one-third ONR7a

Artificial seawater minimal medium (ONR7a) was prepared as described by Dyksterhouse et al. (1995) with modification of the salt concentration, the NaCl and

$MgCl_2.6H_2O$ concentration was reduced by one-third (1/3). The One-third ONR7a media contained per litre of deionized water: 7.60 g of NaCl; 3.73 g of $MgCl_2.6H_2O$; 1.33 g of Na_2SO_4; 0.49 g of $CaCl_2.2H_2O$; 0.43 g of TAPSO (3-[N-tris (hydroxymethyl) methylamino]-2-hydroxypropanesulfonic acid) buffer; 0.24 g of KCl; 0.09 g of NH_4Cl; 29.67 mg of $Na_2HPO_4.7H_2O$; 27.67 mg of NaBr; 10.33 mg of $NaHCO_3$; 9.00 mg of H_3BO_3; 8.00 mg of $SrCl_2$. $6H_2O$; 0.87 mg of NaF; and 0.67 mg of $FeCl_2$. $4H_2O$.

To prevent precipitation of ONR7a during autoclaving, the media was prepared in three separate solutions referred to as solution 1, solution 2 and solution 3. These solutions were autoclaved separately at 121ºC for 15 min then mixed together when cooled to 50°C.They were combined by mixing 100 ml Solution 1, 100 ml Solution 2, 1 ml Solution 3 with 800 ml sterilized distilled water under aseptic conditions.

The composition of each solution was as follows: Solution 1 (1L): 75.97 g of NaCl; 13.27 g of Na_2SO_4; 2.40 g of KCl; 276.67 mg of NaBr; 103.33 mg of NaHCO3; 90.00 mg of H_3BO_3; 8.67 mg of NaF; 0.90 g of NH_4Cl; 296.67 mg of Na_2HPO_4; 4.33 g of TAPSO (pH adjusted to 7.6 with NaOH). Solution 2 (1L): 37.27 g of $MgCl_2$; 4.87 g of $CaCl_2$; 0.08 g of $SrCl_2$ (divalent cation salts). Solution 3 (1L): 0.67 g of $FeCl_2$. $4H_2O$ (OR $FeSO_4$. $7H_2O$).

2.7 Microbial community analysis

To identify key bacterial and archaeal communities involved with the biodegradation of benzene, toluene, isoprene, ethane and propane, both cultivation-dependent (Chapter 5) and independent approaches (DGGE and Illumina MiSeq) were employed. The extraction of the total community nucleic acid was done by the hexadecyltrimethylammonium bromide (CTAB) method (Griffiths et al., 2000). To

extract DNA from sediment slurry microcosms, 1.5 ml aliquots were transferred into 2-ml microfuge tubes. The samples were centrifuged at 11300 x *g* for 5 min to collect the sediment pellets. The sediment pellet (0.5 g) was transferred into sterile beadbeating tubes containing 0.5 g 0.1 mm zirconia/silica beads. To which 0.6 ml CTAB buffer and 0.5 ml Phenol:Cholroform:Isoamyl alcohol (25:24:1) pH 8 were added in a fume cupboard. Bead beating was done for 2 x 30 s runs at 2000 rpm using Precellys® Evolution (Bertin Technologies) bead beater and the tubes were cooled on ice. The samples were centrifuged at 11300 x *g* for 5 min and the upper aqueous layer was transferred to sterile 1.5-ml microfuge tubes under the fume hood. 30% PEG 6000/1.6 M NaCl (1ml) was added to the tubes, inverted a few times and left to stand on the bench overnight. The next morning, samples were centrifuged (11300 x *g* for 5 mins) to get DNA pellet. Ice cold 70% v/v ethanol was used to wash the pellets and air dried for 20 minutes. The DNA pellet extracted were suspended in 50 µl sterile pure water.

In Chapter 3, a DGGE of the PCR-amplified v3 region of bacterial 16S rRNA was carried out to understand the bacterial community change due to hydrocarbon addition; DGGE bands of interest were excised and re-amplified by PCR then Sanger sequenced to identify the main bacterial populations enriched. Ethane and propane degrading bacterial populations were targeted for isolation from sediment slurries (first enrichments, Chapter 3) and genome sequenced to identify the gene cluster encoding enzymes for ethane and propane degradation (Chapter 5). Only ethane and propane-degrading bacterial populations were targeted for isolation because there is little data available on such isolates from estuarine environments. Illumina MiSeq sequencing of the v3-v4 region of bacterial and archaeal *16S rRNA* gene was performed on selected samples as described in section 3.2.4 (Chapter 3)

and section 4.2.6 (Chapter 4) respectively. Only Bacterial *16S rRNA* genes were sequenced in Chapter 3, while both Archaeal and Bacterial *16S rRNA* genes were sequenced in Chapter 4. Detailed methodology is shown in individual chapters (Chapter 3 – 5). Dr Boyd McKew processed the sequences obtained from the MiSeq run from raw sequences to the OTU tables, following the guidelines laid out by Dumbrell et al. (2016) as described in section 3.2.7 (Chapter 3). I performed the subsequent sequence analyses using Statistical Analysis of Metagenomic Profiles, (STAMP) (Parks et al., 2014).

2.8 Statistical analysis

To compare the difference in hydrocarbon degradation between two groups (control microcosms vs test microcosms) a Welch's two-sample t-test was carried out in RStudio (version 0.98.994 - © 2009 – 2013, RStudio Inc.). Test for normality was done in RStudio by Shapiro-Wilk Normality Test (Royston, 1982a, Royston, 1982b, Royston, 1995). The Welch's t-test was also used to determine the difference in abundance (mean proportions) of bacterial genera or OTUs (97% similarity threshold) between controls (No-VOC) and test microcosms carried out in STAMP (Parks et al., 2014). Principal component analysis (PCA) was used to determine the overall bacterial OTUs (97% similarity threshold) compositional differences between samples using STAMP (Parks et al., 2014).

Chapter 3. Aerobic Degradation of Benzene, Toluene, Isoprene, Ethane and Propane

3.1 Introduction

VOCs are commonly found in estuarine environments. Anthropogenic sources include accidental discharge of gasoline (Fu et al., 2008) and release of industrial effluents into water bodies (Yamamoto, 2014, Yamamoto et al., 1997, Dawes and Waldock, 1994). Some VOCs are emitted from marine environments due to biogenic production by algae and methanogens (section 1.2).

They impact the environment in diverse ways (section 1.3). Briefly, benzene and toluene are toxic and carcinogenic (World Health Organization, 2010, World Health Organization, 2000); isoprene, the most abundant BVOC in the atmosphere, has negative impact on atmospheric chemistry (section 1.3.1); ethane and propane contribute to the formation of climate-altering ozone and secondary organic aerosols (Pozzer et al., 2010, Etiope and Ciccioli, 2009, Saito et al., 2000, Musat, 2015, Helmig et al., 2016).

These VOCs provide carbon and energy for some aerobic bacterial communities (Shennan, 2006). During aerobic degradation of VOCs, they use oxygen as a terminal electron acceptor and/or for substrate activation. These reactions are catalysed by oxygenases such as ethane, propane, and isoprene monooxygenase (Ashraf et al., 1994, Coleman et al., 2006, Crombie et al., 2015, Kotani et al., 2006, Kotani et al., 2003, Leahy et al., 2003a, Sharp et al., 2007, Van Hylckama Vlieg et al., 2000, Redmond et al., 2010). The activation of toluene and the benzene ring is achieved by the actions of mono- or di-oxygenases (Wackett and Hershberger, 2000, Cao et al., 2009).

A few bacteria populations are associated with VOC degradation. Isoprene degrading bacteria include *Rhodococcus*, *Norcardia*, *Arthrobacter*, *Alcaligenes*, *Pseudomonas*, *Klebsiella*, *Mycobacterium* and *Stappia* (Cleveland and Yavitt, 1998, van Ginkel et al., 1987, Srivastva et al., 2015, Johnston et al., 2017, Khawand et al., 2016, McGenity et al., 2018, Acuña Alvarez et al., 2009). Those associated with benzene and toluene degradation are *Alcaligenes, Acinetobacter, Pseudomonas, Rhodococcus*, *Norcardia* (Cao et al., 2009). Gram-positive *Corynebacterium*, *Mycobacterium*, *Norcadia* and *Rhodococcus* are the main populations responsible for biodegradation of short-chain alkanes (Shennan, 2006).

Biodegradation of VOCs in estuarine environments has received little attention despite their abundance. Consequently, their cycling in the environment is poorly understood and the identity of bacterial communities or populations involved with the process poorly understood.

Aim

To investigate the aerobic degradation of ethane, propane, benzene, toluene and isoprene in the Colne estuary, UK, particularly to identify key bacterial communities involved with the biodegradation of these volatile hydrocarbons using both cultivation-dependent and independent approaches.

Hypotheses

a. Rates of aerobic degradation of volatile hydrocarbons will be faster at lower concentrations. This was tested by setting up microcosms with varying concentration of each VOC and monitoring degradation over time by GC-FID.

b. The type of VOC degraded will determine the composition of the VOC-degrading bacterial communities. To test the hypothesis, samples from the Colne estuary were amended with individual VOCs in microcosms. OTUs (97% similarity threshold) identified in those enrichments were analysed by PCA to understand the effect of VOC-degraded on the bacterial community composition.

c. Due to salinity and nutrient differences along the Colne estuary, the sample location will influence the composition of the VOC-degrading bacterial community. To test this hypothesis, samples were collected at two locations in the Colne estuary: the head (the Hythe) and middle (Wivenhoe). An individual VOC was added in microcosms. The OTUs identified in enrichments were analysed by PCA to understand the effect of sampling location on the bacterial community composition.

Specific Objectives

3.1 To measure the effect of three different concentrations of benzene, toluene, isoprene, ethane and propane on the rate of aerobic degradation in single-substrate sediment slurry microcosms using GC-FID.

3.2 To enrich and identify the main bacterial populations degrading benzene, toluene, isoprene, ethane using cultivation-independent techniques.

3.2 Materials and Methods

3.2.1 Sample collection and processing

To understand the aerobic degradation of volatile hydrocarbons (substrate) and the effect of substrate concentration on biodegradation in estuarine environments, experiments were set up in static microcosms with samples collected from the Colne estuary, UK. Samples for the main aerobic degradation experiment were collected on the 11th July 2014 from the head (the Hythe) and middle (Wivenhoe) of the Colne estuary, UK. Another experiment referred to as "Effect of VOC Concentration on Degradation" was setup with samples collected on the 22nd January 2016 from the head of the Colne estuary (the Hythe) only (Table 3.1). Sediment and water samples were collected at low tide on the same day. Sample collection, handling and processing for both experiments was done in a similar manner as described below.

Estuarine water samples were collected at low tide, from the middle of the channel, into pre-cleaned sterile 1-litre Duran bottles. These bottles were filled directly to the brim and allowed to overflow, then covered with sterile PTFE-lined rubber stoppers. Sediment samples were collected with plastic cores, 9.8 cm high and 6.5 cm in diameter. Sampling locations were divided longitudinally into three 1 m^2 plots approximately 10 meters apart. Three cores were collected within the first plot (1 m^2) referred to as sample a; three cores were collected within the second plot (1 m^2) referred to as sample b; and three cores were collected within the third plot (1 m^2) referred to as sample c. Samples a, b and c were ~10 m apart at the same tidal height and they were used as biological replicates. Samples were stored in iceboxes (in the dark) at 4^{o}C during transportation to the laboratory. Sediment cores were

transported intact. They were used immediately or stored at 4^{o}C for use the next day.

The top 1.5 cm of the three cores collected within the first 1 m^2 plot (sample a) were pooled into a large sterile pre-weighed 500-ml beaker and covered with aluminium foil. This was repeated for the second (sample b) and third plots (sample c) respectively. The sediment samples from Wivenhoe were labelled, Wa (sample a), Wb (sample b), Wc (sample c); and samples from the Hythe were labelled Ha (sample a), Hb (sample b) and Hc (sample c). Sediment-estuarine water slurries were prepared by making a 1:1 mixture of sediment and water samples collected from the same location (for example, Wivenhoe sediment sample: Wivenhoe water sample). These slurries were stirred continuous using a sterile spatula, magnetic flea and stirrer to obtain a homogenous mixture (master slurry). The six master slurries were: Wa, Wb, Wc, Ha, Hb and Hc. Using a sterile wide-bored pipette, an aliquot was transferred from master slurry into sterile 125-ml serum bottles.

Table 3.1 A summary of aerobic degradation experiments carried out using samples collected along the Colne estuary at the Hythe and Wivenhoe.

Sampling Location	Experiment	Carbon Source	Remarks
Hythe	Main Aerobic Degradation (11th July 2014).	Benzene, toluene, isoprene, ethane, propane	One carbon source per microcosm. Bacterial community analysis carried out
	Effect of VOC Conc. on Degradation (22nd Jan 2016).	Benzene, toluene, isoprene, ethane, propane	One carbon source per microcosm. No bacterial community analysis carried out.
Wivenhoe	Main Aerobic Degradation (11th July 2014).	Benzene, toluene, isoprene, ethane, propane	One carbon source per microcosm. Bacterial community analysis carried out. Not sampled for “Effect of Concentration on VOC Degradation”

VOC = Volatile Organic Compounds

3.2.2 Experiment setup

To identify the main bacterial taxa degrading benzene, toluene, isoprene, ethane or propane in estuarine environments, experiments were set up with samples from the Colne estuary as outlined in Figure 3.1.

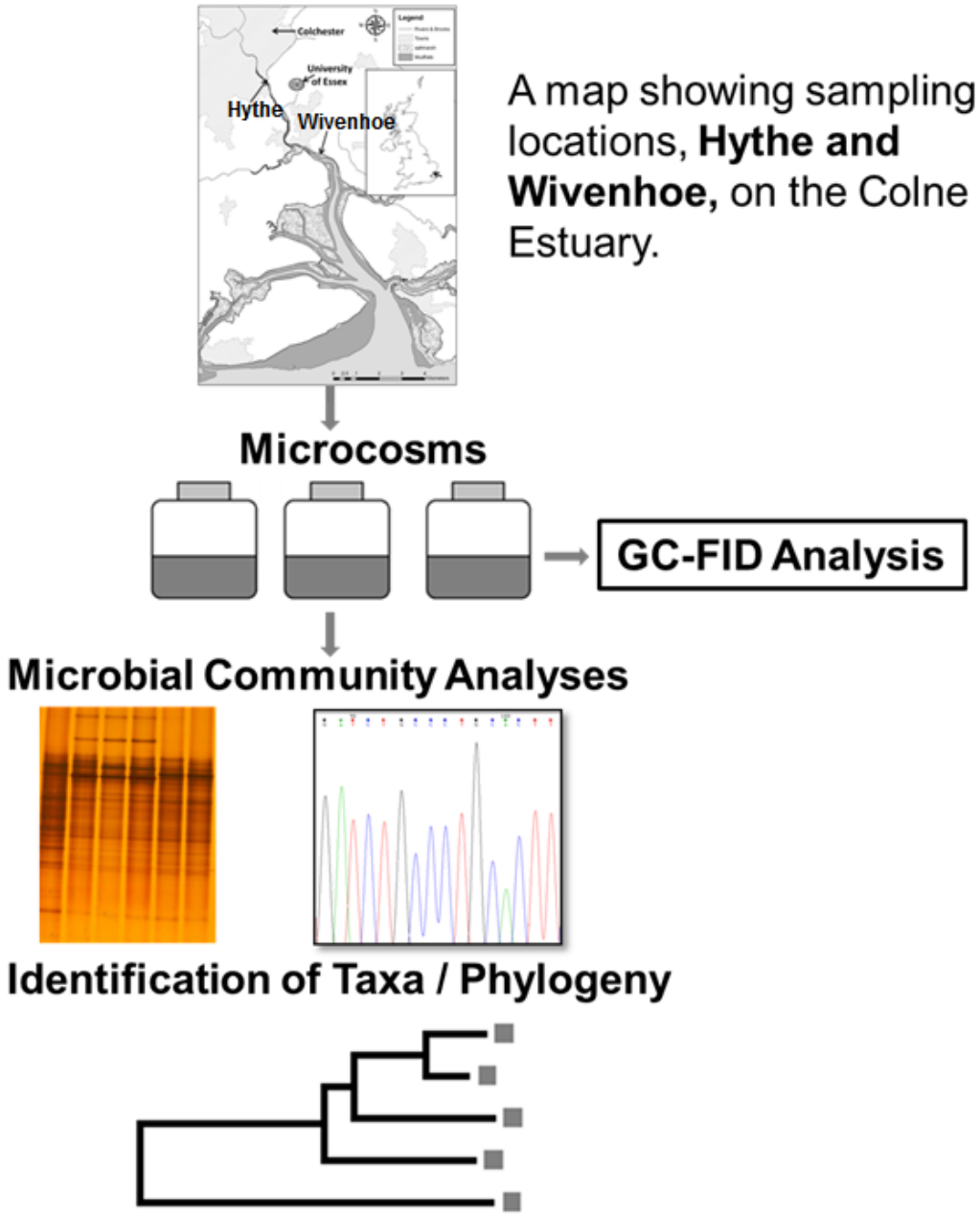


Figure 3.1. A schematic representation of the aerobic degradation experiment.

3.2.2.1 Microcosms design

Microcosms were established in sterile 125-ml serum bottles crimp-sealed with aluminium PTFE-lined septa. Test, killed (or sterile) control and No-VOC control microcosms were designed. All microcosms and controls were set up in triplicate. The same bottle size and volume of samples were used for test microcosms and controls. Samples for killed control were collected a week earlier and they were ready a day before the experiment. They were autoclaved as described by Acuña Alvarez et al. (2009). PTFE-sealed 125-ml serum bottles containing 10 ml of sediment slurry were subjected to a series (three lots) of autoclaving at 121^{o}C for 20 min. After each autoclaving lot, they were incubated at room temperature on the bench for 24 h before the next autoclaving lot. No-VOC controls were designed to ensure the difference in bacterial community was due to hydrocarbon addition. No carbon source was added to these microcosms. Test microcosms and No-VOC controls were sacrificially sampled for bacterial community analyses at whatever time (day) biodegradation was determined in sediment slurry microcosms (First enrichments) and just before the next enrichment in the series was set up.

3.2.2.2 Investigation of benzene-, toluene-, isoprene-, ethane- or propane-biodegradation in sediment slurry microcosms

Samples for this experiment were collected on the 11th July 2014, processed immediately as described in section 3.2.1 and the experiment was set up. Test microcosms contained sediment slurries with a single hydrocarbon substrate (benzene, toluene, isoprene, ethane or propane) to a final volume of 10 ml in 125-ml serum bottles with a large headspace. They were referred to as the first enrichments

(E1), or sediment slurry microcosms; Killed control microcosms and No-VOC controls were also set up as described in section 3.2.2.1. Killed controls contained the same concentration of VOC as the test microcosms.

Single VOCs were added to microcosms as follow: 250 µl of benzene stock (conc. = 1.6 g l^{-1}) was added to give a final concentration of 0.5 mM (39.1 mg l^{-1}) in microcosms.; 1.02 ml of stock toluene (conc. = 0.5 g l^{-1}) was added to give a final concentration of 0.5 mM (46.1 mg l^{-1}) in microcosms. Final concentrations of benzene and toluene in microcosms were as in Shinoda et al. (2004). To maintain a final volume of 10 ml in microcosms, benzene-amended microcosms contained 0.25 ml benzene + 9.75 ml sediment slurries; and toluene-amended microcosms contained 1.02 ml toluene + 8.98 ml sediment slurries. The volumes of the corresponding controls were adjusted accordingly when killed controls (with first enrichments) and sterile controls (with second and third enrichments) were prepared. Isoprene was added at a similar concentration used by Murphy (2017). The isoprene stock was warmed to 30^{o}C for 30 minutes then 1 cm^3 was transferred from the stock headspace into microcosms to give a final concentration of 7.2 x 10^6 ppbv in microcosms. The concentrations of ethane and propane added were like those previously described by Crombie and Murrell (2014) and Arp (1999). Ethane (12.5 cm^3) or propane (12.5 cm^3) was transferred from a latex bladder containing ethane or propane into microcosms to give a final concentration of 10% v/v. Gas filters (Minisart-Plus filters pore size 0.2 µm, Sigma) were used during the final transfer into microcosms to prevent contamination. Microcosms were left on the bench for about 1 hour for the VOCs to equilibrate in microcosms, then the initial GC-FID measurements were taken, and referred to as day-0. All microcosms and controls were set up in triplicate. They were incubated statically in the dark at 12^{o}C. Benzene,

toluene or isoprene concentration were measured every two days while ethane or propane concentration were measured weekly. Benzene, toluene, isoprene, ethane or propane concentrations in the headspace of microcosms were measured as described in section 2.3 (Chapter 2). Calibration standards for the five volatile hydrocarbons (ethane, propane, benzene, toluene, and isoprene) were prepared in sterile distilled water with equal liquid/headspace ratio and were held at the same temperature as the test samples. The GC-FID was calibrated with standards at each measurement. Headspace concentration of each volatile hydrocarbon was measured alongside the triplicate killed control at each time point with GC-FID. Each sample measurement was done in duplicate (technical replicates).

3.2.2.3 Sequential enrichment of aerobic benzene, toluene, isoprene, ethane or propane degrading bacterial communities from the Colne estuary in single-substrate microcosms

To enrich for bacterial communities degrading benzene, toluene, isoprene, ethane or propane from the Colne estuary, subsequent enrichments were established from the first enrichments (E1). They were referred to as second (E2) and third enrichments (E3), respectively. These microcosms were set up when aerobic degradation was confirmed in the first enrichments, i.e. 80% degraded for benzene, toluene and isoprene but less for ethane and propane (when headspace concentration was significantly lower than start concentration). A second enrichment was set up by transferring 1 ml from the first enrichment into 9 ml of one-third strength ONR7a minimal medium. The artificial seawater minimal medium (ONR7a) was prepared as described by Dyksterhouse et al. (1995) with modification of the salt concentration.

The NaCl and $MgCl_2.6H_2O$ concentration were reduced by one-third (1/3) as described in section 2.6 (Chapter 2). Transfer from first enrichments with Benzene, toluene or isoprene was done after 10 days of incubation when more than 80% biodegradation was observed. Transfer from first enrichments with ethane or propane was done after 37 days of incubation when aerobic degradation was observed. Sterile controls (autoclaved one-third ONR7a minimal medium) were established to check abiotic degradation (loss) during the second and third enrichments. They were prepared following a similar procedure as Acuña Alvarez et al. (2009) by autoclaving 10 ml of one-third ONR7a minimal medium at 121^{o}C for 20 min before addition of hydrocarbon substrates. Benzene, toluene, isoprene, ethane or propane were added singly to each new enrichment at similar concentrations described above, section 3.2.2.2. Third enrichment (E3), were setup by transferring an aliquot (1 ml) of E2 into 9 ml of one-third ONR7a minimal medium when evidence of biodegradation was obtained at second enrichment. Second enrichments and third enrichments with benzene, toluene or isoprene and their corresponding sterile controls were incubated at 12^{o}C for 6 days and 5 days respectively while ethane or propane second and third enrichments were incubated at 12^{o}C for 37days and 21 days respectively. Samples were taken at the end of each enrichment for DNA extraction and bacterial community analyses.

3.2.2.4 Measuring the effect of benzene, toluene, isoprene, ethane and propane concentration on biodegradation

To understand how benzene, toluene, isoprene, ethane and propane degradation proceeds at lower concentrations, three concentrations were investigated:

concentration 1 (C) was the highest concentration of VOC, equivalent to those used during the main aerobic degradation experiment described above, section 3.2.2.2; while concentration 2 (C/10) and 3 (C/100) were at 10 and 100-fold lower concentrations, respectively. Each VOC was added as a single carbon source to sediment slurry microcosms. Samples for this experiment were collected on 22nd Jan 2016 from the Colne estuary at the Hythe only and they were processed as described in section 3.2.1. Sediment slurries were made from a 1:1 mixture of sediment to water collected from the Hythe. This site was chosen because rapid degradation was observed with samples from that location during the main aerobic degradation experiment. Microcosms were established in 125-ml serum bottles containing a final volume of 10 ml after carbon source (benzene, toluene, isoprene, ethane or propane) addition.

Single VOCs were added to microcosms as follows: final benzene concentrations of 0.5 mM (C), 0.05 mM (C/10) and 0.005 mM (C/100) were achieved by adding 250 µl, 25 µl and 2.5 µl of benzene stock (conc = 1.6 g/l) to a final volume of 10 ml; final toluene concentration of 0.5 mM (C), 0.05 mM (C/10) and 0.005 mM (C/100) were achieved by adding 1020 µl, 102 µl, 10.2 µl of stock toluene (conc = 0.5 g/l) respectively to a final volume of 10 ml; final isoprene concentrations of 7.2 x 10^6 ppbv (C), 7.2 x 10^5ppbv (C/10) and 7.2 x 10^4 ppbv (C/100) were achieved by transferring 1 cm^3 , 0.1 cm^3, 0.01 cm^3 from the stock headspace into microcosms; final ethane or propane concentration of 10% (v/v) (C), 1% (v/v) (C/10) and 0.1% (v/v) (C/100) were achieved in microcosms by transferring 12.5 cm^3 , 1.25 cm^3 and 0.125 cm^3 respectively from a latex bladder containing ethane or propane into microcosms. All stock solutions were prepared as described in Section 2.4 (Chapter 2) of this thesis. All microcosms and controls were setup in triplicate, and they were

incubated at 12ºC. Benzene and toluene concentrations were measured daily, isoprene was measured every two days, while ethane and propane were measured weekly using GC-FID as described in Section 2.3.

3.2.3 Bacterial community analysis

To identify the main aerobic benzene, toluene, isoprene, ethane or propane-degrading bacterial populations in the Colne estuary, culture-dependent (described in Chapter 5) and culture-independent approaches, such as DGGE and Illumina MiSeq were used. DNA was extracted: i) from sediment slurries at time 0 to explore the bacterial community present at the start of the experiment; ii) from test microcosms at the end of each enrichment to investigate bacterial community change/composition; and iii) from the No-VOC controls at the end of first enrichments to test whether the change in bacterial community was due to addition of VOCs. The hexadecyltrimethylammonium bromide (CTAB) method of DNA extraction was used (Griffiths et al., 2000) as described in section 2.7, Chapter 2.

Denaturing gradient gel electrophoresis (DGGE) was carried out to study changes in bacterial community profiles of Colne estuary samples amended with benzene, toluene, isoprene, ethane or propane. The variable V3 region of bacterial 16S rRNA gene corresponding to positions 341 - 534 in *E.coli* was amplified using F341GC 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3' and R534 5'-ATTACCGCG GCTGCTGG-3’ primers (Muyzer et al., 1993). The PCR amplification was performed using a Perkin Elmer Gene Amp PCR system 700. Each 25 µl reaction mixture was made up of: 0.5 µl of DNA template, 12.5 µl of REDTaq ready mix (Sigma), 1 µl each of forward (F341GC) and reverse

primers (R534), and 10 µl of Milli-Q water. The thermal cycling condition was as follows: Incubation: 94ºC, 5 min; (Denaturing: 94ºC, 1 min; Annealing: 55ºC, 1 min; Extension: 72ºC, 3 min) × 30 cycles. The amplification products were viewed by electrophoresis in 1% (w/v) 1 × TAE (40 mM Tris-acetate, 1mM di-sodium-EDTA, pH 8.0) agarose gel and by ethidium bromide (0.5 µg ml^{-1}) staining. The PCR amplified *16S rRNA* gene was separated by denaturing gradient gel electrophoresis (DGGE). The DGGE was performed as described by McKew et al. (2007). The PCR products with the GC clamp were separated on a gel containing 8% (w/v) polyacrylamide (acrylamide:bisacrylamide, 37:1) and a denaturing gradient from 40% to 60% (100% denaturant in 7 M electrophoresis grade urea and 40% v/v formamide), in 1 × TAE buffer at a constant 100 V and 60 V overnight. The gels were visualized after staining in 1 g l^{-1} silver nitrate solution for 20 min.

Bands on the DGGE gels were counted and scored based on their presence (1) or absence (0) at the same position on the profiles (lanes) of samples on the gel (Krebs, 1999). A matrix of presence / absence of bands was subjected to cluster analysis (see section 3.2.9). The relatedness of microbial communities was expressed by the Sorensen's index of similarity.

DGGE bands of interest (most intense bands) were excised using sterile scalpels and soaked in 20 µl of milliQ water overnight. The DNA was re-amplified by PCR using F341 5'-CCTACGGGAGGCAGCAG-3' and R534 5'-ATTACCGCG GCTGCTGG-3' primers (Muyzer et al., 1993) before sequencing. Each 50 µl reaction mixture was made up of 1 µl of DNA template, 25 µl of REDTaq ready mix (Sigma), 2 µl each of forward (F341) and reverse primers (R534), and 20 µl of Milli-Q water. The thermal cycling programme used was like the one described for DGGE (this section). To purify the PCR product a QIAquick® PCR purification kit was used

(Qiagen, 28104) following the manufacturer's protocol. Briefly, 1 volume of PCR products was mixed with 5 volumes of buffer PB then applied to a QIAquick column and centrifuged (11300 x *g* for 60 s) to bind DNA to the column. The flow through was discarded and 750 µl buffer PE (wash buffer) was reapplied to the QIAquick column and centrifuged (11300 x *g* for 60 s) to wash the DNA. The purified DNA (50 µl) was eluted into a 1.5 ml microcentrifuge tube with Milli-Q water. The purified DNA was sent to either Source Bioscience (Nottingham, UK) or GATC Biotech AG (Cologne, Germany) for DNA sequencing by the Sanger Sequencing method.

3.2.4 Bacterial 16S rRNA amplicon library preparation for Illumina MiSeq sequencing

To achieve a finer resolution of the bacterial community structure and composition, the variable V3 and V4 regions of bacterial 16S rRNA gene were sequenced on Illumina MiSeq platform from time-0, No-VOC controls and third enrichments. The bacterial 16S rRNA gene amplicon sequencing library preparation was done as described by Illumina (2013) in the 16S Metagenomic sequencing protocol (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf).

Bacterial 16S rRNA genes were amplified using the following primer pairs with Illumina overhang adapter sequences. The forward primer with Illumina adapter sequence was (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'); while the reverse primer sequence with Illumina adapter sequence used was (5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAAT CC-3') (Klindworth et al., 2013). PCR was carried out to amplify *16S rRNA* gene from samples as follows: Each 25 µl PCR had the following components: 2.5 µl of DNA template; 5 µl of forward primer (1 µM); 5 µl of reverse primer (1 µM); and 12.5 µl of REDTaq® ReadyMix™ (Sigma-Aldrich Co.). The thermal cycling program was as follows: an initial denaturation at 95^{o}C for 3 min and 25 cycles at 95^{o}C for 30 s, 55^{o}C for 30 s, and 72^{o}C for 30 s, with a final extension at 72^{o}C for 5 min. PCR products were held at 4^{o}C. Then 5 µl of PCR product from each reaction was run on agarose gel (1%) against a GeneRuler DNA Ladder Mix (Thermo Fisher Scientific) to confirm amplification. The PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter UK Ltd, High Wycombe, UK) following Illumina's protocol (Illumina, 2013).

To attach dual indices and Illumina sequencing adapters, Nextera XT dual indexing strategy was used for the Indexing PCR step. Nextera XT Index Kit V2 set D for 96 indexes, 384 samples (FC-131-2004) was supplied by Illumina Cambridge, Ltd, UK. Each 50 µl reaction had 5 µl of bead-purified PCR product (DNA), 5 µl of sample-specific Nextera XT Index Primer 1 (N716, N718, N719, N720, N721, N722, N723, N724, N726, N727, N728, N729), 5 µl of sample-specific Nextera XT Index Primer 2 (S513, S515, S516, S517, S518, S520, S521, S522), 25 µl of REDTaq® ReadyMix™ (Sigma-Aldrich Co.), and 10 µl of PCR-Grade water. The thermal cycling conditions were as follows: an initial denaturation at 95^{o}C for 3 min and 8 cycles at 95^{o}C for 30 s, 55^{o}C for 30 s, and 72^{o}C for 30 s, with a final extension step at 72^{o}C for 5 min. PCR products were held at 4^{o}C. The resulting PCR products (final library) were purified using Agencourt AMPure XP beads (Beckman Coulter UK Ltd, High Wycombe, UK) before quantification. 5 µl of PCR product from each reaction

was run on agarose gel (1%) against a GeneRuler DNA Ladder Mix (Thermo Fisher Scientific) to confirm index addition and verify the size of the PCR products.

3.2.5 Library quantification, normalisation, and pooling

The bacterial amplicon libraries and archaeal amplicon libraries were quantified on a POLAR star Omega (BMG LABTECH GmbH, Germany) plate reader or NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific) using the PicoGreen® dsDNA assay (Fisher Scientific UK Ltd). Using a 384 well optical plate, diluted libraries (1:10), standards of known concentrations of DNA, and two blanks were mixed with an equal volume of Quant-iT PicoGreen dsDNA Assay kit (Invitrogen) prepared as described by the manufacturer. All samples were prepared as analytical duplicates. The POLAR star Omega (BMG LABTECH GmbH, Germany) plate reader was set at 485 nm excitation and 520 nm emission fluorescence intensity. The DNA concentration in nM of each amplicon library was calculated as described in the Illumina protocol (Illumina, 2013) using the following formula: Concentration in nM = [(concentration in ng / µl) / (660g/mol × average library size)] $\times 10^6$. Based on the DNA concentration (nM) determined, bacterial 16S rRNA genes amplicon libraries from four experiments (86 samples) were pooled into a 1.5 ml microfuge tube while Archaeal 16S rRNA gene amplicon libraries from three experiments (49 samples) were pooled into another 1.5 ml microfuge tube. Sample from the following experiments were sequenced: 1. aerobic degradation experiment (bacterial 16S rRNA gene only); 2. the main anaerobic degradation experiment (both bacterial and archaeal 16S rRNA genes); 3. trial anaerobic degradation experiment (both bacterial

and archaeal 16S rRNA genes); and 4. the simple nitrogen flushing experiment (both bacterial and archaeal 16S rRNA genes).

The resulting bacterial amplicon library pool and archaeal library pool were re-quantified using a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific) by the PicoGreen® dsDNA assay (Fisher Scientific UK Ltd). The DNA concentration of the pools were calculated in nM.

Pooled bacterial and archaea libraries (Nextera XT Index Kit v2 set D) were combined in equimolar concentrations with pooled bacterial and archaea libraries (Nextera XT Index Kit v2 set A) from the McGenity group. The size of the resulting pooled libraries was checked by agarose gel (1%) electrophoresis against a GeneRuler DNA Ladder Mix (Thermo Fisher Scientific). The expected size of the PCR product was confirmed by bioinformatics analyses of the 16S rRNA gene of *Haloferax volcanii* strain JCM 8879 (the representative Archaeon) and *Escherichia coli* K-12 (the representative Bacterium) to be 695 bp and 605 bp respectively. The final library concentration was determined by a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific) by the PicoGreen® dsDNA assay (Fisher Scientific UK Ltd). The final library was diluted with 10 mM Tris pH 8.5 to 4 nM.

3.2.6 Sequencing on MiSeq system

The sample libraries were sequenced on an Illumina MiSeq system in the School of Biological Sciences, University of Essex, following the MiSeq workflow described in the MiSeq System Guide (Illumina, 2015). The MiSeq Reagent Kit v3 (600 cycle) (MS-102-3003) and PhiX Control v3 Kit (FC-110-3001) used were supplied by Illumina Cambridge, Ltd, UK. Briefly, the pooled amplicon library was denatured by

combining 5 µl of 4 nM pooled library with 5 µl of fresh 0.2 N NaOH to obtain a 2 nM denatured amplicon library; then diluted to 20 pM by adding 990 µl of pre-chilled Illumina hybridization buffer (HT1) to 10 µl of denatured amplicon library. The denatured library was diluted to a final concentration of 6 pM by adding 180 µl of the 20 pM denatured libraries to 420 µl of pre-chilled Illumina HT1 buffer and placed on ice. The MiSeq run included 20% PhiX which served as internal control for low-diversity libraries. The PhiX was also denatured and diluted to a final concentration of 6 pM. The PhiX library was diluted to 4 nM by combining 2 µl of 10 nM PhiX library with 3 µl of 10 mM Tris pH 8.5; then denatured by combining 5 µl of 4nM PhiX library with 5 µl of fresh 0.2 N NaOH to obtain a 2 nM PhiX library solution, which was diluted to 20 pM PhiX library by adding 990 µl of pre-chilled Illumina HT1 buffer to 10 µl of the denatured PhiX library. A final concentration of 6 pM PhiX library was achieved by adding 180 µl of 20 pM denatured PhiX library to 420 µl of pre-chilled Illumina HT1 buffer and placed on ice. After denaturation and dilution, 120 µl of the 6 pM PhiX control was mixed with 480 µl of the 6 pM sample amplicon libraries. The 600 µl final library mix (20% PhiX control+ 80% amplicon) was loaded onto the MiSeq v3 reagent cartridge in the designated reservoir. Flow cell, PR2 and empty waste bottle were loaded onto the MiSeq machine following the instructions in the MiSeq system guide (Illumina, 2015). A paired-end 300-cycle run was performed, i.e 2 two reads of 301 cycles (2 x 301). Illumina Sequencing Analysis Viewer 2.1.8 was also used to view the sequenced data generated. The cluster density for the run was 471 k/mm^2 and 77.4% of bases with Q≥30 (5.3 GB).

3.2.7 Bioinformatic analyses and quality control of MiSeq data

Dr Boyd McKew processed the sequences obtained from the MiSeq run from raw sequences to the OTU tables, following the guidelines laid out by Dumbrell et al. (2016). Briefly, sequence reads were quality trimmed using Sickle (Joshi and Fass, 2011), error corrected within SPAdes (Nurk et al., 2013) using the BayesHammer algorithm (Nikolenko et al., 2013) and pair-end aligned with PEAR (Zhang et al., 2014) within PANDASeq (Masella et al., 2012). The quality-filtered, error-corrected, and pair-end aligned sequences were then dereplicated, sorted by their abundance, and OTU centroids picked using VSEARCH (Rognes et al., 2016) at the 97% level. All singleton OTUs were removed, along with all chimeric sequences using de-novo and reference-based chimera checking with UCHIME (Edgar et al., 2011). Taxonomy assignment was performed with the RDP Classifier (Wang et al., 2007). The Biological Observation Matrix (BIOM) table (or OTU table) generated was converted to text formatted OTU table for ecological analyses in Statistical Analysis of Metagenomic Profiles, STAMP (Parks et al., 2014) and RStudio (version 0.98.994 - © 2009 – 2013, RStudio Inc.). Out of 86 bacterial and 49 Archaeal samples (for chapters 3 and 4), 0 failed to yield bacterial and 1 failed to yield Archaeal amplicons. OTUs were removed if they: were denovo chimeras, were <200 bp (Archaea) or 400 bp (bacteria). Negative controls and samples with fewer than 1000 reads were removed. One sample (WaEt_E3_O_Main) with 554 OTUs was retained because it had interesting data. Singleton OTUs from remaining samples were removed. This resulted in 1250942 archaeal sequences from 48 samples and 516840 bacterial sequences from 86 samples (see tables in Appendix 2). In this chapter, only bacterial 16S rRNA amplicons were sequenced and the key findings are shown below.

3.2.8 DNA sequence and phylogenetic analysis

DNA sequences from DGGE bands were analysed and edited using Chromas (Technelysium Pty Ltd). Sequence alignment was done using MUSCLE available on MEGA version 6 (Tamura et al., 2013). Sequences were compared with those on the nucleotide database at the National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Jukes-Cantor distance and Neighbour-Joining methods were used to create phylogenetic trees for partial 16S rRNA genes in MEGA 6 with bootstrap values of 1000 (Jukes and Cantor, 1969, Tamura et al., 2013).

3.2.9 Statistical analysis

The significance of hydrocarbon degradation relative to controls was determined as described in section 2.8. Bacterial community analysis was carried out in STAMP (Parks et al., 2014), section 2.8. Correlation analyses was done in Microsoft Excel to compare the degradation of VOCs between high and low concentrations. The significance of the difference was determined by a two-way analysis of variance.

Cluster Analysis: Cluster analysis and cluster diagrams were generated in PRIMER 6 Beta (version 6 β R6, Primer-E Ltd, Plymouth, United Kingdom) from the presence / absence matrix data generated from DGGE gels. The Sorensen's index of similarity (Krebs, 1999) was used to make pairwise comparison between different samples on a DGGE gel. The computation of Sorensen's similarity index was done as follows:

$$S_s = \frac{2a}{2a+b+c}$$

Where

S_s: Sorensen's similarity coefficient; a: Number of bands in sample A and sample B (joint occurences); b: Number of bands in sample B but not in sample A; c: Number of bands in sample A but not in sample B.

Sequential clustering algorithms were used to generate a hierarchical similarity relationship between samples based on the degree of similarities between bands from different profiles (lanes) on a DGGE gel. The following equation represent the algorithms used:

{Similarity between a sample and an existing cluster

= {Arithmetic mean of similarities between the sample and all members of the cluster}

$$S_{J(K)} = (1/t_J\, t_K)(\sum S_{J(K)})$$

Where:

$S_{J(K)}$: Similarity between two clusters J and K; t_J: number of samples in cluster J(≥ 1);

t_K: number of samples in cluster K (≥ 2).

3.3 Results

3.3.1 Influence of concentration on the biodegradation of benzene, toluene, isoprene, ethane and propane in single substrate microcosms

To understand the influence of concentration on benzene, toluene, isoprene, ethane or propane degradation, an experiment was set up with samples from the head of the Colne estuary (the Hythe). Three concentrations designated as C, C/10 & C/100 were added to separate microcosms. Previous experiments showed a hierarchy in degradation (the main aerobic degradation/enrichment experiments), benzene and toluene degraded more rapidly, isoprene degraded within 2 weeks while ethane and propane degradation were usually observed within 21 days of incubation. This explains why each VOC had a different monitoring regime.

For benzene and toluene: 0.005 mM degraded completely in less than a day; 0.05 mM degraded (>95%) by day 2, and effectively complete degradation by day 3; and 0.5 mM degraded (>90%) by day 3 with most degradation occurring between day 2 and 3 (Table 3.2). There was a positive correlation between the degradation at 0.5 mM and 0.05 mM in both cases (toluene and benzene). The correlation was weak for benzene (r = 0.140, P < 0.001) and moderate for toluene (r = 0.690, P < 0.001).

Table 3.2 Aerobic degradation of benzene and toluene at different concentrations in single-substrate sediment slurry microcosms based on the % remaining in the headspace relative to the killed control

	% remaining ± SE					
Time (Day)	**Benzene (C)**	**Benzene (C/10)**	**Benzene (C/100)**	**Toluene (C)**	**Toluene (C/10)**	**Toluene (C/100)**
1	147.6 ±77.56	100.8 ± 15.44	BDL	93.1 ± 24.38	12.1 ± 12.10	BDL
2	123.8 ± 72.42	0.2 ± 0.14	BDL	52.1 ± 17.07	2.6 ± 1.54	BDL
3	0.0 ± 0.00	0.3 ± 0.15	BDL	6.9 ± 5.81	0.0 ± 0.00	BDL

Final concentration of toluene or benzene in microcosms (C = 0.5 mM; C/10 = 0.05 mM; C/100 = 0.005 mM); BDL =below detection limit.

Ethane and propane degradation followed a similar trend with lower concentrations degrading to completion quicker than higher concentrations (Table 3.3). The C/10

(1% v/v) and C/100 (0.1% v/v) concentrations degraded completely by day 20 of incubation while C (10% v/v) required more than 80 days. There was a strong positive correlation between the degradation at 10 % v/v and 1% v/v for ethane (r = 0.843, P < 0.001) and propane (r = 0.836, P < 0.001).

Table 3.3 Aerobic degradation of propane or ethane at different concentrations in single-substrate sediment slurry microcosms based on the % remaining in the headspace relative to the killed controls

	% remaining±SE					
Day	**Ethane (C)**	**Ethane (C/10)**	**Ethane (C/100)**	**Propane (C)**	**Propane (C/10)**	**Propane (C/100)**
9	86.6 ± 6.01	92.4 ± 14.03	90.1 ± 7.98	111.6 ± 12.81	96.2 ± 10.18	103.8 ± 18.60
20	61.0 ± 1.10	0.3 ± 0.10	4.5 ± 0.60	64.0 ± 14.90	0.0 ± 0.00	0.0 ± 0.00
32	38.2 ± 0.54	BDL	BDL	60.2 ± 3.00	BDL	BDL
55	27.4 ± 5.37	BDL	BDL	58.2 ± 5.65	BDL	BDL
87	0.0 ± 0.00	BDL	BDL	81.5 ± 20.88	BDL	BDL
119	BDL	BDL	BDL	64.5 ± 6.50	BDL	BDL

Final concentration of propane or ethane in microcosm (C = 10% v/v, C/10 = 1% v/v, C/100 = 0.1 % v/v); BDL =below detection limit.

At 7.2 x 10^6 ppbv, 7.2 x 10^5 ppbv and 7.2 x 10^4 ppbv, complete degradation of isoprene was observed in all microcosm by day 9 (Table 3.4). Degradation of isoprene correlated positively with concentration (between 7.2 x 10^6 ppbv and 7.2 x 10^5 ppbv, r = 0.892; between 7.2 x 10^6 ppbv and 7.2 x 10^4 ppbv, r = 0.862; between 7.2 x 10^5 ppbv and 7.2 x 10^4 ppbv, r = 0.655; P < 0.001).

Table 3.4 Aerobic degradation of isoprene at different concentrations in single-substrate microcosms based on the % remaining in the headspace relative to the killed control

	% remaining ± SE		
Day	**Isoprene (C)**	**Isoprene (C/10)**	**Isoprene (C/100)**
2	98.0 ± 2.30	104.2 ± 15.88	100.7 ± 6.50
5	74.1 ± 5.31	63.9 ± 10.73	68.8 ± 9.66
9	0.9 ± 0.16	0.0 ± 0.00	0.0 ± 0.00

Final concentration of isoprene in microcosms (C = 7.2 x 10^6 ppbv, C/10 = 7.2 x 10^5 ppbv; C/100 = 7.2 x 10^4 ppbv).

3.3.2 Benzene, toluene, isoprene, ethane and propane biodegradation in single-substrate enrichment series

Sediment slurry microcosms supplemented singly with benzene, toluene, isoprene, ethane or propane were set up to understand VOC degradation in estuarine environments using the concentrations described in Section 3.2.2.2. Here, I consider the degradation in the primary enrichments with the sediment slurries and the subsequent enrichments, which were also used to identify changes in microbial community composition (Sections 3.2.2.2 and 3.2.2.3).

More than 80% benzene (0.5 mM) or toluene (0.5 mM) degradation was achieved within 10 days in sediment slurries (first enrichment). A very short lag phase or no lag phase was observed in some cases (Figure 3.2). The longest lag phase of 2 days was observed in Hythe microcosms with benzene which was followed by rapid benzene degradation. Statistically significant benzene or toluene degradation (Welch two sample t-test: $P < 0.05$) was observed from day 2 of incubation (Figure 3.2).

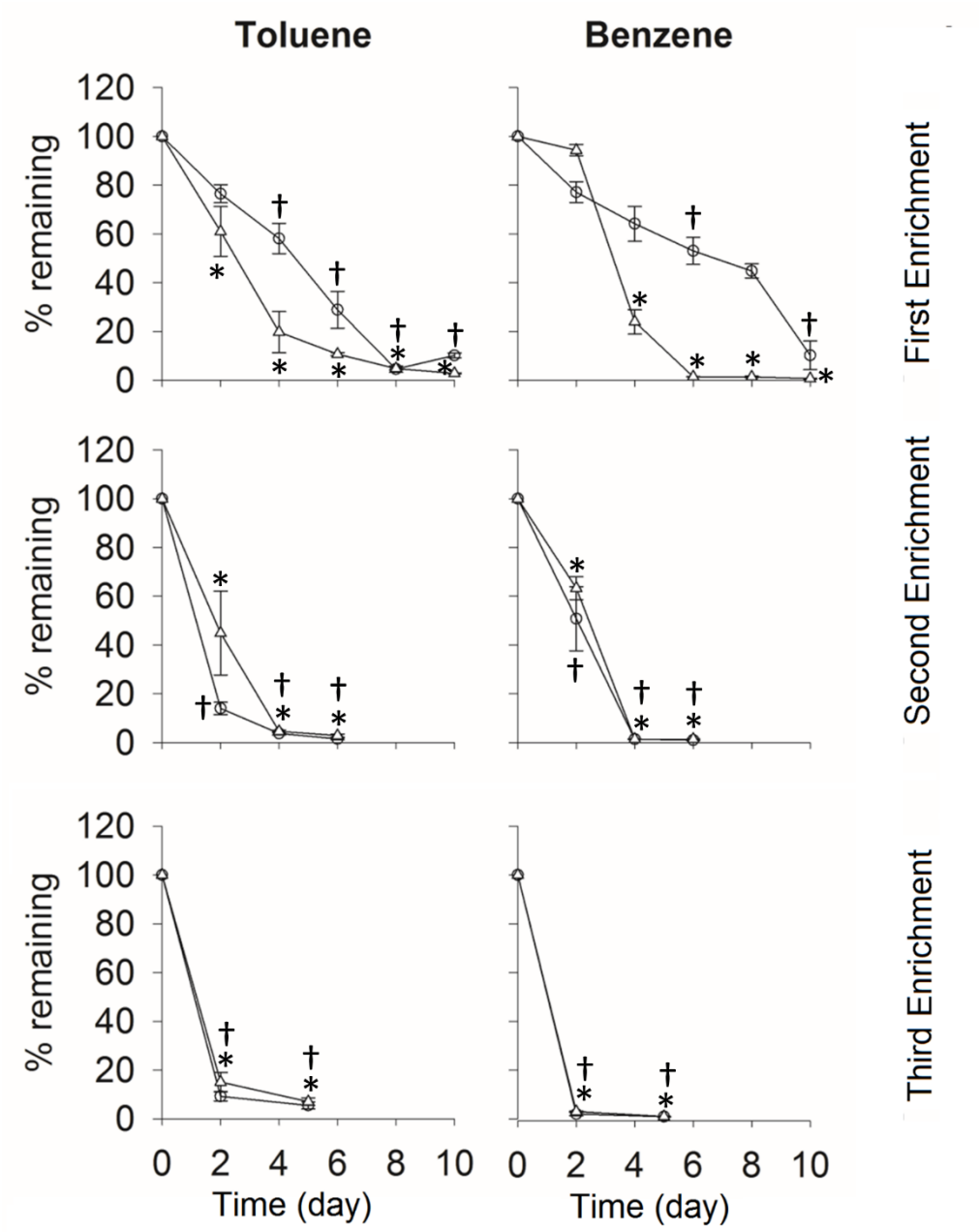


Figure 3.2. Biodegradation of toluene or benzene in Hythe (—△—) and Wivenhoe (—⊖—) enrichments as indicated by the headspace concentration relative to the killed control (first enrichments) or relative to the sterile control (second and third enrichments). Final concentration of benzene and toluene was 0.5 mM. Degradation values were normalized by dividing with mean of the corresponding sterile controls. n = 3; bars show SE of the mean; Star symbols (*) or cross symbols (†) indicate significant difference (Welch two sample t-test: $P < 0.05$) between the headspace concentration of control and test samples for Hythe and Wivenhoe enrichments respectively.

Biodegradation was more rapid in Hythe sediment slurries as compared to the corresponding Wivenhoe sediment slurries (Figure 3.2). Benzene was degraded beyond detection limit in Hythe microcosms by the 6th day of incubation unlike the corresponding Wivenhoe microcosms (Figure 3.2). Similarly, > 80% toluene added in Hythe microcosms was used up by day 4 of incubation and just 40% of the toluene supplied in the corresponding Wivenhoe microcosms was utilized at the same duration of incubation.

Biodegradation became more rapid with enrichments. Second and third enrichments became turbid with time indicating the growth and/or multiplication of benzene or toluene degrading bacteria. In the second and third enrichments, complete benzene degradation was observed in 4 and 2 days of incubation respectively (Figure 3.2). Degradation was very rapid in toluene-enrichments with more than 80% degradation observed in all microcosms within 1 or 2 days of incubation (Figure 3.2).

A longer lag phase of about 10 days was observed with ethane or propane enrichments (Figure 3.3). GC-FID analysis revealed significant ethane (10% v/v) or propane (10% v/v) degradation between 10 and 30 days of incubation at 12^{o}C (Welch two sample t-test: $P < 0.05$). Like benzene and toluene, more rapid ethane or propane consumption was observed in Hythe sediment slurries than in Wivenhoe (Figure 3.3). Significant ethane degradation relative to the killed control (Welch two sample t-test: $P < 0.05$) was observed between 21 and 37 days of incubation in sediment slurries (First enrichments, Figure 3.3). Statistically significant propane degradation (Welch two sample t-test: $P < 0.05$) was observed from day 14 of incubation in Hythe microcosms and day 21 in Wivenhoe microcosms (Welch two sample t-test: $P = 0.077$).

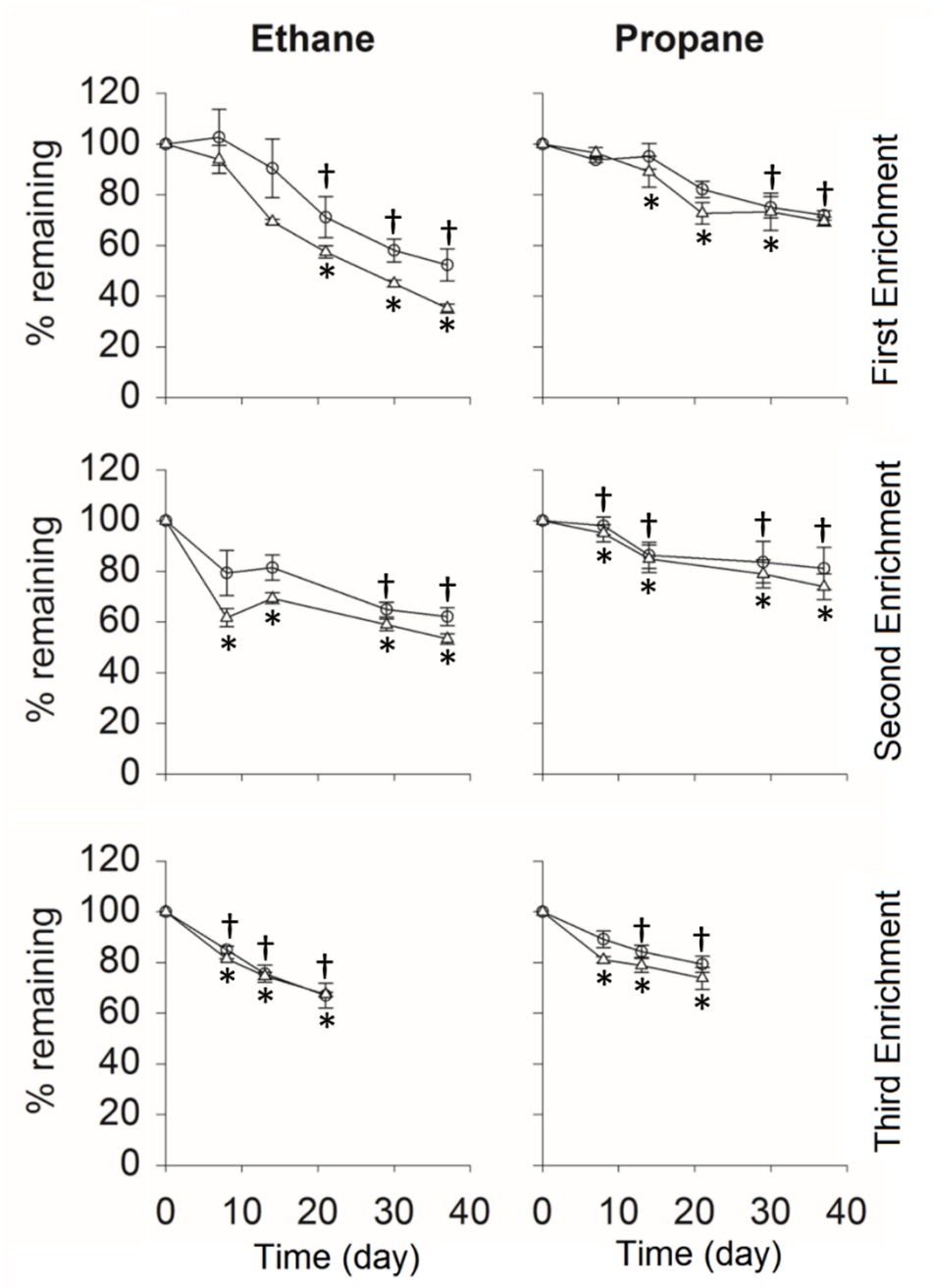


Figure 3.3. Biodegradation of ethane or propane in Hythe (—△—) and Wivenhoe (—⊖—) enrichments as indicated by the headspace concentration relative to the killed control (first enrichments) or relative to the sterile control (second and third enrichments). Final concentration of ethane and propane was 10 % v/v. Degradation values were normalized by dividing with mean of the corresponding sterile controls. n = 3; bars show SE of the mean; Star symbols (*) or cross symbols (†) indicate significant difference (Welch two sample t-test: $P < 0.05$) between the headspace concentration of control and test samples for Hythe and Wivenhoe enrichments respectively.

In second and third enrichments, a quicker onset of statistically significant ethane or propane degradation (Welch two sample t-test: $P < 0.05$) with no lag phase was observed (Figure 3.3). Further evidence of biodegradation was that those enrichments became turbid with time while the corresponding sterile controls remained clear. Third ethane and propane enrichments were sampled at day 21 for molecular analyses when statistically significant biodegradation (Welch two sample t-test: $P < 0.05$) was observed.

In sediment slurries, biodegradation of isoprene in Hythe microcosms was observed from day 2 of incubation with > 90% of the isoprene added (7.2×10^6 ppbv) degraded by day 10 (Figure 3.4). In Wivenhoe microcosms, however, isoprene degradation was slower. Statistically significant isoprene degradation was observed from day 4 in Hythe microcosms (Welch two sample t-test: $P < 0.05$), whereas no statistically significant isoprene degradation was observed in Wivenhoe microcosms (at $P < 0.05$) until the third enrichment (Figure 3.4). This reinforces the trend observed with the biodegradation of the other 4 volatile hydrocarbons (benzene, toluene, ethane and propane), that VOCs could degrade more rapidly at the head of the Colne estuary than the more marine middle of the estuary at Wivenhoe. More rapid isoprene degradation was observed at the second and third enrichments respectively. Isoprene was below detection in the headspace of the second and third enrichments by day 8 (Hythe only) and 5 (both Hythe and Wivenhoe) respectively (Figure 3.4) for Hythe microcosm.

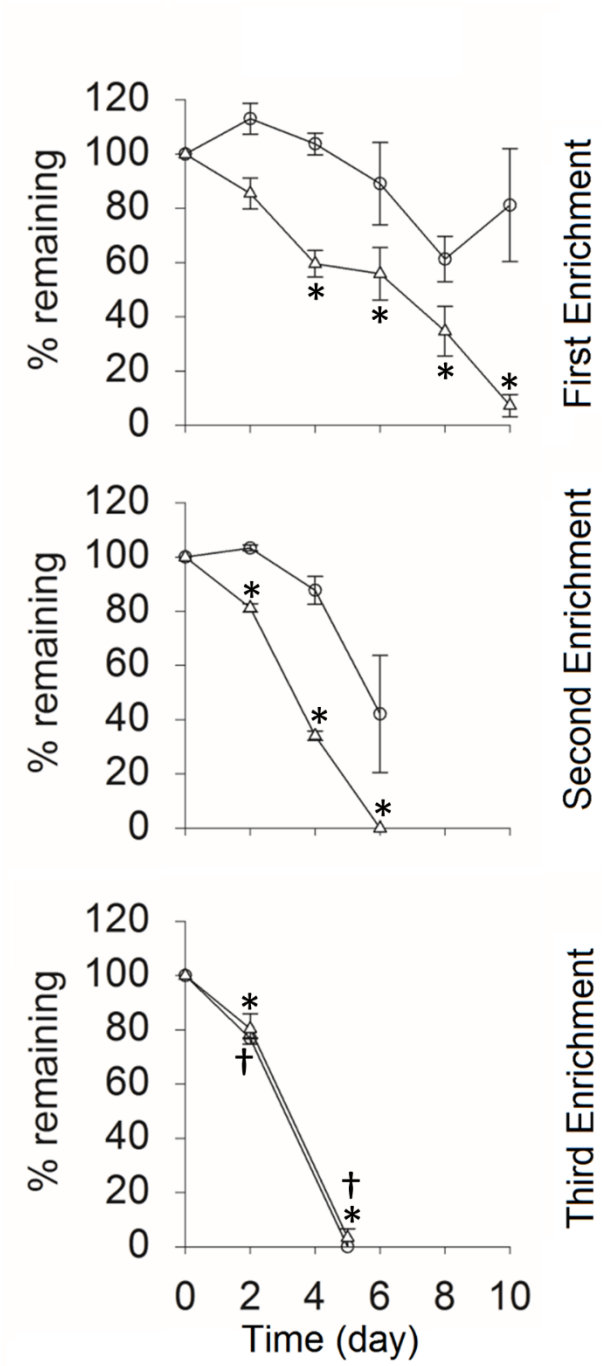


Figure 3.4. Biodegradation of isoprene in Hythe (—△—) and Wivenhoe (—⊖—) enrichments as indicated by the headspace concentration relative to the killed control (first enrichments) or relative to the sterile control (second and third enrichments). Final concentration of isoprene was 7.2 x 10^6 ppbv. Degradation values were normalized by dividing with mean of the corresponding sterile controls. *n* = 3; bars show SE of the mean; Star symbols (*) or cross symbols (†) indicate significant difference (Welch two sample t-test: $P < 0.05$) between the headspace concentration of control and test samples for Hythe and Wivenhoe enrichments respectively.

3.3.3 Bacterial community profiles of microcosms after benzene, toluene, isoprene, ethane and propane degradation based on bacterial *16S rRNA* gene analyses by DGGE and Illumina MiSeq

To assess the extent to which different VOCs affected the bacterial community composition in enrichments with inocula from two different locations on the Colne estuary, two approaches analysing *16S rRNA* genes were adopted. Firstly, DGGE was used to give a broad overview of community changes, with some identification by sequencing of key bands. However, to obtain finer resolution and definitively identify differentially enriched microbes, Illumina MiSeq analysis was performed.

A shift in bacterial community profile was observed in all third enrichments amended with single-substrate VOCs as evidenced by the DGGE profiles (Figure 3.5 & 3.6). The bacterial community profile of triplicate microcosms was very similar (>90% similarity) (Figure 3.5b & 3.6b). This VOC-driven shift in bacterial community composition was confirmed by PCA analysis of bacterial OTUs (97% similarity clustering) obtained from MiSeq data (Figure 3.7). The communities at time 0 (Ht0 & Wt0) and in No-VOC controls at day 37 (Ht37 & Wt37) were similar, as evidenced from their clustering, and were distinct from communities amended with volatile hydrocarbons (Figure 3.7). Thus, the shift in bacterial community composition was driven by the volatile hydrocarbon degraded.

The PCA also showed that location from which samples were collected had minimal effect on the bacterial community composition except for samples amended with benzene where OTUs enriched in Wivenhoe microcosms were different from those enriched in Hythe microcosms (Figure 3.7).

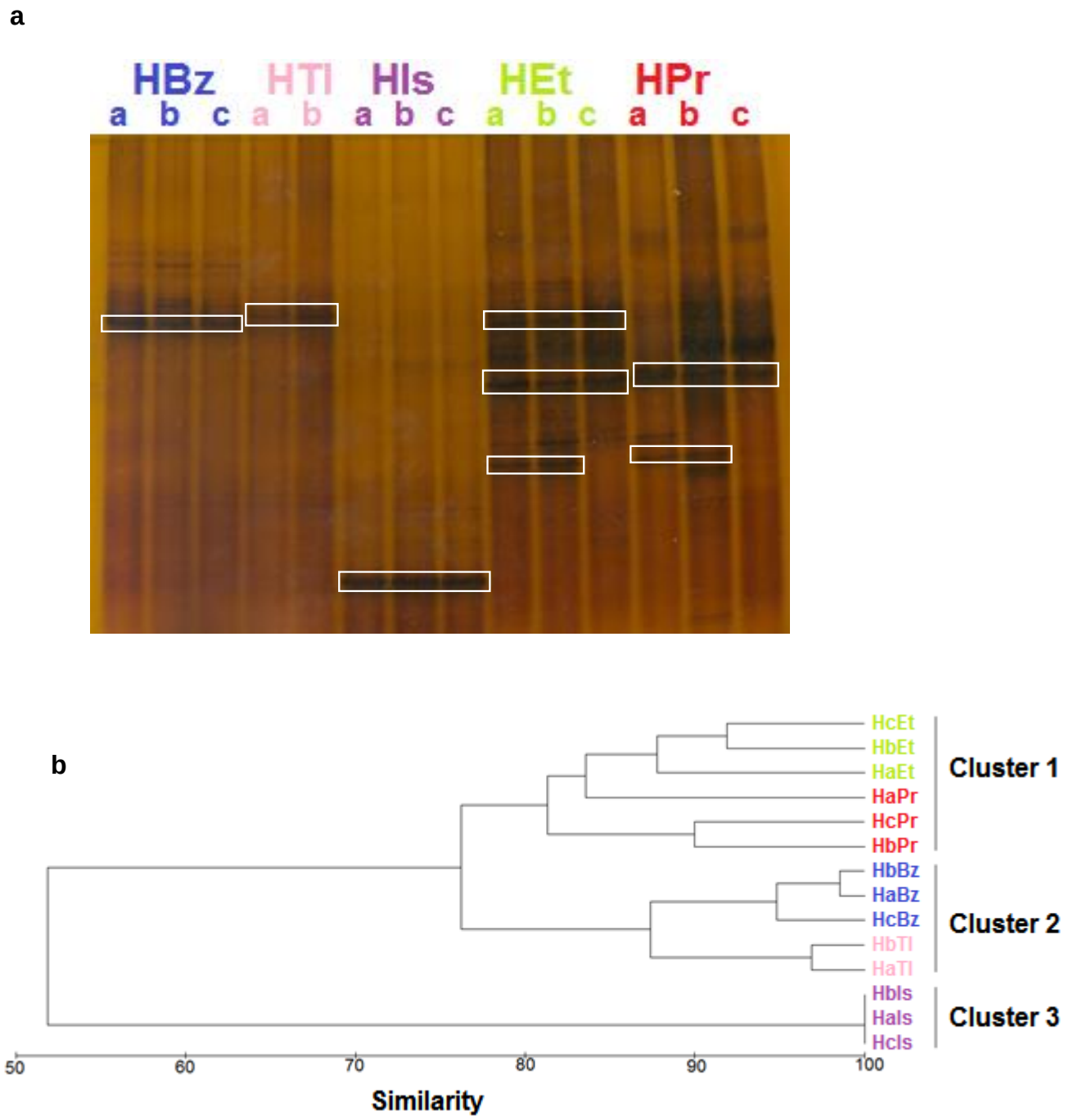
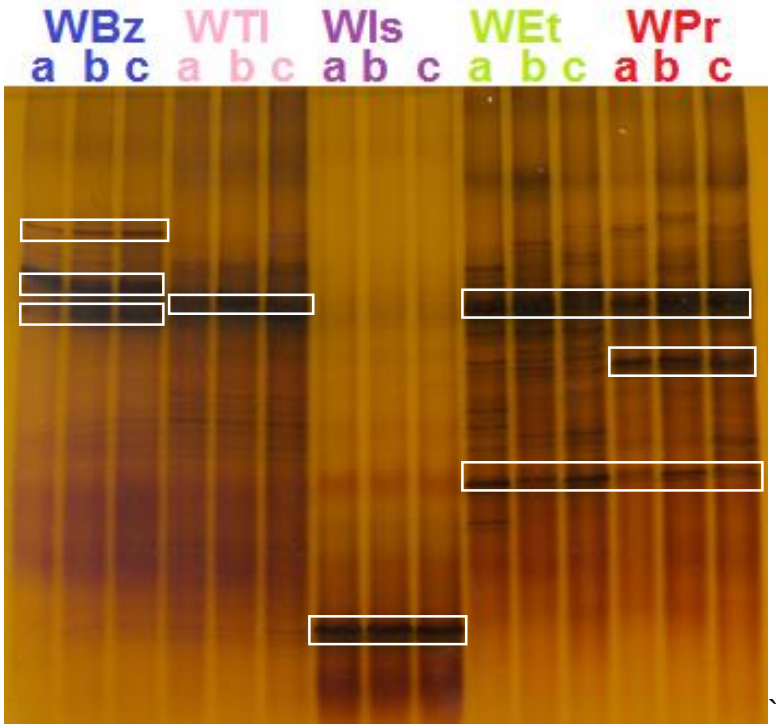


Figure 3.5. DGGE profiles of third enrichments (E3) showing changes in bacterial community structure with single-substrate volatile hydrocarbons in Hythe microcosms. a) DGGE gel showing bacterial populations enriched (thick bands) at the third enrichment (E3) with benzene (Bz), toluene (Tl), isoprene (Is), ethane (Et) or propane (Pr) as a sole carbon source; Boxes indicate major changes observed in profiles. b) A cluster diagram indicating the relationship between bacterial community compositions enriched with single substrate hydrocarbons. The cluster diagram was generated in PRIMER 6 Beta from a presence /absence matrix data obtained from the DGGE gel. H represents Hythe microcosms; a, b & c are biological replicates, *n* =3 except HTl where *n* = 2.

DGGE analysis showed that different classes of volatile hydrocarbons selectively enriched distinct bacterial communities. Isoprene-degrading communities were distinct, ethane and propane degrading communities shared >80% similarity while toluene and benzene degrading communities shared >85% similarity (Figure 3.5b & 3.6b). Interestingly, communities degrading ethane, propane, toluene and benzene share high similarity (>75 % similarity) as shown in Figures 3.5b and 3.6b. PCA of MiSeq data, also highlighted that the isoprene-enriched community was distinct, but in this case the toluene-enriched community clustered with those enriched with ethane and propane (Figure 3.7), and communities enriched with benzene were distinct.

a



b

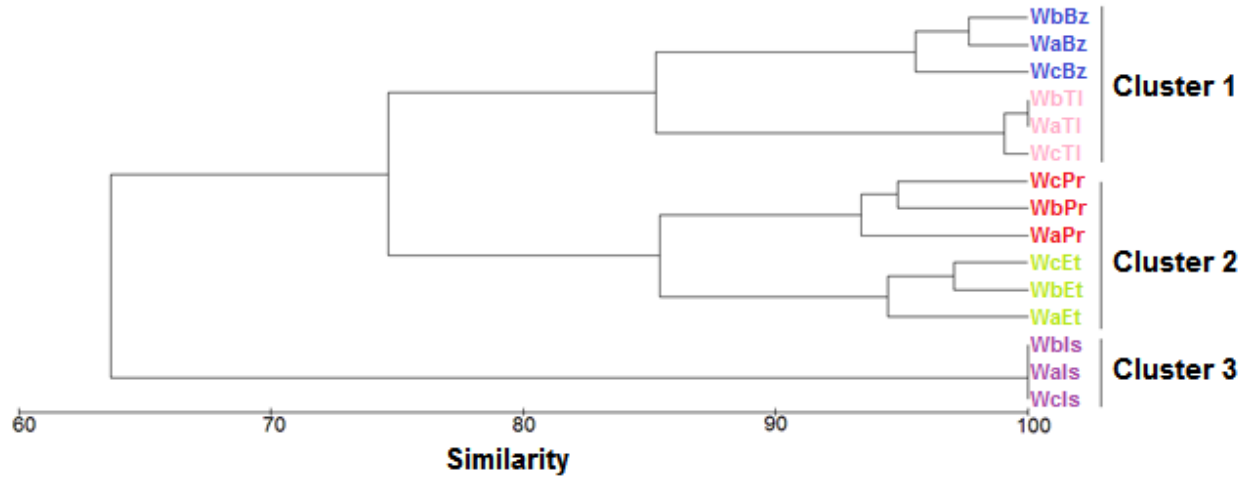


Figure 3.6. DGGE profiles of third enrichments (E3) showing changes in bacterial community composition with single-substrate volatile hydrocarbons in Wivenhoe sediment slurry microcosms. a) DGGE gel showing bacterial populations enriched (thick bands) with benzene (Bz), toluene (Tl), isoprene (Is), ethane (Et) or propane (Pr) as a sole carbon source; Boxes indicate major changes observed in profiles. b) Cluster diagram indicating the relationship between bacterial community composition enriched with single substrate hydrocarbons. The cluster diagram was generated in PRIMER 6 Beta from a presence / absence matrix data obtained from the DGGE gel. W represents Wivenhoe microcosms; a, b & c are biological replicates, *n* = 3.

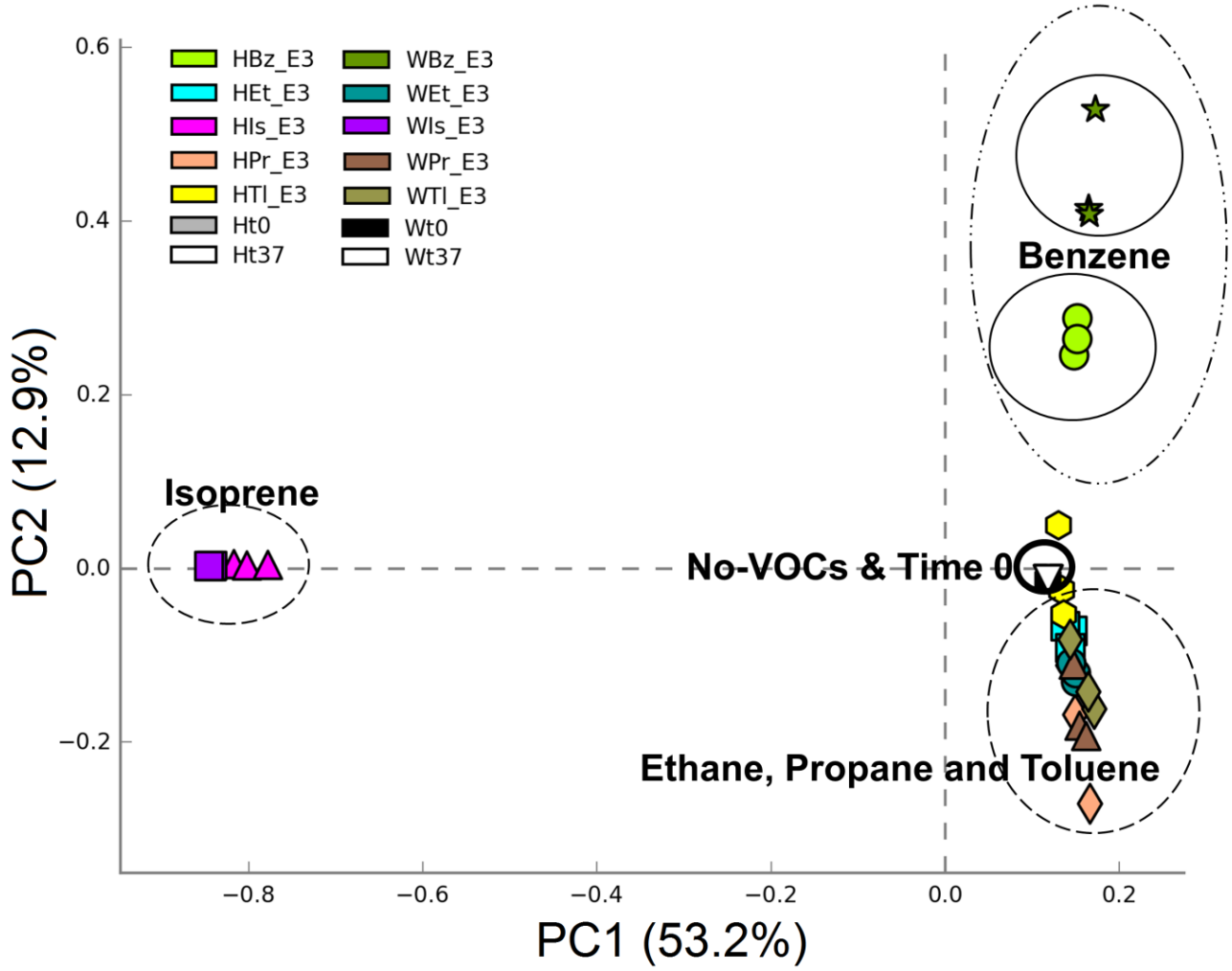


Figure 3.7. Principal component analysis (PCA) of bacterial *16S rRNA* gene OTUs (97% similarity threshold) from the Colne estuary present in Hythe and Wivenhoe enrichments, indicating the relationship of the bacterial community composition of third enrichments with benzene, toluene, isoprene, ethane or propane, to the no-VOC control and communities at time 0. The first two principal components accounting for 66.1% are plotted. All samples presented are the mean of triplicate. Ht0 or Wt0 refers to community at time 0 (t0) from Hythe (H) or Wivenhoe (W); Ht37 or Wt37 refers to Hythe or Wivenhoe No-VOC control enrichments at day 37 (t37) ; HTl_E3 or WTl_E3 refers to Hythe or Wivenhoe enrichments with toluene (Tl) at third enrichment (E3); HBz_E3 or WBz_E3 refers to Hythe or Wivenhoe enrichments with benzene (Bz) at third enrichment (E3); HIs_E3 or WIs_E3 refers to Hythe or Wivenhoe enrichments with isoprene (Is) at third enrichment (E3);HEt_E3 or WEt_E3 refers to Hythe or Wivenhoe enrichments with ethane (Et) at third enrichment (E3); HPr_E3 or WPr_E3 refers to Hythe or Wivenhoe enrichments with propane (Pr) at third enrichment (E3). PCA was performed in STAMP 2.1.3 Parks et al. (2014) with default parameters.

3.3.4 Identification of some bacterial phylotypes in enrichments with volatile hydrocarbons revealed by DGGE analysis

Within the limitations imposed by DGGE, sequence analysis of some dense DGGE bands (Figure 3.8) revealed that *Actinobacteria* were enriched with isoprene; *Gammaproteobacteria* were enriched with benzene or toluene; while both *Betaproteobacteria* and *Gammaproteobacteria* were enriched with ethane or propane (Table 3.5). The densest band (Fig. 3.8) identified in enrichments with isoprene shared 100% identity with *Rhodococcus globerulus* over the 125 bp amplified region compared (Table 3.5). *Pseudomonas* sp. (100% identity) was most likely a key part of the bacterial community enriched with benzene, while *Amphritea atlantica* (99% identity) was enriched with toluene (Table 3.5). A more diverse bacterial community structure was identified in ethane and propane enrichments. Sequenced bands (Figure 3.8) were identified by BLAST analysis as: *Thauera* sp. (95 - 100% identity); *Azoarcus* sp. (93% identity); *Amphritea* sp. (93% identity); *Cycloclasticus* sp. (94% identity) (Table 3.5).

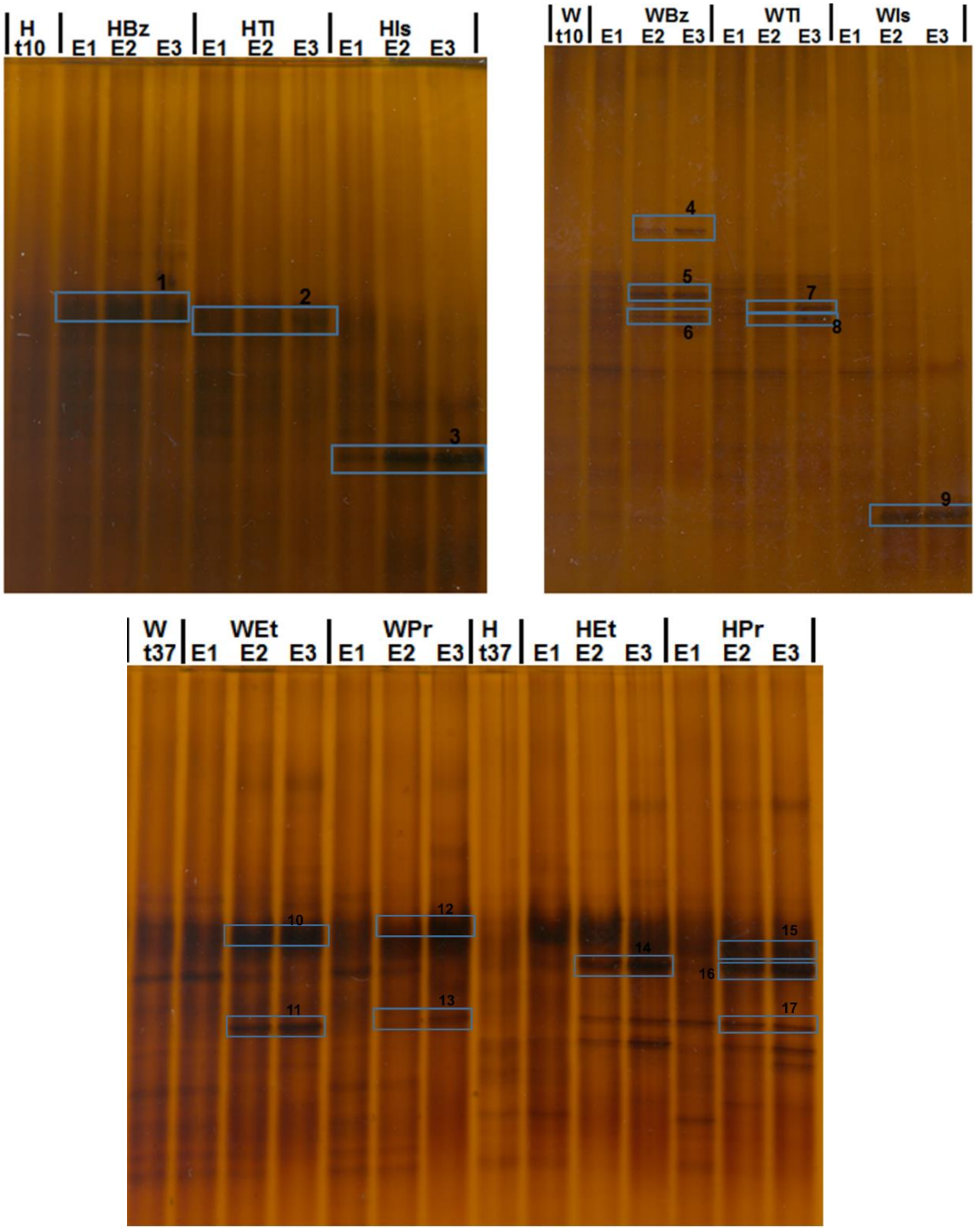


Figure 3.8. DGGE gels of PCR-amplified bacterial *16S rRNA* gene showing a shift in bacterial community composition from first enrichments (E1) to third enrichments (E3) of Hythe (H) or Wivenhoe (W) enrichments with benzene (Bz), toluene (Tl), isoprene (Is), ethane (Et) or propane (Pr). Boxed bands (1 – 17) were excised, PCR-amplified and 16S-rRNA-gene sequenced (Table 3.5). t10 = No-VOC control at day10; t37 = No-VOC control at day 37. The bacterial community profile of triplicate enrichments was almost identical (Figure 3.5 -3.6), therefore, E1 (first enrichment), E2 (second enrichment) and E3 (third enrichments) are pooled triplicate.

Table 3.5 Closest cultured relative of sequenced DGGE bands shown in Figure 3.8 based on BLAST searches against NCBI's 16S rRNA sequence database.

Pooled Sample Code	DGGE Band number	Sequence length (bp)	Bacterial Taxa	Closest relative	% Identity
HBz	1	122	*Gammaproteobacteria*	*Pseudomonas glareae* strain KMM 9500 (NR_145562.1)	100
HTl	2	132	*Gammaproteobacteria*	*Amphritea atlantica* strain M41 (NR_042455.1)	98
HIs	3	123	*Actinobacteria*	*Rhodococcus globerulus* strain DSM 43954 (NR_118617.1)	98
WBz	4	128	*Gammaproteobacteria*	*Pseudomonas helmanticensis* strain OHA11 (NR_126220.1)	100
WBz	5	140	*Gammaproteobacteria*	*Pseudomonas helmanticensis* strain OHA11 (NR_126220.1)	100
WBz	6	135	*Gammaproteobacteria*	*Pseudomonas flavescens* strain NBRC 103044 (NR_114195.1)	100
WTl	7	136	*Gammaproteobacteria*	*Amphritea atlantica* strain M41(NR_042455.1)	99
WTl	8	135	*Gammaproteobacteria*	*Amphritea atlantica* strain M41(NR_042455.1)	100
WIs	9	125	*Actinobacteria*	*Rhodococcus globerulus* strain DSM 43954 (NR_118617.1)	100
WEt	10	101	*Gammaproteobacteria*	*Amphritea japonica* strain JAMM 1866 (NR_041616.1)	100
WEt	11	111	*Betaproteobacteria*	*Thauera phenylacetica* strain B4P 1(NR_027224.1)	95
WPr	12	118	*Gammaproteobacteria*	*Amphritea japonica* strain JAMM 1866 (NR_041616.1)	93
WPr	13	116	*Gammaproteobacteria*	*Cycloclasticus pugetii* strain PS-1 (NR_025955.1)	94
HEt	14	117	*Betaproteobacteria*	*Thauera humireducens* strain SgZ-1 (NR_109534.1)	95
HPr	15	117	*Betaproteobacteria*	*Thauera terpenica* strain 58Eu (NR_025284.1)	98
HPr	16	117	*Betaproteobacteria*	*Thauera terpenica* strain 58Eu (NR_025284.1)	100
HPr	17	120	*Betaproteobacteria*	*Azoarcus communis* strain SWub3(NR_024850.1)	93

3.3.5 Novel bacterial phylotypes enriched with benzene, toluene, isoprene, ethane and propane in single-substrate enrichments as revealed by Illumina MiSeq sequencing of bacterial *16S rRNA* gene

Although DGGE provides valuable information about enriched phylotypes, not all members of the enrichments are resolved and sequenced, and so MiSeq analysis was performed. Novel bacterial phylotypes were identified from single substrate-enrichments belonging mainly to *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* (Table 3.6, Figure 3.9 – 3.12).

Sequences identified in Hythe and Wivenhoe microcosms with benzene indicated that *Pseudomonas* was enriched significantly, suggesting that this genus was primarily responsible for benzene degradation at both locations of the Colne estuary (Figure 3.9 c & d). *Pseudomonas* was clearly a dominant member of the bacterial community identified in the Hythe (54.5%) and Wivenhoe (71.8%) benzene enrichments (Figure 3.9 c & d). At the OTU level, OTU_8 and OTU_12 were enriched significantly (Figure 3.11 c & d). BLAST analyses revealed that OTU_8 shared 98% sequence identity with *Pseudomonas benzenivorans*, while OTU_12 shared 99% sequence identity with *Pseudomonas borbori* R-20821 (Table 3.6).

Sequences identified in Hythe and Wivenhoe microcosms amended with toluene significantly enriched two bacterial phylotypes: *Amphitrea* in both Hythe and Wivenhoe microcosms and *Bradymonas* in Hythe microcosms only (Figure 3.9 a & b). *Amphritea* was the most abundant genus identified in the Hythe (12.9%) and Wivenhoe (45.4%) toluene enrichments, suggesting an important role in toluene biodegradation (Figure 3.9 a & b). OTU_7 was enriched in both Hythe and Wivenhoe microcosms while OTU_131 was enriched in Hythe microcosms only (Figure 3.11 a & b). BLAST analysis revealed that OTU_7 shared 98% sequence identity with

Amphritea atlantica strain M41 and OTU_131 shared 99% sequence identity with *Pseudomonas sagittaria* strain CC-OPY-1 (Table 3.6).

Table 3.6 Identity of OTUs demonstrating increased abundance in enrichments with volatile hydrocarbons relative to no-VOC controls, based on BLAST searches against NCBI's 16S rRNA sequence database.

Enrichment	OTU	Bacterial Taxa	Closest Relative	% Identity
HIs, WIs	OTU_4	*Actinobacteria*	*Rhodococcus globerulus* strain DSM 43954 (NR_118617.1)	99
HPr, WPr, HTl, WTl	OTU_7	*Gammaproteobacteria*	*Amphritea atlantica* strain M41 (NR_042455.1)	98
HBz, WBz	OTU_8	*Gammaproteobacteria*	*Pseudomonas benzenivorans* strain DSM 8628 (NR_116904.1)	98
HEt	OTU_10	*Betaproteobacteria*	*Azoarcus olearius* strain DQS-4 (NR_108183.1)	95
HBz, WBz	OTU_12	*Gammaproteobacteria*	*Pseudomonas borbori* strain R-20821 (NR_042450.1)	99
WEt	OTU_24	*Gammaproteobacteria*	*Cycloclasticus spirillensus* strain M4-6 (NR_115117.1)	95
			Cycloclasticus endosymbiont of *Bathymodiolus heckerae* clone O (KX509816.1)	97
WEt	OTU_72	*Alphaproteobacteria*	*Roseibacterium elongatum* (NR_121734.1)	99
HIs	OTU_117	*Actinobacteria*	*Rhodococcus erythropolis* strain ATCC 4277 (NR_119125.1)	99
HTl	OTU_131	*Gammaproteobacteria*	*Pseudomonas sagittaria* strain CC-OPY-1 (NR_118347.1)	99
WEt	OTU_246	*Gammaproteobacteria*	*Marinobacterium aestuariivivens* strain DB-1 (NR_148849.1)	94

HBz, HEt, HIs, HPr, HTl refers to Hythe enrichments with benzene (Bz), ethane (Et), isoprene (Is), propane (Pr) or toluene (Tl) as sole carbon source while WBz, WEt, WIs, WPr, & WTl refers to Wivenhoe with the respective carbon source.

Rhodococcus was the dorminant phylotype in Hythe (92.7% of the community) and Wivenhoe (97.3% of the community) enrichments with isoprene as sole carbon source (Figure 3.9 e & f). A small proportion of another Actinobacteria genus was identified in the Hythe only (Figure 3.9e). This dominance by *Rhodococcus* was also shown on the DGGE gel by a single thick band (Figure 3.5 – 3.6). The OTUs enriched were OTU_4 (Hythe and Wivenhoe) and OTU_117 (Hythe only) (Figure 3.11e & f). BLAST analysis revealed OTU_4 shared 99% sequence identity with *Rhodococcus globerulus* strain DSM 43954 while OTU_117 shared 99% sequence identity with *Rhodococcus erythropolis* strain ATCC 4277 (Table 3.6).

The following genera were identified in enrichments with ethane as sole carbon source: *Azoarcus* (Hythe only), *Cycloclasticus* (Wivenhoe only), *Pseudoruegeria* (Wivenhoe only) and *Marinobacterium* (Wivenhoe only) (Figure 3.10 a & b). *Azoarcus* formed only a small proportion (6.9%) of the bacterial community. Interestingly, *Cycloclasticus* was a dominant member of the bacterial community identified in Wivenhoe, constituting 32.7% of the community enriched (Figure 3.10b). Unique OTUs were selectively enriched from each sampling location as follows: OTU_10 *Azoarcus olearius* (95% identity, Hythe only), OTU_24 *Cycloclasticus spirillensus* (95% identity, Wivenhoe only), OTU_72 *Roseibacterium elongatum* (99% identity, Wivenhoe only) and OTU_246 *Marinobacterium aestuariivivens* (94% identity, Wivenhoe only) (Figure 3.12 a & b). Interestingly, OTU_24 (97% identity) shared closer sequence identity with the ethane-degrading *Cycloclasticus* endosymbiont of *Bathymodiolus heckerae* clone O reported recently by Rubin-Blum et al. (2017); Table 3.6).

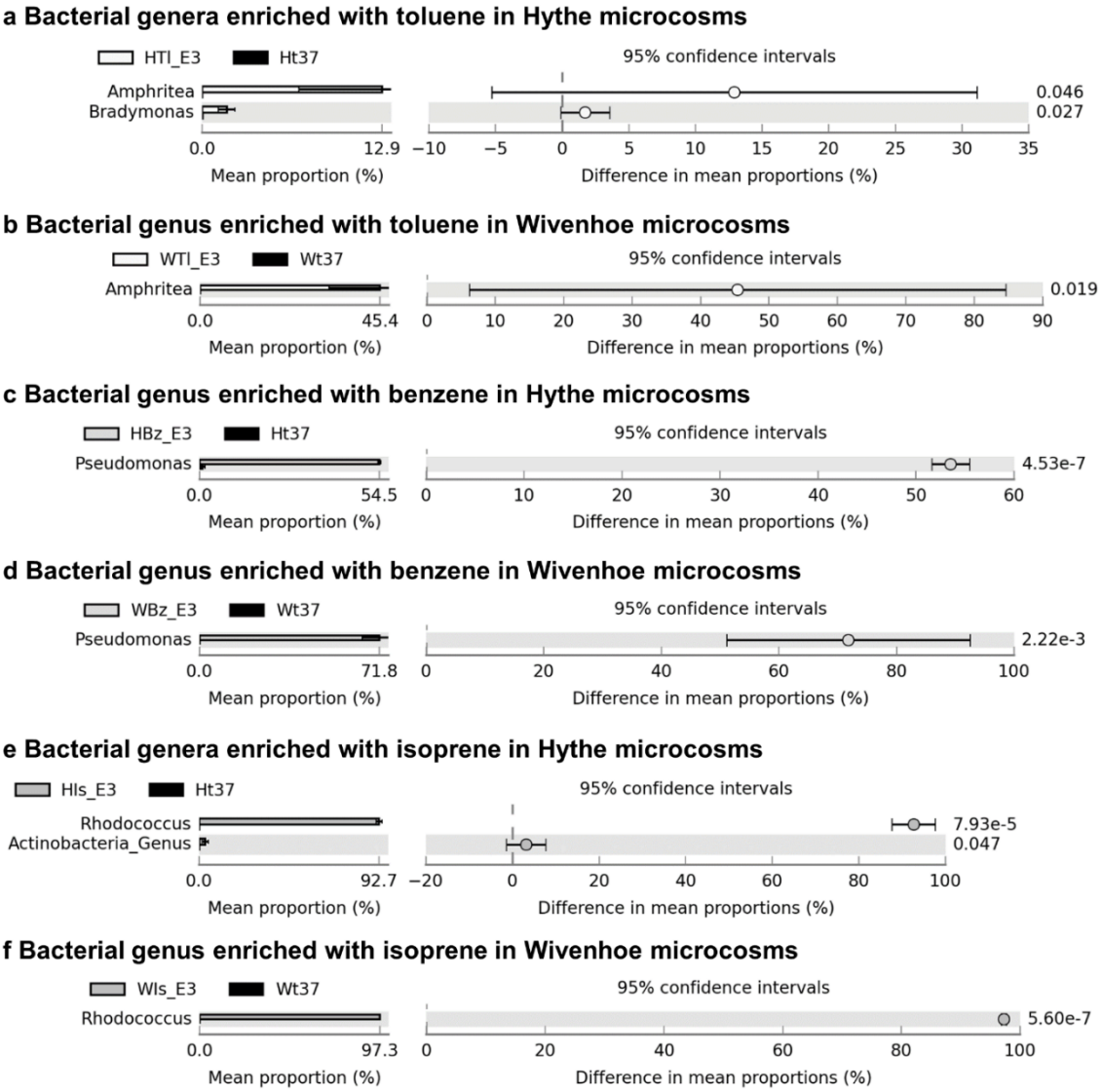


Figure 3.9. The main bacterial genera enriched with toluene (a,b), benzene (c,d) or isoprene (e,f) in aerobic enrichments relative to the no-VOC controls (Ht37, Wt37) of samples from the Colne estuary collected at the Hythe and Wivenhoe. Analysis was performed in STAMP 2.1.3 Parks et al. (2014) with default parameters, except for filtering out: *P* value < 0.05; difference between proportions < 0.2 or difference between ratios < 1.5. Data were sorted according to effect size. Significant difference in mean proportions of genera between control and test microcosms was determined by a one-sided Welch's t-test. *P value* (corrected) is shown on the right. Note the difference in the scale of the x axes. All samples presented are the mean of triplicates. Ht37 or Wt37 refers to Hythe or Wivenhoe No-VOC controls at day 37 (t37); HTl_E3 or WTl_E3 = Hythe or Wivenhoe enrichments with toluene (Tl) at third enrichment (E3); HBz_E3 or WBz_E3 refers to Hythe or Wivenhoe enrichments with benzene (Bz) at third enrichment (E3); HIs_E3 or WIs_E3 refers to Hythe or Wivenhoe enrichments with isoprene (Is) at third enrichment (E3).

Amphritea, mainly represented by OTU_7, was significantly enriched in both Hythe and Wivenhoe enrichments with propane as the sole carbon source (Figure 3.10 c & d & 3.12 c & d). *Amphritea* identified was only a small part of the bacterial community identified in Hythe (1.7%) and Wivenhoe (19.8%) (Figure 3.10c & d). OTU_7 was also enriched in microcosms with toluene as the sole carbon source and shared high sequence identity (98% identity) with *Amphritea atlantica* strain M41(Table 3.6). From the data presented, the *Amphritea* identified prefer toluene as a carbon source and grew better in the more marine Wivenhoe microcosms (Figure 3.9 a & b; Figure 3.10 c & d).

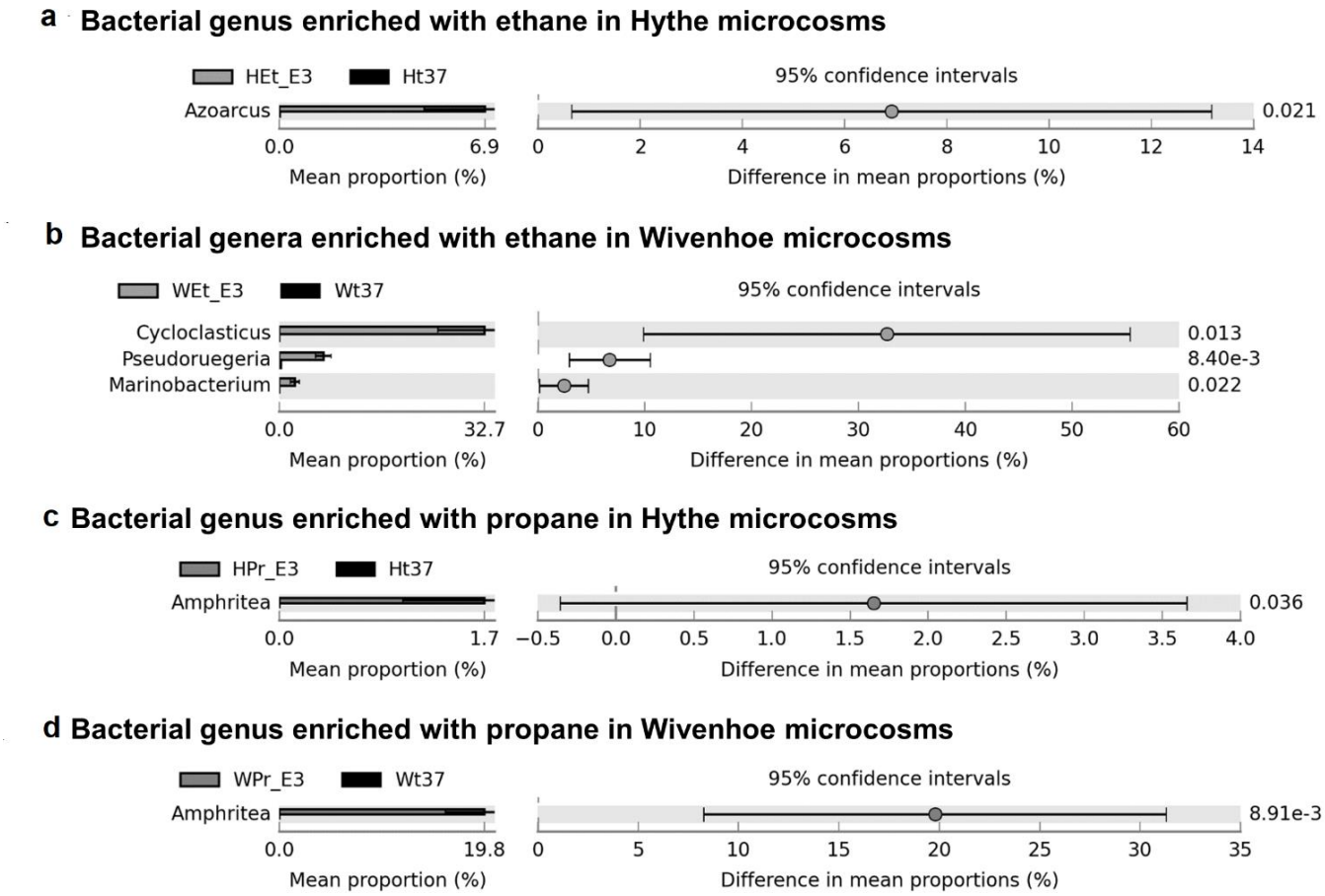


Figure 3.10. The main bacterial genera enriched with ethane (a,b) or propane (c,d) in aerobic enrichments relative to the no-VOC controls (Ht37, Wt37) of samples from the Colne estuary collected at the Hythe and Wivenhoe. Analysis was performed in STAMP 2.1.3 Parks et al. (2014) with default parameters, except for filtering out: *P value* < 0.05; difference between proportions < 0.2 or difference between ratios < 1.5. Data were sorted according to effect size. Significant difference in mean proportions of genera between controls and test enrichments was determined by a one-sided Welch's t-test. *P value* (corrected) is shown on the right. Note the difference in the scale of the x axes.All samples presented are the mean of triplicates. Ht37 or Wt37 refers to Hythe or Wivenhoe No-VOC controls at day 37 (t37); HEt_E3 or WEt_E3 refers to Hythe or Wivenhoe enrichments with ethane (Et) at third enrichment (E3); HPr_E3 or WPr_E3 refers to Hythe or Wivenhoe enrichments with propane (Pr) at third enrichment (E3).

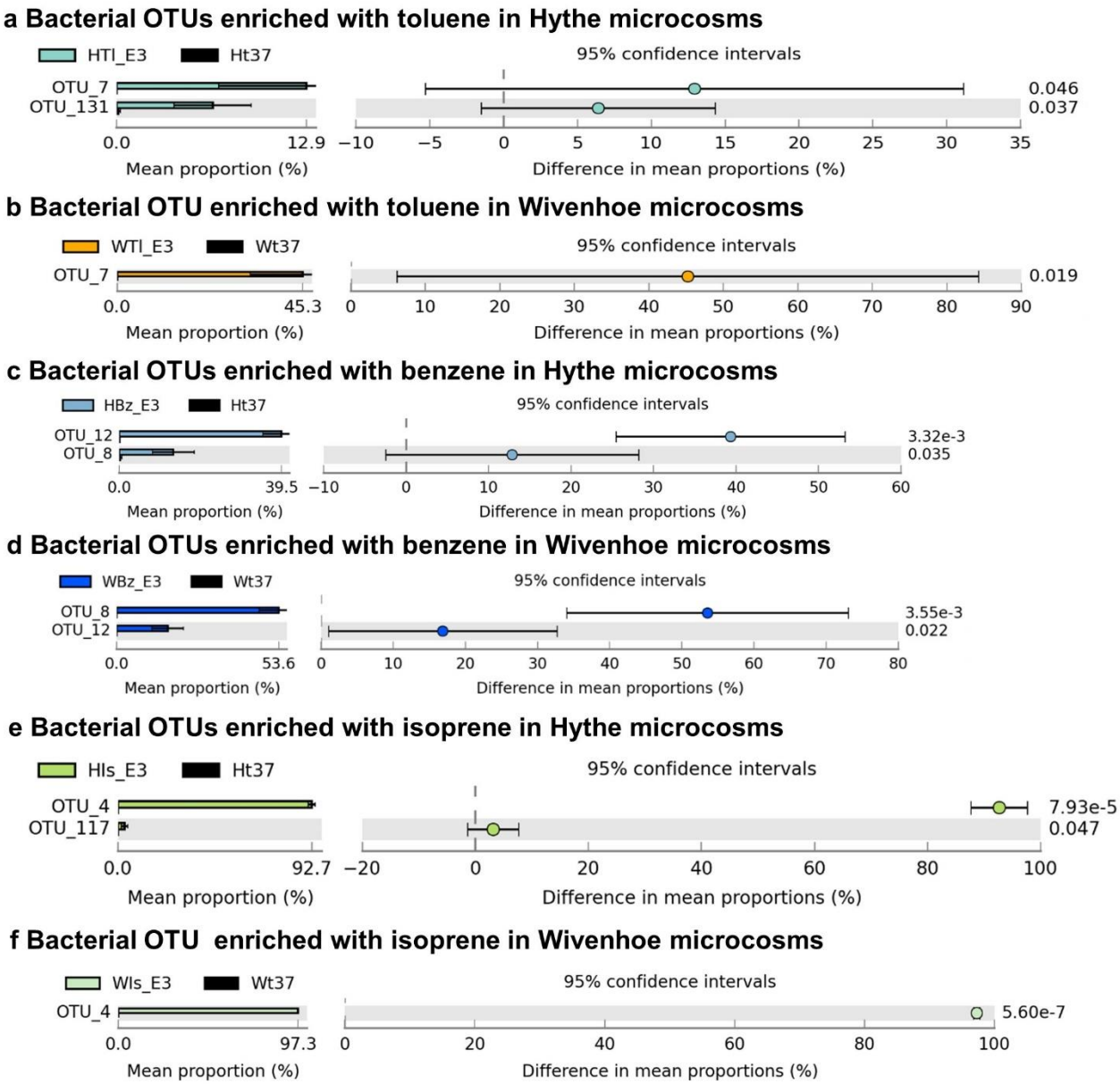


Figure 3.11. The OTUs identified in toluene (a,b); benzene (c,d); or isoprene (e,f) enrichments relative to the no-VOC controls (Ht37, Wt37). Analysis was performed in STAMP 2.1.3 Parks et al. (2014) with default parameters, except for filtering out: *P value* < 0.05; difference between proportions < 0.2 or difference between ratios < 1.5. Data were sorted according to effect size. Significant difference in mean proportions of genera between control and test enrichments was determined by a one-sided Welch's t-test. *P value* (corrected) is shown on the right. Note the difference in the scale of the x axes. All samples presented are the mean of triplicates. Ht37 or Wt37 refers to Hythe or Wivenhoe No-VOC controls at day 37 (t37); HTl_E3 or WTl_E3 = Hythe or Wivenhoe enrichments with toluene (Tl) at third enrichment (E3); HBz_E3 or WBz_E3 refers to Hythe or Wivenhoe enrichment with benzene (Bz) at third enrichment (E3); HIs_E3 or WIs_E3 refers to Hythe or Wivenhoe enrichments with isoprene (Is) at third enrichment (E3).

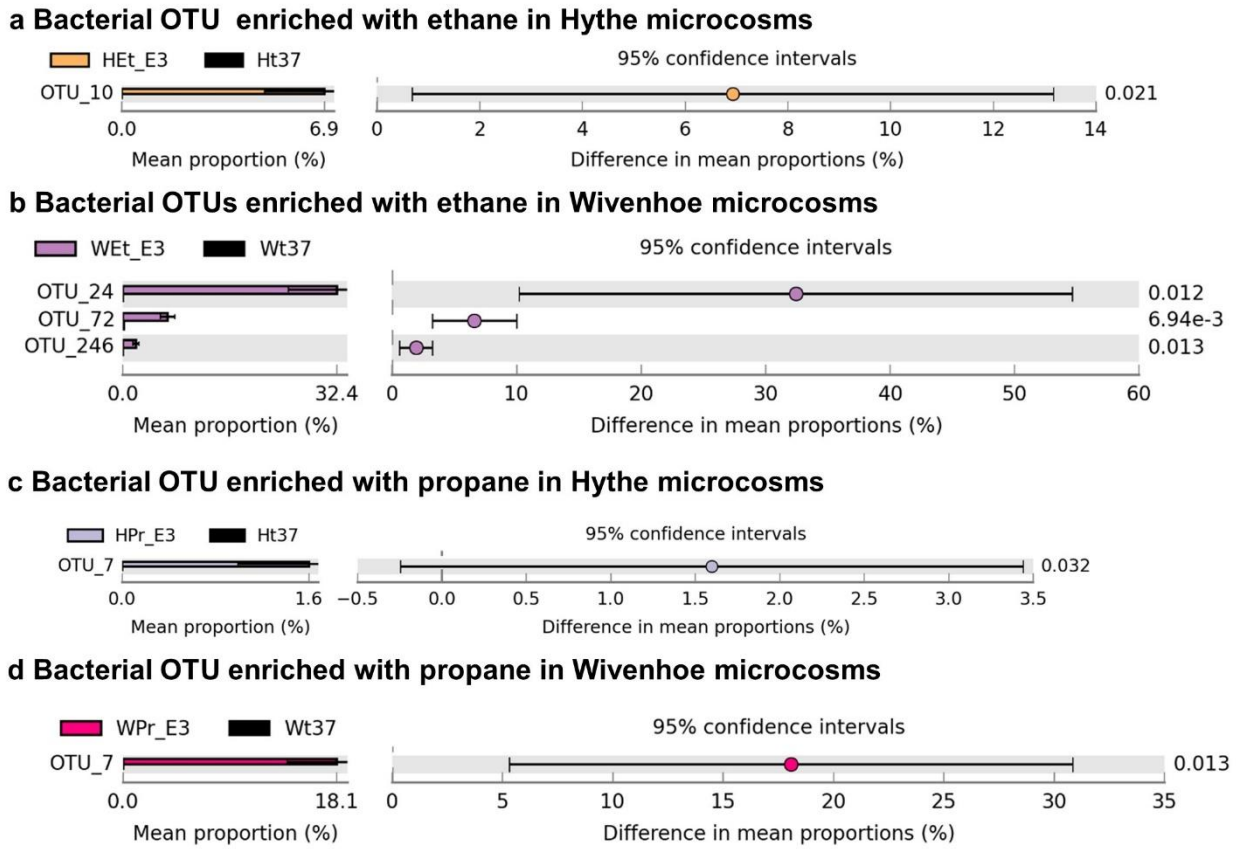


Figure 3.12. The OTUs identified in ethane (a,b) or propane (c,d) enrichments relative to the no-VOC controls (Ht37, Wt37). Analysis was performed in STAMP 2.1.3 Parks et al. (2014) with default parameters, except for filtering out: *P* value < 0.05; difference between proportions < 0.2 or difference between ratios < 1.5. Data were sorted according to effect size. Significant difference in mean proportions of genera between control and test enrichments was determined by a one-sided Welch's t-test. *P value* (corrected) is shown on the right. Note the difference in the scale of the x axes.All samples presented are the mean of triplicates (*n*=3). Ht37 or Wt37 refers to Hythe or Wivenhoe No-VOC controls at day 37 (t37); HEt_E3 or WEt_E3 refers to Hythe or Wivenhoe enrichments amended with ethane (Et) at third enrichment (E3); HPr_E3 or WPr_E3 refers to Hythe or Wivenhoe enrichments amended with propane (Pr) at third enrichment (E3).

3.3.6 Bacterial *16S rRNA* phylogeny of sequences identified in benzene, toluene, isoprene, ethane and propane enrichments obtained by Illumina sequencing and DGGE gel band sequencing

Two main bacterial phyla were enriched in this study, *Actinobacteria* and *Proteobacteria*. To understand the phylogenetic affiliation of the dominant OTUs across techniques (DGGE and MiSeq), across enrichments and with the wider literature, phylogenetic trees were constructed (Figure 3.13 – 3.15). Where possible most of the sequences used for phylogenetic analysis were type strains.

OTU_4 and OTU_117, which dominated in isoprene enrichments, were analysed with other 16S rRNA gene sequences of *Rhodococcus*. It was observed that OTU_4 was closely related to *Rhodococcus globerulus* while OTU_117 was closely related to *Rhodococcus erythropolis* and *Rhodococcus* sp. i26 (Figure 3.13). *Rhodococcus* sp. i26 was isolated from isoprene enrichments with samples collected from the Colne estuary by Acuña Alvarez et al. (2009). OTU_4 and OTU_117 also clustered with previously sequenced DGGE bands excised from DGGE profiles of isoprene enrichments (DGGE_HIs_3 & DGGE_WIs_9) shown in Figure 3.15.

OTU_10, a *Betaproteobacteria* that was identified in Hythe enrichments with ethane (Figure 3.12 a), is related to *Azoarcus communis,* but more closely related to *Azoarcus* sp. b303 that was isolated from penicillin-production waste-water treatment plant and the receiving water in China (Figure 3.14). OTU_72, an *Alphaproteobacteria* that was identified in Wivenhoe enrichments with ethane is related to *Roseibacterium elongatum* (Figure 3.14). OTU_10 and OTU_72 clustered with other sequenced DGGE bands (Figure 3.15) identified in ethane (DGGE_WEt_11) or propane (DGGE_HPr_15, DGGE_HPr_16, DGGE_HPr_17) enrichments (Figure 3.9).

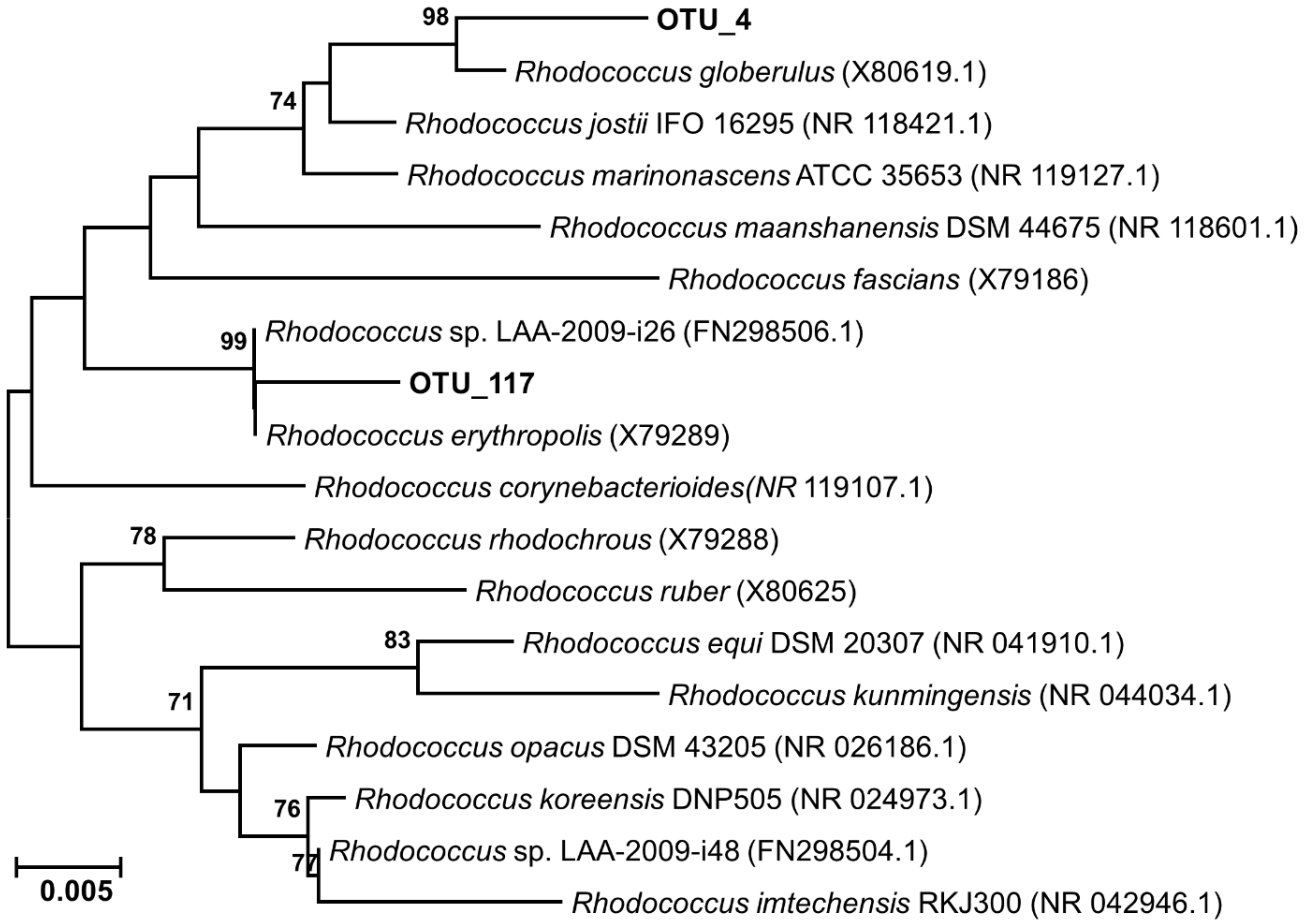


Figure 3.13. Phylogenetic relationships of OTUs related to *Rhodococcus* sp. based on *16S rRNA* gene sequence. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. Bootstrap values ≥70% are shown. Evolutionary analyses were conducted in MEGA6.

OTU_7, OTU_8 and OTU_12 were *Gammaproteobacteria* identified in enrichments with toluene, propane or benzene (Figure 3.11 – 3.12). They clustered with both cultivated and uncultured groups (Figure 3.14). OTU_7 is related to *Amphritea atlantica* strain M41; OTU_8 is related to the cultured relative *Pseudomonas flavescens*; and OTU_12 is related to *Pseudomonas borbori*. OTU_8 and OTU_12 also clustered with other sequenced DGGE bands identified from DGGE profile of benzene enrichments (DGGE_HBz_1, DGGE_WBz_4, DGGE_WBz_5, and DGGE_WBz_6) obtained from this study (Figure 3.15). OTU_131 also identified in enrichments with toluene was shown to be closely related to the cultured strains *Pseudomonas sagittaria* strain CC-OPY-1.

OTU_24 and OTU_246 were both identified in Wivenhoe enrichments with ethane (Figure 3.12 b). OTU_24 is closely related to a mix of cultured and uncultured *Cycloclasticus* sp. while OTU_246 is closely related to the cultured *Marinobacterium aestuariivivens* strain DB-1 (Figure 3.14).

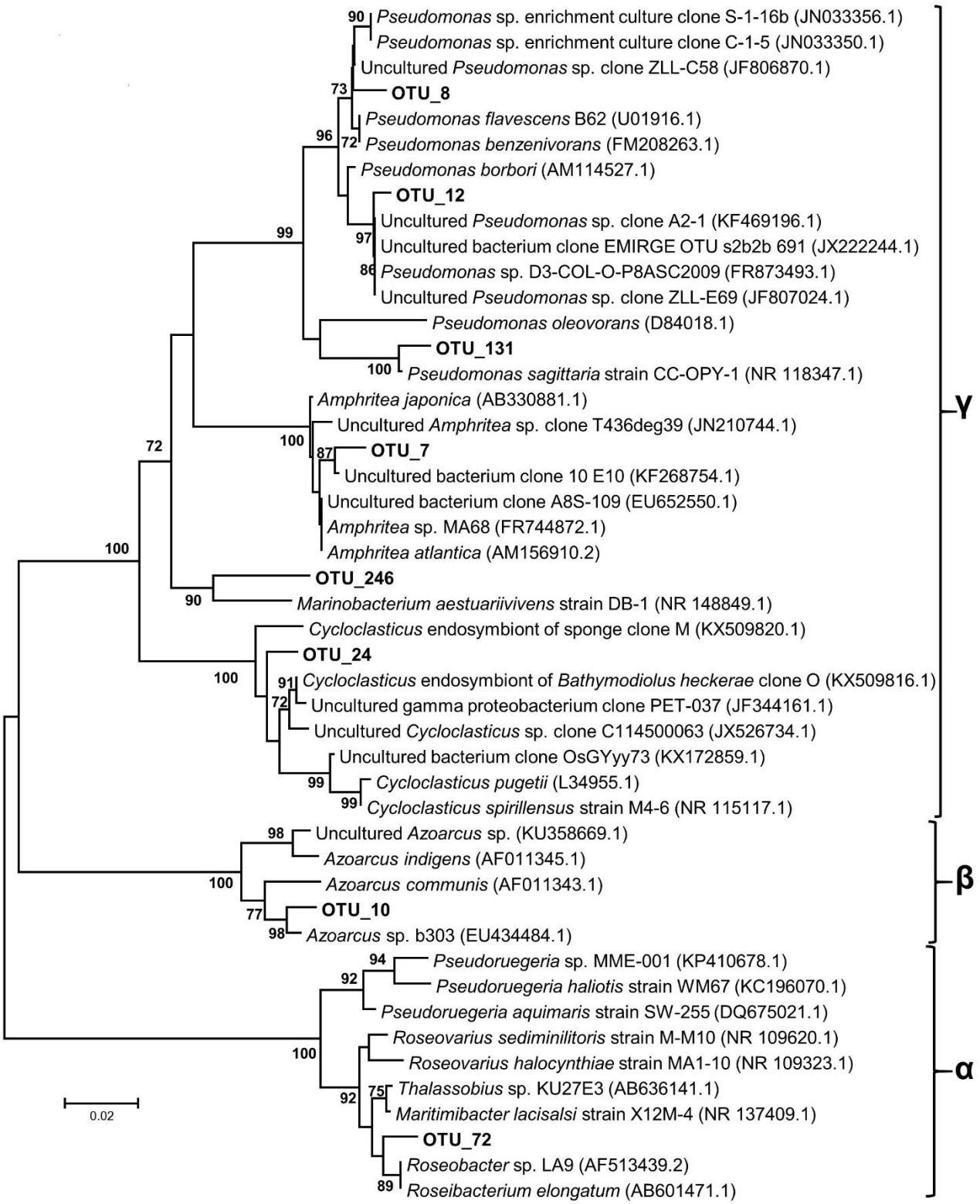


Figure 3.14. Phylogenetic relationships of *Alpha*-, *Beta*- and *Gamma- Proteobacteria* identified in aerobic enrichments with other species. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap values ≥70% are shown. Evolutionary analyses were conducted in MEGA6.

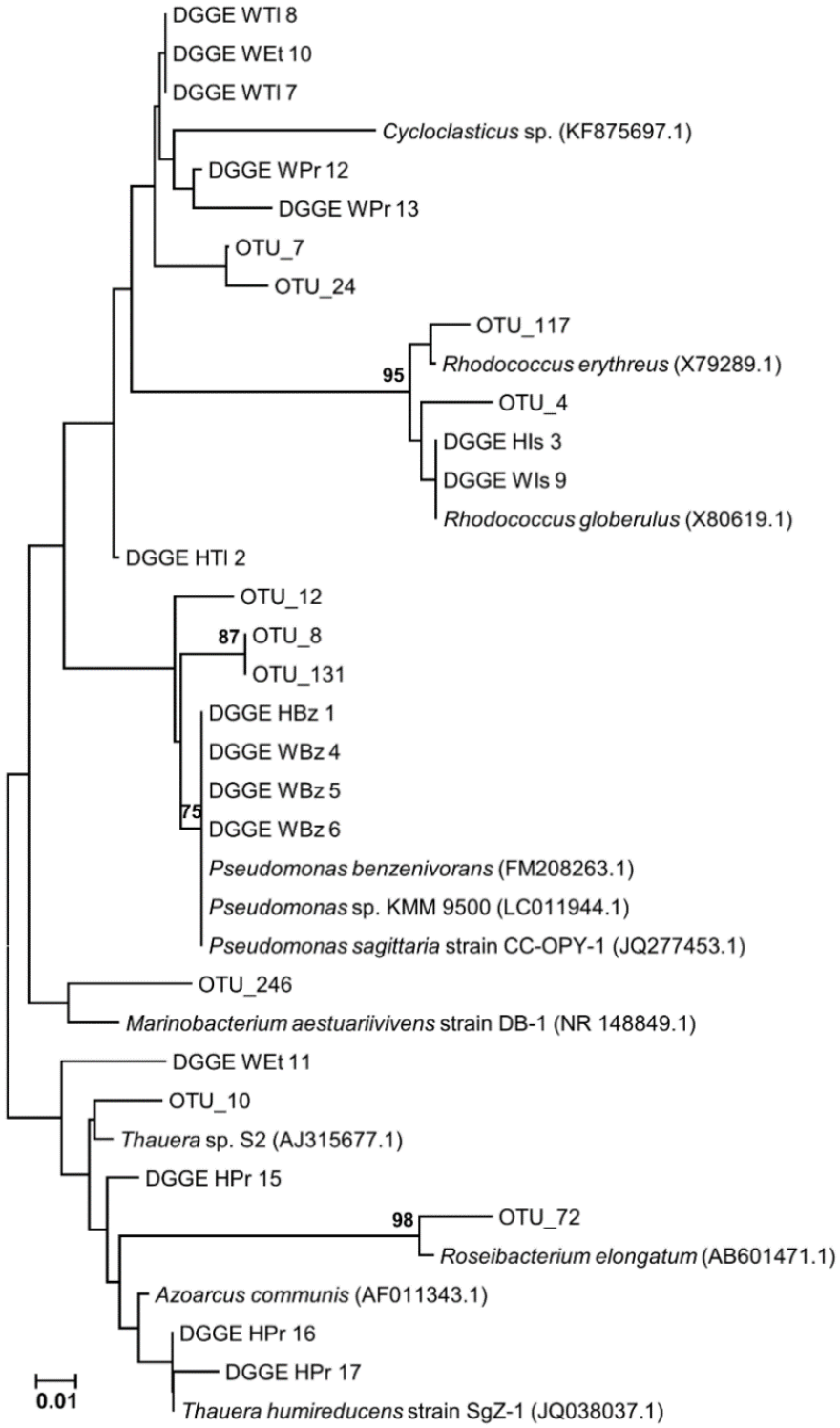


Figure 3.15. Phylogenetic relationships of taxa enriched with single substrate volatile hydrocarbons identified from this study (OTUs and DGGE bands). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. Bootstrap values ≥70% are shown. Evolutionary analyses were conducted in MEGA6.

3.4 Discussion

3.4.1 Bacterial consumption of benzene, toluene, isoprene, ethane and propane in enrichments from the Colne estuary

The biodegradation of benzene, toluene, isoprene, ethane and propane in enrichments was exemplified by the shift in bacterial community structure and diversity (Figures 3.5 – 3.8) which was likely driven by the volatile hydrocarbon degraded. OTUs (97% similarity threshold) in time 0 samples and no-VOC controls were similar but different from those enrichments in which a VOC was added (Figure 3.7). A typical example could be seen in enrichments with isoprene or benzene where OTUs identified were distinct from time 0 and the no-VOC controls. A similar shift in bacterial community structure driven by the class of hydrocarbon degraded was observed with oil-metabolizing marine bacteria from the Thames estuary (McKew et al., 2007). The corresponding reduction in bacterial diversity/composition observed at third enrichments may be due to dilution of growth factors or selection of particular organisms by the volatile hydrocarbon degraded (Figure 3.9 – 3.10). Acuña Alvarez et al. (2009) also observed a reduction in bacterial biodiversity and composition after isoprene degradation.

Biodegradation was reported as percentage of VOC remaining relative to the sterile control because the goal of this project was primarily to identify the VOC-degrading populations and not to determine absolute consumption of each carbon source. VOC removal was observed in all cases and specific taxa were identified with specific VOCs. This approach is consistent with similar studies reporting biodegradation of hydrocarbons and other organic carbon in estuarine environments (McKew et al., 2007, McKew et al., 2013, Acuña Alvarez et al., 2009). Alternatively, the degradation

data could be reported as absolute concentration. This is particularly useful when the goal of the study is to calculate consumption of substrates.

The biodegradation of all five volatile hydrocarbons over a range of concentrations (Tables 3.2 – 3.4) suggests that they would be degraded *in situ* and so represent part of the carbon pool sustaining bacterial communities in estuarine environments. Volatile hydrocarbons produced by microalgae or methanogens are biodegraded in the marine environment as a carbon and energy source (Steinke et al., 2011, McGenity et al., 2012, Booge et al., 2018, Shennan, 2006, Xie et al., 2013).Acuña Alvarez et al. (2009) showed the ubiquitous marine *n*-alkane degrading *Alkanivorax borkumensis* could rely on isoprene as its carbon and energy source in the absence of an oil spill. Other VOCs, like ethane and propane, fuel hydrocarbonolcastic populations like *Cyclocasticus* in marine environments (Rubin-Blum et al., 2017). In the Colne estuary, measurement of biogenic isoprene production by microalgae has been reported (Acuña Alvarez et al., 2009, Exton et al., 2012) but information about the other VOCs is lacking. Nevertheless, ethane, propane, benzene and toluene are commonly found in estuaries, including those in the UK (Xie et al., 2013, Yamamoto, 2014, Yamamoto et al., 1997, Dawes and Waldock, 1994).

The changes in bacterial community structure observed with VOC addition indicates an impact which may affect ecosystem functioning. A possible explanation could be succession in the community where VOC degraders increased with enrichment (Figure 3.8), while the other populations decreased in relative abundance below 1% of the community (DGGE detection limit) (Green et al., 2010, Muyzer et al., 1993). During the Deepwater Horizon event in the Gulf of Mexico, trapped propane and ethane sustained a low-diversity bacterial bloom (comprising mainly *Cycloclasticus* and *Colwellia*) that primed the bacterial community for degradation of other

hydrocarbons in the aging plume (Valentine et al., 2010). Johnston et al. (2017) reported a bacterial community change from time point 0, timepoint (12 days) and timepoint 2 (15 days) of a DNA-SIP isoprene degrading enrichment.

The occurrence of bacterial populations with the ability to degrade benzene, toluene, isoprene, ethane and propane suggests prior exposure to these VOCs in estuarine environments. Possible sources include biogenic production, anthropogenic inputs etc. Biogenic isoprene production by microphytobenthic communities has been shown in marine environment (Acuña Alvarez et al., 2009, Bonsang et al., 1992, Booge et al., 2018, Milne et al., 1995, Moore et al., 1994, Shaw et al., 2003, Shaw et al., 2010) . Ethanogenesis and propanogenesis by methanogens in sediments is most likely the source of ethane and propane in estuarine environments (Oremland, 1981, Xie et al., 2013). Exposure to benzene or toluene would have been from anthropogenic sources (e.g. industrial or household effluents) because the Colne estuary is in a residential area (Nedwell et al., 2016). Effluents from sewage treatment works (STW) located near the head of the estuary (the Hythe) may be a source of benzene or toluene into the estuary. This prior exposure is most likely the reason why benzene or toluene degradation proceeded with a short lag phase (Figure 3.2). Yamamoto et al. (1997) encountered toluene (conc. > 100 µg l^{-1}) and benzene (conc. > 10 µg l^{-1}) in all rivers of Osaka and their estuaries, mainly from domestic and industrial input. In the UK, Dawes and Waldock (1994) implicated anthropogenic sources (mainly industrial) as the source of benzene and toluene in estuaries. Benzene-containing products include detergents, gasoline, household cleaning products etc (Resource4Leukemia.com, 2012) while those with toluene are spray paints, liquid or aerosol multi-purpose cleaners, paint thinners, floor polish etc (Aller Air, 2012).

On the other hand, the bacterial populations identified could be sustained *in situ* by other carbon sources beside prior exposure to those VOCs studied. The suite of gases (including VOCs) produced in marine environments play diverse ecological roles including serving as food for heterotrophic bacterial communities (Steinke et al., 2011). That is the reason why short chain hydrocarbon (C_2 -C_4)-degrading bacterial populations are metabolically versatile possessing more than one enzyme system (Mendes et al., 2015, Redmond et al., 2010, Shennan, 2006, Van Beilen and Funhoff, 2007, Coleman et al., 2006, Leahy et al., 2003a). They are usually undetectable *in situ* unless there is an oil spill (Valentine et al., 2010, Joye et al., 2016, Joye et al., 2011, Head et al., 2006). A typical example can be seen with the rapid adaptation of bacterial communities to ethane, propane and butane consumption after exposure (Mendes et al., 2015, Valentine et al., 2010). Other well-known hydrocarbonoclastic taxa like *Alcanivorax borkumensis* are fuelled by alga-derived-VOCs like isoprene in the absence of an oil spill, (Acuña Alvarez et al., 2009). This may be why *Alcanivorax* spp are commonly found in close proximity to algae (McGenity et al., 2012, Green et al., 2004, Radwan et al., 2010).

In sediment slurries (1st enrichments), the onset of aerobic biodegradation of all volatile hydrocarbons was quicker in the Hythe than Wivenhoe presumably because the former is richer in Nitrogen (N) and Phosphorus (P) than the latter. This agrees with a previous observation by Acuña Alvarez et al. (2009). Close to the head of the estuary (the Hythe) inorganic N and P concentrations could be as high as 1 mM nitrate and 50 µM phosphate respectively (Nedwell et al., 2016). These nutrients become less concentrated towards the middle of the estuary (Wivenhoe) due to removal by biogeochemical processes including biodegradation, mixing with marine water and increased volume of water. The river and a sewage treatment work (STW)

located near the Hythe are responsible for nutrient loading in the estuary (McMellor and Underwood, 2014). Such a nutrient-rich environment at the Hythe may sustain a more active bacterial community *in situ* than in Wivenhoe.

In general, biodegradation was quicker with the second and third enrichments, indicating that VOC-utilising bacterial populations were established and responded swiftly to the availability of each VOC in fresh minimal marine medium (1/3 ONR7a). The rapid degradation without lag phase implies that enzymes required for benzene, toluene, isoprene, ethane or propane activation and consumption were induced in single-substrate microcosms during the first enrichments. All oxygenases and monooxygenases required for benzene and toluene (Cao et al., 2009); ethane and propane (Shennan, 2006) and isoprene (McGenity et al., 2018) oxidation are inducible. Other possible reasons include an increase in the number of VOC-degrading population transferred from the previous enrichment to the next, enhanced growth due to exposure to a VOC, reduction in competing populations, etc.

3.4.2 Benzene-utilising bacterial communities in the Colne estuary

This study has shown that bacterial communities inhabiting estuarine environment degrade benzene rapidly. Aerobic benzene degradation was rapid and complete within 2 – 6 days in sediment slurries. This observation is in agreement with benzene degradation in soils and aquifer material (Xie et al., 2011, Alvarez and Vogel, 1991). In aquifer material slurries with benzene as a sole carbon source, complete degradation was observed in two days following a two-day lag phase (Alvarez and Vogel, 1991). Benzene removal in soil microcosms was also observed within 2 – 6

days (Xie et al., 2011). This indicates that benzene biodegradation in estuarine environment (marine) may proceed at similar rates as terrestrial environments.

The rapid degradation of benzene over a range of concentration has the potential for application in bioremediation after an acute pollution event. At 0.005 mM concentration in sediment slurry microcosms, complete benzene degradation was observed in less than a day. This concentration is several orders of magnitude higher than benzene concentration (<0.13 nM) commonly encountered in UK estuaries (Dawes and Waldock, 1994). However, petrochemical accidents occasionally release tons of benzene into the environment (Fu et al., 2008). In 2005, approximately 100 tons of a mix of benzene, nitrobenzene and other toxic chemicals were released into the Songhua River, China, due to an explosion at a petrochemical plant (Fu et al., 2008). A combination of volatilisation, photolysis and biodegradation were required to restore the environment to its health. After volatilization and photolysis, biodegradation is usually the main environmental process that completes the restoration (Vogt et al., 2011, Rabus et al., 2016a).

Benzene degradation was generally more rapid at the low-salt, nutrient-rich head of the estuary as compared with the more marine middle of the estuary. Possible factors driving the difference were salinity and/or nutrient difference at both sites along the estuary as explained above. There is a community and legacy effect of the samples, but the cultivation conditions were similar, so degradation became the same with more enrichments (Figure 3.2). Benzene degradation rate can be influenced by several factors including salinity, pH, substrate concentration and availability, oxic or anoxic nature of the environment etc (Aburto et al., 2009). Benzene degradation was shown after a 5 weeks lag phase in microcosms made of samples from the hypersaline Great Salt Plains in north-central Oklahoma, USA

(Nicholson and Fathepure, 2005) demonstrating the impact of salinity on bacterial communities. After the lag time, complete benzene degradation was observed within 7 days in microcosms with 1, 2, and 2.5 M NaCl; up to 2 weeks with 0, 0.5, and 3 M NaCl; and 4 weeks with 4 M NaCl.

The benzene-degrading bacterial community in the Colne estuary is distinct, dominated by the *Gammaproteobacteria* genera *Pseudomonas* as evidenced by DGGE and Illumina MiSeq data. *Pseudomonas* formed 54.5% of the benzene degrading community encountered in the Hythe microcosms and 71.8% of the community in Wivenhoe. This genus is known to have species that degrade a wide range of hydrocarbons including benzene (Cao et al., 2009, Shennan, 2006, Niepceron et al., 2010), and although better known for inhabiting soil, they have been shown to inhabit estuarine environments (Niepceron et al., 2010) . Therefore, it is likely that they contribute to benzene degradation in the Colne Estuary. At the Hythe, phylotypes identified as *Pseudomonas glareae* strain KMM 9500 (DGGE data), *Pseudomonas borbori* R-20821 (OTU_12) and *Pseudomonas benzenivorans* (OTU_8) were implicated in benzene degradation. At Wivenhoe, the strains identified were *Pseudomonas helmanticensis* strain OHA11(DGGE data), *Pseudomonas flavescens* strain NBRC 103044 (DGGE Data), *Pseudomonas benzenivorans* (OTU_8), *Pseudomonas borbori* R-20821(OTU_12). Aburto et al. (2009) observed that *Pseudomonas* spp. dominated a benzene-contaminated sandstone aquifer. *Pseudomonas* sp. was also reported to dominate groundwater microcosms (Ri-) by Fahy et al. (2006). A pure culture, *Pseudomonas* sp. CFS-215, was shown to degrade all BTX including benzene (Alvarez and Vogel, 1991). The strain's ability to degrade benzene was enhanced in the presence of trace amounts of toluene

(Alvarez and Vogel, 1991). *Pseudomonas borbori* isolated from a nitrifying inoculum (Vanparys et al., 2006) is the closest cultured relative of OTU_12.

Pseudomonas benzenivorans (OTU_8) and *Pseudomonas borbori* R-20821 (OTU_12) are putative benzene degraders identified in high abundance from enrichments (Figure 3.11 c & d). *Pseudomonas benzenivorans* can grow on benzene, toluene or *para*-cresol as a sole source of carbon and energy (Lang et al., 2010). Fahy et al. (2008) isolated benzene degrading *Pseudomonas* strains closely related to *Pseudomonas borbori* from a benzene contaminated ground water aquifer. Several benzene degrading *Pseudomonas* species have been isolated and studied extensively for possible application in bioremediation of benzene polluted sites (Alagappan and Cowan, 2003, Irie et al., 1987, Kim et al., 2005, Kitayama et al., 1996, Leahy et al., 2003b). However, little is known about these putative benzene degrading *Pseudomonas* from estuarine environments. This study has shown that benzene degradation is estuarine environments is also carried out by *Pseudomonas* species. With the information provided, further studies may look at the sources and concentration of benzene in estuarine environments with *Pseudomonas* species targeted for isolation.

3.4.3 Toluene-utilising bacterial communities at the Colne estuary

Biodegradation of toluene was rapid and complete at all sites studied along the Colne estuary. It was more rapid in the second and third enrichments, which is most likely due to acclimation and proliferation of toluene degrading microbes during the first enrichment (sediment slurries). Toluene degradation in sediment slurries was complete within 3 days. Similar toluene degradation rates were reported by Alvarez et al. (1991) in aquifer material slurries. A 2-day lag phase was observed followed by

complete toluene degradation in 2 days in microcosms amended with toluene as a sole carbon source (Alvarez et al., 1991). The onset of toluene degradation was quicker than benzene at first enrichments (Figure 3.2). The rapid toluene degradation could be due to the ease of toluene activation by monooxygenase and dioxygenase enzymes in many bacterial populations including *Pseudomonas* sp and *Burkholderia* sp. (Cao et al., 2009). The benzene ring is more resistant to biodegradation due to the energy input required to overcome the resonance stability of the benzene ring caused by the stability of the pi-electron cloud (Weelink et al., 2010). The finding of this project agrees with the general knowledge that toluene is the most readily degraded simple aromatic hydrocarbon (Cao et al., 2009). *Gammaproteobacteria* was the main toluene degrading taxon identified by DGGE and MiSeq. *Amphitrea* was the main genus implicated in toluene degradation along the Colne estuary (Hythe and Wivenhoe), based on its high relative abundance in toluene-degrading enrichments, with a small proportion of *Bradymonas* and *Pseudomonas* encountered at the Hythe only (Figure 3.10). *Pseudomonas* is known for its toluene degrading capability mostly isolated from gasoline polluted environments (Cao et al., 2009) but there is no evidence of toluene or hydrocarbon degradation by *Bradymonas* to my knowledge.

Amphitrea formed 12.9% of the toluene-degrading bacterial communities at the Hythe and 45.4% in Wivenhoe, respectively. It was also identified in propane enrichments (Figure 3.10 c & d). To my knowledge, no study has reported propane or toluene degradation by *Amphritea* sp. Koo et al. (2015) found *Amphritea* as transient responders to perturbation with crude oil, in a study designed to evaluate the changes in the indigenous bacterial communities in Gulf of Mexico (GoM) coastal sediment. *Amphritea* increased in relative abundance by 2 weeks but returned to

the basal level 3 weeks after oil treatment (Koo et al., 2015). *Amphritea* could have been utilising the volatile components, which are more transient under environmental conditions, even though toluene was below detection limit in the oil according to the data provided by ZymaX Laboratory in Escondido, CA (Oil Spill Academic Task Force, 2010).

Evidence from this thesis suggests *Amphritea* is responsible for toluene and propane degradation in estuarine environments. *Amphritea* is in the order *Oceanospirillales*, an order with many putative hydrocarbon degraders, together with *Neptunomonas*, *Oceanospirillum* and *Marinobacterium* (Coulon et al., 2012, Gartner et al., 2008, Koo et al., 2015, Miyazaki et al., 2008, Yakimov et al., 2004). Most *Oceanospirillales* prefer high salt environments suggesting why the proportion of *Amphritea* identified in the toluene degrading enrichment was higher in Wivenhoe than the Hythe. Sequenced DGGE bands and OTU_7 shared very high identity with *Amphritea atlantica* strain M41 (98 – 99% identity) from Logatchev hydrothermal vent field (Gartner et al., 2008). The strain grew well at a temperature range of 4- 40^{o}C and a salinity range of 0.3- 9% (Gartner et al., 2008). The salinity gradient at the Colne estuary favour the existence of *Amphritea* at both Hythe (salinity = 0.5 - 5) and Wivenhoe (salinity = 3 - 31) (Acuña Alvarez et al., 2009, Exton et al., 2012).

3.4.4 Ethane-utilising bacterial communities in the Colne estuary

Biodegradation of ethane and propane was observed after a 14-day lag phase suggesting that short-chain alkane-degrading bacterial populations were present in low numbers at the start of the experiment. They had to acclimatize by producing enzymes required for metabolisms before the onset of biodegradation. Followed by

an increase in the abundance of degrading populations. The soluble diiron monooxygenase enzymes, such as propane monooxygenase, required for the activation of propane and ethane are inducible (Sharp et al., 2007, Kotani et al., 2006, Kotani et al., 2003, Coleman et al., 2006).

The *prmABCD* gene cluster encoding propane monooxygenase was identified in the genomes of ethane (and propane)-degrading *Rhodococcus* strain I and *Mycobacterium* strain B isolated from those enrichments (Chapter 5). This means that some bacterial species in the estuary have the genetic capacity to use ethane and propane. Such ability may be acquired by lateral or vertical gene transfer in the environment. For instance, *Rhodococcus* spp. can degrade a wide range of pollutants due to their ability to acquire catabolic genes by lateral gene transfer (Larkin et al., 2005). The onset of degradation was quicker in second and third enrichment (Figure 3.3) confirming the establishment of ethane- or propane-degrading populations.

At high concentration (10% v/v) ethane degradation was not complete (Figure 3.3, First enrichment) or took longer (119 days, Table 3.3) but at lower concentrations (1% v/v or 0.1% v/v), it was complete by day 20 in sediment slurries (Table 3.3). This implies that bacterial communities are acclimated to using ethane at lower concentrations in environment. Xie et al. (2013) reported biogenic ethane concentration in anoxic estuarine sediments of between 0.08 µmol l^{-1} to 1.8 µmol l^{-1} slurry which is several orders of magnitudes smaller than the smallest concentration (0.1% v/v) employed during this study. Higher ethane concentration could be toxic to certain members of the bacterial community thereby reducing the bacterial populations with ethane degrading potential.

Putative ethane-utilising bacterial communities were identified in ethane enrichments as revealed by MiSeq and DGGE. Previous studies focused on petrogenic ethane from polluted sites or hydrocarbons seeps (Redmond et al., 2010, Rubin-Blum et al., 2017, Dworkin and Foster, 1958). The ethane degrading bacterial community at the Colne estuary are members of *Betaproteobacteria* and *Gammaproteobacteria.* Only *Betaproteobacteria* were identified in the Hythe (salinity < 5) while both *Betaproteobacteria* and *Gammaproteobacteria* were encountered in Wivenhoe (salinity = 3 - 31). This suggest that salinity of the sampling location had an influence on the ethane-degrading bacterial communities enriched. *Azoarcus* was the main phylotype (by Illumina MiSeq data) identified in the Hythe ethane enrichment and OTU_10 related to *Azoarcus communis*, a betaproteobacterium isolated from a French refinery oily sludge (Reinhold-Hurek et al., 1993). OTU_10 also shared 95% identity with *Azoarcus olearius* isolated from an oil contaminated soil located in Kaoshiung City, Taiwan (Chen et al., 2013). Since the level of identity is low, the *Azoarcus* identified in the Colne estuary is new, utilising ethane as a source of carbon and energy. *Azoarcus* commonly isolated from oil polluted environments could be living off ethane (Reinhold-Hurek et al., 1993, Chen et al., 2013) . This strain was enriched only in Hythe microcosms, presumably because it is not typically found in marine environments (Reinhold-Hurek et al., 1993, Chen et al., 2013). *Azoarcus indigens* strain HZ5 is known to degrade the pesticide, beta-cypermethrin (Ma et al., 2013) and *Azoarcus tolulyticus* Tol-4 has been implicated with anaerobic toluene degradation (Migaud et al., 1996). However, to my knowledge, this is the first time a putative *Azoarcus* is linked to ethane degradation in an estuarine environment.

The other *Betaproteobacteria* identified in ethane enrichments were *Thauera* as revealed by DGGE. Phylotypes similar to *Thauera humireducens* strain SgZ-1 (95% identity) and *Thauera phenylacetica* strain B4P 1 (95% identity) were identified in the Hythe and Wivenhoe respectively. However, these phylotypes were not identified in high abundance from Illumina MiSeq data suggesting a limited role in ethane degradation. Members of the genera *Thauera* degrade hydrocarbons aerobically and anaerobically under nitrate reducing conditions (Cooley et al., 2009, Dubbels et al., 2009, Foss and Harder, 1998, Heider et al., 1998, Kube et al., 2004, Leuthner and Heider, 1998). *Thauera butanivorans* contain the soluble butane monooxygenase (sBMO) that degrade C2 – C9 alkanes (Cooley et al., 2009).

Putative obligate hydrocarbonoclastic bacteria were identified in ethane-enrichments for Wivenhoe including *Alphaprotobacteria* (*Pseudoruegeria* and *Roseibacterium*) and *Gammaproteobacteria* (*Cycloclasticus* and *Marinobacterium*). It suggests biogenic ethane fuels putative hydrocarbonoclastic bacteria like *Cycloclasticus* and *Marinobacterium* in estuarine environments (Yakimov et al., 2007, McGowan et al., 2004). *Cycloclasticus* was the dominant phylotype identified, forming 32.7% of the ethane degrading bacterial community, with only a small proportion of *Marinobacterium* (2.3%) and *Pseudoruegeria* (6.8%). Until recently, *Cycloclasticus* was thought to be an obligate polyaromatic hydrocarbon degrader (Yakimov et al., 2007). However, there is growing evidence that *Cycloclasticus* could be sustained by short-chain alkanes like ethane and propane (Rubin-Blum et al., 2017, Valentine et al., 2010). To my knowledge, this is the first-time *Cylcoclasticus* has been identified in an estuarine environment with ethane as a sole source of carbon. A survey of the bacterial communities to determine the impact of the Deepwater Horizon oil spill event in the Gulf of Mexico water column revealed a low-diversity bloom of ethane

and propane degrading bacteria dominated by *Cycloclasticus*, *Colwellia* and members of the *Oceanospirillaceae* (Valentine et al., 2010). *Cycloclasticus* was suspected to be utilising ethane and propane because it bloomed with their presences in the water column (Valentine et al., 2010). More recently, short-chain alkanes including ethane, propane were reported to fuels symbiotic *Cycloclasticus* in Camp Knoll (Rubin-Blum et al., 2017). Rubin-Blum et al. (2017) reported that, these *Cycloclasticus* sp. in symbiotic relationship with *BathyImodiolus heckeIrae* mussels or poecilosclerid sponges, had lost their ability to degrade PAHs. The symbiotic *Cycloclasticus* did not contain the genes that encode vital enzymes required for PAH degradation (Rubin-Blum et al., 2017). Instead, they used ethane, propane and other short chain hydrocarbons as their carbon and energy source. The *Cycloclasticus* phylotype identified in ethane enrichments shared 97% identity with a *Cycloclasticus* symbiont from an asphalt-rich, deep-sea oil seeps at Campeche Knolls in the southern Gulf of Mexico (Rubin-Blum et al., 2017). OTU_24 shared 95% sequence identity with a PAH-degrading *Cycloclasticus spirillensus* strain M4-6, isolated from marine macrofaunal burrow sediments (Figure 3.14). This strongly indicates that the ethane-degrading *Cycloclasticus* encountered at the Colne estuary is new and would merit further investigation. Previous investigators encountered PAH degrading *Cycloclasticus* in oiled mesocosms from the Colne estuary (Coulon et al., 2012). It will be interesting to know whether these ethane-degrading *Cycloclasticus* also have PAH utilising capabilities.

The putative ethane utilising phylotypes revealed by this study are new because they share low sequence identity with those on NCBI's database as exemplified by the key OTUs enriched: OTU_24 or *Cycloclasticus spirillensus* (95% identity) and OTU_246 or *Marinobacterium aestuariivivens* (94% identity). The diverse ethane

degrading community identified could imply that ethane degradation in the marine environment requires interspecies cooperation, that even in bottles there is the opportunity for niche partitioning (e.g. biofilms on the glass *versus* planktonic) or competition (McGenity et al., 2012).

3.4.5 Propane-utilising bacterial communities in the Colne estuary

Novel members of *Betaproteobacteria* and *Gammaproteobacteria* were identified as putative propane utilising taxa along the Colne estuary. Analyses of the PCR amplified and sequenced v3 region of 16S rRNA gene obtained from DGGE band revealed a partitioning of *Betaproteobacteria* at the Hythe and *Gammaproteobacteria* rRNA at the marine end of the estuary at Wivenhoe. *Thauera terpenica* strain 58Eu (98 -100% identity) and *Azoarcus communis* strain SWub3 (93% identity) were the main *Betaproteobacteria* identified in the Hythe. On the other hand, *Amphritea japonica* strain JAMM 1866 (93% identity) and *Cycloclasticus pugetii* strain PS-1, (94% identity) were the *Gammaproteobacteria* identified in Wivenhoe. Evidence obtained from the Illumina MiSeq sequenced v3-v4 region of bacterial 16S rRNA gene revealed that, *Gammaproteobacteria* was the only propane degrading taxon along the Colne estuary with *Amphritea* as a sole genus. Even though *Amphritea* formed only a small part of the bacterial community composition with a low mean proportion observed in Hythe (1.7%) and Wivenhoe (19.8 %) microcosms respectively. *Amphritea atlantica* strain M41 (98% identity) or OTU_7 was the main phylotype identified in the Hythe and Wivenhoe. *Amphritea* has hydrocarbon degrading potentials as discussed in section 3.4.3.

3.4.6 Isoprene-utilising bacterial communities in the Colne estuary

In sediment slurries, complete isoprene degradation was generally observed within the first 10 days of incubation (Figure 3.4 & Table 3.4) at 7.2 x 10^6 ppbv final concentration. The extent of isoprene degradation correlated positively with concentration (section 3.3.1). This suggests that under similar conditions, isoprene biodegradation may be directly proportional to the concentration of isoprene available. The concentrations employed in this study were shown to be more suitable for isoprene enrichments (Murphy, 2017) than the higher concentrations (89.1 ppmv) employed by Acuña Alvarez et al. (2009). Previous studies using different concentrations of isoprene observed complete isoprene degradation in microcosms within similar durations (at the Hythe in particular) (Acuña Alvarez et al., 2009, Johnston et al., 2017).

Isoprene biodegradation became more rapid from first to third enrichment suggesting putative degraders were established. At the third enrichment, complete isoprene degradation was observed by day 5 with a bacterial community change shown on DGGE profiles as dense bands (Figure 3.8, 3.5a & 3.6a). On the contrary, isoprene degradation rate was reported to decrease after the second enrichment due to a trade-off between larger inoculum of isoprene degraders with dilution of growth factors from the environment (Acuña Alvarez et al., 2009).

Isoprene-degrading communities were distinct, dominated by *Rhodococcus* sp. (*Actinobacteria*) as evidenced by DGGE and MiSeq data. *Rhodococcus globerulus* strain DSM 43954 (99% identity) was the dominant *Actinobacteria* identified in isoprene enrichments. *Rhodococcus* formed 92.7% of the bacterial community composition from the Hythe and 97.3% of Wivenhoe respectively. Another phylotype identified as *Rhodococcus erythropolis* strain ATCC 4277 was enriched in a small

proportion at the Hythe only. Acuña Alvarez et al. (2009) reported a more diverse enrichment of isoprene degraders from the Colne estuary with *Rhodococcus* dominating in sediment microcosms and *Mycobacterium* dominating in estuarine water microcosms. The impact of isoprene on the bacterial community composition was shown by a near complete elimination of other members except *Rhodococcus.* *Rhodococcus* is commonly retrieved from both terrestrial and marine isoprene enrichments (McGenity et al., 2018). *Rhodococcus* has been enriched and isolated from soils, fresh water lakes, leaves, estuarine and marine environments (Acuña Alvarez et al., 2009, Crombie et al., 2015, Johnston et al., 2017, Khawand et al., 2016, Van Hylckama Vlieg et al., 2000, Murphy, 2017) suggesting a key role in the global cycling of isoprene. However, a recent study Johnston et al. (2017) using DNA-SIP could not identify *Rhodococcus* amongst the active isoprene degraders at the Colne estuary.

Genomic and transcriptomic evidence show that *Rhodococcus* has isoprene degrading strains some of which have been isolated (Acuña Alvarez et al., 2009, Crombie et al., 2015, Johnston et al., 2017, Khawand et al., 2016). *Rhodococcus* (AD45) isolated from fresh water lakes is helping scientist understand the pathway for isoprene degradation. New *isoA* gene primers were designed for use in culture independent assays based on sequenced data generated from isoprene degrading *Rhodococcus*, *Gordonia* etc (Khawand et al., 2016). It is interesting that certain ethane and propane degrading *Rhodococcus* species from the Colne estuary do not possess the gene cluster required for isoprene metabolism (Chapter 5). Whereas, Johnston et al. (2017) isolated *Rhodococcus* and *Mycobacterium* with the ability to metabolise isoprene and propane.

3.5 Conclusion

This study has demonstrated that VOC degraders are widespread in estuarine environments and they are vital biological sinks for benzene, toluene, ethane, propane and isoprene, thereby, contributing to the biogeochemical cycling of climate-active or toxic volatile hydrocarbons. These processes are essential for mitigating the impact of VOCs on the environment by reducing the amount emitted and remediating polluted ecosystems. Without such services, our environments and its inhabitants could face the devastating consequences highlighted in section 1.3. More so, they contribute to carbon cycling in marine environments.

It shows that putative VOC-degrading bacterial communities in estuarine environments have evolved to utilize specific classes of hydrocarbons: benzene degradation by *Pseudomonas* sp; isoprene degradation by *Rhodococcus* sp; toluene and propane degradation by *Amphritea* sp. Ethane degrading communities partitioned into low NaCl-ethane degrading communities like *Azoarcus* sp. and marine-ethane degraders dominated by *Cycloclasticus* sp. Several potential volatile hydrocarbon degraders were new, and this is the first indication *Cycloclasticus* from estuarine environments degrades ethane as a carbon and energy source. These findings could have useful biotechnological applications in bioremediation of petroleum hydrocarbon-polluted environments.

Chapter 4. Anaerobic Degradation of Benzene, Toluene, Isoprene, Ethane and Propane

4.1 Introduction

Anaerobic (absence of oxygen) locations are prevalent in marine sediments. This is because oxygen is readily removed from the surface through metabolic processes making the bottom anaerobic. In the Colne estuary, <1 mm of the top sediment at the head and 4 – 5 mm near the mouth, is aerobic. (Nedwell et al., 2016). The rest of the sediment that lie beneath the aerobic zone is anaerobic where only microbes (mainly bacteria and archaea) can thrive (Hansson et al., 2018). Anaerobic zones are widely distributed in nature and they have been studied in the Gulf of Mexico, Guaymas Basin, Hydrate Ridge, Gulf of California, Mediterranean lagoons, Baltic sea etc to understand carbon cycling (Kniemeyer et al., 2007, Jaekel et al., 2013, Laso-Perez et al., 2016, Musat and Widdel, 2008, Hansson et al., 2018, Broman et al., 2017a, Broman et al., 2017b). Hydrocarbons are abundant in these anaerobic environments where they serve as a carbon and energy source for some populations. (Rabus et al., 2016a).

Anaerobic biodegradation of hydrocarbons is a crucial natural process that contributes to carbon and sulphur cycling in the marine environment. Unlike aerobic degradation which proceeds rapidly and requires oxygen for both substrate activation and as an energetically rich terminal electron acceptor, anaerobic degradation is slow and requires alternative means of activation (e.g. fumarate addition, methylation, carboxylation and hydroxylation) and terminal electron acceptors, such as sulfate (SO_4^{2-}), iron(III) or nitrate (NO_3^-) (Widdel and Rabus,

2001). Sulfate is the dominant terminal electron acceptor in marine sediments and has been widely used in previous studies (Kniemeyer et al., 2007, Jaekel, 2011, Jaekel et al., 2013).

The anaerobic degradation of most hydrocarbons is poorly understood. To date, for ethane and propane, only sulfate-reducing bacterial communities have been reported, and strain BuS5 is the only pure culture available with the ability to utilize propane or butane *in vitro* (Kniemeyer et al., 2007, Musat, 2015). Information on anaerobic isoprene degradation is scarce, whereas, benzene and toluene degradation have been studied extensively with novel and uncultured anaerobic benzene and toluene degrading microbial communities discovered (Rabus et al., 2016b). Anaerobic toluene degradation is very rapid compared to the biodegradation of benzene with a doubling time of approximately 6 h for the most rapid toluene degrader (Widdel et al., 2010).

Most anaerobic hydrocarbon-degrading microbial communities use a narrow range of hydrocarbons with varying chain length. It is uncommon to obtain a group of organisms with an ability to degrade both aromatic and aliphatic hydrocarbons. To date, members of *Proteobacteria* and *Firmicutes* are the main groups well known to degrade propane, benzene, and toluene coupled with sulfate reduction in the marine environment (Kniemeyer et al., 2007, Widdel et al., 2010).

Studies on the anaerobic degradation of isoprene and other short-chain hydrocarbons in estuarine environments are rare. The goal of this study was to fill this gap in knowledge.

Aim

To investigate the anaerobic biodegradation of ethane, propane, benzene, toluene and isoprene in the Colne estuary, UK, particularly to identify key bacterial communities involved with the biodegradation of these volatile hydrocarbons using cultivation-independent approaches.

Hypotheses

a. The type of VOC degraded will determine the composition of the VOC-degrading microbial community.

b. Anaerobic degradation of hydrocarbons will be slower and more stochastic than aerobic degradation.

Specific Objectives

4.1 To measure the rates of anaerobic degradation of benzene, toluene, isoprene, ethane and propane in sediment slurry microcosms supplemented singly with the hydrocarbons using samples from three different locations along the Colne Estuary.

4.2 To identify the bacterial and archaeal (if any) populations enriched in the presence of benzene, toluene, isoprene, ethane and propane using cultivation-independent techniques, DGGE and Illumina MiSeq analysis of PCR-amplified 16S rRNA genes.

4.2 Materials and Methods

4.2.1 Overview of experiments

Three experiments were carried out to investigate the anaerobic degradation of benzene, toluene, isoprene, ethane and propane. The first experiment was a trial experiment, referred to as the “anaerobic trial experiment”; the second experiment was referred to as the “main anaerobic degradation experiment”, while the third experiment was referred to as the “simple nitrogen-flushing experiment” (Figure 4.1). The goal of the trial and main anaerobic experiments was to understand the fate of individual carbon sources in anoxic sediments. The simple nitrogen flushing experiment was a side-by-side experiment set up to investigate isoprene degradation in sediment slurries under the headspace of nitrogen or oxygen.

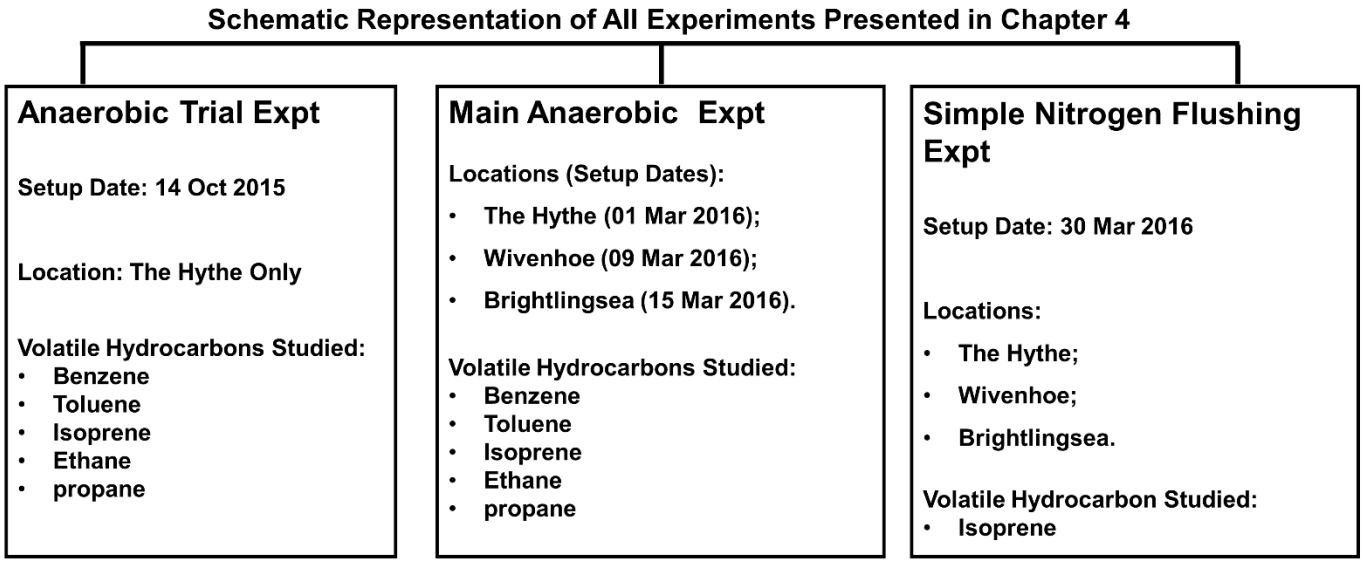


Figure 4.1. Summary of the anaerobic and simple nitrogen flushing experiments performed in chapter 4.

4.2.2 Anaerobic experiment set up and microcosm design

The experiment was set up in triplicate with microcosms established in 125-ml serum bottles and incubated at 20^{o}C, the approximate temperature of the sampling location. Four sets of microcosms made up of test microcosms and three controls were established. The killed control was set up to monitor abiotic loss of VOC over time; a no-VOC control to monitor microbial community change over time without the influence of carbon source and to ensure any community change in test microcosms was due to hydrocarbon addition; and an anaerobic media control to monitor oxygen ingress over time.

4.2.2.1 Sample collection and processing for the anaerobic trial experiment

Sediment samples were collected from the head of the Colne estuary at the Hythe only in October 2015 (Figure 4.1). Plastic cores (9.8 cm high and 6.5 cm in diameter) were used to collect sediment samples at low tide. Three sediment cores were collected within 1 m^2. The cores were transported intact inside ice boxes to the laboratory.

The sediment samples were partially processed on the bench and in an anaerobic cabinet (Whitley Anaerobic Workstation MG500+TG Airlock). The top 1 cm of each core was sliced off and sub-cores were taken using a 50 ml syringe with the tip removed into a sterile 500-ml beaker. Sub-cores from the three cores taken within 1 m^2 were pooled into the beaker, weighed and transferred into the anaerobic cabinet. Sterile anaerobic medium was added to the sediment mud samples (4-part anaerobic medium to 1-part sediment mud) and homogenized with a sterile spatula then stirred continuously with a sterile magnetic flea and magnetic stirrer. The

homogenized mixture was referred to as the master slurry. With a sterile wide-bore 25-ml pipette, 20 ml aliquots of the sediment slurry were added to sterile 125-ml serum bottles (microcosms). Sediment samples to be used as killed control were collected earlier, processed as described above and subjected to a series of autoclaving (121^{o}C for 20 min) as described by Acuña Alvarez et al. (2009). The killed controls were autoclaved three times with a day between each autoclaving lot, incubated on the bench at room temperature, before the next autoclaving in the series. They were autoclaved three times to destroy vegetative cells and spores. The interval between autoclaving was to allow for spore germination at room temperature. The microcosms were ready for gassing before addition of carbon source.

Both flushing and gas-evacuation gassing techniques were used. Initially, headspace of each microcosm was flushed for 1 minute. Using a vacuum pump, gases were evacuated from microcosms and refilled with nitrogen. This procedure was repeated twice. Finally, microcosms were filled with N_2/CO_2 (80/20) to overpressure, 1.5 bar. Then, all microcosms were ready for the addition of the carbon source. Gas filters (Minisart-Plus filters pore size 0.2 µm, Sigma) were used always to prevent contamination.

Carbon sources were added singly to test microcosms and killed control as described below (section 4.2.2.3). They were incubated at 20^{o}C alongside no-VOC controls and the media controls. The media control contained 20 ml anaerobic media. Biodegradation of individual hydrocarbons was monitored by GC-FID as described in section 4.2.4.

Subsequent enrichment series were made by transferring 5 ml from the previous enrichment (first) into 45 ml of anaerobic media, following the gassing procedure described earlier followed by the addition of a single carbon source. Sterile controls (rather than controls with killed sample) were used to monitor abiotic loss from second enrichments. They were prepared in the same way as the test bottles but contained 50 ml of anoxic media and no inoculum. Sterile controls were autoclaved as described by Acuña Alvarez et al. (2009) before gassing and addition of carbon source.

4.2.2.2 Sample collection and processing for the main anaerobic degradation experiment

Sediment samples were collected along the Colne estuary from three locations in March 2016 (Figure 4.1). They were collected from the head (the Hythe), mid (Wivenhoe) and mouth (Brightlingsea) of the estuary. Experiments were set up independently for each sampling location: the Hythe (1 March), Wivenhoe (9 March); and Brightlingsea (15 March) as outlined in Figure 4.1.

Before collecting sediment cores, surface sediments (at least the top 2 cm) were removed to expose anaerobic sediment mud. Plastic cores (9.8 cm high and 6.5 cm in diameter) were used to collect sediment samples at low tide. Replicates cores were collected at each location i.e. 1 core within 1 m^2 = sample a; 1 core within 1 m^2 = sample b; 1 core within 1 m^2 = sample c. Samples a, b and c were ~10 m apart at the same tidal height. The cores were transported intact inside ice boxes to the laboratory.

The samples were partially processed on the bench and in an anaerobic cabinet as described above (section 4.2.2.1). With a sterile wide-bored 25-ml pipette, 50 ml aliquots of the sediment slurry were added to sterile 125-ml serum bottles (microcosms). Sediment slurry was stirred throughout the time of pipetting to ensure homogeneity. Samples to be used as killed controls were collected earlier and prepared as described above. All microcosms were ready for gassing before addition of carbon source. The gassing was done as described in section 4.2.2.1. Carbon sources were added to test microcosms and killed controls as described in section 4.2.2.3. Test microcosms, killed controls, no-VOC controls and media controls were incubated at 20^{o}C. Degradation of individual hydrocarbons was monitored by GC-FID as described in section 4.2.4. Calibration standards for the five volatile hydrocarbons (ethane, propane, benzene, toluene, and isoprene) were prepared in sterile distilled water with equal liquid/headspace ratio and were held at 20^{o}C. GC-FID was calibrated with standards at each measurement. Headspace concentration of each volatile hydrocarbon was measured alongside the triplicate killed control at each time point with GC-FID. Each sample measurement was done in duplicate.

4.2.2.3 Addition of carbon sources to microcosms

All microcosms were amended with a single carbon source. Benzene and toluene were added neat to give a final concentration of 0.5 mM in microcosms. To a 50-ml microcosm, 2.2 µl of benzene was added neat using a 10 µl gas tight glass syringe to achieve 0.5 mM final concentration. For toluene, 2.7 µl was added neat to a 50-ml microcosm using a gas tight glass syringe to give a final concentration of 0.5 mM. Isoprene gas (100 µl) from the isoprene stock headspace was added to give a final

concentration of 7.2 x 10^6 ppbv. The volume of propane or ethane gas added was 12.5 ml to give a final concentration of 10% v/v propane or ethane in the microcosms.

4.2.2.4 Anaerobic media

The anaerobic medium was prepared by combining the basal medium with seven different stock solutions (Widdel, 2010). In part, the medium was heat sterilised and in part filter sterilized. The anaerobic medium contained per litre of anoxic water: NaCl,12 g; $MgCl_2.6H_2O$, 2 g; $CaCl_2.2H_2O$, 0.15 g; NH_4Cl ,0.3 g; KH_2PO_4, 0.2 g; KCl, 0.5 g; Na_2SO_4, 4 g; Resazurin (0.5 g l^{-1}), 1 ml; Trace elements A (non-chelated),1 ml; Trace elements B, 1 ml; $NaHCO_3$-solution (1 M), 30 ml; Vitamin mixture,1 ml; Thiamine solution,1 ml; B12-solution, 1 ml; Na_2S-solution (0.2 M), 5 ml. The pH was adjusted to between 7.1 – 7.6 with 1 M HCl.

The basal medium contained per litre of anoxic water: NaCl, 12 g; $MgCl_2.6H_2O$, 2 g; $CaCl_2.2H_2O$, 0.15 g; NH_4Cl, 0.3 g; KH_2PO_4, 0.2 g; KCl, 0.5 g; Na_2SO_4, 4 g and Resazurin (0.5 g l^{-1}),1 ml. It was heat sterilized by autoclaving at 121^{o}C for 15 min.

Trace elements mixture A (non-chelated) contained: distilled water, 987 ml; HCl (25% = 7.7 M), 13 ml; H_3BO_3, 10 mg; $MnCl_2.4H_2O$, 5 mg; $FeSO_4.7H_2O$, 2100 mg; $CoCl_2.6H_2O$, 190 mg; $NiCl_2.6H_2O$, 24 mg; $CuCl_2.2H_2O$, 2 mg and $ZnSO_4.7H_2O$, 144 mg. Trace elements mixture B contained: distilled water, 1000 ml; NaOH, 400 mg; $Na_2SeO_3.5H_2O$, 6 mg; $Na_2MoO_4.2H_2O$, 36 mg; $Na_2WO_4.2H_2O$, 8 mg. The trace elements were autoclaved anoxically under N_2.

Bicarbonate ($NaHCO_3$) solution (1 M) was prepared by dissolving 84 g of $NaHCO_3$ in 500 ml distilled water then made up to a final volume of 1000 ml and autoclaved

under a headspace of CO_2. The sodium sulfide solution (0.2 M) was made by dissolving 48 g of $Na_2S.9H_2O$ in 1 l of distilled water by stirring under N_2 atmosphere. The solution was autoclaved under a headspace of nitrogen.

All the vitamin solutions were filter-sterilized (Minisart-Plus filters pore size 0.2 µm, Sigma). Three separate vitamin solutions were prepared as described by Widdel (2010). Vitamin mixture contained: NaH_2PO_4 + Na_2HPO_4 (10 mM total P; pH 7.1), 100 ml; 4-Aminobenzoic acid, 4 mg; D(+)-Biotin,1 mg ; Nicotinic acid ,10 mg; D(+)-Pantothenic acid, Ca-salt, 5 mg and Pyridoxine dihydrochloride; 15 mg. The thiamine solution contained: H_3PO_4 + NaH_2PO_4 (10 mM total P; pH 3.4), 100 ml and ThiamineHCl, 10 mg. The Vitamin B_{12} Solution contained: distilled water, 100 ml and cyanocobalamin, 5 mg.

4.2.3 Sample collection, processing and set up of the simple nitrogen flushing experiment

This experiment was set up at the end of March 2016 (Figure 4.1). Sediment samples were collected from the head (the Hythe), mid (Wivenhoe) and mouth (Brightlingsea) of the Colne estuary on the same day to investigate isoprene biodegradation under near *in-situ* conditions with no basal medium added. Microcosms were amended with isoprene as a sole source of carbon and energy.

Plastic cores (9.8 cm high and 6.5 cm in diameter) were used to collect sediment samples at low tide. Replicates cores were collected at each location i.e. 3 cores within 1 m^2 = sample a; 3 cores within 1 m^2 = sample b; 3 cores within 1 m^2 = sample c. Samples a, b and c were ~10 m apart at the same tidal height. The cores were transported intact inside ice boxes to the laboratory.

Without cutting off the top 1cm of cores, sub-cores were taken using a 50 ml syringe and transferred into sterile beakers. Sub-cores of the three sediment cores collected within 1 m^2 were pooled into one beaker, weighed and sterile distilled water was added to the sediment mud samples (1-part sterile distilled water to 1-part sediment mud) and homogenized. With a sterile wide-bore 25-ml pipette, 10 ml aliquots or 50 ml aliquots of the sediment slurry were transferred to sterile 125-ml serum bottles. Serum bottles with 10 ml sediment slurries were for aerobic microcosms while those with 50 ml sediment slurries were for anaerobic microcosms (Nitrogen flushed).

Anaerobic microcosms (headspace approximately 75 ml) were flushed with nitrogen for 10 minutes before the addition of carbon source, whereas aerobic microcosms had a bigger headspace (approximately 105 ml) and they were not flushed with nitrogen. Microcosm were flushed by a continuous flow of nitrogen through its headspace and out through an outlet (syringe needle plugged into the rubber septa). Gas filters (Minisart-Plus filters pore size 0.2 µm, Sigma) were used to prevent contamination.

Two sets of controls were used: a sterile control to monitor abiotic loss of isoprene and a no-VOC control to ensure microbial community change was due to hydrocarbon addition. Serum bottles containing 10 ml and 50 ml distilled water were sterilized by autoclaving (121^{o}C for 20 min) before addition of carbon source as described by Acuña Alvarez et al. (2009). These were referred to as sterile controls for aerobic and anaerobic microcosms, respectively. Sterile controls for anaerobic microcosms were flushed with nitrogen for 10 minutes as described above before addition of isoprene. No-VOC controls were established in 125-ml serum bottles with 10 ml sediment slurries for aerobic microcosm and 50 ml sediment slurries for anaerobic microcosm without addition of isoprene.

All microcosms were set up in triplicate and incubated at 12^{o}C. Isoprene degradation was monitored by GC-FID as described in section 4.2.4. Microbial community analysis was carried by Illumina MiSeq as described in section 3.2.4 – section 3.2.7 (Chapter 3) and section 4.2.6, when isoprene concentration in the headspace was below detection limit (aerobic) or at the end of the experiment (anaerobic).

4.2.4 GC-FID analysis

Degradation was monitored using GC-FID (section 2.3). An aliquot (100 µl) of the headspace gas was collected and injected manually with a gas tight SGE syringe. Test microcosms, controls and calibration standards were analysed at each measurement. Syringes were flushed with nitrogen between measurements.

4.2.5 Microbial community analysis

To identify the bacterial and archaeal communities enriched with volatile hydrocarbons (benzene, toluene, isoprene or propane), Illumina MiSeq sequencing was used. DGGE analyses of PCR amplified bacterial 16S rRNA gene was done for a few samples obtained during the anaerobic trial experiment as described in section 3.2.3 (Chapter 3). The extraction of the total community nucleic acid was done by the hexadecyltrimethylammonium bromide (CTAB) method (Griffiths et al., 2000) described in section 2.7, Chapter 2.

4.2.6 Illumina bacterial and archaeal 16S rRNA amplicon sequencing library preparation

To understand the bacterial and archaeal composition and community structure, the variable V3 and V4 regions of archaeal and bacterial *16S rRNA* gene was sequenced on the Illumina MiSeq platform, from time-0, No-VOC controls and selected samples (with evidence of anaerobic degradation). The bacterial 16S rRNA amplicon library preparation was done as described in section 3.2.4 (Chapter 3). Therefore, only details of the Archaeal library preparation are described here.

The Archaeal 16S rRNA amplicon sequencing library preparation was performed following Illumina (2013) protocol.

Archaeal 16S rRNA gene were amplified using primer pairs with Illumina overhang adapter sequences. The Archaeal 16S rRNA primer pair used were 344 F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACGGGGYGCAGCAGGCGCGA - 3') (Raskin et al., 1994); and 915R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTGCTCCCCCGCCAATTCCT-3') (Stahl and Amann, 1991). PCR was carried out to amplify bacterial and archaeal *16S rRNA* gene from samples using similar volumes as described in section 3.24 (Chapter 3), except with a different thermocycling programme for archaea. The thermal cycling program for amplification of archaeal 16S rRNA was as follows: an initial denaturation at 95^{o}C for 5 min and 32 cycles at 95^{o}C for 45 s, 60^{o}C for 45 s, and 72^{o}C for 60 s, with a final extension step at 72^{o}C for 5 min. PCR products were held at 4^{o}C. Then 5 µl of PCR product from each reaction was run on agarose gel (1%) electrophoresis to confirm amplification. The PCR products obtained were purified using Agencourt AMPure XP PCR purification beads

(Beckman Coulter UK Ltd, High Wycombe, UK) following Illumina's "16S Metagenomic Sequencing Library Preparation" document (Illumina, 2013).

Nextera XT dual indexing strategy was used for the Indexing PCR step. Nextera XT Index Kit V2 set D for 96 indexes, 384 samples (FC-131-2004) was supplied by Illumina Cambridge, Ltd, UK. Each 50 µl reaction had 5 µl of bead purified PCR product (DNA), 5 µl of sample specific Nextera XT Index Primer 1 (N716, N718, N719, N720, N721, N722, N723, N724, N726, N727, N728, N729), 5 µl of sample-specific Nextera XT Index Primer 2 (S513, S515, S516, S517, S518, S520, S521, S522), 25 µl of REDTaq® ReadyMix™ (Sigma-Aldrich Co.) and 10 µl of PCR Grade water. The thermal cycling conditions were as follows: an initial denaturation at 95^{o}C for 3 min and 8 cycles at 95^{o}C for 30 s, 55^{o}C for 30 s, and 72^{o}C for 30 s, with a final extension step at 72^{o}C for 5 min. PCR products were held at 4^{o}C. The resulting PCR products were purified using Agencourt AMPure XP PCR Purification beads (Beckman Coulter UK Ltd, High Wycombe, UK). PCR product (5 µl) from each reaction was used for agarose gel (1%) electrophoresis to confirm index addition. The library quantification, normalization and pooling were done as described in section 3.2.5 (Chapter 3). Sequencing on MiSeq system and bioinformatics analyses were also performed as described in sections 3.2.6 and 3.2.7 of Chapter 3, respectively. Except, only forward reads were used during bioinformatics analyses of Archaea sequences due similar reasons cited by Clark et al. (2017). The forward and reverse sequences were unable to be pair-end aligned due to the length of the amplicon. All analyses were based on forward sequences only. The quality control of the MiSeq data is described in section 3.2.7 (Chapter 3). Further downstream analyses were done in Statistical Analysis of Metagenomics Profiles (STAMP) (Parks et al., 2014).

4.2.7 DNA sequence and phylogenetic analysis

OTUs of interest were compared with those on the nucleotide database at the National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Jukes-Cantor distance and Neighbour-Joining methods were used to create phylogenetic trees for partial 16S rRNA genes in MEGA 6 with bootstrap values of 10000 (Jukes and Cantor, 1969, Tamura et al., 2013).

4.2.8 Statistical analysis

Analyses of hydrocarbon degradation and OTUs (97% similarity threshold) abundance done as described in section 2.8.

4.3 Results

To understand the anaerobic degradation of benzene, toluene, propane, ethane or isoprene in estuarine sediments, two anaerobic experiments (trial and main) were performed in single-substrate microcosms with sediment samples collected from the Colne estuary, UK.

Table 4.1 Degradation of VOCs in anaerobic enrichments from the Colne estuary. Final concentrations in enrichments: benzene and toluene = 0.5 mM; isoprene = 7.2 x 10^6 ppbv; ethane and propane = 10% v/v. Hythe, Wivenhoe and Brightlingsea were the sampling locations on the Colne estuary. SE= standard error of the mean.

VOC	Experiment (sampling location)	Enrichment	Duration of incubation (days[a])	% Degraded (± SE)	MiSeq Sequenced (Yes/No)
Benzene	Trial (Hythe)	First (*n*=3)	14	100 (± 0.1)	No
	Trial (Hythe)	Second (*n*=3)	58	100 (± 0.0)	Yes
	Trial (Hythe)	Third (*n*=3)	287	47 (± 8.4)	No
Toluene	Trial (Hythe)	First (*n*=3)	14	100 (± 0.0)	No
	Trial (Hythe)	Second (*n*=3)	58	100 (± 0.0)	No
	Trial (Hythe)	Third (*n*=3)	30	100 (± 0.0)	No
	Main (Hythe)	First (*n*=3)	56	100 (± 0.0)	Yes
	Main (Hythe)	Second (*n*=2)	157	16 (± 3.4)	No
	Main (Wivenhoe)	First (*n*=3)	41	100 (± 0.0)	No
	Main (Wivenhoe)	Second (*n*=3)	157	0 (±.0.0)	No
	Main (Brightlingsea)	First (*n*=3)	35	100 (± 0.0)	No
	Main (Brightlingsea)	Second (*n*=3)	157	11 (± 5.4)	No
Isoprene	Trial (Hythe)	First (*n*=3)	124	58 (± 3.1)	Yes
	Trial (Hythe)	Second (*n*=2)	293	25 (± 4.8)	No
	Main (Hythe)	First (*n*=3)	276	0 (±.0.0)	No
	Main (Wivenhoe)	First (*n*=3)	269	0 (± 0.0)	No
	Main (Wivenhoe)	First (*n*=3)	261	0 (± 0.0)	No
Ethane	Trial (Hythe)	First (*n*=2)	356	0 (± 0.0)	No
	Main (Hythe)	First (*n*=2)	213	9 (± 0.9)	No
	Main (Wivenhoe)	First (*n*=2)	269	9 (± 7.3)	No
	Main (Brightlingsea)	First (*n*=2)	261	0 (± 0.0)	No
Propane	Trial (Hythe)	First (*n*=3)	356	54 (± 3.7)	No

Length of incubation shown[a]. Irregularity in duration of incubation due to differences in experiment setup date and response of VOCs to anaerobic degradation.

4.3.1 Anaerobic benzene or toluene biodegradation observed in single substrate enrichments

Complete anaerobic degradation of benzene was observed within 14 days of the first enrichment (trial experiment) relative to killed controls (Table 4.1). Benzene degradation became slow with increased sequential enrichment. Benzene degradation was not observed within the first 8 days of the second enrichment; however, complete degradation was observed at day 58 when the next GC-FID measurement was taken. At third enrichments, only 47 (± 8) % benzene was degraded after incubation for 287 days (Table 4.1).

During the main anaerobic experiment, benzene degradation was observed in all enrichments and complete degradation observed in Wivenhoe enrichments (Figure 4.2). A lag phase of 56 days was observed in Hythe enrichments (Figure 4.2a). However, the % remaining in the headspace of Hythe enrichments relative to the killed control at day 213 was 30 ± 3.0 % (Figure 4.2a). In Wivenhoe and Brightlingsea enrichments, the % remaining in the headspace relative to the killed control was 0 ± 0.0 and 21 ± 19.0 at day 210 and 301 respectively (Figure 4.2 b, c).

During the trial and main anaerobic experiments, complete toluene degradation was observed in all first enrichments (Table 4.1). Toluene degradation was slow with increased sequential enrichment. For instance, complete degradation of toluene was achieved within 14 days of the first enrichment, and 30 days at the third enrichment (Anaerobic trial experiment, Table 4.1). Complete degradation of toluene occurred earlier at the second enrichment because it was below detection limit at day 58 when a measurement was made.

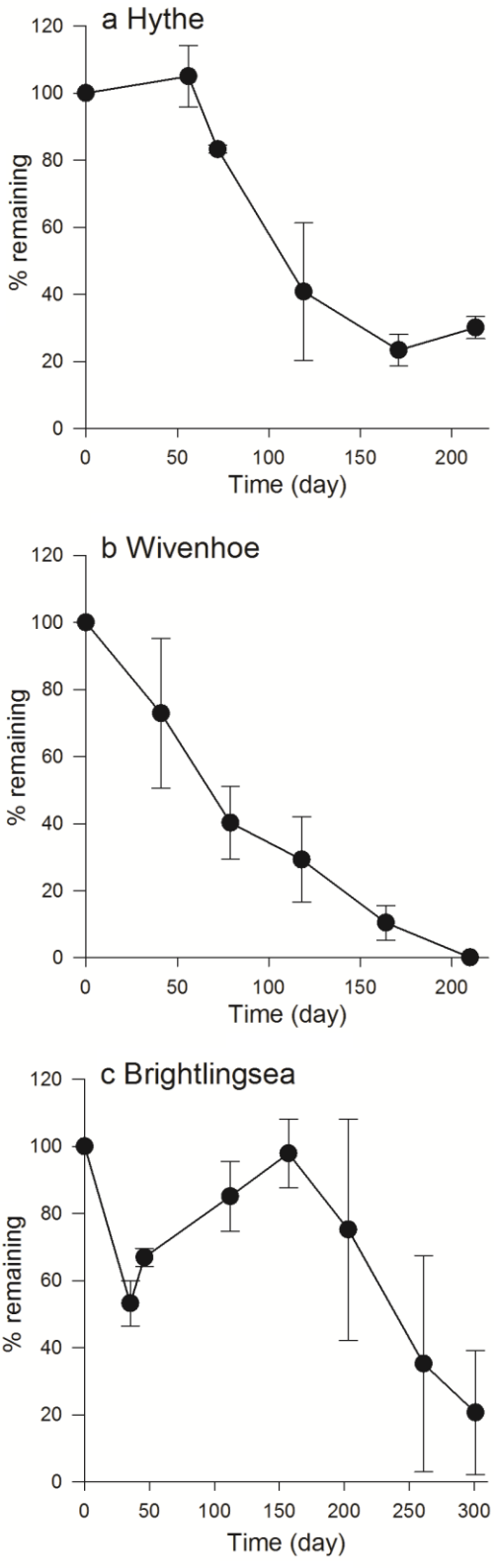


Figure 4.2. Biodegradation of benzene in Hythe (a), Wivenhoe (b) and Brightlingsea (c) first enrichments as indicated by the percentage remaining in the headspace relative to the killed control. *n* = 3; bars show SE of the mean.Final concentration of benzene was 0.5 mM. For

the main anaerobic experiment, complete toluene degradation was observed by day

56, 41, and 35 in Hythe, Wivenhoe and Brightlingsea anaerobic enrichments respectively (Table 4.1). Intermediate measurements were not taken; however, it is likely that complete anaerobic toluene degradation occurred earlier in all microcosms. At the second enrichment, no toluene degradation was observed in any Wivenhoe enrichment after 157 days of incubation. Only 16.4 (± 3.4) % and 11.2 (± 5.4) toluene was degraded in Hythe and Wivenhoe enrichments respectively (Table 4.1).

4.3.2 Anaerobic propane biodegradation demonstrated in estuarine sediments, but no evidence of anaerobic ethane biodegradation

Propane degradation was observed during the trial and main anaerobic experiments, but ethane degradation was not observed. During the trial experiment, 54.1 ± 3.7 % propane was degraded in 356 days, but no ethane degradation was observed in any enrichment for the same duration (Table 4.1). During the main experiment, propane degradation was observed after a long lag phase of at least 164 days in Hythe and Wivenhoe enrichments (Figure 4.3 a & b). No lag phase was observed in Brightlingsea enrichments, but degradation levelled out after 73 days and no further degradation was observed (Figure 4.3). In Hythe microcosms, the concentration of propane in the headspace indicated by the percentage remaining relative to the killed control was significantly different from day 213 through today 316 ($P < 0.05$) when the experiment ended (Figure 4.3 a). In Wivenhoe microcosms, however, significant propane degradation was observed at day 210 ($P < 0.05$) and day 269 ($P = 0.05$) (Figure 4.3 b).

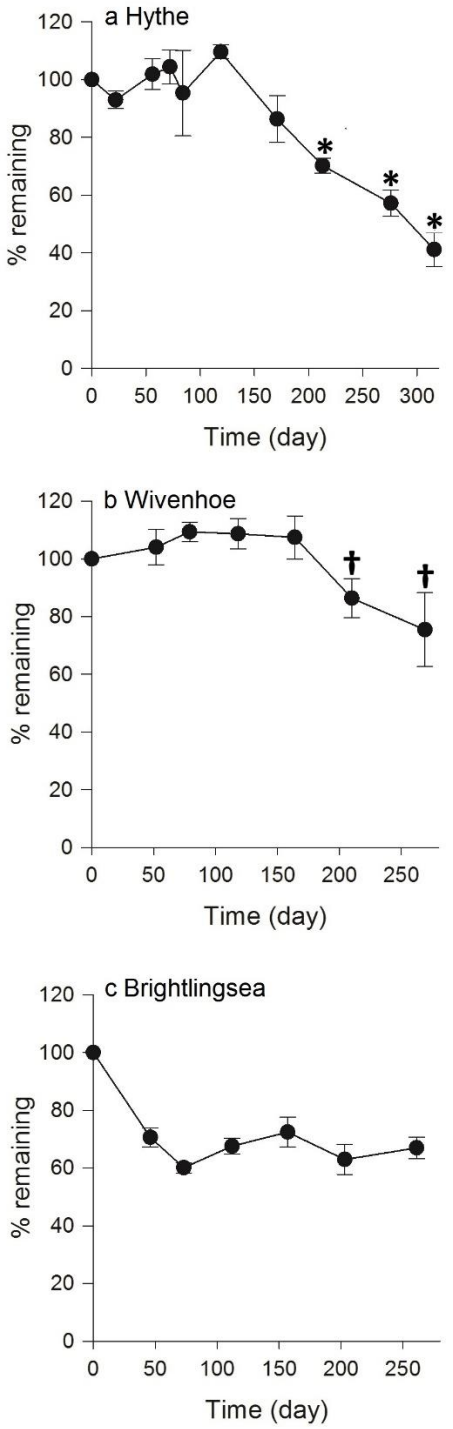


Figure 4.3. Biodegradation of propane in Hythe (a), Wivenhoe (b) and Brightlingsea (c) first enrichments as indicated by the percentage remaining in headspace relative to the killed control. *n* = 3; bars show SE of the meanFinal concentration of propane was 10 % v/vNote the difference in the scale of the x axes Star symbols (*) or cross symbols (†) indicate significant difference (Welch two sample t-test: $P < 0.05$) between the headspace concentration of killed control and test enrichments for Hythe and Wivenhoe, respectively.

No significant propane degradation was observed in Brightlingsea microcosms up to day 261 (P = 0.3096) when the last GC-FID measurements were made (Figure 4.3 c).

4.3.3 Anaerobic isoprene biodegradation in estuarine sediment

Anaerobic isoprene degradation was investigated in three experiments: trial anaerobic, main anaerobic, and the simple nitrogen flushing experiments. Isoprene degradation was observed during the trial and simple nitrogen flushing experiments (Table 4.1 & Figure 4.4 b). However, no isoprene degradation was observed in any enrichment for the duration of the main anaerobic experiment: Hythe (276 day), Wivenhoe (269 days) and Brightlingsea (261 days) (Table 4.1). During the trial experiment, 58.3 ± 3.1 % isoprene was degraded in 124 days at first enrichments and 25 ± 4.8 % in 293 days at the second enrichment (Table 4.1).

During the simple nitrogen flushing experiment, isoprene degradation was more rapid in aerobic enrichments (Figure 4.4). By day 22, the isoprene concentration in the headspace of aerobic enrichments from the Hythe was below detection limit (Figure 4.4a). In Wivenhoe and Brightlingsea microcosms, the isoprene in the headspace was below detection limit by day 38 of incubation (Figure 4.4a). In contrast, no significant isoprene degradation was observed in either Brightlingsea or Wivenhoe enrichments flushed with nitrogen after 280 days of incubation (Figure 4.4b). Isoprene degradation was observed in Hythe enrichments flushed with nitrogen (Figure 4.4b) relative to the killed controls. The residual isoprene (% remaining) after 280 days was 46 ± 16 %.

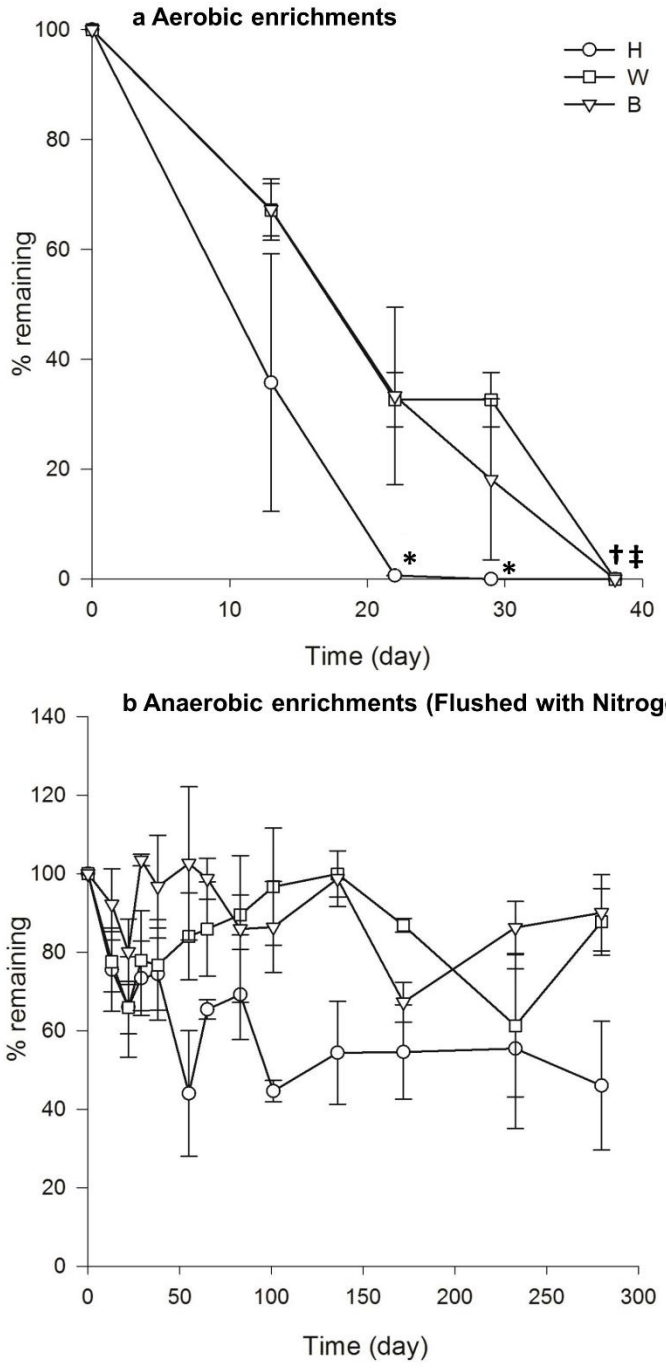


Figure 4.4. Biodegradation of isoprene in Hythe, Wivenhoe and Brightlingsea first enrichments as indicated by the % remaining in the headspace relative to the sterile controls. *n* = 3; bars show SE of the meanFinal concentration of isoprene was 7.2 x 10^6 ppbvNote the difference in the scale of the x axesStar symbols (*), cross symbols (†) or double dagger (‡) indicate significant difference (Welch two sample t-test: $P < 0.05$) between the headspace concentration of sterile control and test enrichments for Hythe, Wivenhoe and Brightlingsea respectively.

However, high variability in degradation was observed between replicates. For instance, at day 280, the percentage isoprene remaining in the headspace of one

replicate enrichment relative to the killed control was 15.3 %, whereas the other two replicates microcosms had 71.4% and 51.3% isoprene remaining.

4.3.4 Microbial community structure after the anaerobic degradation of benzene, toluene, propane and isoprene in single-substrate enrichments based on Illumina MiSeq sequenced data and DGGE

To understand the changes in microbial community structure of anaerobic enrichments post-VOC treatment, principal component analysis (PCA) of Bacterial or Archaeal OTUs (Figure 4.5) was performed in STAMP (Parks et al., 2014).

For the trial experiment, bacterial OTUs from some enrichments showed a shift in bacterial community structure driven by the VOC degraded. The bacterial community composition in Hythe second enrichment with benzene was distinct from that of the no-VOC control (Figure 4.5a). One microcosm with isoprene (HaIs) also had a distinct bacterial OTU composition (Figure 4.5a and 4.6a) from all other microcosms. On the contrary, two microcosms with isoprene as a sole carbon source (HbIs and HcIs) clustered with the No-VOC control (Figure 4.5a), suggesting no apparent differences in their bacterial OTU composition, which agreed with DGGE data (Figure 4.6 a). The PCA analyses also revealed that benzene in contrast to isoprene caused a change in the archaeal community structure and composition. The benzene amended enrichments had a distinct archaeal OTU composition but there was no difference between isoprene amended enrichments and no-VOC controls (Figure 4.5 b).

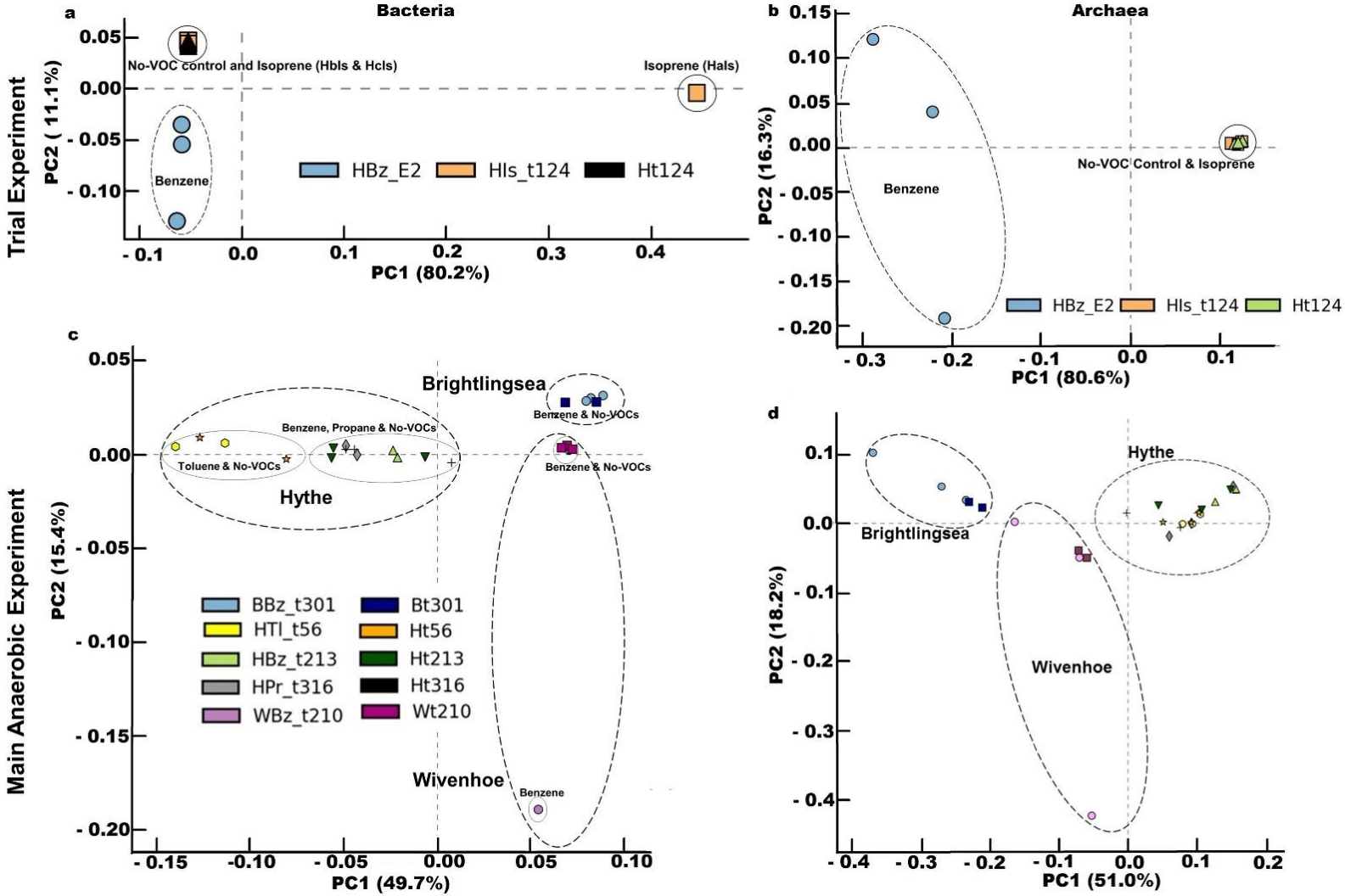


Figure 4.5. Principal component analysis (PCA) of bacterial (a&c) and archaeal (b&d) 16S rRNA gene OTUs (97% similarity threshold) from the Colne estuary indicating changes in microbial community structure due to benzene, toluene, isoprene, or propane addition as single substrates relative to the no-VOC control.

a.) Bacterial community change due to isoprene or benzene addition (Trial Expt). The first two principal components accounting for 91.3% are plotted. Ht124 = Hythe (H) No-VOC controls at day 124; HBz_E2 = Hythe second enrichments with benzene; HIs_t124 = Hythe enrichments with isoprene (Is) _Day 124. HaIs, HbIs and HcIs are replicates; b.) Archaeal community change due to isoprene or benzene addition (Trial Expt). The first two principal components accounting for 96.9% are plotted. Same abbreviations as (a); c.) Bacterial community change due to benzene, toluene or propane addition (Main Expt). The first two principal components accounting for 65.1% are plotted. BBz_t301 = Brightlingsea enrichments with benzene (Bz)_Day 301; Bt301 = Brightlingsea No-VOC controls at day 301; HTl_t56 = Hythe enrichments with toluene (Tl)_Day 56; Ht56 = Hythe No-VOC controls at day 56; HBz_t213 = Hythe enrichments with benzene_Day213; Ht213 = Hythe No-VOC controls at day 213; HPr_t316 = Hythe enrichments with propane (Pr)_Day 316; Ht316 = Hythe No-VOC controls at day 316; WBz_t210 = Wivenhoe enrichments with benzene _Day 210; Wt210 = Wivenhoe No-VOC controls at day 210; d.) Archaeal community change due to benzene, toluene or propane addition (Main Expt). The first two principal components accounting for 69.2% are plotted. Same abbreviations as (c); Brightlingsea, Hythe, and Wivenhoe were the sampling locations. PCA was performed in STAMP 2.1.3 (Parks et al., 2014) with default parameters.

For the main experiment, despite evidence for anaerobic degradation (Table 4.1, Figure 4.2 - 4.3), the microbial communities showed minor changes from the corresponding no-VOC controls, and clustered in the PCA plots according to the sample location, i.e. the Hythe, Wivenhoe and Brightlingsea (main anaerobic degradation experiment, Figure 4.5 c & d). A notable exception was one of the Wivenhoe enrichment for Bacteria (Figure 4.5 c) and two for Archaeal communities (Figure 4.5 d). The OTU composition of these enrichments was distinct from the no-VOC controls. The replicates that degraded most VOC also had the biggest shift in communities (Figure 4.5a, c & d). One Wivenhoe enrichment with the distinct Archaeal community was identified as WaBz_t210 and analysed separately in STAMP (Parks et al., 2014).

From the trial experiment, DGGE analyses of PCR amplified bacterial 16S rRNA genes was performed on samples from the enrichments with benzene and isoprene. DGGE bands were not excised and sequenced because Illumina MiSeq sequencing was performed for all samples. DGGE analyses of Archaeal communities was not

done since Illumina MiSeq provided a finer resolution and more holistic view of the microbial community composition/structure of enrichments.

A shift in bacterial community structure and dense bands were observed on DGGE profiles suggesting enhanced growth by a few bacterial populations on benzene or isoprene (Figure 4.6). One enrichment with isoprene (HaIs_t124) had a distinct bacterial community profile from the other replicates (HbIs_t124 and HcIs_t124) and the no-VOC controls (Ht124). Three boxed bands were unique to the enrichment because they were denser than those found on the profiles of the corresponding samples (Figure 4.6a). Two of these bands denatured high up the gel, suggesting that the bacterial phylotypes had AT-rich sequences (or low GC-content).

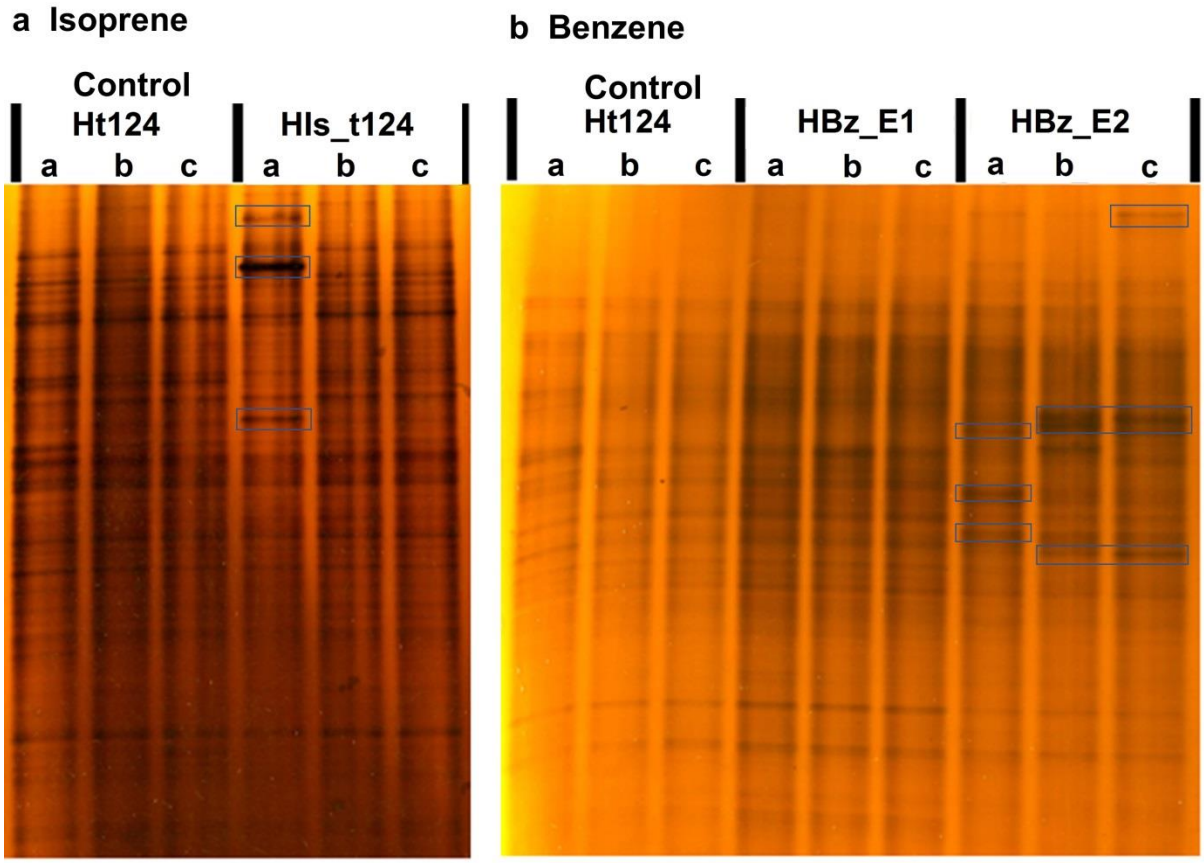


Figure 4.6. DGGE gel showing bacterial community change due to isoprene (a) or benzene (b) addition in anaerobic enrichments. a) DGGE profiles showing bacterial populations enriched (thick bands) from first anaerobic enrichments with Isoprene (Is) as a sole carbon source. b.) DGGE profiles showing bacterial populations enriched in first enrichments (E1) and second (E2) with benzene (Bz). Ht124 = Hythe no-VOC controls at day 124; HIs_t124 = Hythe enrichments with isoprene (Is) _Day 124. HBz_E1 = Hythe first enrichments with benzene; HBz_E2 = Hythe second enrichments with benzene. Each lane represents PCR amplified 16S rRNA genes from an enrichment. Blue boxed bands show enriched bacterial populations. a, b & c are replicates.

In enrichments with benzene, a change in the bacterial community structure and reduction in composition was observed on DGGE profiles (Figure 4.6b). Dense DGGE bands were boxed and they represent bacterial populations exhibiting increased abundance in the presence of benzene in second enrichment (HBz_E2). Compared to the no-VOC control (Ht124), the intensity of those boxed bands increased between the first (HBz_E1) and second enrichment (HBz_E2).

Table 4.2 Identity of Bacterial OTUs demonstrating increased abundance in anaerobic enrichments with volatile hydrocarbons based on BLAST searches against NCBI's 16S rRNA sequence database.

OTU	Taxa	Closest relative	% Identity	Expt
OTU_58	*Deltaproteobacteria*	*Pelobacter acetylenicus* strain WoAcy1 (NR_029238.1)	95	Anaerobic trial experiment
OTU_95	*Epsilonproteobacteria*	*Sulfurimonas paralvinellae* strain GO25 (NR_041439.1)	93	
OTU_239	*Deltaproteobacteria*	*Pelobacter acetylenicus* strain WoAcy1 (NR_029238.1)	95	
OTU_552	*Epsilonproteobacteria*	*Sulfurimonas autotrophica* strain OK10 (NR_028643.1)	97	
OTU_42	*Chloroflexi*	*Thermomarinilinea lacunifontana strain SW7 (NR_132293.1)*	89	Main anaerobic experiment
OTU_47	*Actinobacteria*	*Ilumatobacter fluminis strain YM22-133 (NR_041633.1)*	97	
OTU_60	*Verrucomicrobia*	*Luteolibacter algae strain A5J-41-2 (NR_041624.1)*	95	
OTU_61	*Deltaproteobacteria*	*Desulfatiglans parachlorophenolica strain DS (NR_126176.1)*	89	
OTU_137	*Epsilonproteobacteria*	*Arcobacter marinus strain CL-S1 (NR_116342.1)*	95	
OTU_162	*Actinobacteria*	*Rugosimonospora africana strain Delta3 (NR_044610.1)*	89	
OTU_191	*Deltaproteobacteria*	*Desulfatiglans parachlorophenolica strain DS (NR_126176.1)*	93	
OTU_249	*Deltaproteobacteria*	*Desulfatiglans parachlorophenolica strain DS*	93	
OTU_363	*Deltaproteobacteria*	*Desulfobacca acetoxidans strain DSM 11109 (NR_074955.1)*	81	
OTU_569	*Epsilonproteobacteria*	*Sulfurimonas gotlandica strain GD1 (NR_121690.1)*	93	
OTU_741	*Deltaproteobacteria*	*Smithella propionica strain LYP (NR_024989.1)*	90	

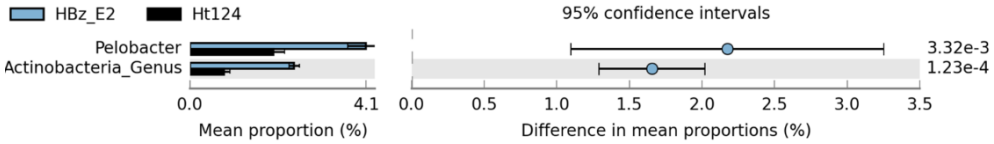
4.3.5 Microbial phylotypes identified in anaerobic benzene and toluene enrichments

From the trial experiment, bacterial and archaeal phylotypes were enriched with benzene as a sole carbon source. *Pelobacter* and an uncharacterised actinobacterial genus were the main bacterial genera identified in Hythe enrichments (Figure 4.7). Only 4.1% of the bacterial community was occupied by *Pelobacter* while the uncharacterised actinobacterial genus formed 2.4%. At the OTU level, OTU 239 and 58 identified shared 95% sequence identity with *Pelobacter acetylenicus* strain WoAcy1 (Table 4.2, Figure 4.11 a). *Methanomassiliicoccus* was a dominant member of the archaeal community, making 64.1% (Figure 4.8a). OTU_20, OTU_25, OTU_60, OTU_62 and OTU_135 enriched were *Euryarchaeota* with no close relatives on the NCBI 16S ribosomal RNA sequence database (Table 4.3). However, OTU_60, OTU_62 and OTU_135, were 83 to 86% similar to *Methanomassiliicoccus* and so are putative methanogens.

From the main experiment, bacterial phylotypes were enriched in small proportions (usually < 1% of the community) with benzene. No dorminant phylotypes were found in any enrichment. In Brightlingsea enrichments, the two genera, *Blastospirellula* and *Longilinea* enriched constituted only 0.6 % and 0.9% of the bacterial community respectively (Figure 4.7b). *Arcobacteria* (0.9%) and *Caloribacterium* (0.4%) were identified in Hythe (Figure 4.7c) while *Luteolibacter* (1%) was identified in Wivenhoe enrichments (Figure 4.7d).

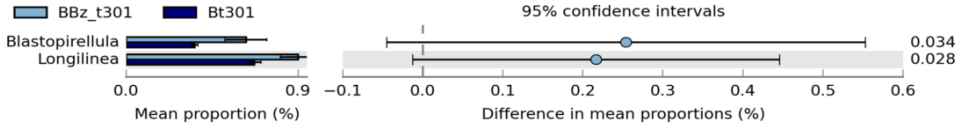
Trial Experiment

a Bacterial genera enriched with benzene in Hythe microcosms

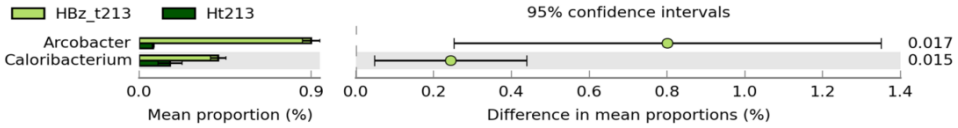


Main Anaerobic Experiment

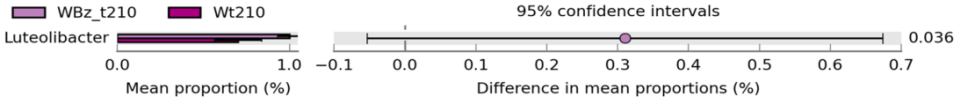
b Bacterial genera enriched with benzene in Brightlingsea microcosms



c Bacterial genera enriched with benzene in Hythe microcosms



d Bacterial genera enriched with benzene in Wivenhoe microcosms



e Bacterial genera enriched with toluene in Hythe microcosms

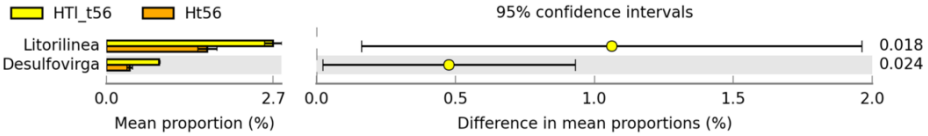


Figure 4.7. Bacterial genera enriched with benzene (a - d) or toluene (e) in anaerobic microcosms relative to No-VOC controls. a) HBz_E2 vs Ht124; b) BBz_t301 vs Bt301. c) HBz_t213 vs Ht213. d) WBz_t210 vs Wt210; e) HTl_t56 vs Ht56. Analysis was performed in STAMP 2.1.3 (Parks et al., 2014) with default parameters, except for filtering out were: *P* value < 0.05; difference between proportions < 0.2 or difference between ratios < 1.5. Data were sorted according to effect size. Significant difference was determined by a one-sided Welch's t-test between mean proportions. *P* value (corrected) is shown on the right. Note the difference in the scale of the x axes. All samples presented are the mean of triplicate. Ht124 = Hythe No-VOC controls at day 124; HBz_E2 = Hythe second enrichments with benzene; BBz_t301 = Brightlingsea enrichments with benzene (Bz) _Day 301; Bt301 = Brightlingsea No-VOC controls at day 301; HBz_t213 = Hythe enrichments with benzene_Day213; Ht213 = Hythe No-VOC controls at day 213; WBz_t210 = Wivenhoe enrichments with benzene _Day 210; Wt210 = Wivenhoe No-VOC controls at day 210; HTl_t56 = Hythe enrichments with toluene (Tl) _Day 56; Ht56 = Hythe No-VOC controls at day 56.

At the OTU level, OTU_569 identified in Hythe enrichments was 1.5% of the bacterial community post anaerobic benzene degradation (Figure 4.11c). This OTU shared 93% sequence identity with *Sulfurimonas gotlandica* strain GD1 (NR_121690.1) (Table 4.2). Apart from OTU_47 which shared 97% sequence identity with *Ilumatobacter fluminis* strain YM22-133 (NR_041633.1), other OTUs were different from cultured strains (Table 4.2).

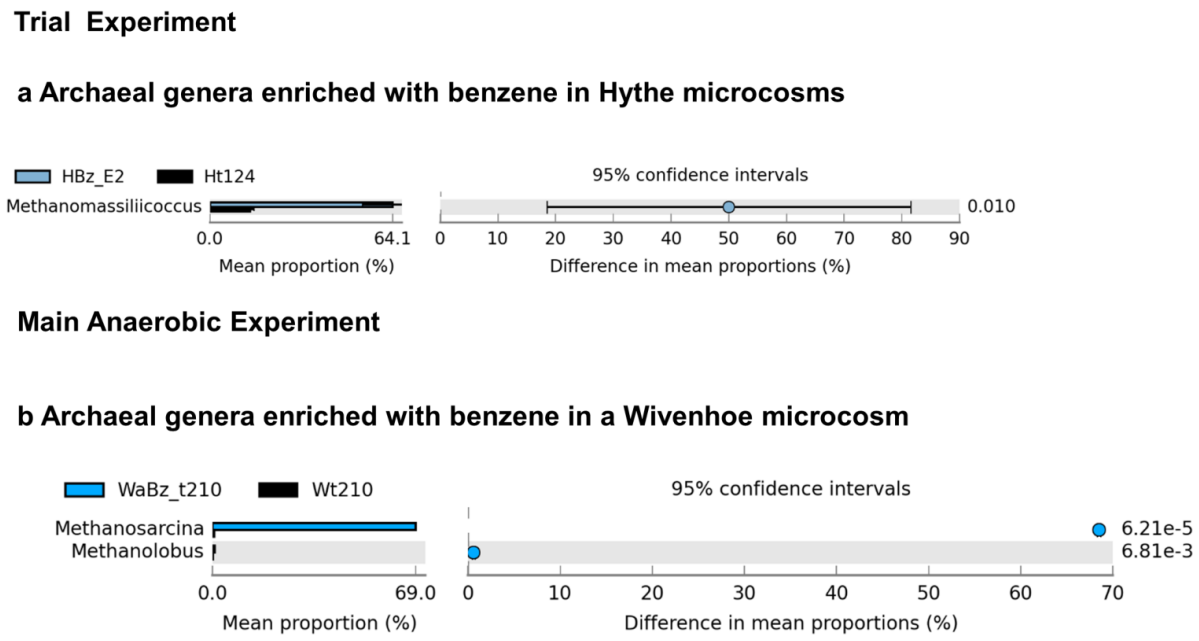


Figure 4.8. The archaeal genera enriched with benzene in anaerobic microcosms relative to the No-VOC controls. a) HBz_E2 vs Ht124; b) WaBz_t210 vs Wt210. Analysis was performed in STAMP 2.1.3 (Parks et al., 2014) with default parameters, except for filtering out were: *P value* < 0.05; different between proportions < 0.2 or difference between ratios < 1.5. Data were sorted according to effect size. Significant difference was determined by a one-sided Welch's t-test between mean proportions. *P value* (corrected) is shown on the right. Note the difference in the scale of the x axes. All samples are the mean of triplicate except where indicated. Ht124 = Hythe No-VOC controls at day 124; HBz_E2 = Hythe second enrichments with benzene; WaBz_t210 = one Wivenhoe enrichments with benzene _Day 210 (*n* = 1); Wt210 = mean of replicates Wivenhoe No-VOC controls at day 210 (*n* = 2).

At the genus level, no Archaeal phylotypes were identified in Brightlingsea and Hythe enrichments with benzene from the main experiment, but OTUs were enriched in small proportions (0 – 1.3%) (Figure 4.9). In Wivenhoe enrichments, Archaeal phylotypes were identified in one sample (WaBz_t210) at the genus and OTU levels (Figure 4.8b and 4.11d). The sample was identified by PCA to have a distinct archaeal community composition (Figure 4.5d). Most notable was that *Methanosarcina* constituted 69% of this single benzene-enriched microcosm compared with less than 1% in the no-VOC control (Figure 4.8b). The dominant, OTU_39 identified from that enrichment was 97% similar to *Methanosarcina semesiae* strain MD1 (Figure 4.9d, Table 4.3). In addition, *Methanolobus* was 1.2% of the Archaeal community from the enrichment (Figure 4.8b).

The Archaeal OTUs identified in enrichments of the main anaerobic experiment, were identified as belonging to two main phyla, *Euryarchaeota* and *Crenarchaeota* (Table 4.3). Although OTU_1114 and OTU_1252 were identified in Brightlingsea enrichments with benzene, each of those OTUs formed only a very small proportion (< 0.0% each) of the archaeal community composition (Figure 4.9 b).

In Hythe enrichments with benzene, OTU_37, a *Crenarchaeota* and OTU_90, a *Euryarchaeota*, were identified (Figure 4.9c). Another *Crenarchaeota*, OTU_12, was identified in Hythe enrichments with toluene as a sole carbon source (Figure 4.9e). Again, these OTUs formed only a small proportion of the corresponding archaeal communities and ranged between < 1.0 – 3.0% (Figure 4.9c & e).

Only bacterial and archaeal *16S rRNA* gene from Hythe enrichments with toluene (main experiment only) were sequenced. *Litorilinea* (2.7%) and *Desulfovirga* (<1.0%) were the bacterial genera identified in those enrichments (Figure 4.7e), while

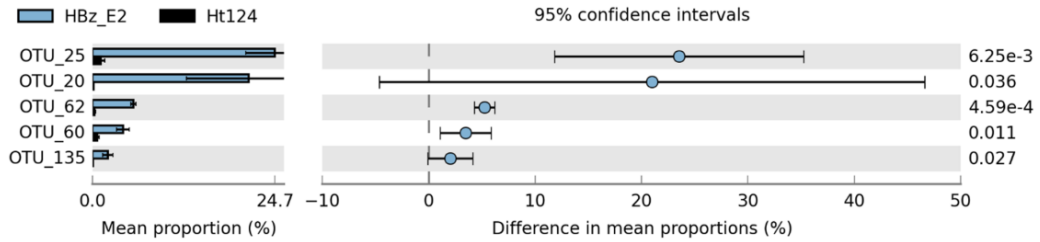
OTU_42 and OTU_61 were the main OTUs identified (Figure 4.11f). The OTUs were different from those on NCBI's database. OTU_42 had 89% sequence identity with *Thermomarinilinea lacunifontana* strain SW7 and OTU_61 had 89% sequence identity with *Desulfatiglans parachlorophenolica* strain DS (Table 4.2).

Table 4.3 Identity of archaeal OTUs demonstrating increased abundance in anaerobic enrichments with volatile hydrocarbons based on BLAST searches against NCBI's 16S rRNA sequence database.

OTU	Taxa	Closest relative	% Identity	Expt
OTU_20	*Euryarchaeota*	*Thermogymnomonas acidicola* strain IC-189 (NR_041513.1)	78	Anaerobic trial experiment
		Uncultured archaeon clone 113A57 (EF687626.1)	97	
OTU_25	*Euryarchaeota*	*Methanothermus fervidus* strain DSM 2088 (NR_102926.1)	77	
OTU_60	*Euryarchaeota*	*Methanomassiliicoccus luminyensis* strain B10 (NR_118098.1)	86	
		Uncultured archaeon clone SBAK-shallow-05	98	
OTU_62	*Euryarchaeota*	*Methanomassiliicoccus luminyensis* strain B10 (NR_118098.1)	83	
OTU_135	*Euryarchaeota*	*Methanomassiliicoccus luminyensis* strain B10 (NR_118098.1)	85	
OTU_9	*Euryarchaeota*	*Methanosarcina subterranea strain HC-2* (NR_134763.1)	98	Main anaerobic experiment
		Uncultured *Methanosarcina sp.* clone TS1A083 (JF789590.1)	99	
OTU_12	*Crenarchaeota*	*Staphylothermus hellenicus* strain DSM 12710 (NR_074532.1)	80	
		Uncultured archaeon clone E3 NEREIS-BAL (JF931524.1)	98	
OTU_17	*Euryarchaeota*	*Methanohalophilus levihalophilus* strain GTA13 (NR_125474.1)	95	
OTU_37	*Crenarchaeota*	*Thermocladium modestius* strain IC-125 (NR_040779.1)	86	
		Uncultured Crenarchaeota (AM942174.2)	100	
OTU_39	*Euryarchaeota*	*Methanosarcina semesiae* strain MD1 (NR_028182.1)	97	
OTU_56	*Euryarchaeota*	*Methanomassiliicoccus luminyensis* strain B10 (NR_118098.1)	92	
OTU_90	*Euryarchaeota*	*Thermogymnomonas acidicola* strain IC-189 (NR_041513.1)	79	
		Uncultured archaeon clone M05-A96 (AB328132.1)	100	
OTU_1114	*Euryarchaeota*	*Methanococcus aeolicus* strain Nankai-3 (NR_074182.1)	83	
OTU_1252	*Euryarchaeota*	*Methanothermococcus okinawensis* strain IH1 (NR_102915.1)	80	

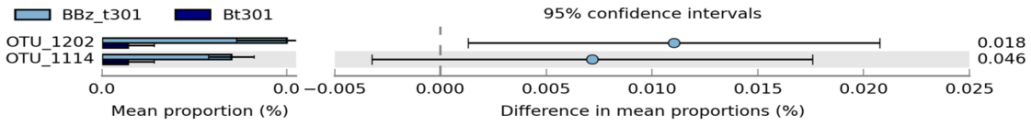
Anaerobic Trial Experiment

a Archaeal OTUs enriched with benzene in Hythe microcosms

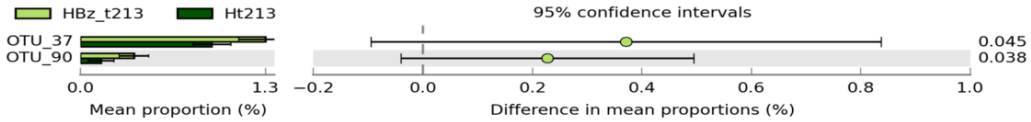


Main Anaerobic Experiment

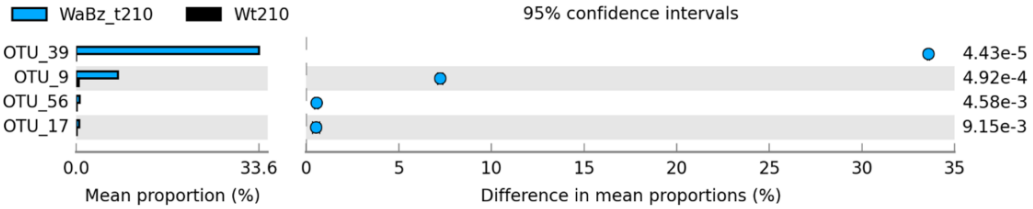
b Archaeal OTUs enriched with benzene in Brightlingsea microcosms



c Archaeal OTUs enriched with benzene in Hythe microcosms



d Archaeal OTUs enriched with benzene in a Wivenhoe microcosm



e Archaeal OTUs enriched with toluene in Hythe microcosms

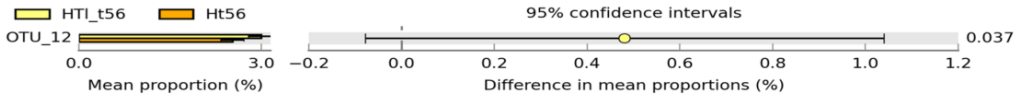


Figure 4.9. The archaeal OTUs enriched in anaerobic microcosms with benzene or toluene relative to No-VOC controls. a) HBz_E2 vs Ht124; b) BBz_t301 vs Bt301; c) HBz_t213 vs Ht213; d) WaBz_t210 vs Wt210; e) HTl_t56 vs Ht56. Analysis was performed in STAMP 2.1.3 (Parks et al., 2014) with default parameters, except for filtering out were: P value < 0.05; different between proportions: < 0.2. Data were sorted according to effect size. No filtering out was done for Brightlingsea enrichments (a). Significant difference was determined by a one-sided Welch's t-test between mean proportions. P value (corrected) is shown on the right. Note the difference in the scale of the x axes. Values are the mean of triplicate except where indicated. Ht124 = Hythe No-VOC controls at day 124; HBz_E2 = Hythe second enrichments with benzene; WaBz_t210 = one Wivenhoe enrichments with benzene _Day 210 (n = 1); Wt210 = Wivenhoe No-VOC controls at day 210 (n = 2); BBz_t301 = Brightlingsea enrichments with benzene (Bz) _Day 301; Bt301 = Brightlingsea No-VOC controls at day 301; HBz_t213 = Hythe enrichments with benzene_Day213; Ht213 = Hythe No-VOC controls at day 213; HTl_t56 = Hythe enrichments with toluene (Tl) _Day 56; Ht56 = Hythe No-VOC controls at day 56.

4.3.6 Microbial phylotype in anaerobic enrichments with propane

To minimise the cost of MiSeq sequencing, only samples from Hythe enrichments with propane (main anaerobic experiment only) were sequenced. Bacterial phylotypes were identified in enrichments both at the genus and OTUs levels (Figure 4.10 and 4.12e). On the contrary, neither archaeal genera nor OTUs were enriched, therefore, STAMP analysis returned no plot. The bacterial genera enriched with propane as a sole carbon source were *Smithella* and *Tepidisphaera* which made only 0.4% and 0.9% of the bacterial community respectively (Figure 4.10).

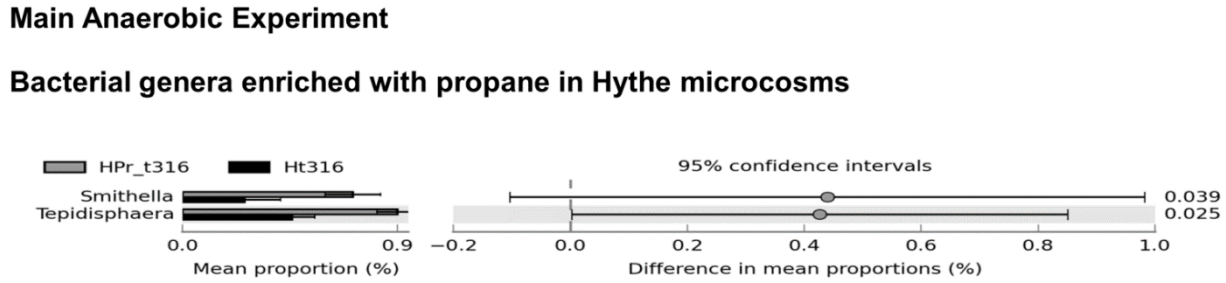


Figure 4.10. The bacterial genera enriched with propane in anaerobic microcosms relative to the No-VOC controls. HPr_t316 vs Ht316. Analysis was performed in STAMP 2.1.3 (Parks et al., 2014) with default parameters, except for filtering out were: *P* value < 0.05; different between proportions: < 0.2. Data were sorted according to effect size. Significant difference was determined by a one-sided Welch's t-test between mean proportions. *P* value (corrected) is shown on the right. Values are the mean of triplicate. HPr_t316 = Hythe enrichments with propane (Pr) _Day 316; Ht316 = Hythe No-VOC controls at day 316.

OTU_741, OTU_162 and OTU_249 (Figure 4.11e) were the main OTUs, identified as *Smithella propionica* strain LYP (90% identity); *Rugosimonospora africana* strain Delta3 (89% identity) and *Desulfatiglans parachlorophenolica* strain DS (93% identity), respectively (Table 4.2).

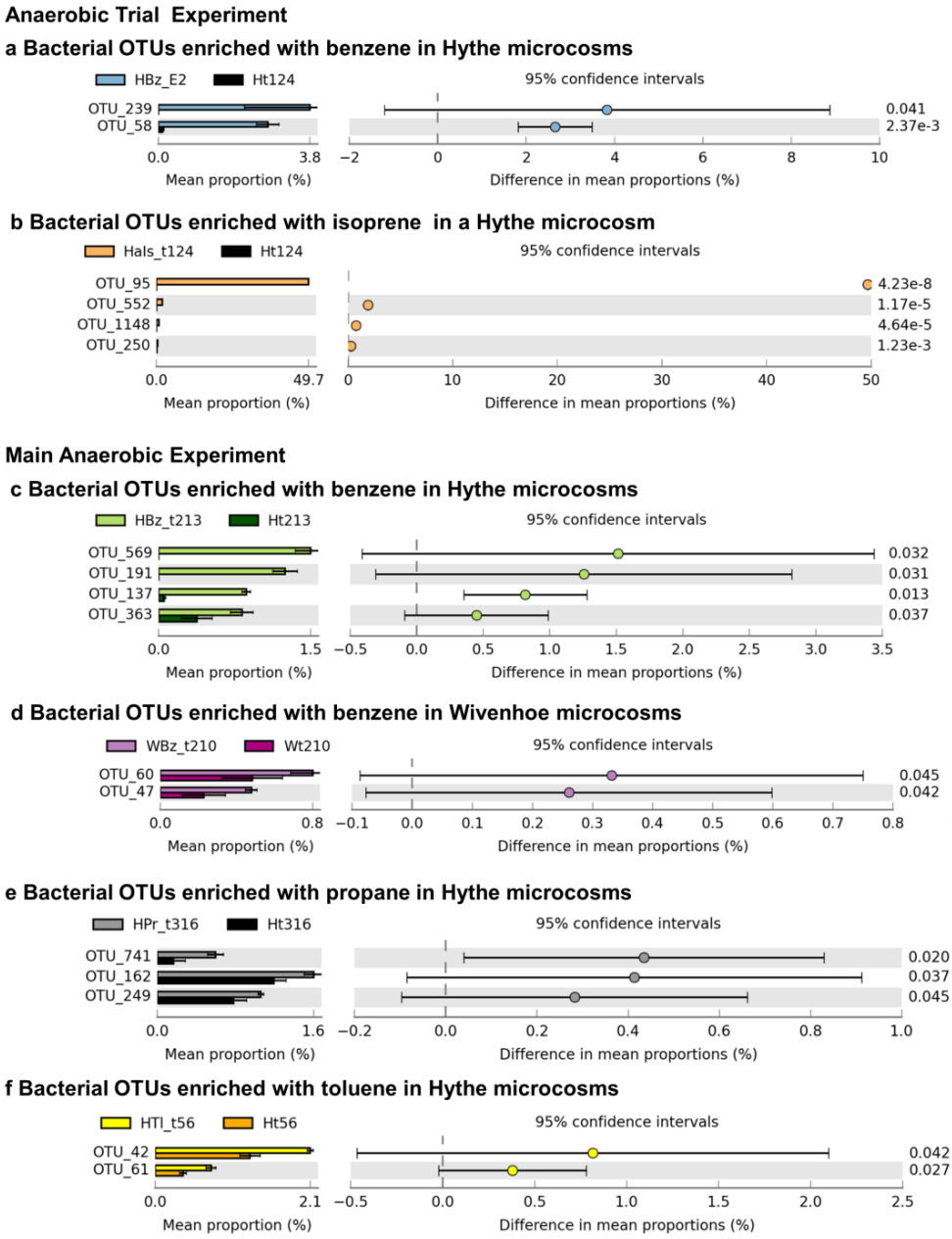


Figure 4.11. The Bacterial OTUs enriched in anaerobic microcosms with benzene, isoprene, propane or toluene relative to the No-VOC controls. a) HBz_E2 vs Ht124; b) HaIs_t124 vs Ht124; c) HBz_t213 vs Ht213; d) WBz_t210 vs Wt210; e) HPr_t316 vs Ht316; f) HTl_t56 vs Ht56. Analysis was performed in STAMP 2.1.3 (Parks et al., 2014) with default parameters, except for filtering out were: *P* value < 0.05; difference between proportions: < 0.2. Data were sorted according to effect size. Significant difference was determined by a one-sided Welch's t-test between mean proportions. *P* value (corrected) is shown on the right. Note the difference in the scale of the x axes. Values are the mean of triplicate determinations except where indicated. Ht124 = Hythe No-VOC controls at day 124; HBz_E2 = Hythe second enrichments with benzene; HaIs_t124 = Hythe enrichment with isoprene_day124 (*n* = 1); HBz_t213 = Hythe enrichments with benzene_Day213; Ht213 = Hythe No-VOC controls at day 213; WBz_t210 = Wivenhoe enrichments with benzene _Day 210; Wt210 = Wivenhoe No-VOC controls at day 210; HPr_t316 = Hythe enrichments with propane (Pr) _Day 316; Ht316 = Hythe No-VOC controls at day 316; HTl_t56 = Hythe enrichments with toluene (Tl) _Day 56; Ht56 = Hythe No-VOC controls at day 56.

4.3.7 Microbial phylotype identified in anaerobic isoprene enrichments

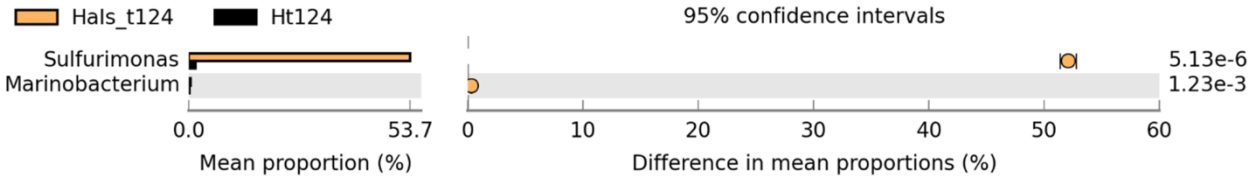
From the Hythe enrichments with isoprene (trial experiment), bacterial phylotypes were identified but no archaea were found. The PCA (Figure 4.5 a) and DGGE data (Figure 4.6 a) indicated changes in bacterial community composition after isoprene degradation from a single replicate microcosm (HaIs). But the Archaeal OTU composition showed no difference with the no-VOC controls indicating no archaeal communities change due to isoprene addition (Figure 4.5 b). Archaeal community abundance data analysed in STAMP (both at genus and OTU levels) using identical parameters to those shown in the figure legend (Figure 4.12), returned no plot confirming that no OTUs or genera were enriched significantly.

Sulfurimonas was the dominant bacterial phylotype identified in HaIs making 53.7% of the community in that enrichment (Figure 4.12 a). *Marinobacterium* was also identified but formed only 1% of the bacterial community (Figure 4.12 a). The two main OTUs were OTU_95 and OTU_552 which shared 93% sequence identity with *Sulfurimonas paralvinellae* strain GO25 and 97% sequence identity with *Sulfurimonas autotrophica* strain OK10, respectively (Table 4.2).

Sequences identified in anaerobic (nitrogen flushed) and aerobic enrichments with isoprene showed very low abundance, between 0 – 1.7% of the community (Figure 4.12b & Figure 4.13); and no archaeal genera or OTU were identified. The bacteria genera identified in aerobic microcosms with isoprene were *Sideroxydans* (1.5%), *Gallionella* (< 1%), *Sulfuritalea* (< 1%) and *Sulfurimonas* (< 1%) (Figure 4.12b). The only OTU identified in aerobic microcosms was OTU_803 (0.5%) which shared 96% sequence identity with *Sideroxydans lithotrophicus* strain ES-1 (Figure 4.13a).

Anaerobic Trial Experiment

a Bacterial genera enriched with isoprene in a Hythe microcosm



Simple Nitrogen Flushing Experiment

b Bacterial genera enriched with isoprene in Hythe aerobic microcosm

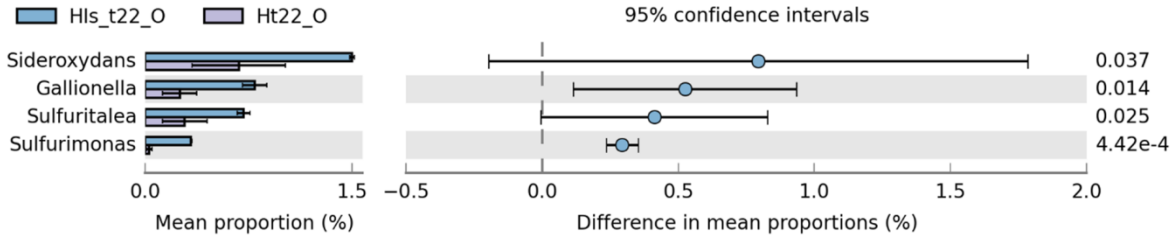


Figure 4.12. The bacterial genera enriched with isoprene in microcosms relative to the No-VOC controls. a) Anaerobic enrichment: HaIs_t124 vs Ht124. b) Aerobic enrichments: HIs_t22_O vs Ht22_O. Analysis was performed in STAMP 2.1.3 (Parks et al., 2014) with default parameters, except for filtering out were: *P* value < 0.05; different between proportions < 0.2 or difference between ratios < 1.5. Note the difference in the scale of the x axes. Values are the mean of triplicate except where indicated. HaIs_t124 = Hythe enrichment with isoprene_day124 (*n* = 1); Ht124 = Hythe No-VOC controls at day 124; HIs_t22_O = Aerobic Hythe enrichments with isoprene at day 22; Ht22_O = Hythe No-VOC control aerobic enrichments at day 22.

Sequenced data was obtained from just one nitrogen-flushed Hythe microcosms (HcIs_t280_N), the replicate had 46% remaining relative to the killed control after 280 days of incubation. Notably, the main OTU identified in HcIs_t280_N was OTU_167 (Figure 4.13), which had 94% sequence identity to *Pelobacter acetylenicus*. OTU_167 was 1.7% of the bacterial community, whereas the other OTUs were well below 1% (Figure 4.13).

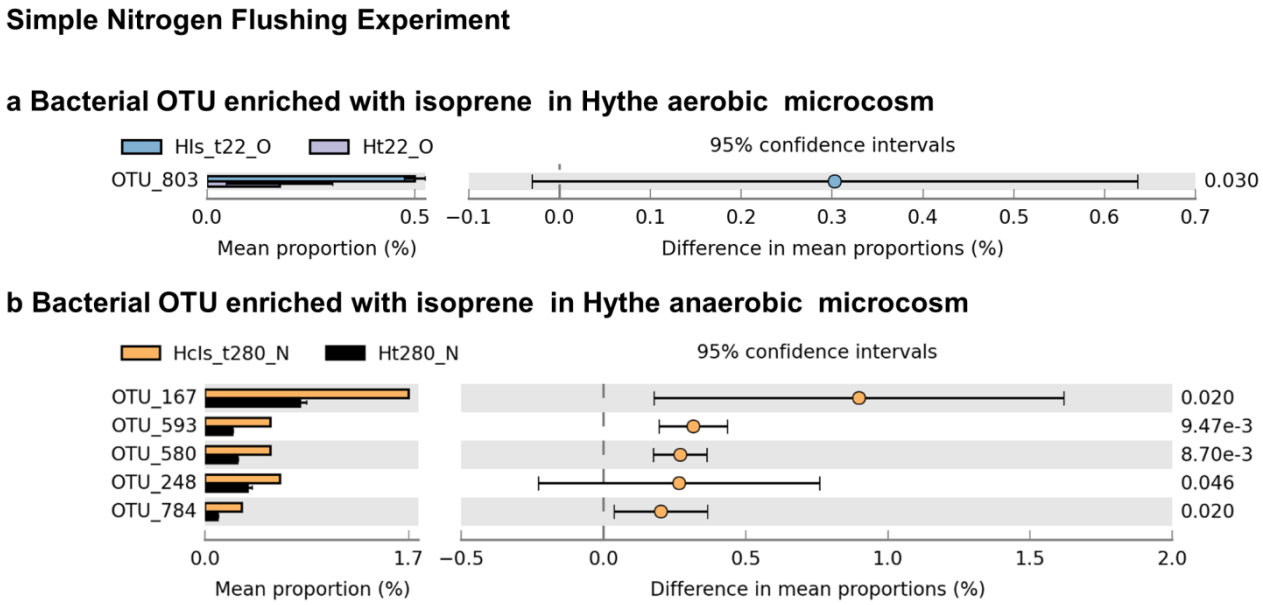


Figure 4.13. The bacterial OTUs enriched with isoprene in microcosms relative to no-VOC controls. a) Aerobic enrichments: HIs_t22_O vs Ht22_O.; b) Anaerobic enrichment: HcIs_t280_N) vs Ht280_N. Analysis was performed in STAMP 2.1.3 (Parks et al., 2014) with default parameters, except for filtering out were: *P* value < 0.05; different between proportions: < 0.2. Data were sorted according to effect size. Significant difference was determined by a one-sided Welch's t-test between mean proportions. *P* value (corrected) is shown on the right. Note the difference in the scale of the x axes. Values are the mean of triplicate except where indicated. HIs_t22_O = Aerobic Hythe enrichments with isoprene as a sole carbon source at day 22; Ht22_O = Hythe No-VOC control aerobic enrichments at day 22; HcIs_t280_N = nitrogen-flushed Hythe enrichment with isoprene as a sole carbon source at day 280 (*n*=1); Ht280_N = Nitrogen-flushed Hythe No-VOC controls at day 280.

4.3.8 Phylogenetic affiliation of OTUs enriched with benzene, toluene, isoprene or propane in single substrate microcosms

Phylogenetic analyses indicated that the bacterial and archaeal OTUs identified in enrichments clustered with each other and a mix of uncultured and cultured microbes (Figure 4.14 – 4.15). Archaea phylotypes identified in anaerobic enrichments were members of *Euryarchaeota* and *Crenarchaeota* (Figure 4.14 & Table 4.3). The bacterial phylotypes were mainly affiliated with *Deltaproteobacteria* and *Epsilonproteobacteria* (Figure 4.15).

Most OTUs identified in enrichments with benzene were affiliated with putative methanogens. OTUs 39, 9 and 17 identified in the single Wivenhoe enrichment with benzene (WaBz_t210, Figure 4.9d) during the main anaerobic experiment were affiliated with *Methanosarcina* and *Methanohalophilus*. OTU 56, obtained from the same Wivenhoe enrichment was affiliated with *Methanomassiliicoccus luminyensis.* OTUs 20, 25, 60, 62, 90 and 135 identified in Hythe enrichment with benzene during the trial experiment (HBz_E2, Figure 4.9a) and main experiment (HBz_t213, Figure 4.9c), formed a distinct clade. They were more closely related to each other (Figure 4.14) but also affiliated with *Methanomassiliicoccus luminyensis* (Figure 4.14), thus methanogen.

OTU 37, also identified in Hythe enrichment with benzene (HBz_t213, Figure 4.9c) was affiliated with other *Crenarchaeota* (Figure 4.14) while OTU 12 identified in Hythe enrichments with toluene clustered with uncultured Archaea from mangrove sediments in a distinct clade.

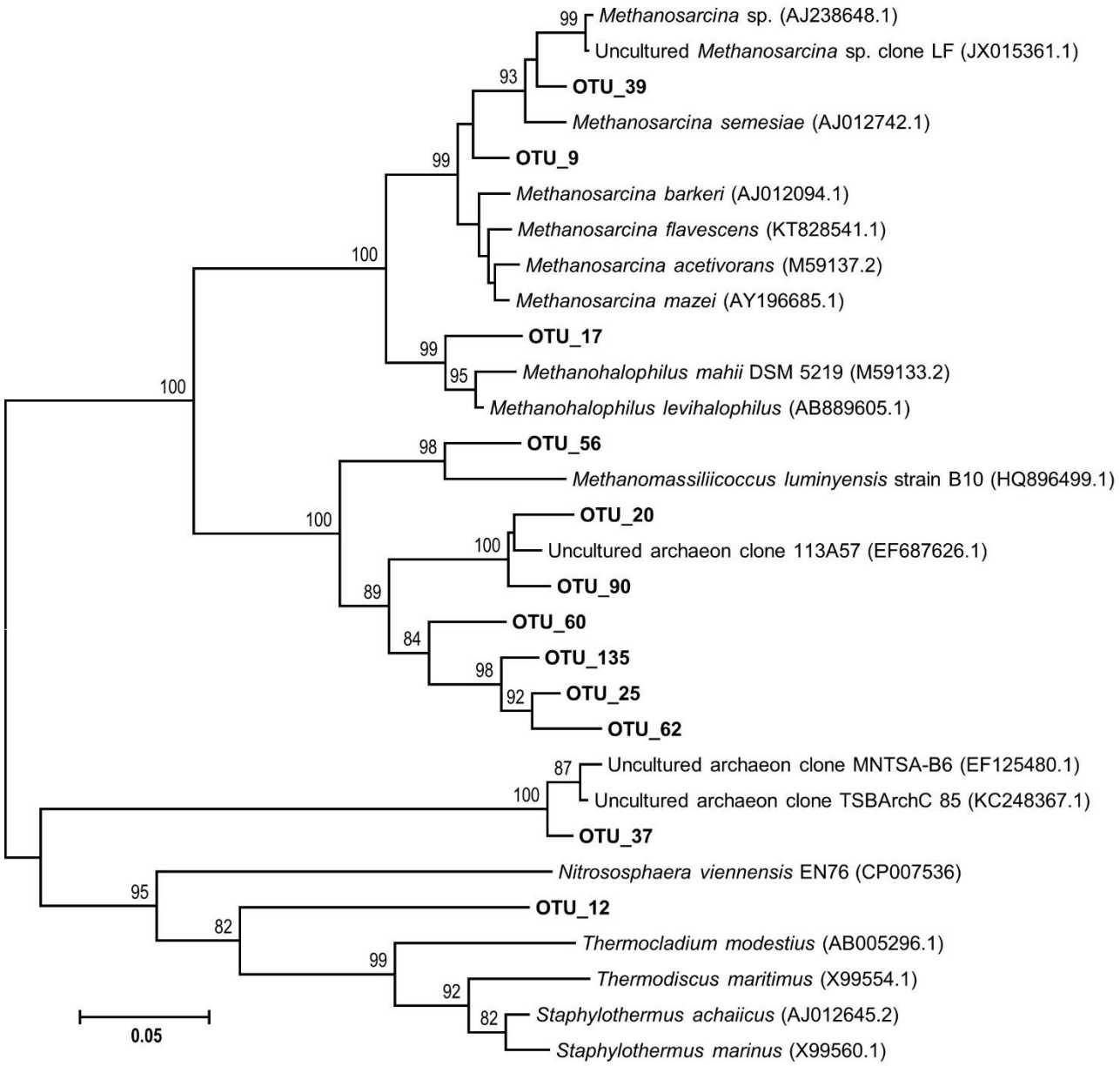


Figure 4.14. Phylogenetic relationships of the main archaeal OTUs identified in benzene, toluene, isoprene or propane enrichments of the trial and main anaerobic experiment microcosms. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicates trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. Bootstrap values ≥70% are shown. Evolutionary analyses were conducted in MEGA6.

Bacterial OTUs were affiliated with putative strict anaerobes from the class *Deltaproteobacteria* able to use organic carbon sources coupled with sulfate reduction. OTUs 191, 249, and 61 identified in Hythe enrichments with benzene, propane and toluene respectively (Figure 4.11 c, e – f), during the main anaerobic experiment, clustered with each other and *Desulfatiglans parachlorophenolica* (Figure 4.15). OTU 58 and 239 identified in Hythe enrichments with benzene (Figure 4.11a) during the trial experiment clustered with OTU 167 obtain with isoprene from Hythe microcosms flushed with nitrogen (Figure 4.13 b) and *Pelobacter* (Figure 4.15).

All *Epsilonproteobacteria* identified in this study were phylogenetically related and could play a role in benzene or isoprene biodegradation. OTUs 95, 552 and 569 were related to each other and *Sulfurimonas* (Figure 4.15). OTU 95 which was dominant in isoprene enrichments (Figure 4.11 b) cluster with OTU 569 identified in benzene enrichments (Figure 4.11 c). Both OTUs were identified from the Hythe during the trial and main anaerobic experiments respectively. OTU 137 also identified in Hythe enrichments with benzene was affiliated with members of the genus *Arcobacter* (Figure 4.15).

Other OTUs from enrichments were phylogenetically diverse, related to *Chloroflexi*, *Verrucomicrobia* and *Actinobacteria* (Figure 4.15). OTUs and their affiliations were as follows: 42 (uncultured *Chloroflexi*); 60 (*Verrucomicrobia*); 47 and 162 (*Actinobacteria*). OTU 42 and 162 were identified in Hythe enrichments with toluene and propane respectively (Figure 4.11 e & f). OTUs 47 and 60 were identified in the same enrichment (Wivenhoe) with benzene (Figure 4.11 d).

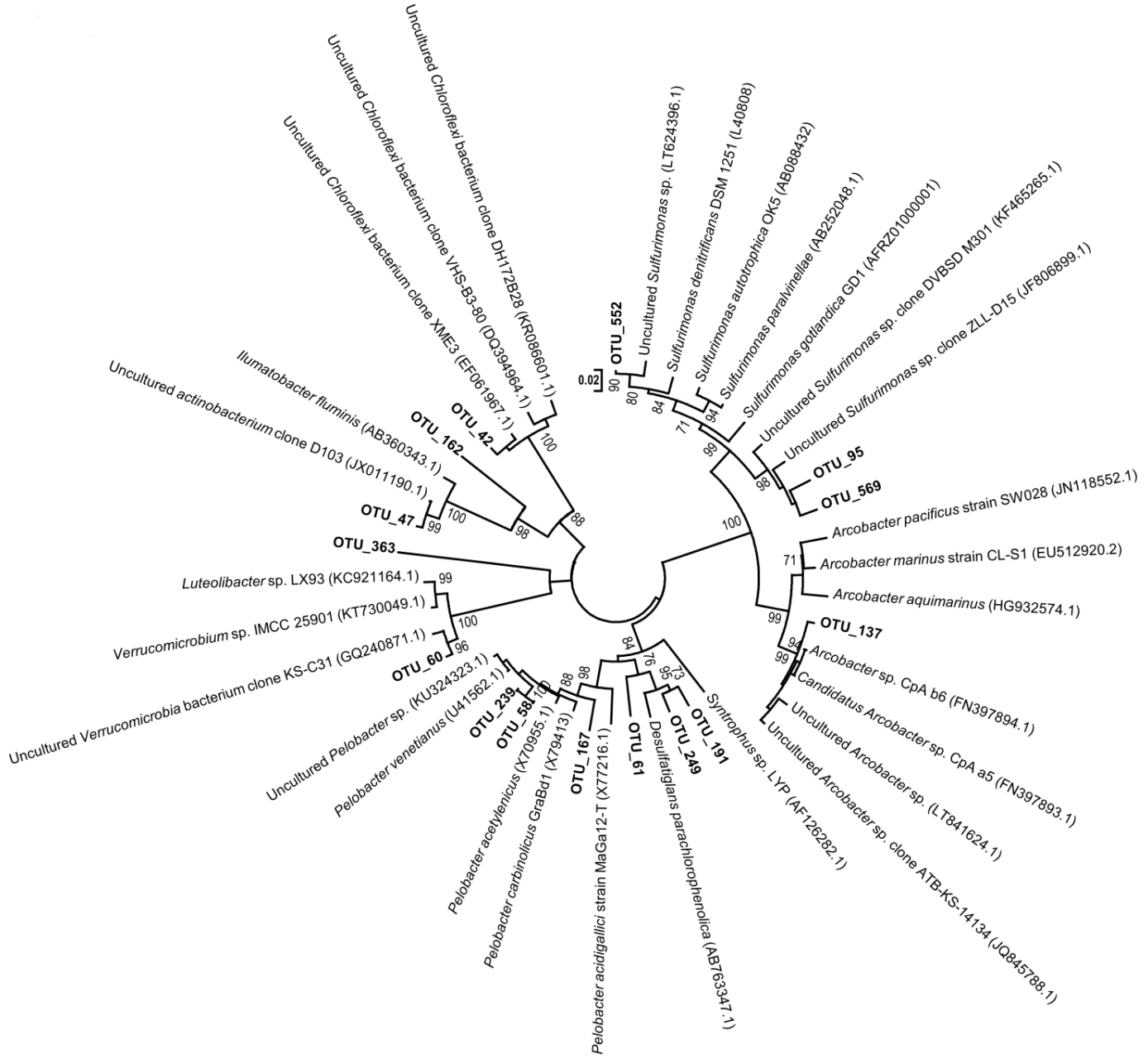


Figure 4.15. Phylogenetic relationships of bacterial OTUs from the phylum *Actinobacteria*, *Chloroflexi*, *Verrucomicrobia*, class *delta- and epsilon- Proteobacteria* identified in anaerobic enrichments amended with benzene, toluene, isoprene or propane. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicates trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. Bootstrap values ≥70% are shown. Evolutionary analyses were conducted in MEGA6.

4.4 Discussion

Benzene, toluene, ethane, propane, and isoprene are degraded rapidly in the presence of oxygen as demonstrated in Chapter 3. However, these hydrocarbons persist in anoxic sediment due to the absence of microorganisms able to use them or unsuitable physicochemical parameters such as temperature, salinity, pH that are prevalent in anoxic environments. Despite progress in microbial ecology because of reduced cost of sequencing, the identity of anaerobic volatile hydrocarbon degrading microbial communities in estuarine sediments has been poorly described. This chapter has provided a glimpse into the identity of the possible bacterial and/or archaeal populations involved with anaerobic degradation.

4.4.1 Anaerobic biodegradation of benzene and toluene in estuarine sediments

Benzene degradation was demonstrated in all experiments (trial and main), usually after a long lag phase (main anaerobic experiment), which indicates that anaerobic benzene degradation capability exists in estuarine sediments. Benzene is the most stable monoaromatic hydrocarbon and is well known to resist activation under anoxic conditions due to the large resonance energy caused by the stability of its π electron cloud (Weelink et al., 2010). Despite the challenge of activating the benzene ring in the absence of oxygen, anaerobic benzene degradation has been shown in marine, coastal and estuarine environments previously (Yu et al., 2012, Lovley et al., 1995). Microbial communities at the Colne estuary are in contact with benzene from anthropogenic sources mainly through effluents from the sewage treatment plant near the estuary (Nedwell et al., 2016).

Benzene was degraded below detection in the first enrichment, but degradation became slower due to dilution with subsequent enrichment series. A similar trend was reported by Musat and Widdel (2008). This is due to the dilution of relevant nutrients (growth factors) present in the sediment slurry (first enrichment) or the removal of debris required for attachment by microbial populations or consortia (Vogt et al., 2011). Bacterial communities often function in a network in their natural ecosystem, dislodgement of such consortia due to dilution may also account for the slow biodegradation.

Novel Bacterial and Archaeal phylotypes affiliated with putative degraders (e.g. *Desulfatiglans*) and methanogens (e.g. *Methanomassiliicoccus*) were identified in benzene enrichments (Figure 4.14 & 4.15). The key bacterial OTUs shared between 81% - 97% sequence identity with those currently on NCBI's database indicating that the phylotypes were new and poorly understood (Table 4.2). However, these phylotypes were identified in low proportions indicating that there were no dominant anaerobic benzene degrading populations or syntrophic approach to its degradation (McInerney et al., 2009). Benzene may be a transient carbon source, introduced in small quantities in estuarine sediments, with only few species able to use it as a supplementary source of carbon and energy in anoxic sediments.

The microbial populations encountered were partitioned according to the sampling location. This differences in salinity and nutrient gradient along the estuary is most likely the reason why OTUs clustered according to location rather than the carbon source degraded (Figure 4.5c&d). Niche portioning amongst anaerobic toluene degraders was reported by Kuppardt et al. (2014) in a BTEX contaminated aquifer. From Hyhe enrichments, a diverse bacterial taxon including *Pelobacter* (*Deltaproteobacteria*), *Desulfatiglans* (Deltaproteobacteria), *Desulfobacca*

(*Deltaproteobacteria*), *Arcobacteria* (*Epsilonproteobacteria*), *Sulfurimonas* (*Epsilonproteobacteria*) and *Caloribacterium* (*Firmicutes*) were identified. From Wivenhoe enrichments, *Luteolibacter* (*Verrucomicrobia*) and *Ilumatobacter* (*Actinobacteria*) were identified. In Brightlingsea enrichments, *Blastospirellula* (*Planctomycetes*) and *Longilinea* (*Chloroflexi*). *Euryarchaeota* was the main archaea phylum identified in enrichments also partitioned according to location as follows: *Methanomassiliicoccus* (Hythe only), *Methanosarcina* and *Methanolobus* (Wivenhoe only).

The most notably enriched bacterial genus with benzene in the trial experiment was the *Deltaproteobacteria*, *Pelobacter* (95% identity) along with the archaeal genus *Methanomassiliicoccus* (83-86% identity) and other novel *Euryarchaeota* (Table 4.3). Berlendis et al. (2010) implicated *Pelobacter* (*Deltaproteobacteria*) along with *Thermotogales* and *Methanolobus* in having a role in anaerobic BTEX (benzene, toluene, ethylbenzene, xylenes) degradation in enrichment from an underground gas storage aquifer. In both this thesis and the paper of Berlendis et al. (2010) the *Pelobacter* phylotype identified was 95% identical in *16S rRNA* gene sequence to *Pelobacter acetylenicus,* an obligate anaerobe that grows on acetylene and a few other carbon sources by fermentation (Schink, 1985). The increase in relative abundance of methanogenic taxa suggests that they are involved in syntrophic biodegradation of benzene, utilising for example acetate or H_2 and CO_2, or methylated compounds in the case of *Methanosarcina*. In the case of *Methanomassiliicoccus,* a novel pathway with methanol as electron acceptor and hydrogen as electron donor may be employed (Kröninger et al., 2016).

From the main experiment, several OTUs were identified in the presence of benzene in low abundance, belonging to the *Epsilonproteobacteria* genera *Sulfurimonas* and

Arcobacter, and the *Deltaproteobactria* genus *Desulfatiglans* which suggests the involvement of several microbes in benzene degradation. *Epsilonproteobacteria* in general are commonly associated with anaerobic hydrocarbon enrichments and their precise role is not fully understood although acetate scavenging has been proposed (Starke et al., 2016). *Desulfatiglans* species can degrade 4-chlorophenol (Li et al., 2015). A *Desulfatiglans* phylotype was also identified in toluene enrichments in the main experiment.

The microbial community identified in this study suggests that several microbes are involved with anaerobic benzene degradation in estuarine sediments. In addition to the previously known taxa, commonly identified in anaerobic benzene degrading enrichments namely *Deltaproteobacteria* (Oka et al., 2008, Phelps et al., 1996, Musat and Widdel, 2008), *Epsilonproteobacteria* (Phelps et al., 1998)*, Firmicutes* (Sakai et al., 2009) and *Chloroflexi* (Phelps et al., 1998) members of phylum *Verrucomicrobia*, *Actinobacteria*, or *Planctomycetes* were *also* identified.

Anaerobic degradation of toluene was complete and more rapid than benzene. Enrichments were turbid, and biofilms observed in microcosms. This is because of the ease of toluene activation by microbial communities. Activation of the benzene ring is more difficult than activating toluene (methyl benzene) (Rabus et al., 2016b), with three proposed mechanisms (hydroxylation, carboxylation and methylation; (Coates et al., 2002)). Indeed Aganbi (2008) observed no benzene degradation in enrichments from the Thames Estuary in contrast to degradation within 50 days for toluene. Due to the ease of toluene activation in the marine environment, toluene activating enzymes are model enzymes for BTEX activation studies (Rabus et al., 2016b).

Toluene degradation in estuarine sediments involves many microbes including *Desulfovirga* and *Desulfatiglans* (OTU_61, *Desulfatiglans parachlorophenolica* strain DS 89% identity). The only archaeal phylotype identified in toluene enrichments was OTU_12, a *Crenarchaeota* that shared only 80% identity with *Staphylothermus hellenicus* strain DSM 12710. The sulfate reducing rod, *Desulfovirga* was enriched recently in a toluene degrading sulfate reducing consortia of a polluted aquifer (Kuppardt et al., 2014). *Desulfovirga* is known for the ability to use C1-C12 straight-chain fatty acids, other organic acids, and alcohols as carbon sources and electron donors (Kuever et al., 2015, Tanaka et al., 2000). *Desulfatiglans* was also identified in benzene enrichments but their role in toluene degradation is not clear.

4.4.2 Anaerobic biodegradation of ethane and propane in estuarine sediments

In both the trial and main experiments, no evidence of anaerobic ethane degradation was observed. Musat (2015) discusses anaerobic oxidation of short-chain alkanes and reports that propane and butane are more readily oxidised anaerobically than ethane (e.g. (Bose et al., 2013, Mastalerz et al., 2009)). The small size of the ethane molecule and the stability of the C-H is usually cited as the reason for the resistance to biodegradation in anaerobic ecosystems (Singh et al., 2017). Adams et al. (2013) reported anaerobic ethane degradation in enrichments made with sediment samples from a hydrothermal vent system of the Middle (MV, Juan de Fuca Ridge) at 55^{o}C, even at that temperature, propane was the preferred substrate.

Indeed, propane was degraded in the main experiment and trial experiment. The main bacterial OTUs enriched on propane were ~90% similar to *Smithella, Rugosimonospora* and *Desulfatiglans.* The latter was also enriched with benzene

and toluene, indicating a potentially versatile sulfate reducer able to degrade a range of chemical structures directly or a microbe that is growing syntrophically on acetate (Suzuki et al., 2014) produced by fermentative microbes such as *Smithella*. *Smithella* has been implicated in the anaerobic degradation of alkanes using syntrophic association with methanogens (Gray et al., 2011, Tan et al., 2014).

4.4.3 Anaerobic biodegradation of isoprene in estuarine sediments

In a side-by-side experiment (simple nitrogen-flushing experiment), it was shown that aerobic degradation is relatively rapid and complete, whereas anaerobic degradation was equivocal. Anaerobic degradation of isoprene was shown from the Hythe in the nitrogen-flushing experiment (the location where aerobic degradation was fastest; Fig. 4.4a; (Acuña Alvarez et al., 2009) and in the trial experiment (Table 4.1), but not at other locations or in the main experiment. To date, anaerobic isoprene degradation has not been shown (McGenity et al., 2018). This first indication of isoprene degradation must be considered as preliminary, because of its stochastic nature.

Sulfurimomas was the dominant phylotype identified in anaerobic isoprene enrichment in the trial experiment (Figure 4.12 a). The dominant OTU_95 is related to the cultured *Sulfurimonas paralvinellae* strain GO25 (93% identity) and OTU_552 is related to *Sulfurimonas autotrophica* strain OK10 (97% identity). This dominance by *Sulfurimonas* spp. in an isoprene enrichment suggests it is involved with anaerobic degradation, even though this observation was made in a single microcosm. Although originally recognised as sulfur-oxidising (lithotrophic) species, representatives of the epsilonproteobacterial genus *Sulfurimonas* are metabolically

versatile, widespread in marine environments and some can use organic compounds as electron donors for growth (Han and Perner, 2015). They have AT-rich sequences, for example, the G+C content of *Sulfurimonas autotrophica,* strain OK genomic DNA is 35.2 mol% (Sikorski et al., 2010). This explains why thick bands (high intensity) were found high up on the DGGE gel for HaIs microcosm (Figure 4.6a). More work will be required to understand the role, if any, of *Sulfurimonas* in anaerobic isoprene degradation. Since *Sulfurimonas* is not a sulfate reducer, it is highly likely that it was using a different oxidant.

Oxygen contamination is a possibility, but it wasn't observed in the enrichment from which the *Sulfurimonas* was identified. Resazurin was added in the enrichment to check oxygen contamination. As a redox indicator, resazurin (dark blue) is reduced in two steps; irreversibly to resorufin (pink) by losing oxygen, then reversibly in the absence of oxygen to dihydroresorufin (colourless) (Tratnyek et al., 2001). In the presence of oxygen, dihydroresorufin is re-oxidized to resorufin and the pink colour reappears (Gerhardt et al., 1981). For the duration of incubation, the pink colour did not re-appear in any enrichment suggesting oxygen was absent or too little to be detected. The sensitivity of resazurin is not in doubt because it has been used successfully to detect microbial activity in anaerobic sediment (Liu and Strachan, 1979, Tratnyek et al., 2001). Therefore, it is highly unlikely that oxygen was responsible for the oxidation of isoprene.

On the other hand, *Sulfurimonas* thrives in sulfidic conditions and could have been using the sufide (made from sulfate reduction or added to the media) as its electron donor rather than isoprene (Han and Perner, 2015). As an unsaturated hydrocarbon, isoprene can serve as an electron acceptor. In fact, isoprene (1.6 µmole h^{-1}) is degraded reductively by homoacetogens (mainly *Acetobacterium* spp.) to 2-methyl-

1-butene (>97%), 3-methyl-1-butene (≤2%), 2-methyl-2-butene (≤1%) in the presence of HCO_3^- with H_2 as the electron donor (Kronen et al., 2019).

Pelobacter phylotype (OTU 167) identified in one isoprene enrichment flushed with nitrogen was related to others identified in benzene enrichments suggesting a potential role in fermentative isoprene degradation (Figure 4.15). OTU 95 (*Sulfuromonas*) was also related to OTU 569 identified from benzene enrichments so potential acetate scavengers (Vogt et al., 2011).

Anaerobic oxidation of isoprene coupled with SO_4^{2-}, NO_3^- or Fe^{3+} reduction remains an unresolved question. Kronen et al. (2019) did not observe isoprene oxidation in the presence of any of those oxidants for a year. Further work would include stable isotope probing of DNA from enrichments using ^{13}C-labelled hydrocarbons, following single enrichments to try to obtain pure cultures or stable consortia. Complementary analysis, such as measuring consumption of electron acceptors like sulfate or the production of methane should also be performed alongside these experiments.

4.5 Conclusion

The anaerobic biodegradation of benzene, toluene, and propane was demonstrated. For the first time there was an indication of anaerobic isoprene degradation with a corresponding enrichment of *Sulfurimonas*. Novel bacterial and archaeal phylotypes were identified in enrichments in low abundance. Overall, the stochastic nature of these anaerobic enrichments made it difficult to conclusively assign degradative function to particular taxa. However, this study has provided a new insight into possible microbial populations that play a role in the cycling of volatile hydrocarbons in anoxic sediments. Based on the phylotypes identified, syntrophic interaction is

suspected to play a key in the anaerobic degradation of volatile hydrocarbons in estuarine sediments.

Chapter 5. Isolation and Genome Sequence Analysis of Propane and Ethane Degrading *Rhodococcus wratislaviensis* strain I and *Mycobacterium hodleri* strain B from the Colne Estuary

5.1 Introduction

Biogenic ethane and propane produced in estuarine sediments by methanogens (Xie et al., 2013, Oremland, 1981) fuel diverse bacterial populations. These populations in estuarine environments are understudied. Research effort in the past focused on the anaerobic oxidation of short chain alkanes at marine hydrothermal vents and cold marine seeps (Singh et al., 2017). On the contrary, microorganisms that use hydrocarbon gas in soil have been characterized and are dominated by the slow growing *Corynebacterium*, *Mycobacterium*, *Norcardia*, *Rhodococcus* (CMNR) group (Shennan, 2006).

Short chain alkane degraders activate their substrates by inserting an atom of oxygen in it through the action of monooxygenases (Shennan, 2006). An organism's ability to utilize a short-chain hydrocarbon depends on its expression of a suitable monooxygenase (Shennan, 2006). These monooxygenases usually have a wide substrate range with varying activities (Van Beilen and Funhoff, 2007, Martin et al., 2014). For instance, ethane can be oxidized by ethane monooxygenase (Redmond et al., 2010), propane monooxygenase or butane monooxygenase (Dubbels et al., 2009, Sluis et al., 2002, Kulikova, 1995, Shennan, 2006) . Substrate's carbon-chain length influence enzyme activity (Coleman et al., 2012, Martin et al., 2014). Activities

of a monooxygenase (Smo enzyme) produced by transformed *Mycobacterium smegmatis* mc^2-155 decreased with substrate-size increase, from C_2 (ethane: 0.57 nmol/min/mg of protein) through C_3 (propane: 0.12 nmol/min/mg of protein) to C_4 (butane: 0.04 nmol/min/mg of protein) (Martin et al., 2014).

Propane monooxygenase is a multicomponent soluble diiron monooxygenase that activates ethane or propane. (Leahy et al., 2003a, Coleman et al., 2006, Shennan, 2006) . It has been purified and fully characterized using molecular techniques (Kotani et al., 2003). The propane monooxygenase from *Gordonia* sp. TY-5 has three components, a hydroxylase, an oxidoreductase and a coupling protein (Kotani et al., 2003). It is encoded by the *prmABCDE* gene cluster that is transcribed polycistronically (Kotani et al., 2003, Kotani et al., 2006, Sharp et al., 2007).

Genome analysis has revealed that genes encoding propane monooxygenase are often located on a chromosome with *prmA*, *prmB*, *prmC*, *prmD* closely spaced and located upstream of the *prm* genes while four open reading frames (including *prmE* and *groEL1*) are downstream (Kotani et al., 2003, Sharp et al., 2007). The role of each gene in the prmABCDE operon has been proven experimentally as follows: *prmA* encodes the large subunit of the hydroxylase; *prmB* encodes the NADH-dependent acceptor oxidoreductase (reductase); *prmC* encodes the small subunit of hydroxylase; *prmD* encodes the coupling protein; *prmE* encodes alcohol dehydrogenase and *groL1* encodes 60-kDa chaperonin GroEL (Sharp et al., 2007, Kotani et al., 2006, Kotani et al., 2003). Some bacterial strains have more than one propane monooxygenase gene cluster. *Gordonia* sp. TY-5 and *Mycobacterium* sp. TY-6 contain one propane monooxygenase gene cluster while *Pseudonorcadia* sp. TY-7 has two kinds of propane monooxygenase gene clusters (Kotani et al., 2006, Kotani et al., 2003). However, there are variations in gene organization within the

prm gene cluster of *Gordonia* sp. TY-5 and *Mycobacterium* sp. TY-6 (Kotani et al., 2006).

The pathway for aerobic oxidation of short chain alkanes by alkanotrophs proceeds from substrate activation by a monooxygenase using reactive oxygen species to overcome the low reactivity of alkane molecules to yield an alkanol (Rojo, 2009). Ethane-degrading microbes utilize ethane through oxidation to the primary alcohol, ethanol, followed by conversion to acetaldehyde and acetate (Shennan, 2006). This pathway was demonstrated experimentally in the fungus, *Acremonium* sp. (Davies et al., 1976). The propane degradation pathway generated great interest and argument amongst scientists for decades. However, there has been a general consensus that propane oxidation proceeds through three possible pathways: terminal oxidation of propane to 1-propanol; subterminal oxidation of propane to 2-propanol and simultaneous oxidation of propane to both 1-propanol and 2-propanol (Ashraf et al., 1994, Kulikova, 1995, Shennan, 2006). All three pathways have been proven in bacterial strains. *Mycobacterium vaccae* JOB5 and *Mycobacterium sp*. TY-6 are examples of bacterial strains that metabolize propane through the terminal oxidation pathway (Perry, 1980, Kotani et al., 2006). *Pseudonocardia* sp. TY-7 and *Rhodococcus rhodochrous* PNKbl use both terminal and subterminal oxidation pathways for propane metabolism (Woods and Murrell, 1989, Ashraf and Murrell, 1992, Ashraf and Murrell, 1990a, Kotani et al., 2006).

Propane degradation begins with the oxidation of propane at the terminal or subterminal carbon atom (Figure 5.1). The reaction is catalysed by propane monooxygenase in the presence of NADH and O_2 (Shennan, 2006, Kulikova, 1995). Bacterial strains using the terminal oxidation pathway oxidize propane to 1-propanol, propanal (propionaldehyde) and propanoic (propionic) acid, then enter the

tricarboxylic cycle. Subterminal oxidation proceeds from propane to 2-propanol (isopropanol), then to propanone (acetone) and could continue by different routes to acetate, the central metabolite. The sub-terminal propane oxidation pathway through acetone oxidation to methanol (Figure 5.1) was demonstrated in *Gordonia* sp. Strain TY-5 (Kotani et al., 2007) . A key enzyme required for the process is acetone monooxygenase, which oxidizes acetone to methyl acetate. The *acmA* and *acmB* genes that encodes acetone monooxygenase and methyl acetate hydrolase were responsible for the metabolism of methyl acetate to acetic acid and methanol respectively (Figure 5.1) (Kotani et al., 2007).

To date only a few ethane or propane-degrading bacterial genomes have been sequenced, mostly from soil environments (Shennan, 2006, Musat, 2015, Singh et al., 2017). The focus of this study was to isolate autochthonous ethane and propane-degrading bacteria from the Colne estuary. These groups were targeted for isolation from sediment slurries at the end of the first enrichments (section 3.2.2.2, Chapter 3). Isoprene utilising bacteria were isolated previously by Acuña Alvarez et al. (2009) from the same sampling location, therefore, they were not targeted for isolation. Similarly, toluene and benzene were not targeted for isolation, however, the isolates obtained were tested for the ability to degrade isoprene, benzene, or toluene.

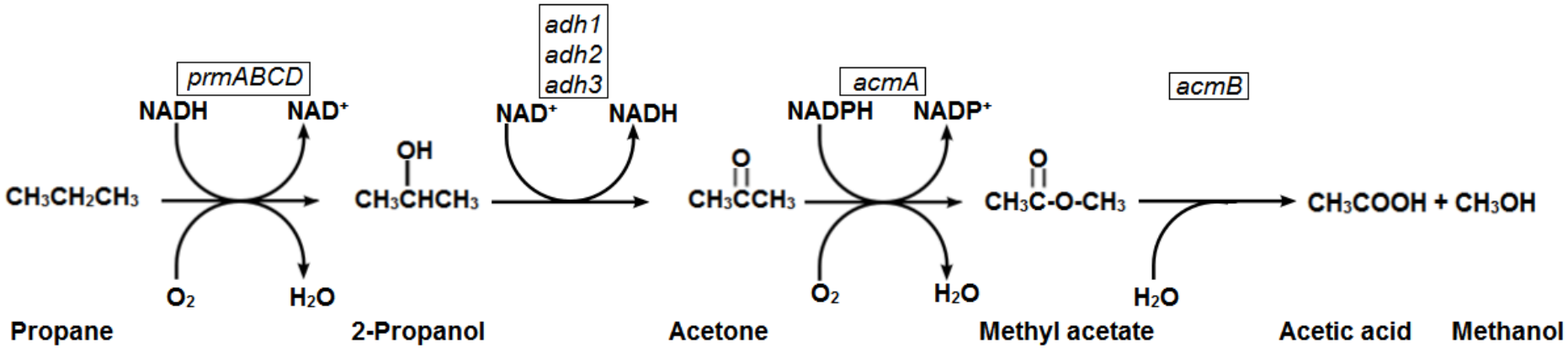


Figure 5.1. Propane metabolism in *Gordonia* sp. strain TY-5. The genes involved in each step of the reaction are indicated in the boxes: *prmABCD* gene cluster encodes propane monooxygenase; *acmA* encodes acetone monooxygenase and *acmB* encodes methyl acetate hydrolase. Redrawn from Kotani et al. (2007).

Aim

To isolate ethane and propane degrading microbes from aerobic microcosms of samples from the Colne estuary, sequence their genomes and from the genome analysis, predict the possible metabolic pathways.

Hypothesis

Ethane and propane-degrading bacterial isolates will possess a soluble diiron centre monooxygenase enzyme for substrate activation since this is the most typical mechanism for short alkane activation.

Specific Objectives

5.1 Isolate and characterize aerobic ethane and propane-degrading bacteria.

5.2 Sequence the genomes of bacterial strains isolated and identify the ethane or propane metabolic gene cluster.

5.2 Materials and Methods

5.2.1 Isolation and identification of ethane and propane degrading bacterial strains from the Colne estuary sediment slurries

To isolate ethane and propane degrading bacteria from the Colne estuary, several bacterial isolates were obtained from sediment slurry microcosms (aka first enrichment, section 3.2.2.2 of Chapter 3) with propane or ethane. Isolation was carried out at the end of the first enrichment (day 37). This was a different stage to my bacterial community analysis. The bacterial community analyses described in section 3.2.4 of Chapter 3 were performed at the end of the third enrichment.

A 10-fold serial dilution of samples from triplicate microcosms was carried out, then an aliquot of 0.1 ml from each dilution was streaked onto plates of 1/3 ONR7a medium containing 15 g l^{-1} agar. Plates were incubated in a desiccator with an ethane or propane-rich atmosphere at room temperature for 7 days. The head space of desiccators was made propane or ethane-rich by flushing with ethane or propane for 5 min from a gas cylinder under the fume cupboard. Distinct colonies were picked and sub-cultured as described above until discrete pure colonies were obtained. Screening for isolates that can use ethane or propane was done on solid agar by streaking individual colonies onto plates of 1/3 ONR7a medium containing 15 g l^{-1} agar. Each isolate was incubated in a desiccator with an ethane or propane-rich atmosphere; or on the bench in the laboratory with no carbon source; at room temperature for 7 days. Isolates that grew well under the atmosphere of ethane or propane but could not in the absence of a carbon source were selected for cultivation in liquid medium. Ten isolates, referred to as isolate A to J, were cultivated in 1/3 ONR7a liquid medium with ethane or propane as a sole carbon and energy source. The inoculum was prepared as follows: a loopful of each isolate was

transferred from a culture plate into a sterile 50-ml falcon tube containing 10 ml of sterile 1/3 ONR7a media (section 5.2.2) and vortexed to homogenize. The broth cultures were set up in 125-ml serum bottles containing a mixture of 1 ml of inoculum and 9 ml of 1/3 ONR7a media (Figure 5.6 b & c). Ethane (12.5 cm^3) or propane (12.5 cm^3) were transferred from a latex bladder into the culture bottle to give a final concentration of 10% v/v. Gas filters (Minisart-Plus filters pore size 0.2 µm, Sigma) were used during the final transfer into microcosms to prevent contamination. Sterile controls had no bacterial strain and they were prepared by autoclaving 10 ml of one-third ONR7a minimal medium at 121^oC for 20 min before addition of hydrocarbon substrates (ethane or propane). All liquid culture bottles and the corresponding controls were incubated at 12^oC in triplicate for 34 days. Consumption was monitored using the GC-FID as described in Chapter 2, Section 2.3. Two isolates (B and I) grew well on both ethane and propane, therefore, they were selected for further studies. The two strains were tested for the ability to utilize ethane and propane at low concentration, i.e 1.6% v/v final concentrations in liquid culture. They were also tested for their ability to utilize isoprene, benzene and toluene as single substrates in liquid culture at the same concentrations described in section 3.2.2.2 (Chapter 3). The liquid cultures were set up in 125-ml serum bottle containing a mixture of 1 ml of inoculum prepared as described above and 9ml of 1/3 ONR7a media (final volume 10 ml) with a large headspace. At low concentrations, 2 cm^3 ethane or propane was added into the culture bottles at a final concentration of 1.6 % v/v ethane or propane. Gas filters (Minisart-Plus filters pore size 0.2 µm, Sigma) were used during the final transfer into microcosms to prevent contamination. Sterile controls with carbon source but no bacterial strains added were prepared for each carbon source. They were prepared in a similar manner and contained the same

volume of 1/3ONR7a except that no inoculum was added. Liquid culture bottles and the corresponding controls were incubated at 12^{o}C for 48 days.

Isolates B and I were identified by 16S rRNA sequencing. Briefly, 2 ml was transferred from a 20-day old broth culture into 2 ml microfuge tubes using sterile pipettes and centrifuged (11300 x *g* for 60 s) to obtain cell pellets. DNA was extracted from the pellets following CTAB method (Griffiths et al., 2000). The DNA extracted was used as template in the PCR amplification targeting the bacterial *16S rRNA* gene with the 27F/1492R primer pair (Lane, 1991). The primer sequences were: 27F 5'-AGAGTTTGATCCTGGCTCAG-3'; and 1492R: 5'-GGTTACCTTGTTACGACTT-3'. Each 50 µl reaction mixture was made up of 1 µl of DNA template, 25 µl of REDTaq ready mix (Sigma), 2 µl each of forward (27F) and reverse primers (1492R), and 20 µl of Milli-Q water. The thermocycling programme used was as follows: Incubation: 95^{o}C, 5 min; (Denaturing: 94^{o}C, 30 s; Annealing: 57^{o}C, 30 s; Extension: 72^{o}C, 90 s) × 30 cycles with a final extension at 72^{o}C for 10 min. The amplification products were viewed by electrophoresis in 1% (w/v) 1 × TAE (40 mM Tris-acetate, 1 mM di-sodium-EDTA, pH 8.0) agarose gel and by ethidium bromide (0.5 µg ml^{-1}) staining. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN) following manufacturer's protocol as described in section 3.2.3 of Chapter 3. Both forward and reverse DNA strands where sequenced using the 27F, 1492R primer pair. DNA samples were sent to Source Bioscience (Nottingham, UK) for sequencing. DNA sequences were analysed and edited using Chromas (Technelysium Pty Ltd). Sequence alignment was done using MUSCLE (Edgar, 2004) available in MEGA versions 6 (Tamura et al., 2013). Sequences were compared with those in the nucleotide database at the National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST)

(Altschul et al., 1990). Jukes-Cantor distance and Neighbour-Joining methods were used to create phylogenetic trees for partial *16S rRNA* genes in MEGA 6 with bootstrap values of 1000 (Jukes and Cantor, 1969, Tamura et al., 2013, Saitou and Nei, 1987, Felsenstein, 1985). Following sequence and phylogenetic analyses, the two main bacterial strains were identified as *Mycobacterium hodleri* strain B (isolate B) and *Rhodococcus wratislaviensis* strain I (isolate I), Figure 5.2 – 5.3.

5.2.2 Preparation of artificial seawater minimal media, one-third ONR7a

Artificial seawater minimal media (ONR7a) was prepared as described by Dyksterhouse et al. (1995) with modification of the sodium and magnesium salt concentration as described in Chapter 2, Section 2.12. Solid one-third ONR7a medium was prepared by replacing the distilled water with molten bacteriological agar (Oxoid, UK). The bacteriological agar was washed with acetone to remove any nutrients or impurities before use. Briefly, to prepare 1000 ml agar at a final concentration of 15 g/l, 18 g of bacteriogical agar (Oxoid, UK) was weighed into a 2000-ml Duran bottle. 18g agar was added to account for loss during the agar wash. Distilled water (500 ml) and acetone (20 ml) were added and stirred continuously on a magnetic stirrer for 30 minutes. The mixture was allowed to stand for 30 minutes. Then the supernatant was poured out carefully to minimise the loss of agar. The agar was washed two more times with 500 ml distilled water without the addition of acetone. After the last wash, the volume was made up to 1000 ml before autoclaving. One-third ONR7a Media with 1% (w/v) Yeast Extract was also prepared to support a more rapid growth and proliferation of the ethane and propane utilising bacterial isolates. The Yeast Extract (Oxoid, UK) served as a nutrients source. This

was done by mixing 100 ml Solution 1, 100 ml Solution 2, and 1 ml Solution 3 with 800 ml of agar/yeast extract solution (Chapter 2, section 2.6).

5.2.3 Preparation of bacterial cells for genome sequencing

Cells for genome sequencing were prepared following the guideline provided by MicrobesNG (Birmingham, UK). A single colony of each bacterial strain to be sequenced was mixed with 100 µl of sterile 1x Phosphate buffered saline (PBS). The 1x PBS buffer was prepared as described by Sambrook et al. (1989). To make 1 litre of 1x PBS, 800 ml of distilled water was used to dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in a volumetric flask. The pH of the solution was adjusted to 7.4 with concentrated HCl, and deionised water was added to make a total volume of 1 litre. The buffer was sterilized by autoclaving (15 min, 121°C) and stored at room temperature. The bacterial strain from the 100 µl culture described above was streaked onto an agar plate (1/3 ONR7a media with 1% yeast extract) (Figure 5.6a). After incubation at room temperature for 7 days, all the bacterial culture from the plate were transferred into the barcoded bead tube supplied by MicrobesNG (Birmingham, UK) using a large sterile loop. Tubes were vortexed and most of the liquid component was removed. Bead tubes were sealed and sent to MicrobesNG as recommended in the protocol.

5.2.4 Genome sequencing and annotation

The genomes of *Rhodococcus wratislaviensis* strain I and *Mycobacterium hodleri* strain B were sequenced on HiSeq 2500 platform (Illumina sequencer) using standard Nextera protocol at the University of Birmingham MicrobesNG, IMI - School

of Biosciences (Birmingham, UK). Each genome had 30x coverage. Each run produced 2 x 250bp paired-end reads. The bioinformatic analyses was carried out by MicrobesNG (Birmingham,UK) including: *De novo* assembly of reads was done using SPAdes (Bankevich et al., 2012); the reads were trimmed using Trimmomatic (Bolger et al., 2014) and quality was assessed using MicrobesNG in-house sottware (Samtools, BedTools and bwa-mem). The genome sequences obtained from MicrobesNG were uploaded to RAST (Rapid Annotation using Subsystem Technology) in gbank format for annotation (Aziz et al., 2012, Devoid et al., 2013, Disz et al., 2010, Overbeek et al., 2014). ClassicRAST was the annotation scheme used as implemented by the RAST pipeline (Overbeek et al., 2014). The “Compare Regions” tool in SEED was used to compare the region containing the propane (and ethane) metabolic gene cluster in *R. wratislaviensis* strain I and *M. hodleri* strain B with homologous regions identified in related bacterial strains (Overbeek et al., 2014). The annoted protein and amino acid sequences from *R. wratislaviensis* strain I and *M. hodleri* strain B were compared with those on the protein database at the National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (Protein BLAST) (Altschul et al., 1990). The propane monooxygenase gene cluster identified in RAST was analyzed phylogenetically. Poisson correction method and Neighbour-Joining methods were used to create phylogenetic trees for the amino acid sequences in MEGA 6 with bootstrap values of 10000 (Tamura et al., 2013, Saitou and Nei, 1987, Felsenstein, 1985, Zuckerkandl and Pauling, 1965).

5.2.5 Statistical analysis of degradation data

Significant difference in concentration of ethane or propane degraded by bacterial isolates relative to the sterile controls was determined by Welch's two-sample t-test carried out in RStudio (version 0.98.994 - © 2009 – 2013, RStudio Inc.).

5.3 Results

5.3.1 Identity of ethane and propane utilising bacterial isolates revealed by *16S rRNA gene* analysis

In order to isolate ethane and propane utilising bacteria from the Colne estuary, several bacterial isolates were obtained from sediment slurry microcosms enriched with ethane or propane as sole carbon and energy source (Chapter 3). Two strains referred to as strain B and strain I grew well on both ethane and propane (Figure 5.4 and 5.5). Strain I was isolated from Wivenhoe microcosms with ethane as the sole carbon source while strain B was isolated from Hythe microcosms with propane as the sole carbon source. Plates showing both strains are displayed in Figure 5.6a.

The *16S rRNA* gene sequence of strain B was 99% similar to that of *Mycobacterium hodleri* strain DSM 44183. Phylogenetic analysis showed that strain B was closely related to the type strain, *M. hodleri* (X93184) (Figure 5.2), so it is referred to as *Mycobacterium hodleri* strain B. Strain I also share 99% *16S rRNA* gene sequence identity with *Rhodococcus wratislaviensis* strain NCIMB 13082 (NR_026524.1) and it is phylogenetically related to the type strain, *R. wratislaviensis* (Z37138) (Figure 5.3). As a result, strain I is referred to as *Rhodococcus wratislaviensis* strain I.

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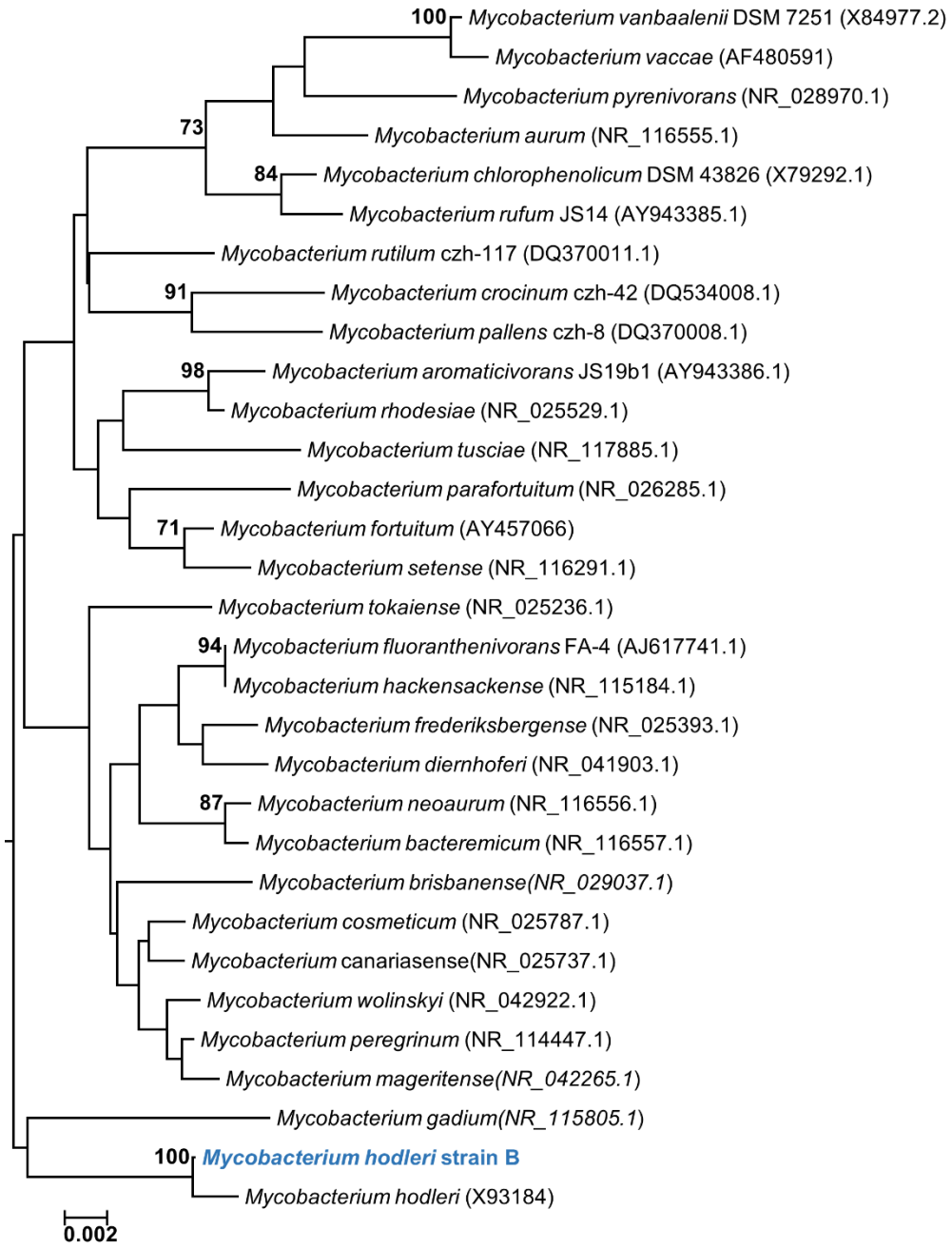

Figure 5.2. Phylogenetic tree based on *16S rRNA* gene sequence of *Mycobacterium hodleri* strain B (blue) and selected organisms. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. Only bootstrap values ≥ 70% are shown. Evolutionary analyses were conducted in MEGA6.

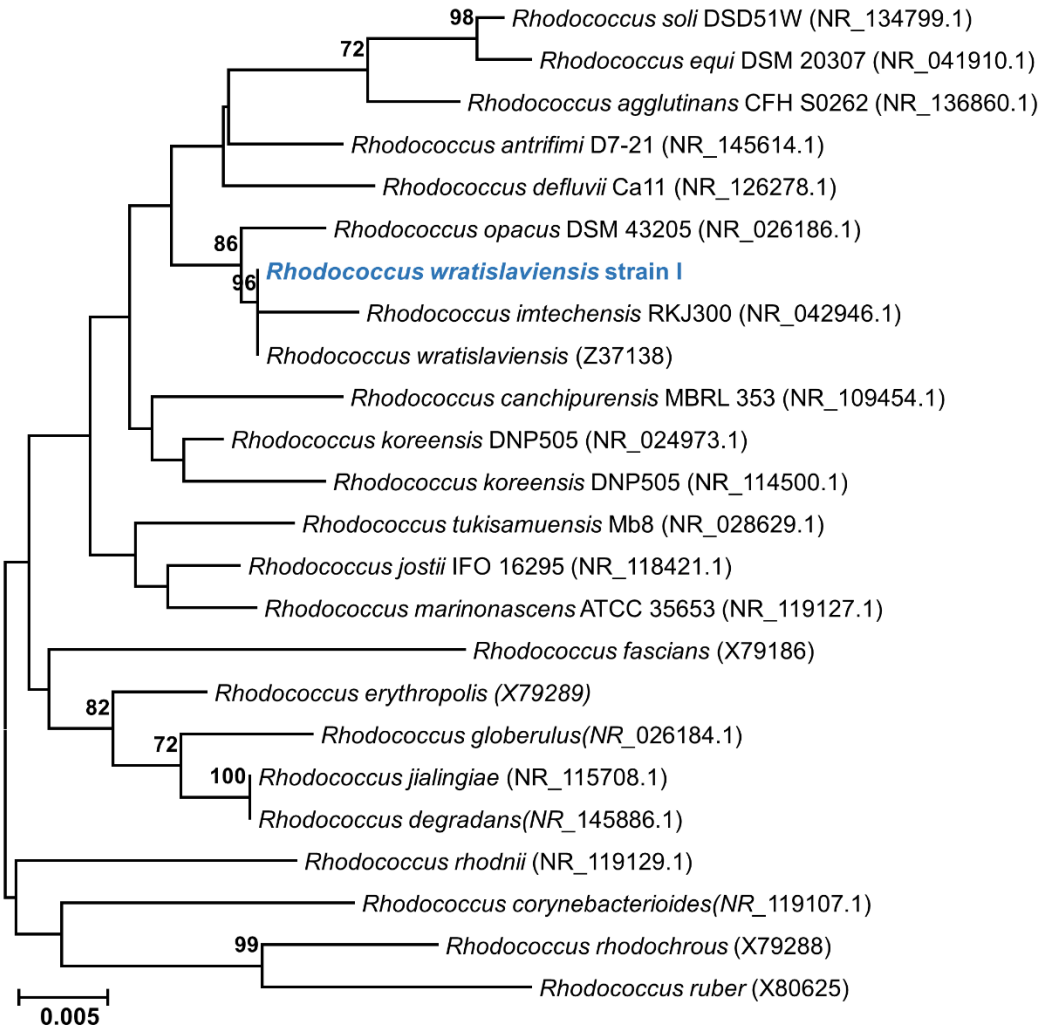

Figure 5.3. Phylogenetic relationship based on *16S rRNA* gene sequence of *Rhodococcus wratislaviensis* strain I (blue) and selected organisms. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. Only bootstrap values ≥ 70% are shown. Evolutionary analyses were conducted in MEGA6.

5.3.2 Degradation of propane and ethane by *Mycobacterium* and *Rhodococcus* species

M. hodleri strain B and *R. wratislaviensis* strain I degraded ethane or propane as a sole source of carbon and energy (Table 5.1, Figure 5.4). At a lower concentration, both isolates degraded ethane below detection limit (section 2.3) in 19 days (Table 5.1). At day 19, significant propane degradation was also observed in liquid culture relative to the sterile control ($P < 0.05$) with 45 (±5.8) % degraded by *M. hodleri* strain B and 42 (±1.8) % by *R. wratislaviensis* strain I (Table 5.1). Propane degradation was not complete. Residual propane (% remaining) at 48 days was 9.3 (±2.5) and 31.4 (±1.6) in *M. hodleri* strain B and *R. wratislaviensis* strain I cultures respectively (Figure 5.4).

Table 5.1 Ability of *Mycobacterium hodleri* strain B and *Rhodococcus wratislaviensis* strain I to utilize benzene, toluene, isoprene, ethane or propane as a sole source of carbon and energy in 1/3 ONR7a broth after 19 days of incubation .

Hydrocarbon	Concentration	% Degradation (± SE) by *Mycobacterium* sp. strain B	% Degradation (± SE) by *Rhodococcus* sp strain I
Ethane	1.6% v/v [a]	100 (± 0.0)	100 (± 0.0)
Propane	1.6% v/v [a]	45 (± 5.8)	42 (± 1.8)
Isoprene	7.2 x 10^{-6} ppbv	5 (± 7.9)	54 (± 19.6)
Benzene	0.5 mM	21 (± 2.1)	100 (± 0.0)
Toluene	0.5 mM	100 (± 0.0)	100 (± 0.0)

Lower ethane or propane concentrations were tested[a] .

M. hodleri strain B and *R. wratislaviensis* strain I were able to degrade other volatile hydrocarbons including benzene and toluene (Table 5.1). *M. hodleri* strain B could not degrade isoprene (Table 5.1). Isoprene degradation by *R. wratislaviensis* strain I was not confirmed by a corresponding turbidity in liquid cultures (Fig 5.6 b &c).

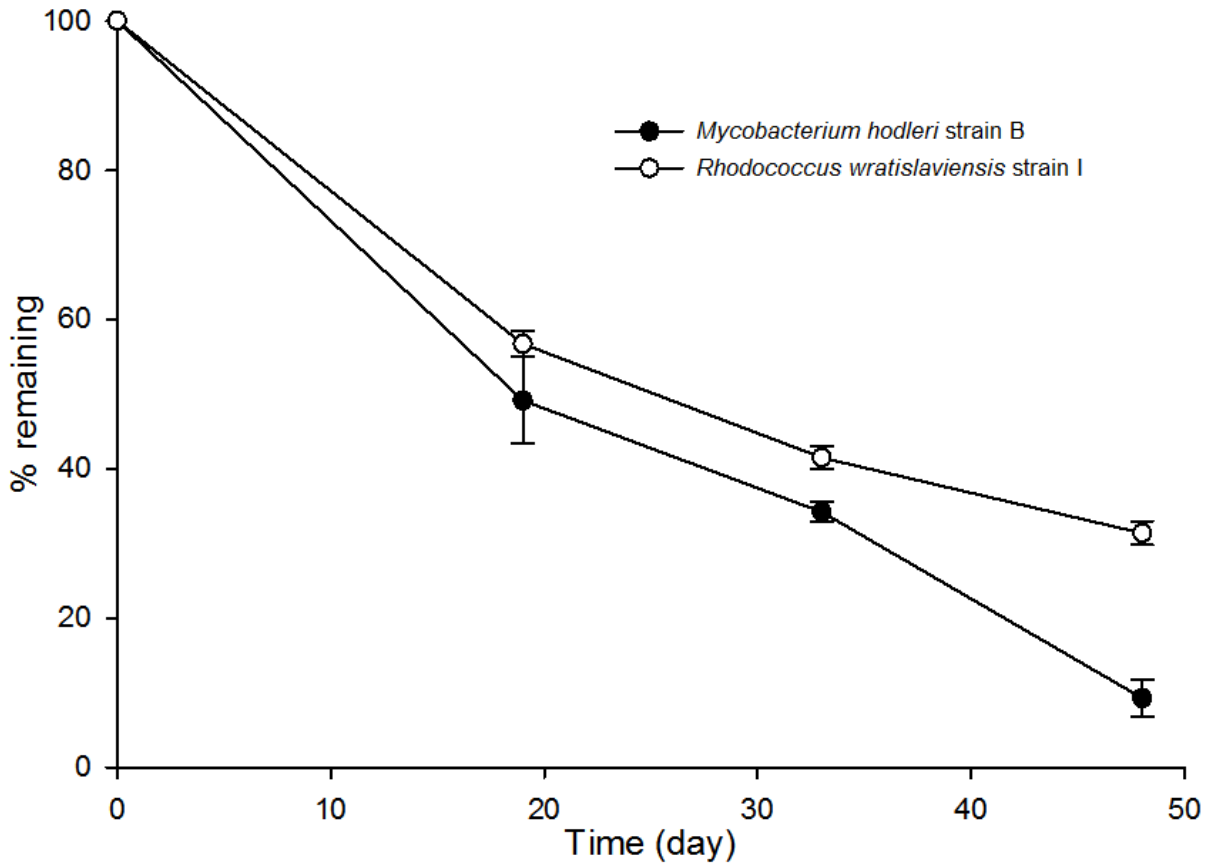

Figure 5.4. Degradation of propane by strain B and I. Values were normalized with the control and are presented as the mean ± standard error, *n* = 3.

To determine the growth of *R. wratislaviensis* strain I and *M. hodleri* strain B on ethane or propane in minimal media (1/3 ONR7a), turbidity measurements were performed at day 26 of incubation. *R. wratislaviensis* strain I grew better on ethane or propane than *M. hodleri* strain B (Figure 5.5).

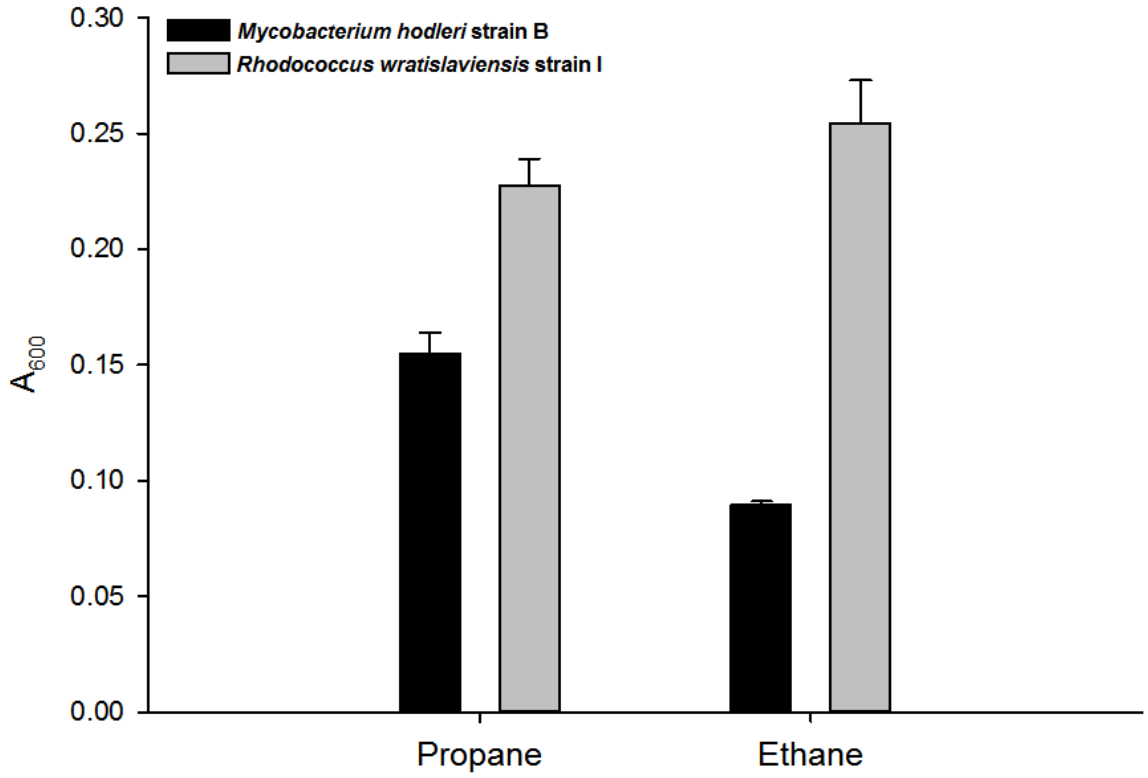

Figure 5.5. Growth of *Mycobacterium hodleri* strain B and *Rhodococcus wratislaviensis* strain I on minimal salt medium (1/3 ONR7a) with ethane or propane as sole carbon source after incubation at 12°C for 26 days. Absorbance was measured at 600 nm (A_{600}). Concentration of ethane or propane was 10% v/v.

5.3.2 General genome features for *Rhodococcus wratislaviensis* strain I and *Mycobacterium hodleri* strain B

The genome of *R. wratislaviensis* strain I is approximately 8.77 Mbp in size with a GC content of 67.1 mol% and a total of 8462 predicted protein coding sequences (Table 5.2). The genome of *M. hodleri* strain B is approximately 6.99 Mbp in size with a GC content of 67.5 mol% and a total of 6702 predicted protein coding sequences (Table 5.2.).

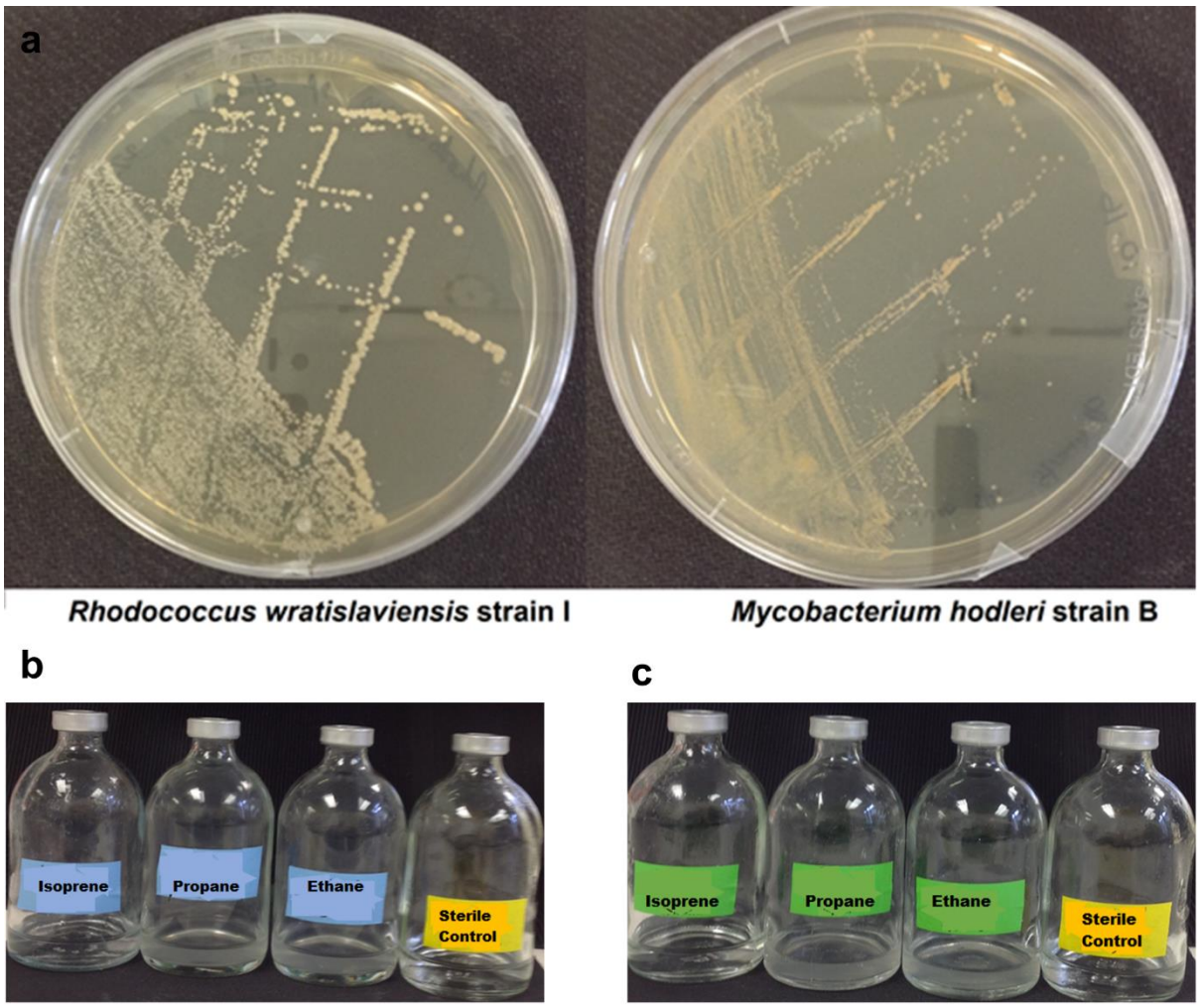

Figure 5.6. Axenic culture of *Rhodococcus wratislaviensis strain I* and *Mycobacterium hodleri* strain B cultivated on 1/3 ONR7a minimal media with 1% yeast extract (a); *R. wratislaviensis* strain I (b) & *M. hodleri* strain B (c) cultivated on ethane, propane, or isoprene in 1/3 ONR7a minimal salt medium.

Table 5.2 Genome features of *Rhodococcus wratislaviensis* strain I and *Mycobacterium hodleri* strain B.

Genome Feature	*R. wratislaviensis* strain I	*M. hodleri* strain B
Size (bp)	8,771,474	6,987,084
GC content (%)	67.1	67.5
Number of Contigs	320	220
Number of Coding Sequences	8462	6702

Key features of the *R. wratislaviensis* strain I and *M. hodleri* strain B genomes were compared with other *Mycobacterium* and *Rhodococcus* species respectively (Table 5.3 and 5.4). Their genome size and G+C content were similar to related strains. The G+C content of *M. hodleri* Strain B (67.5%), was similar to *M. smegmatis* Strain MC^2 155 (67.4%) (Table 5.3). The G+C content of *R. wratislaviensis* strain I (67.1%) was also similar to *Rhodococcus* sp RHA1 (67.0) and *R. opacus* PD630 (67.5%), (Table 5.4).

Table 5.3 Comparison of *Mycobacterium hodleri* strain B genome with selected *Mycobacterium* strains.

Organism	Size (Mbp)	GC Content (%)	Reference
Mycobacterium hodleri Strain B	6.99	67.5	This Study
Mycobacterium sp. AT1	7.07	67.2	Johnston et al. (2017)
Mycobacterium avium subsp. hominissuis Strain H87	5.63	68.8	Zhao et al. (2017)
Mycobacterium rufum JS14	6.18	69.3	Kwak et al. (2016)
Mycobacterium smegmatis Strain (MC^2 155)	6.99	67.4	Mohan et al. (2015)
Mycobacterium cosmeticum DSM 44829	6.46	68.2	Croce et al. (2014)
Mycobacterium phlei Type Strain RIVM601174	5.68	69.2	Abdallah et al. (2012)
Mycobacterium marinum	6.64	62.5	Stinear et al. (2008)

5.3.3 Genome sequence of the *prmABCDE* gene cluster

The *prmABCDE* gene cluster was identified in both *R. wratislaviensis* strain I and *M. hodleri* strain B genomes by a BLAST search against available sequence databases and phylogenetic analysis (Figure 5.10 and 5.11). The *prmABCDE* gene cluster that encodes propane monooxygenase was present in both strains but differed in organization (Figures 5.7 and 5.8).

Table 5.4 Comparison of *Rhodococcus wratislaviensis* strain I genome with selected *Rhodococcus* strains.

Organism	Size (Mbp)	GC Content (%)	Reference
Rhodococcus wratislaviensis Strain I	8.77	67.1	This study
Rhodococcus sp. AD45	6.80	61.7	Crombie et al. (2015)
Rhodococcus sp. JG-3	5.28	64	Goordial et al. (2015)
Rhodococcus opacus R7	10.1	66	Orro et al. (2015)
Rhodococcus opacus PD630	9.17	67.5	Chen et al. (2014)
Rhodococcus sp. BCP1	6.23	70.4	Cappelletti et al. (2013)
Rhodococcus sp. Strain JVH1	9.19	66.9	Brooks and Van Hamme (2012)
Rhodococcus sp RHA1	9.70	67.0	McLeod et al. (2006)
Rhodococcus erythropolis strain PR4	6.90	62.3	Sekine et al. (2006)

In *R. wratislaviensis* strain I, the *prmABCDE* gene cluster was located on the negative strand of contig 80 (Figure 5.7). Eight putative open reading frames were on the same strand. The *prmABCD* genes were located upstream (5') of the propane monooxygenase gene closely spaced. The alcohol dehydrogenase gene (*prmE*), *orf1*, *orf2* and *groEL1* genes were located downstream of the propane

monooxygenase gene. The *orf1* and *orf2* genes were identified as a metal-dependent hydrolase and hypothetical proteins respectively (Table 5.6). This operon is very similar in *R.* jostii RHA1, with sequence identities ranging from 97 – 100% (Figure 5.7).

The *prmABCDE* gene cluster of *M. hodleri* strain B was located on a negative strand in contig 23 (Figure 5.8). The *prmABCD* genes were located upstream of the propane monooxygenase gene, closely spaced together. The alcohol dehydrogenase gene (*prmE*), hypothetical protein, *groEL1* and a putative helix-turn-helix (HTH) type transcriptional regulator were located downstream (Figure 5.8, Table 5.5). The structure of the *prmABCDE* in *M. hodleri* strain B is different from *M. smegmatis* strain MC^2 155 with sequence identities ranging from 58 to 97% (Figure 5.8). The *prmC* gene in *M. smegmatis* strain MC^2 155 shared 89% identity with *M. hodleri* strain B and the gene is split in two (Figure 5.8). Their alcohol dehydrogenase gene (*prmE*) share only 58% sequence identity (Figure 5.8).

A protein BLAST search against available sequence databases revealed that the *prmABCDE* gene cluster in *R. wratislaviensis* strain I and *M. hodleri* strain B were soluble diiron monooxygenases. A phylogenetic tree based on the amino acid sequence of the α-subunit of hydroxylases in *M. hodleri* strain B, *R. wratislaviensis* strain I and other soluble diiron monooxygenases (SDIMO) showed that the *prmA* gene of *R. wratislaviensis* strain I was homologous to that from *R. jostii* RHA1, while the *prmA* gene of *M. hodleri* strain B was homologous to that from *M. smegmatis* str MC^2 155 (Figure 5.9).The amino acid sequence *of prmB, C, D, E* and *GroEL* genes were also homologous to other propane monooxygenase subunit genes sequenced from other microbial strains (Figure 5.10).

The presence of a *prmABCDE* gene cluster which encode propane monooxygenase accounts for the ability of *R. wratislaviensis* strain I and *M. hodleri* strain B to utilize both ethane and propane. No gene encoding isoprene monooxygenase was found in the two genomes.

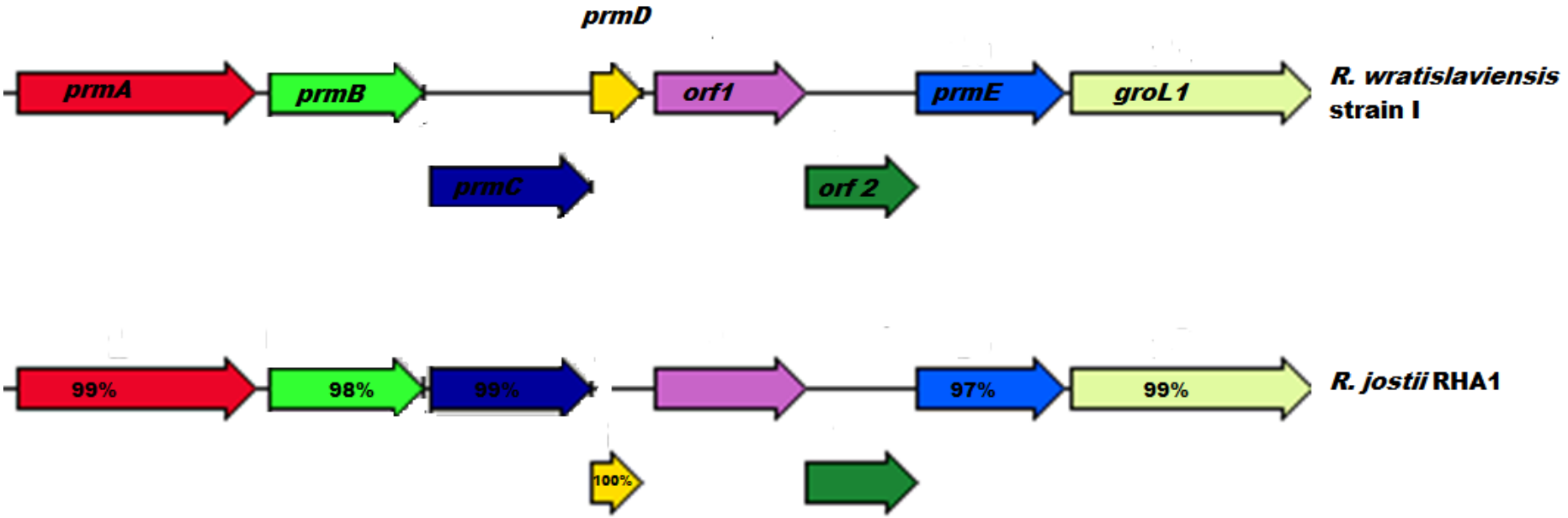

Figure 5.7. The region of the *Rhodococcus wratislaviensis* strain I chromosome (contig 80) containing propane (and ethane) metabolic genes (top) and a homologous region identified on the chromosome of *Rhodococcus jostii* RHA1 (bottom). Sets of genes with similar sequence are the same colour. Percentage identities of deduced amino acid sequences between *R. wratislaviensis* strain I and *Rhodococcus jostii* RHA1 propane monooxygenase (PMO)-related genes is shown.

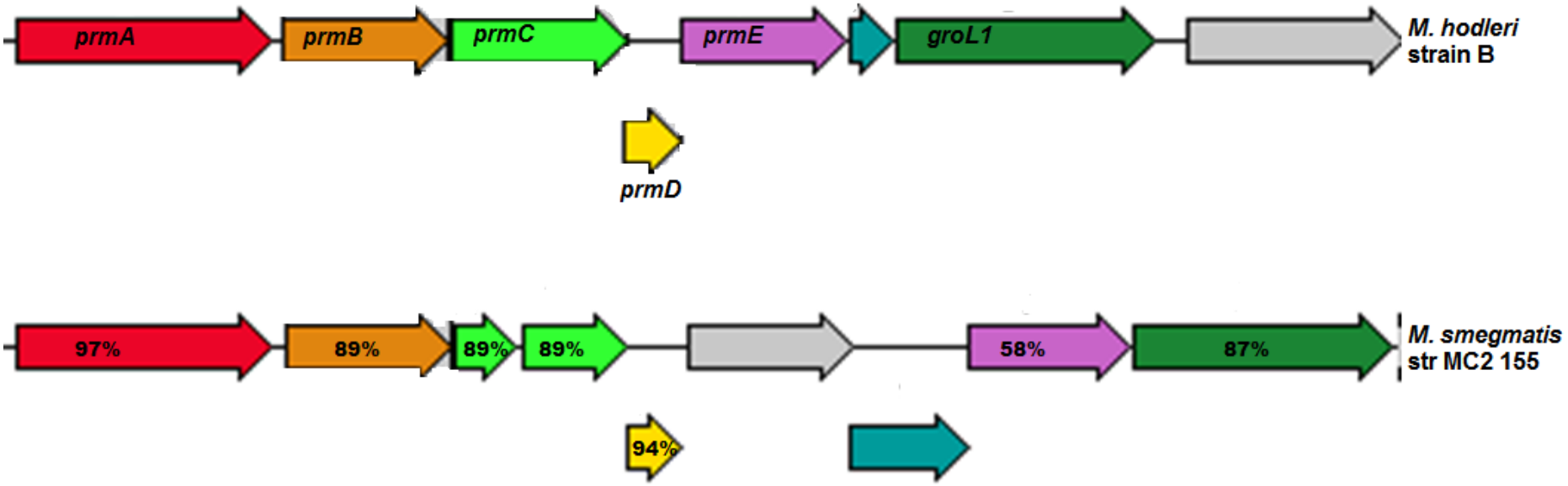

Figure 5.8. The region of the *Mycobacterium hodleri* strain B chromosome (contig 23) containing propane (and ethane) metabolic genes (top) and a homologous region identified on the chromosome of *Mycobacterium smegmatis* str MC2 155 (bottom). Sets of genes with similar sequence are grouped with the same color. Percentage identities of deduced amino acid sequences between *Mycobacterium hodleri* strain B and *Mycobacterium smegmatis* str MC2 155 propane monooxygenase (PMO)-related genes is shown.

Table 5.5 *Overview of the* prmABCDE *operon in* Mycobacterium hodleri *strain B.*

ID	Start	Stop	Nucleotide Size	Strand	Function
fig|6666666.244804.peg.2350	77197	74429	2769	-	Putative HTH-type transcriptional regulator Rv0890c/MT0914
fig|6666666.244804.peg.2351	79050	77404	1647	-	Heat shock protein 60 family chaperone GroEL
fig|6666666.244804.peg.2352	79346	79083	264	-	FIG01128099: hypothetical protein
fig|6666666.244804.peg.2353	80428	79382	1047	-	Putative alcohol dehydrogenase (*prmE*)
fig|6666666.244804.peg.2354	80781	80425	357	-	Propane monooxygenase coupling protein (*prmD*)
fig|6666666.244804.peg.2355	81884	80778	1107	-	Propane monooxygenase small subunit (*prmC*)
fig|6666666.244804.peg.2356	82955	81909	1047	-	Propane monooxygenase reductase (*prmB*)
fig|6666666.244804.peg.2357	84655	83030	1626	-	Propane monooxygenase hydroxylase large subunit (*prmA*)

HTH = helix-turn-helix

Table 5.6 Overview of the *prmABCDE* operon in *Rhodococcus wratislaviensis* strain I.

ID	Start	Stop	Nucleotide Size	Strand	Function
fig|6666666.244803.peg.7856	8159	6510	1650	-	Heat shock protein 60 family chaperone GroEL
fig|6666666.244803.peg.7857	9226	8201	1026	-	Putative alcohol dehydrogenase (*PrmE*)
fig|6666666.244803.peg.7858	9966	9223	744	-	FIG01128099: hypothetical protein
fig|6666666.244803.peg.7859	11009	9963	1047	-	Predicted metal-dependent hydrolase of the TIM-barrel fold
fig|6666666.244803.peg.7860	11430	11089	342	-	Propane monooxygenase coupling protein (*prmD*)
fig|6666666.244803.peg.7861	12533	11427	1107	-	Propane monooxygenase small subunit (*prmC*)
fig|6666666.244803.peg.7862	13627	12584	1044	-	propane monooxygenase reductase (*prmB*)
fig|6666666.244803.peg.7863	15347	13713	1635	-	Propane monooxygenase hydroxylase large subunit (*prmA*)
fig|6666666.244803.peg.7864	15719	17347	1629	+	Possible transcriptional regulator

TIM = triosephosphate isomerase

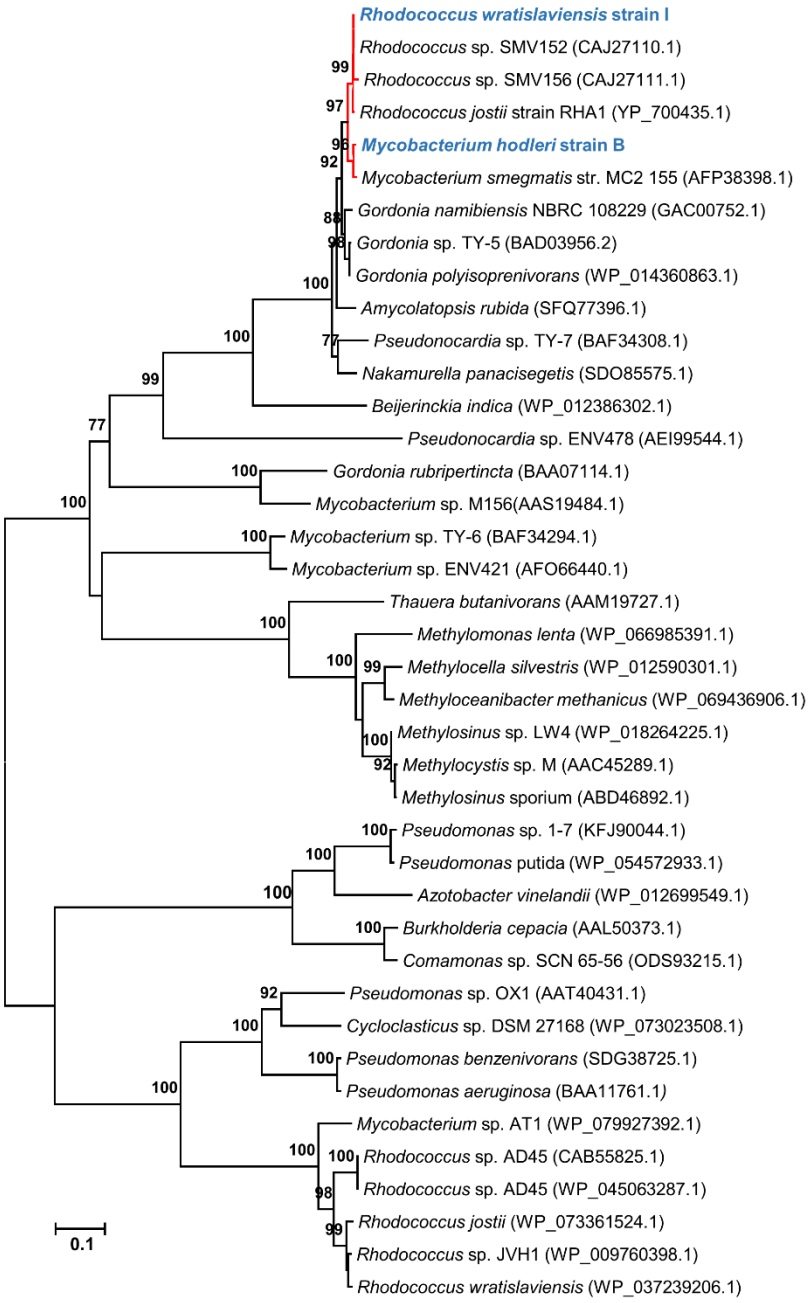

Figure 5.9. Phylogenetic relationship between the α-subunit of hydroxylases of Soluble Diiron Monooxygenases (SDIMO) of bacterial species. Red = *prmA* amino acid sequences related to those of *Mycobacterium hodleri* strain B (blue); *Rhodococcus wratislaviensis* strain I (blue). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Only bootstrap values ≥ 70% are shown. Evolutionary analyses were conducted in MEGA6.

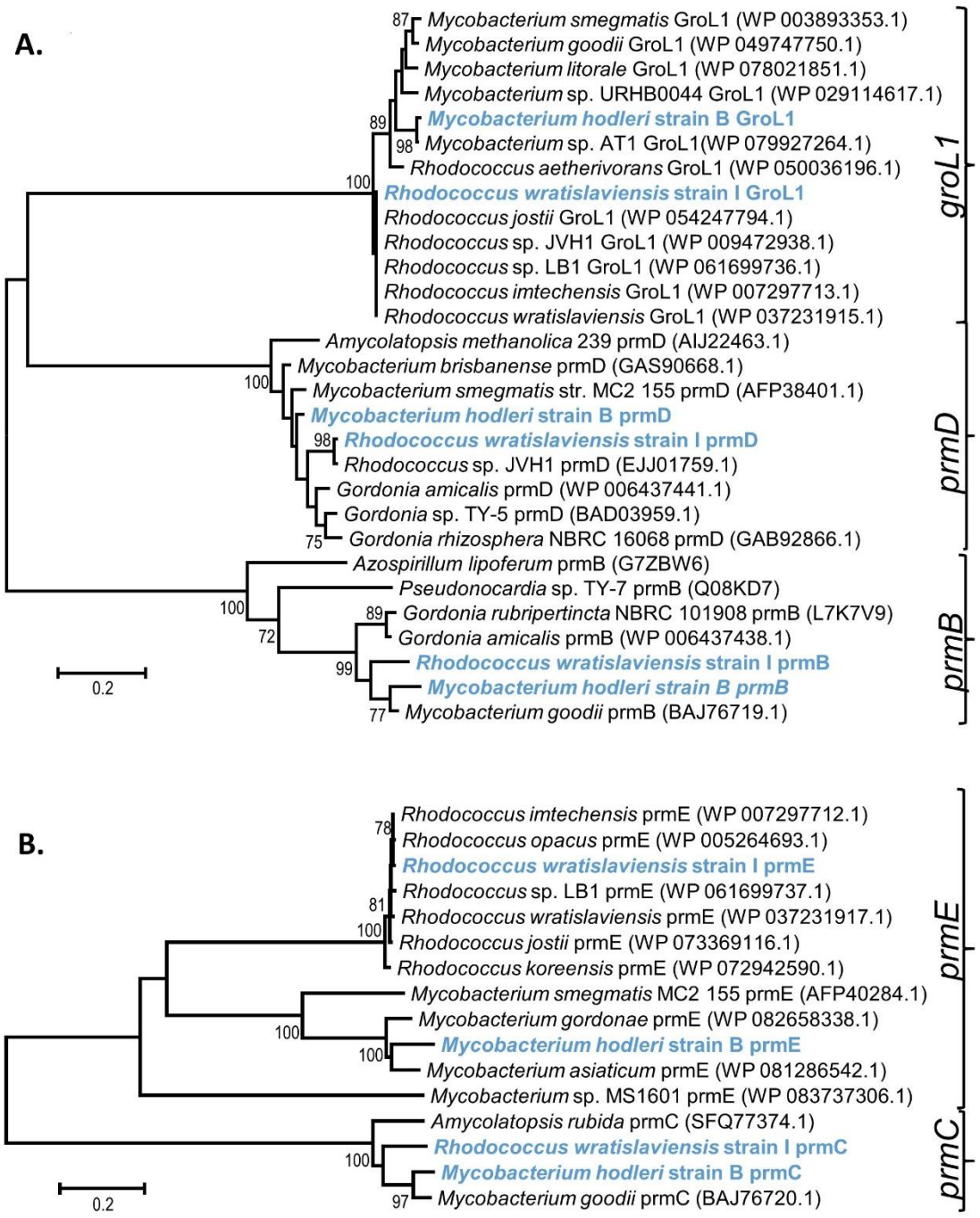

Figure 5.10. Phylogenetic relationship of the *prmBCDE & groL1* (A: *prmBD & groL1; B: prmCE*) from *Mycobacterium hodleri* strain B (blue) and *Rhodococcus wratislaviensis* strain I (blue) with the *prmBCDE* in other bacterial species. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Only bootstrap values ≥ 70% are shown. Evolutionary analyses were conducted in MEGA6.

5.4 Discussion

This study isolated *M. hodleri* strain B and *R. wratislaviensis* strain I from the Colne estuary. Both strains degraded ethane and propane as a source of carbon and energy. *Mycobacterium* and *Rhodococcus* are known to play an important role in isoprene degradation in the estuary (Acuña Alvarez et al., 2009, Johnston et al., 2017). They inhabit a wide range of ecosystems and can metabolize several organic compounds (Larkin et al., 2005, Hruska and Kaevska, 2012) . Most of the aerobic short alkane degrading bacteria isolates available are from the Gram positive CMNR group where *Mycobacterium* and *Rhodococcus* belong (Shennan, 2006).

It is interesting that both *Mycobacterium* and *Rhodococcus* were not identified from third enrichments amended singly with ethane or propane as revealed by Illumina MiSeq 16S rRNA sequencing (Figure 3.10, Chapter 3). The non-detection of these groups with enrichments is most likely due to bacterial community change because of succession from first to third enrichments. On DGGE profiles of ethane and propane enrichments GC-rich populations were seen in first but not in second and third enrichments (Figure 3.8). Dilution of relevant growth factors in sediment free, third enrichment, favored specialist and not generalists like *Rhodococcus* and *Mycobacterium*. Nevertheless, *Mycobacterium* and *Rhodococcus* may be autochthonous to the Colne estuary as demonstrated by this study (Table 3.5, Figure 3.9 e & f , Figure 5.2 -5.3) and other studies investigating isoprene degradation (Acuña Alvarez et al., 2009, Johnston et al., 2017). Johnston et al. (2017) observed that *Mycobacterium* was outcompeted by bacterial species with higher growth rate on isoprene with time of incubation (at day 15).

The presence of genes that encode propane monooxygenase in these strains suggests that propane (and ethane) oxidation was catalysed by the enzyme. Ethane is oxidized by soluble/particulate methane monooxygenase, ethane monooxygenase, propane monooxygase and butane monooxygenase (Van Beilen and Funhoff, 2007, Redmond et al., 2010). Apart from propane monooxygenase, genes encoding the other enzymes were not found in the two genomes, suggesting it was responsible for ethane oxidation. It is well known that propane monooxygenase is induced when bacterial strains are cultivated on ethane or propane (Woods and Murrell, 1989, Sharp et al., 2007, Davies et al., 1976) which results in the terminal or sub-terminal oxidation of propane (Shennan, 2006). In *Pseudonocardia* TY-7, both ethane and propane induce the expression of propane monooxygenase and subsequent oxidation by the enzyme (Kotani et al., 2006). The upstream arrangement of the *prmABCD* gene cluster in *R. wratislaviensis* strain I and *M. hodleri* strain B was similar to previously reported propane-degrading bacterial strains (Kotani et al., 2006, Kotani et al., 2003, Kotani et al., 2007, Sharp et al., 2007, Cappelletti et al., 2015). The main variation was observed downstream of *prm* genes in *M. hodleri* strain B and unlike *R*. *wratislaviensis* strain I, lacked the predicted metal-dependent hydrolase of the TIM-barrel fold (Table 5.5 and Table 5.6). Kotani et al. (2006) showed a variation in the gene arrangement of *Mycobacterium* sp. TY-6 in the *prm* cluster as *prmA*, *prmC*, *prmD* and *prmB*.

The *prmABCDE* gene cluster in *R. wratislaviensis* strain I and *M. hodleri* strain B encode a soluble diiron monooxygenases. This was confirmed by phylogenetic analysis as shown in Figure 5.9 - 5.10. Their genes were homologous to other soluble diiron monooxygenase (Leahy et al., 2003a).The *prmE* gene which encodes alcohol

dehydrogenase of *R. wratislaviensis* strain I shared 97% sequence identity with *R. jostii* RHA1 (Figure 5.7) and clustered with other well-known NAD^+-dependent secondary alcohol dehydrogenases like that from propane-grown *R. rhodochrous* PNKb1 (Figure 5.11) (Ashraf and Murrell, 1990b, Ashraf and Murrell, 1990a).Therefore, propane metabolism could proceed through the terminal and/or sub-terminal oxidation pathway as shown in other *Rhodococcus* species (Ashraf and Murrell, 1990b, Ashraf and Murrell, 1990a, Woods and Murrell, 1989, Sharp et al., 2007, Cappelletti et al., 2015).

The *prmE* gene of *M. hodleri* strain B is different from *R. wratislaviensis* strain I and shared only 58% sequence identity with *M. smegmatis* strain MC^2 155 (Figure 5.8 & 5.10B). Alcohol dehydrogenase (encoded by *prmE*) is important for the oxidation of 1 - or 2- propanol, a key step for propane metabolism. Its properties determine the propane degradation pathway (Kotani et al., 2003). Therefore, *M. hodleri* strain B will have a propane utilisation pathway that is different from the other strains. Other strains like *Mycobacterium* sp TY-6, *M*. *vaccae* JOB5 and *R. rhodochrous* PNKb1 oxidize propane through the terminal oxidation pathway (Kotani et al., 2006, Ashraf and Murrell, 1990b).Propane degradation through acetone oxidation to methanol (Kotani et al., 2007) (Figure 5.1) is unlikely in *R. wratislaviensis* strain I and *M. hodleri* strain B. This is because the *AcmAB* gene cluster that encodes acetone monooxygenase was not found in them.

R. wratislaviensis strain I and *M. hodleri* strain B were metabolically versatile with the ability to utilize benzene and toluene suggesting their broader importance in the estuarine ecosystem. *Rhodococcus* i24 isolated from the same estuary could not degrade benzene and toluene, however, ethane and propane were not tested (Acuña

Alvarez et al., 2009). This means Strain I and B are metabolically more versatile than other autochthonous populations. *M. hodleri* strain B could not degrade isoprene and *R. wratislaviensis* strain I used it poorly (Table 5.1) because their genomes lacked the genes that encode isoprene monooxygenase. On the contrary, genomes of *Gordonia* sp. i37 and *Mycobacterium* sp. AT1 isolated from the Colne estuary contained genes that encoded isoprene monooxygenase and propane monooxygenase-like enzymes (Johnston et al., 2017). Both strains consumed isoprene and propane, therefore, overlapping roles for isoprene and propane monooxygenase were proposed (Johnston et al., 2017).

In the absence of isoprene monooxygenase, propane monooxygenase may oxidize isoprene. Isoprene oxidation by propane monooxygenase is possible in *Gordonia* sp. i37 because its *prmA* gene is upregulated 36-fold and 18-fold when grown on propane and isoprene respectively (Johnston et al., 2017). Therefore, the propane monooxygenase in *R. wratislaviensis* strain I could be responsible for isoprene activation. The poor degradation of isoprene is the cells inability to metabolise the toxic epoxides produced due to lack glutathione-*S*-transferase, resulting in cell death (Crombie et al., 2015). These findings point to the fact that indigenous *Actinobacteria* utilising trace gases in estuarine environments are more diverse than previously thought. Some strains of which are specialized to utilising specific volatile hydrocarbons.

At high concentration, *R. wratislaviensis* strain I degrades ethane or propane better than *M. hodleri* strain B under laboratory conditions (Figure 5.5). *Rhodococcus* species occupy several niches due to their ability to degrade a wide range of carbon sources including short chain ethane and propane (Cappelletti et al., 2015, Larkin et al., 2005,

Martinkova et al., 2009, McGowan et al., 2004, Peng et al., 2006). Their large genomes have several catabolic genes acquired through lateral transfer conferring the competitive advantage over other species like *Mycobacterium* with smaller genomes (Larkin et al., 2005, Larkin et al., 2010).

5.5 Conclusion

This study has isolated, genome sequenced and identified the gene cluster responsible for ethane and propane degradation in *R. wratislaviensis* strain I and *M. hodleri* strain B, suggesting an ecological role in the cycling of volatile hydrocarbons in estuarine environments. The genome sequences obtained has added to the number of environmental species available and contributes to the understanding of the catabolic capacities of *Mycobacterium* and *Rhodococcus* species from estuarine environments.

Chapter 6. Quantification of *isoA* Gene Expression in Sediments of the Colne Estuary by Reverse Transcriptase Quantitative PCR

6.1 Introduction

Isoprene is an abundant volatile hydrocarbon produced by algae, bacteria, animals (including humans) and higher plants in response to environmental stressors like high temperature and reactive oxygen species (ROS) (McGenity et al., 2018). It offers protection against ROS and provides thermotolerance (Loivamaki et al., 2007, Sharkey et al., 2008). The amount of isoprene emitted is influenced by physical and biological factors such as light, temperature, wind speed, photosynthesis and bacterial activity (Dani and Loreto, 2017, Booge et al., 2018).

Phototrophs are the main isoprene producers in marine environments and several microalgal species have been identified and characterized (Booge et al., 2018, Exton et al., 2013, Exton et al., 2012, Shaw et al., 2003, Shaw et al., 2010). Isoprene production by these species show seasonal and diel variation influenced by temperature and sunlight (Broadgate et al., 1997, Exton et al., 2012, Liakakou et al., 2007, Loivamaki et al., 2007). They use either the mevalonate or non-mevalonate biosynthetic pathways (Figure 1.1). Both pathways require isoprene synthase, the only enzyme known to convert DMAPP to isoprene (McGenity et al., 2018).

The gene encoding isoprene synthase display a diel variation in expression, being enhanced during the day and reduced at night (Loivamaki et al., 2007). Consequently, more isoprene is produced during the day (Sharkey and Monson, 2017, Sharkey et al., 2008). The amount of isoprene produced is measured indirectly by determining the concentration of chlorophyll *a* (Chl *a*) (Shaw et al., 2010). This is due to the positive correlation between isoprene and Chl *a* concentrations (Bonsang et al., 1992, Milne et al., 1995, Moore et al., 1994); and the ease for measuring the later. The diel production of isoprene in water at the Colne estuary is influenced mainly by changes in temperature (Exton et al., 2012). More isoprene is produced in the day than at night with a peak of 408 pmol l^{-1} observed at 16:00 which coincides with increase in water temperature and light intensity (Exton et al., 2012). Some of the isoprene is emitted to the atmosphere where they impact the chemistry in diverse ways (section 1.3.1).

The amount of isoprene emitted from marine environments to the atmosphere is reduced due to consumption by bacterial communities (Acuña Alvarez et al., 2009, Booge et al., 2018). Recent studies at the Colne estuary demonstrate isoprene consumption by diverse bacterial communities dominated by *Actinobacteria* (Acuña Alvarez et al., 2009, Exton et al., 2012, Johnston et al., 2017). In a DNA-SIP experiment, a technique that links the identity of active microbial communities to substrate consumption, carried out with samples from the Colne estuary supplied with ^{13}C-labelled isoprene in microcosms, *Mycobacterium* and *Microbacterium* were the main phylotypes implicated with isoprene degradation (Johnston et al., 2017). Isoprene-degrading bacterial populations including *Rhodococcus* and *Mycobacterium* were

enriched, and some isolates obtained from the Colne estuary (Acuña Alvarez et al., 2009).

Isoprene degradation is catalysed by isoprene monooxygenase (IsoMO). In *Rhodococcus* strain AD45, the enzyme is encoded by the *isoABCDEF* gene cluster (Crombie et al., 2015, Van Hylckama Vlieg et al., 2000). All known isoprene utilising bacteria also possess the *isoABCDEF* gene cluster (Crombie et al., 2015, Van Hylckama Vlieg et al., 2000 , Acuña Alvarez et al., 2009, Johnston et al., 2017, Khawand et al., 2016, McGenity et al., 2018, Murphy, 2017). Crombie et al. (2015) demonstrated that *isoA*, the gene encoding the alpha subunit of isoprene monooxygenase is essential for isoprene consumption in *Rhodococcus* sp AD45. This was followed by the design of *isoA* primer pair using sequences of *Rhodococcus* sp. and *Gordonia* sp. (Khawand et al., 2016). The *isoA* primer pair has become a valuable tool for application in culture independent studies (Khawand et al., 2016, Johnston et al., 2017, McGenity et al., 2018, Murphy, 2017).

Gene expression studies have shown that the ability to use isoprene is an inducible trait (Johnston et al., 2017, Crombie et al., 2015). Crombie et al. (2015) demonstrate this in a in a substrate-switch time-course experiment with *Rhodococcus* sp. AD45. The substrates were isoprene plus succinate or succinate only. Isoprene consumption does not take place until succinate is used up. Then it proceeds at a reduced rate indicating a switch from succinate, the preferred substrate, to isoprene. Isoprene metabolic gene products including *isoA* were detected in the medium containing isoprene but absent in the one with succinate only.

This chapter was predicated on the knowledge that isoprene production by photosynthetic algae happens in sediments of the Colne estuary, therefore, isoprene degraders could be utilising it (Acuña Alvarez et al., 2009, Exton et al., 2013, Exton et al., 2012). The study of *isoA* gene expression as a measure of *in situ* isoprene consumption has not been reported. The *psbA* gene coding for protein D1 of photosystem II reaction centre was used as the positive control because of its presence in most photosynthetic microbes from marine environments (Zeidner et al., 2003) and isoprene production by phototrophs is positively correlated to *Chl a.*

Hypothesis

Isoprene monooxygenase genes will be more highly expressed during the day, when more isoprene is being produced by phototrophic microalgae, compared with the night, in an estuarine intertidal sediment.

Specific Objective

6.1 To measure the diel variation in the expression of isoprene monooxygenase (*isoA*) genes *in-situ* in the Colne estuary sediments using reverse transcription quantitative PCR.

6.2 Materials and Methods

6.2.1 Diel sediment sample collection

Sediment samples were collected on the 24th and 25th August 2016 at the Colne estuary in Wivenhoe. Two-hourly sampling over a 24-hour period was carried out from 10.20 to 08.20 and are referred to as t1 - t12. Since naked RNA is vulnerable to degradation by endogenous ribonucleases (RNases) found in all living cells, care was taken to ensure neither endogenous nor exogenous RNases were introduced during sampling, RNA extraction or processing (Bustin and Nolan, 2009). Sterile nuclease free 50 ml falcon tubes were used during sample collection and sterile nuclease free gloves were worn at all time. RNaseZAP™ Cleaning agent for removing RNase (Sigma) was used for cleaning work area and other sterile equipment (autoclaved) such as spatula, before use.

Samples were collected from the Colne estuary at Wivenhoe near an overhanging oak tree. Wivenhoe is located about the middle of the Colne estuary (Figure 2.1). The mudflat sampling location was sandwiched between vegetation including sea aster (*Aster tripolium*), sparse *Spartina alterniflora*, and Sea purslane (*Sesuvium portulacastrum*) (Figure 6.1). Small brackish water snail, *Hydrobia* spp. were abundant on the mudflat. The sampling location was divided into four quadrats of 0.25 m^2 each (Figure 6.1a). These quadrats (biological replicates) were represented as q1 (quadrat 1), q2 (quadrat 2), q3 (quadrat 3) and q4 (quadrat 4). At each time point, sediment samples were picked using sterile 50 ml falcon tubes from the top 2 cm of each quadrat, mixed immediately with 2 ml of RNA*later* (Sigma) and shaken vigorously to homogenize (Figure 6.1b). At each time, a new 50 ml falcon tube was used to collect a sample from

each quadrat, and sediment samples were collected in a clockwise fashion (Figure 6.1b). Samples were allowed to stand at environmental temperature for 15 min to equilibrate before freezing on dry ice in iceboxes.

At each time of sampling, light intensity was measured in quadruplets (within each quadrat) using a hand-held light meter (Sky Instruments, UK) and the air temperature of sediments was measured with a thermometer. Light intensity was measured in W m^{-2} but converted to mmol m^{-2} s^{-1} by multiplying each value by 4.6 (Skye Instruments Ltd UK). Samples were held on dry ice in iceboxes overnight. They were transported to the laboratory in ice boxes on dry ice and transferred immediately for storage at -80ºC.

a

b

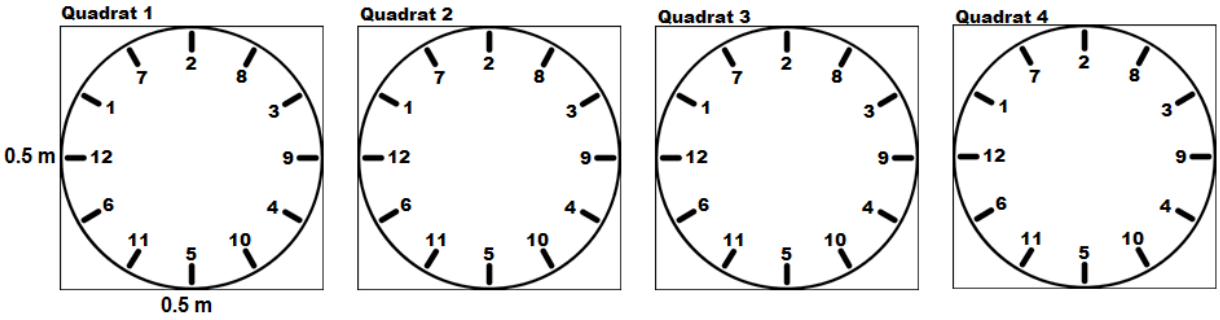

Figure 6.1. The sampling location on the Colne Estuary in Wivenhoe showing the plot divided into four quadrats (a); A schematic diagram showing how sampling was done within each quadrat (b). Samples were collected in a clockwise direction. Number represents the order of sampling, from 1 (t1) to 12 (t12). The total number of sediment samples collected was 48.

6.2.2 Sample processing

The sediment samples were processed on the 21st March 2017 for RNA extraction. Frozen sediment samples were allowed to thaw on ice and centrifuged at 11300 x *g* for 5 min to collect the sediment pellets. The liquid component comprising of RNA*later* and water were discarded.

6.2.3 RNA extraction

RNA was extracted using the RNA PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories, Inc) following manufacturer's instructions. Samples were treated in small batches of 4 (q1 – q4) for every time point.

One gram of sediment sample was added to a 15 ml bead tube and the following solutions were added sequentially as described in the manufacturer's protocol: 2.5 ml of bead solution (a buffer used to disperse cells and sediment particles); 0.25 ml of solution SR1 (containing SDS and other disruption agents which aid in complete cell lysis); 0.8 ml of solution SR2 (a precipitation reagent used to remove non-DNA organic and inorganic material including cell debris and proteins), and 3.5 ml of phenol:chloroform:isoamyl alcohol (pH 6.5 – 8.0, Sigma). Cells were lysed by vortexing the bead tubes on the MO BIO Vortex Adapter at maximum speed for 15 min and separated by centrifugation at 2500 x *g* for 10 min at room temperature. The upper aqueous phase was carefully transferred (avoiding the interphase and lower phenol layer) to a clean 15 ml collection tube. The phenol:chloroform:isoamyl alcohol was discarded. Secondary precipitation of protein debris and cellular debris was achieved by

adding solution SR3 (1.5 ml) to the aqueous phase; vortexed to mix and incubated at 4°C for 10 min. Debris was removed by centrifugation at 2500 x *g* for 10 min at room temperature. The supernatant containing the nucleic acid was transferred to a new 15 ml collection tube without disturbing the pellet. Five ml of solution SR4 (100% Isopropanol) was added to the collection tube containing the supernatant, vortexed to mix, and incubated at room temperature for 30 min. Nucleic acids were precipitated by centrifugation (2500 x g for 30 min at room temperature). The Isopropanol supernatant was discarded by decanting and inverting the 15 ml collection tubes on a paper towel for 5 minutes. Then 1 ml of solution SR5 (a proprietary salt solution) was added to the 15 ml collection tube. The pellets were suspended completely by repeatedly pipetting and vortexing to disperse the pellet. The RNA isolation sample (suspended pellets above) was added onto the RNA capture column and allowed to gravity flow through the column. The nucleic acids were bound to the column matrix of the capture column, then washed with a second volume of solution SR5 (1 ml) to ensure unbound contaminants were removed from the sample column prior to the elution of RNA. The RNA capture column was transferred to a new 15 ml collection tube. One millilitres of solution SR6 (RNA elution buffer) was added to the RNA capture column to elute the bound RNA into the 15 ml collection tube. The eluted RNA was transferred to a 2.2 ml collection tube and 1 ml of solution SR4 was added. The collection tube was inverted to mix its content and incubated at -20°C for 10 minutes. The 2.2 ml collection tube was centrifuged at 13,000 x *g* for 15 min at room temperature to pellet the RNA. The supernatant was decanted, and the 2.2 ml collection tube was inverted onto a paper towel for 10 min to air dry the pellet. The RNA pellet was resuspended in 100 µl of solution SR7

(RNase/DNase-Free water). The extracted RNA was preserved in several aliquots at -80^{o}C to minimize the effect of freeze thaw.

6.2.4 DNAse treatment

A one in ten (1:10) dilution of the extracted RNA in nuclease free water (Fisher, catalogue number AM9932) was done, followed by TURBO™ DNAse treatment to remove any residual DNA. Residual DNA was removed from 50 µl sample using TURBO DNA-free™ Kit (Ambion) as described in the manufacturer's protocol. Briefly, 50 µl of the diluted RNA sample was mixed with1 µl TURBO DNase and 10x TURBO DNase buffer by pipetting with a micropipette up and down ten times. The mixture was incubated at 37 °C for 30 min, then 5 µl of DNase inactivation reagent was added and mixed thoroughly by pipetting up and down ten times. The mixture was incubated at room temperature for 5 min, then centrifuged at 10,000 × *g* for 1.5 min. The RNA containing supernatant was transferred into a fresh tube, ready for use.

6.2.5 The PCR amplification of 16S rRNA

A control PCR was carried out to check if complete removal of DNA was achieved after the DNase treatment step using primer pairs for amplification of 16S rRNA (Muyzer et al., 1993). The PCR amplification was performed using a Perkin Elmer Gene Amp PCR system 700. Each 25 µl reaction mixture was made up of 0.5 µl DNase treated RNA as template, 12.5 µl REDTaq ready mix (Sigma), 1 µl each of forward (F341) and reverse primers (R534), and 10 µl of Milli-Q water. The cycling conditions were as follows:

94ºC, 5 min; (Denaturing: 94ºC, 1 min; Annealing: 55ºC, 1 min; Extension: 72ºC, 3 min) × 30 cycles. The amplification products were viewed on 1% (w/v) agarose gel electrophoresis in 1 × TAE (40 mM Tris-acetate, 1mM di-sodium-EDTA, pH 8.0) and staining with ethidium bromide (0.5 µg ml^{-1}).

6.2.6 Reverse transcriptase (RT) - PCR

To investigate the diel expression of *isoA* gene in sediments, relative quantification approach was used with *16S rRNA* gene as reference gene. *psbA* gene was used as the positive control. The *isoA* gene encodes the alpha subunit of isoprene monooxygenase while *psbA* encode the protein D1 of photosystem II reaction centre. A two-step RT-PCR assays strategy was employed.

First-strand cDNA synthesis was done using Superscript™ III Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. The 20 µl reaction mixture contained 7 µl of nuclease-free water (Fisher, catalogue number AM9932), 1 µl of 100 µM random hexamers (ThermoFisher Scientific, VX18080044), 1 µl of 10 mM dNTPs Mix (ThermoFisher Scientific) , 1 µl of 200 U of Superscript III reverse transcriptase (Invitrogen), 1 µl of DTT, 4 µl of 5x first-strand buffer and 5 µl of RNA template . RT reactions were performed in a Gene Amp® PCR system 9700 Thermocycler (Applied Biosystems) for 50 min at 50°C, followed by termination at 70°C for 15 min. Three microliters (3 µl) of RT products (cDNA) was subsequently used in qPCR assays or stored at -20ºC.

All RT-PCR reactions were prepared to a final volume of 20 µl in a 96-well plate, comprising 3 µl of cDNA, 0.8 µl of 10 µM forward and reverse primers, 5.4 µl of

nuclease free water and 10 µl of 2 x SensiFast SYBR® No-ROX Mix (Bioline). Each plate contained a dilution series of a positive control. Negative controls (No-Template Control) were set up in triplicate for each target with no cDNA template. Positive controls were amplicon of target gene obtained from aerobic enrichments to serve as a check on each RT-PCR run. These positive controls were DNA (not cDNA).

The primer sets used were as follows: *psbA* (Forward 5' – GTNGAYATHGAYGGNATHMGNGARCC – 3'; Reverse 5' - GRAARTTRTGNGCRTTNCKYTCRTGC-AT – 3') (Zeidner et al., 2003); *isoA* (forward: 5´-TGCATGGTCGARCAYATG - 3´ ; Reverse Primer: 5´-GRTCYTGYTCGAAGCACCACTT-3´) (Khawand et al., 2016) and *16S rRNA* (Bact1369F 5' – CGGTGAATACGTTCYCGG – 3'; Prok1492R 5' – GGWTACCTTGTTACGACTT – 3') (Suzuki et al., 2000).

The 96-well plates with reaction mix were briefly centrifuged before transfer to the Bio-Rad CFX96 Real Time System. The quantification of the 16S rRNA gene was done following a two-step cycling protocol (Bioline). The cycling conditions were as follows: polymerase activation (95°C, 2 min), denaturation (95°C, 3 s); Annealing/extension (60°C, 30 s) for 40 cycles; then a melt curve 65°C to 95°C in 0.5°C increments (0.05 min each). A 3-step cycling protocol (bioline) was used for the quantification of *psbA* and *isoA* gene as follows: polymerase activation (95°C, 2 min), denaturation (95°C, 5 s); Annealing (60°C, 10 s); Extension (72°C, 20 s) for 40 cycles; 95°C for 0.10 min then a melt curve 65°C to 95°C in 0.5°C increments (0.05 min each).

6.2.7 Data analyses

CFX Manager Version 3.1.1517.0823 was used to visualize and analyse the quantification and melt curve data obtained.

6.3 Results

6.3.1 Physico-chemical characteristics

On the day of sampling, 24th August 2016, the sun rose at 05.54 and set at 19.59 as reflected by the changes in light intensity (Figure 6.2a). On 25th August 2016, the sun rose at 05.55 before the last two samples were collected at 06.20 and 08.20 respectively. A temperature range of 18 to 31^{o}C was measured during sampling while the light intensity ranged from 0 – 730 mmol m^{-2} s^{-1} (Figure 6.2). The first samples were collected at low tide (Figure 6.2b) when the first three quadrats were in shade while the 4th quadrat was in dappled sunlight. At 12:20, two quadrats were under dappled sunlight while the other two were in shade accounting for the large standard error due to variation in light intensity between replicates (Figure 6.2a). All quadrats were under full sunlight at 14.20 and 16.20 with occasional clouds cover. Even though the sampling location was chosen beyond tidal influence, water started covering quadrats at 16.20 when samples were collected because there was high tide at 16.26 (Figure 6.2b). That was the only time quadrats were partially flooded. Between 18.20 to 08.20, all quadrats were in shade (Figure 6.2a).

The Colne estuary is mesotidal and tidal heights ranged from 0.4 m (22.50) to 3.6 m (16.26) at the time of sampling (Figure 6.2b). Sediment air temperature and light intensity increased with sunrise and decreased with sunset (Figure 6.2a). On the 24th August, the highest temperature (31^{o}C) and light intensity (730 mmol m^{-2} s^{-1}) were observed at 14.20 when all the quadrats were in full sunlight. The air temperature was sustained at 31^{o}C for 2 hours till 16.20 even when the light intensity was on the decline. The air temperature declined to 18^{o}C indicating air's ability to retain the sun's heat. The

light intensity decreased to 0 mmol m^{-2} s^{-1} with sunset at 19.59 and started increasing at 06.20 on the 25th August after sunrise (05.55).

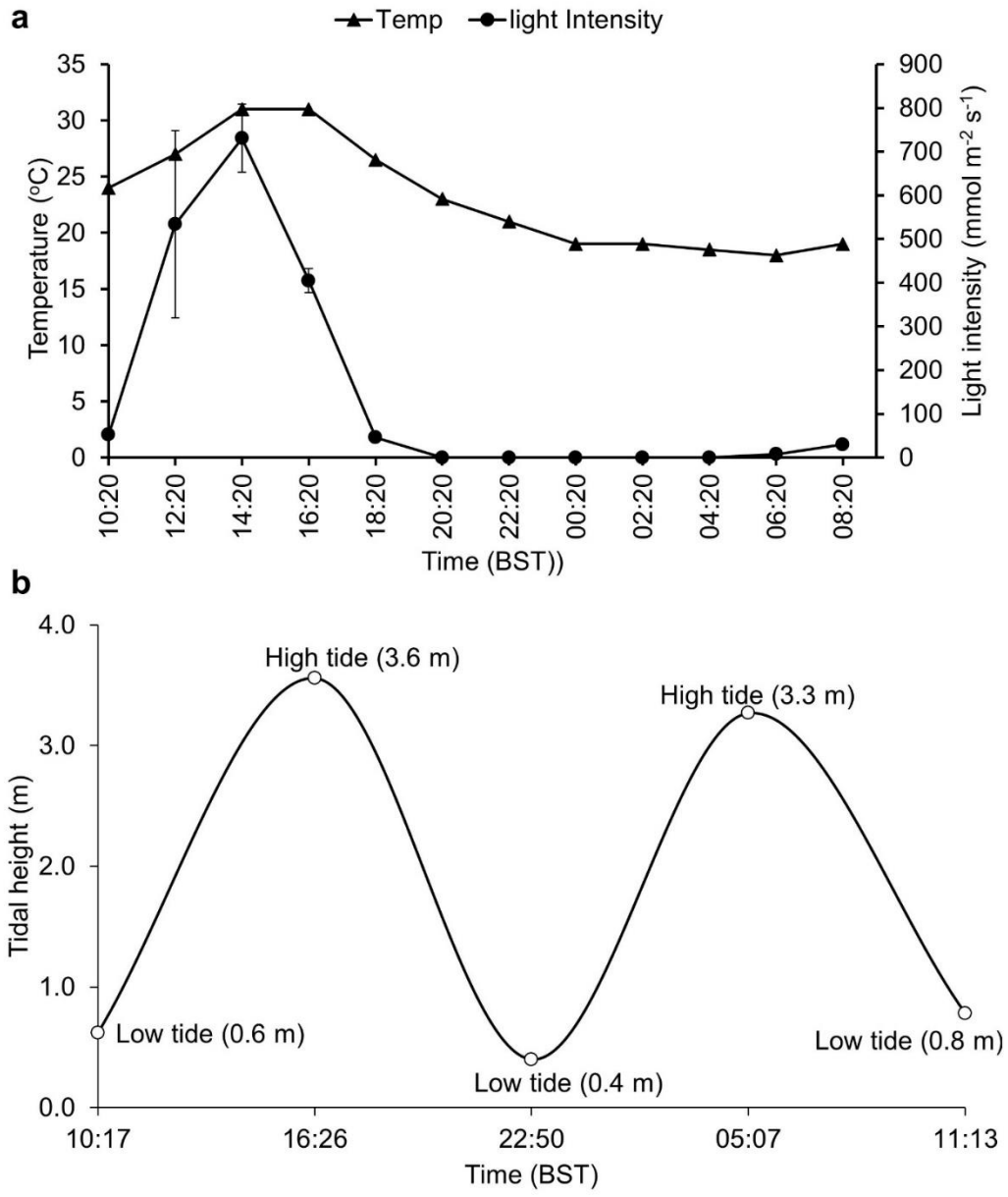

Figure 6.2 Diel variation of sediment air temperature and light intensity (a), and tidal height (b) at Wivenhoe (middle of the Colne estuary) on 24– 25 August 2016. Light intensity (*n* = 4; bars show SE of the mean); BST – British Summer Time.

6.3.2 Total RNA extraction and first-strand cDNA synthesis

RNA was extracted, and cDNA synthesis was confirmed by amplification of *16S rRNA* gene. The total RNA extracted was visualized on a 1% (w/v) agarose gel (Figure 6.3). Although mRNA was not visible on the gel, 23S rRNA and 16S rRNA were seen in most of the samples indicating that RNA was extracted by the procedure. Variation in the total RNA extracted was observed between some replicates and between time points (Figure 6.3). Although genomic DNA was not visible on the agarose gel, DNase treatment was carried out to ensure there were no traces of DNA left in the RNA samples. This was carried out prior to first strand cDNA synthesis by reverse transcription. A 30-cycle PCR amplification of the16S rRNA gene was carried out after DNase treatment and no PCR products were detected in any sample (Figure 6.4). However, the right PCR product size (ca. 200 bp) was shown in the positive control confirming the removal of any residual DNA from samples (Figure 6.4).

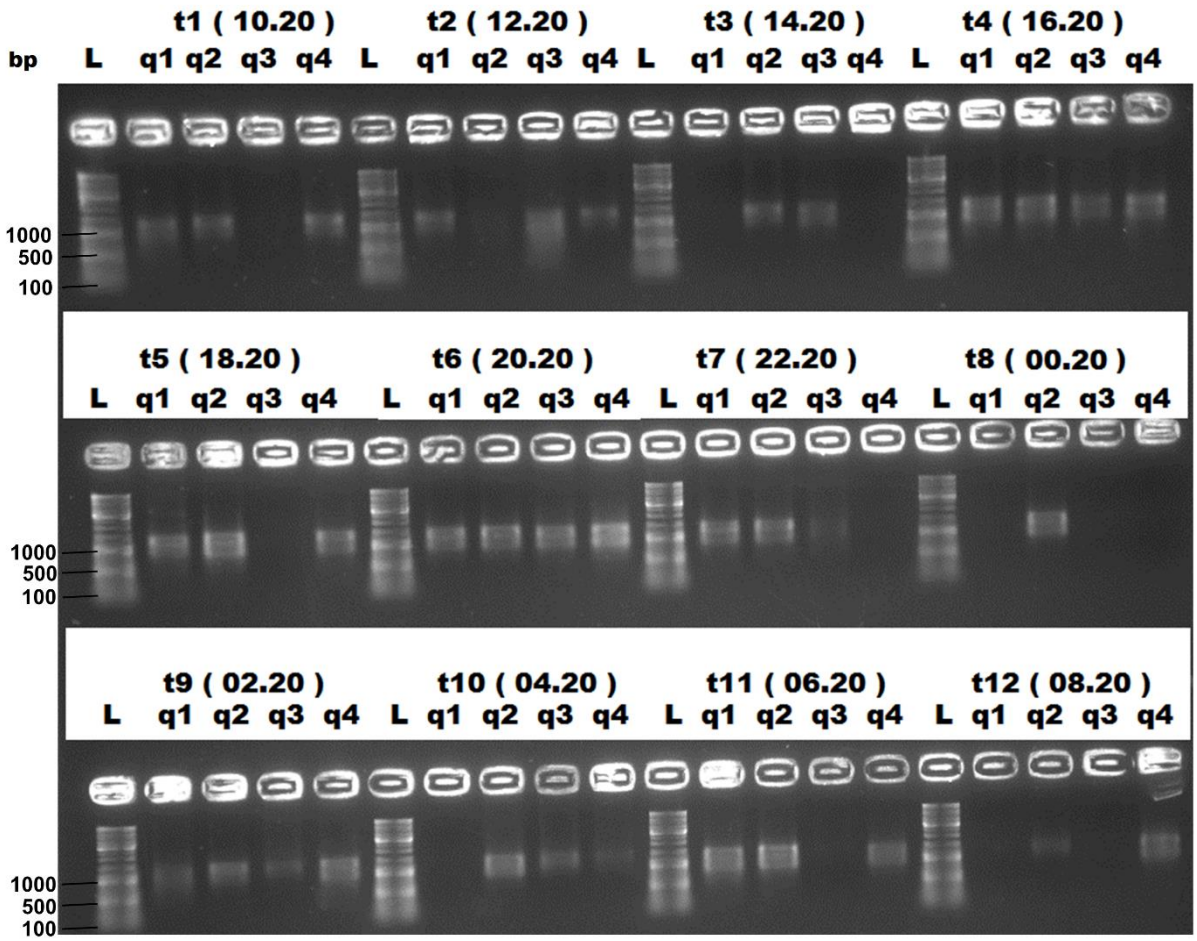

Figure 6.3. Total RNA extracted from sediments. t1 – t12 represents time samples were collected with actual times in brackets; q1 (quadrat 1); q2 (quadrat 2); q3 (quadrat 3) and q4 (quadrat 4) are replicates; L represents GeneRuler™ DNA Ladder Mix (Thermo Scientific); 1% (w/v) Agarose gel.

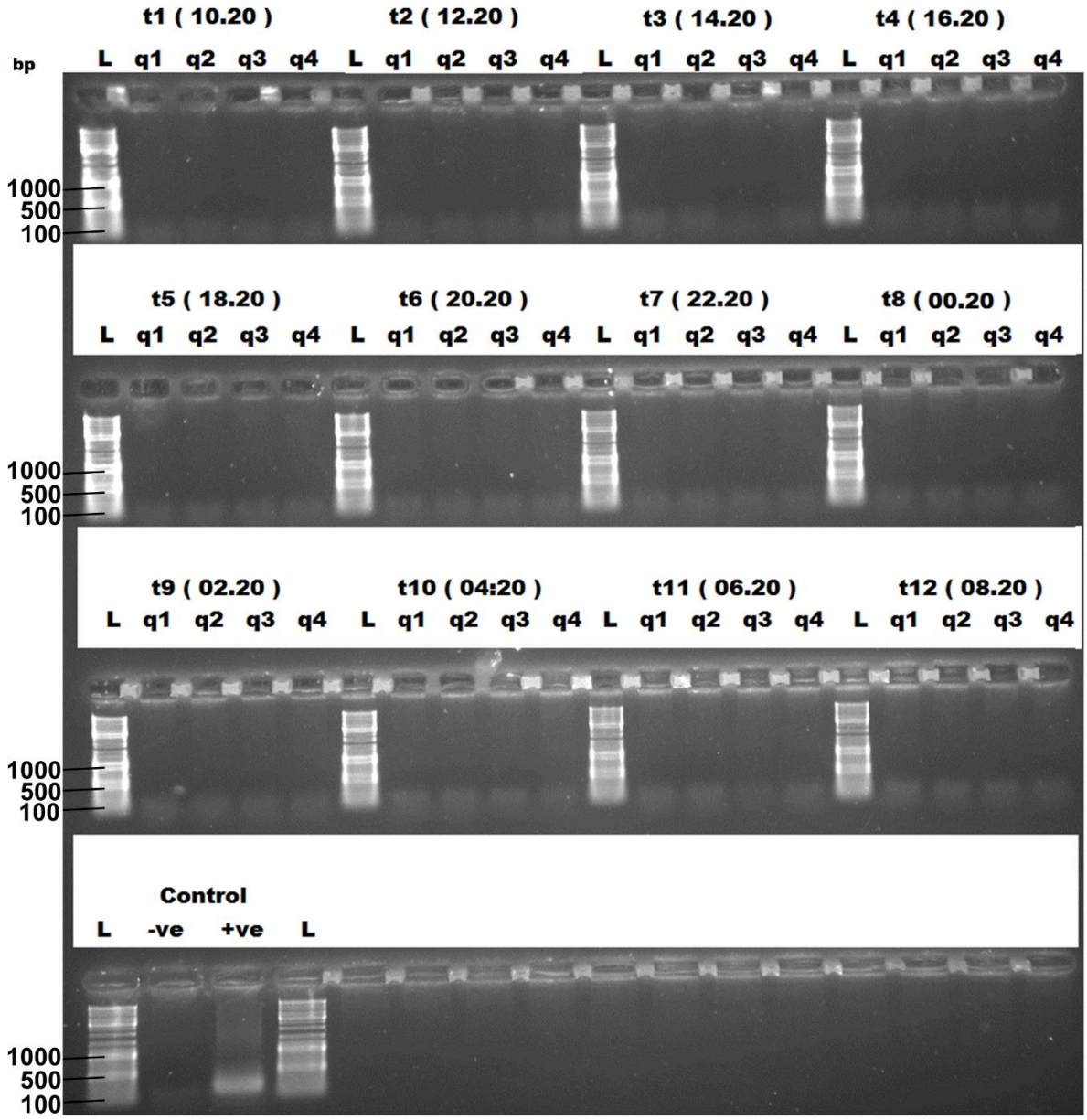

Figure 6.4. PCR product of *16S rRNA* gene in samples post DNAse treatment. No PCR products were seen in any sample after a 30 cycles PCR. t1 – t12 represents time samples were collected with actual times enclosed in brackets; q1 (quadrat 1); q2 (quadrat 2); q3 (quadrat 3) and q4 (quadrat 4) represent replicates; L represents GeneRuler™ DNA Ladder Mix (Thermo Scientific). Positive control was DNA from a sediment samples while the negative sample had no DNA template; 1% (w/v) Agarose gel.

6.3.3 Attempt to determine *isoA* gene expression in estuarine sediment

Amplification of the *isoA* gene was observed in three samples, collected from quadrats 2 and 3, after 40 cycles but not in the no-template controls (NTC) (Table 6.1, Figure 6.5). Samples collected from q3 at 14.20 and 16.20, under full sunlight, showed *isoA* gene amplification with Cq values of 39.13 and 38.15 respectively. Only one sample collected from q2 at night (00.20) showed *isoA* gene amplification with a Cq value of 37.81 to (Table 6.1).

Table 6.1 Cq values and potential diel *isoA* gene expression in sediments of the Colne estuary at Wivenhoe, determined by RT-qPCR

Time of Sampling	Gene of interest (GOI): Cq of *isoA* gene	Normalizer = Mean Cq of *16S rRNA* gene ± SE (*n*=4)	GOI / (Normalizer)	Day / Night *isoA* gene expression
10:20	nil	19.7 ± 2.6	nil	nil
12:20	nil	16.7 ± 1.3	nil	nil
14:20	**39.13 (q3 only)**	**21.1 ± 3.2**	**1.85**	**1.4**
16:20	**38.15 (q3 only)**	**13.8 ± 0.9**	**2.76**	**2.1**
18:20	nil	17.2 ± 3.9	nil	nil
20:20	nil	13.0 ± 0.5	nil	nil
22:20	nil	19.9 ± 5.0	nil	nil
00:20	**37.81 (q2 only)**	**28.9 ± 5.7**	**1.31**	**1.0**
2:20	nil	31.1 ± 4.8	nil	nil
4:20	nil	21.7 ± 3.7	nil	nil
6:20	nil	16.0 ± 2.3	nil	nil
8:20	nil	23.9 ± 5.4	nil	nil

nil means no data obtained , *isoA* gene amplified in samples from quadrats 2 & 3 only

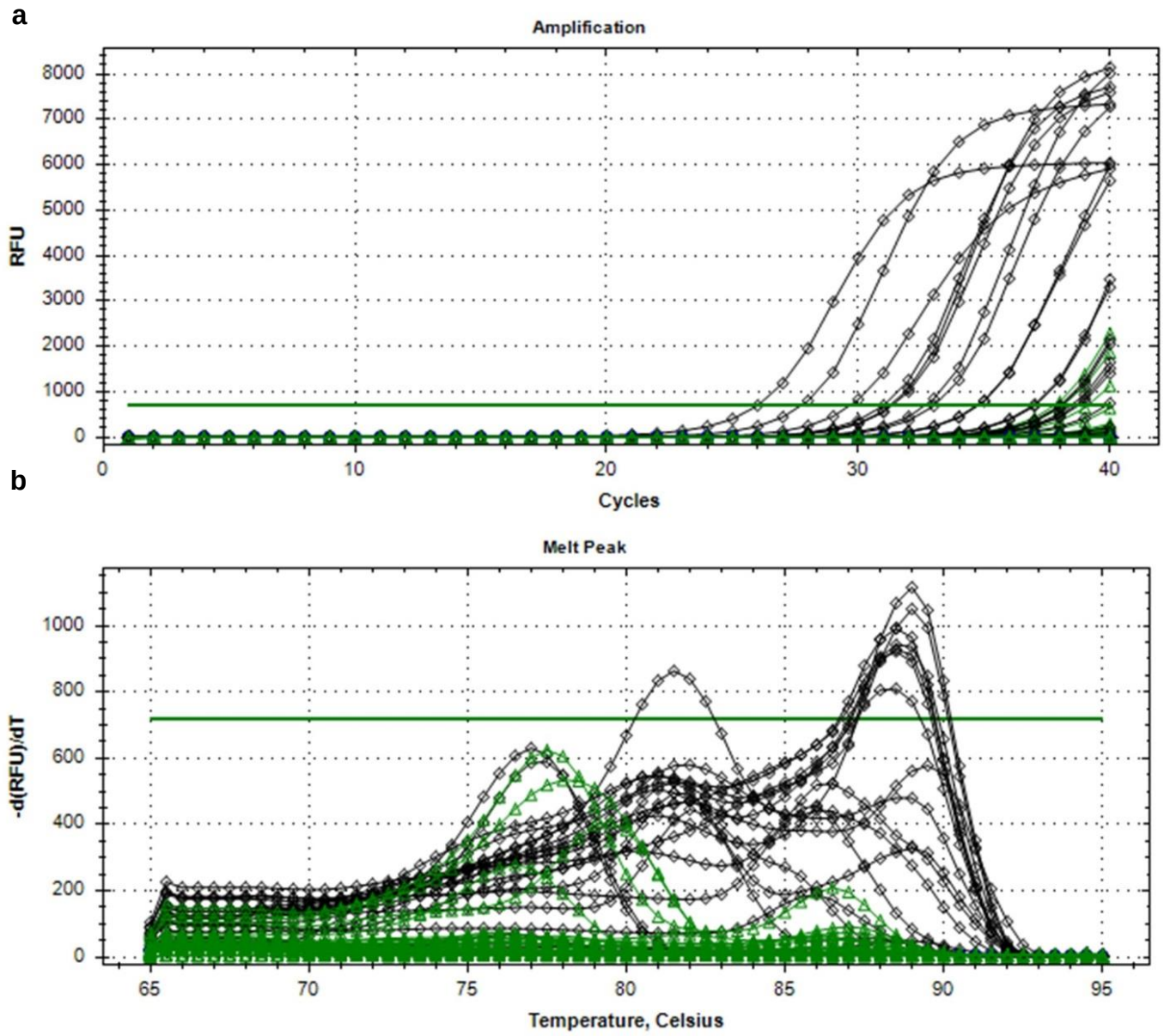

Figure 6.5.The *isoA* gene amplification on Bio-Rad CFX96 Real Time System. (a) The amplification plot shows 40 cycles of cDNA (sediment), DNA from isoprene enrichments (positive control) and NTC samples. (b) The first negative differential of fluorescence with respect to temperature plotted against temperature. Traces of samples are green (triangle), positive controls are black (diamond), NTC are blue (circle).

The *isoA gene* was amplified in all positive control samples indicating that primer pair worked (Figure 6.5). However, melt curve analysis, showed three peaks indicating non-specific products were formed (Figure 6.5b).

The *16S rRNA* gene was amplified in all samples (Figure 6.6) making it a suitable reference gene. Melt peak analysis also indicated that specific PCR products were formed (Figure 6.6b). On the contrary, *psbA* gene was not detected in any sample through RT-qPCR (Figure 6.7).

In sediments, the *isoA* gene expression was two times higher in the day than at night in quadrat 2 and 3. The level of *isoA* gene expression in quadrat 3 (16.20) coincided with full sunlight and the highest temperature of 31^{o}C at the time of sampling (Figure 6.2). A 1.5-fold change in *isoA* gene expression was also observed at 14.20 in quadrat 3 (Table 6.1) which also coincided with a high temperature of 31^{o}C, and the highest light intensity of 730 mmol m^{-2} sec^{-1} at the time of sampling (Figure 6.2).

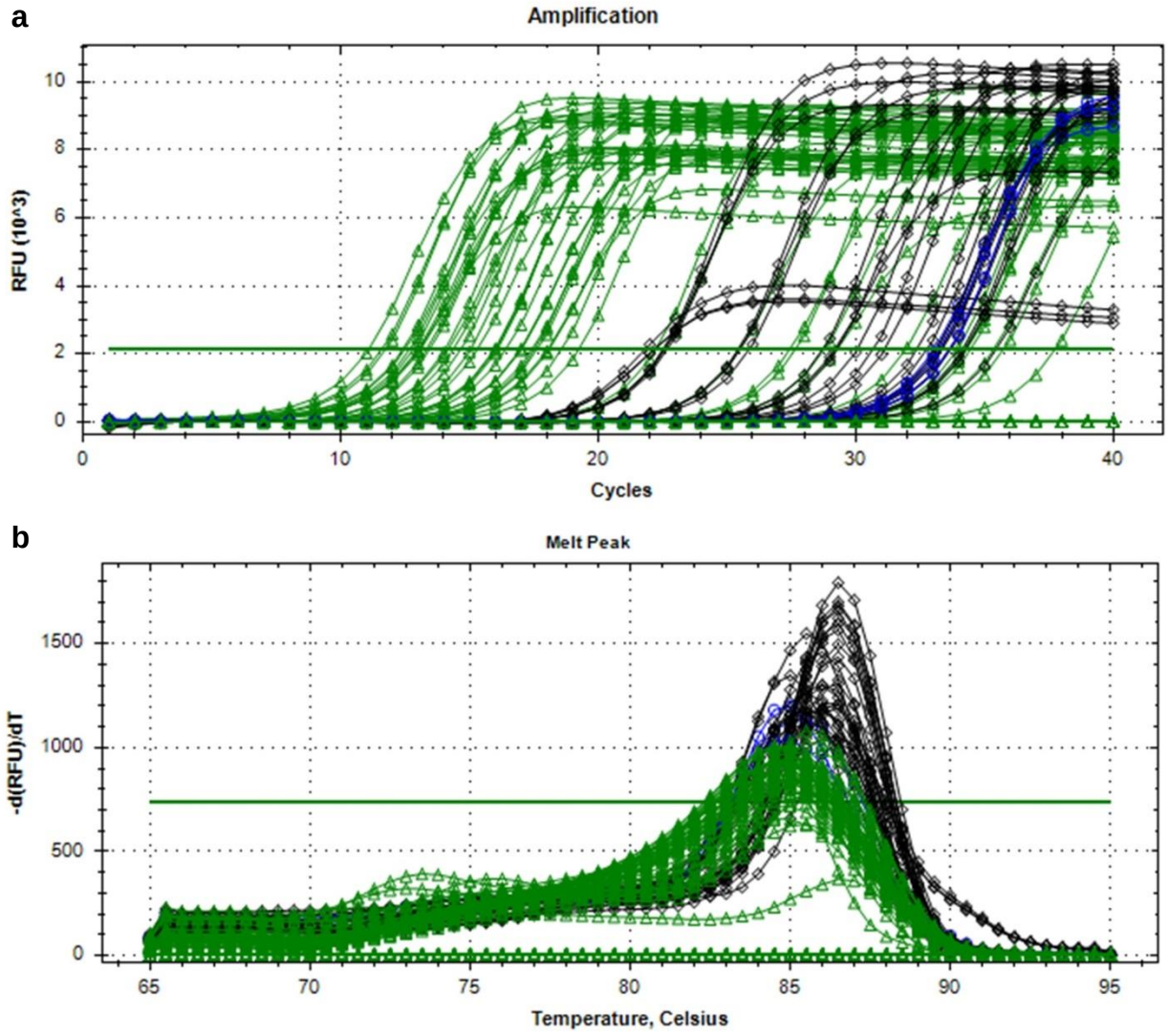

Figure 6.6. *16S rRNA* gene amplification on Bio-Rad CFX96 Real Time System. (a) The amplification plot shows 40 cycles of cDNA (sediment), DNA, NTC samples. Mean Cq values of samples shown on table 6.1 (b) The first negative differential of fluorescence with respect to temperature plotted against temperature. Traces are colour coded as follows: Traces of samples are green (triangle), positive controls are black (diamond), NTC are blue (circle).

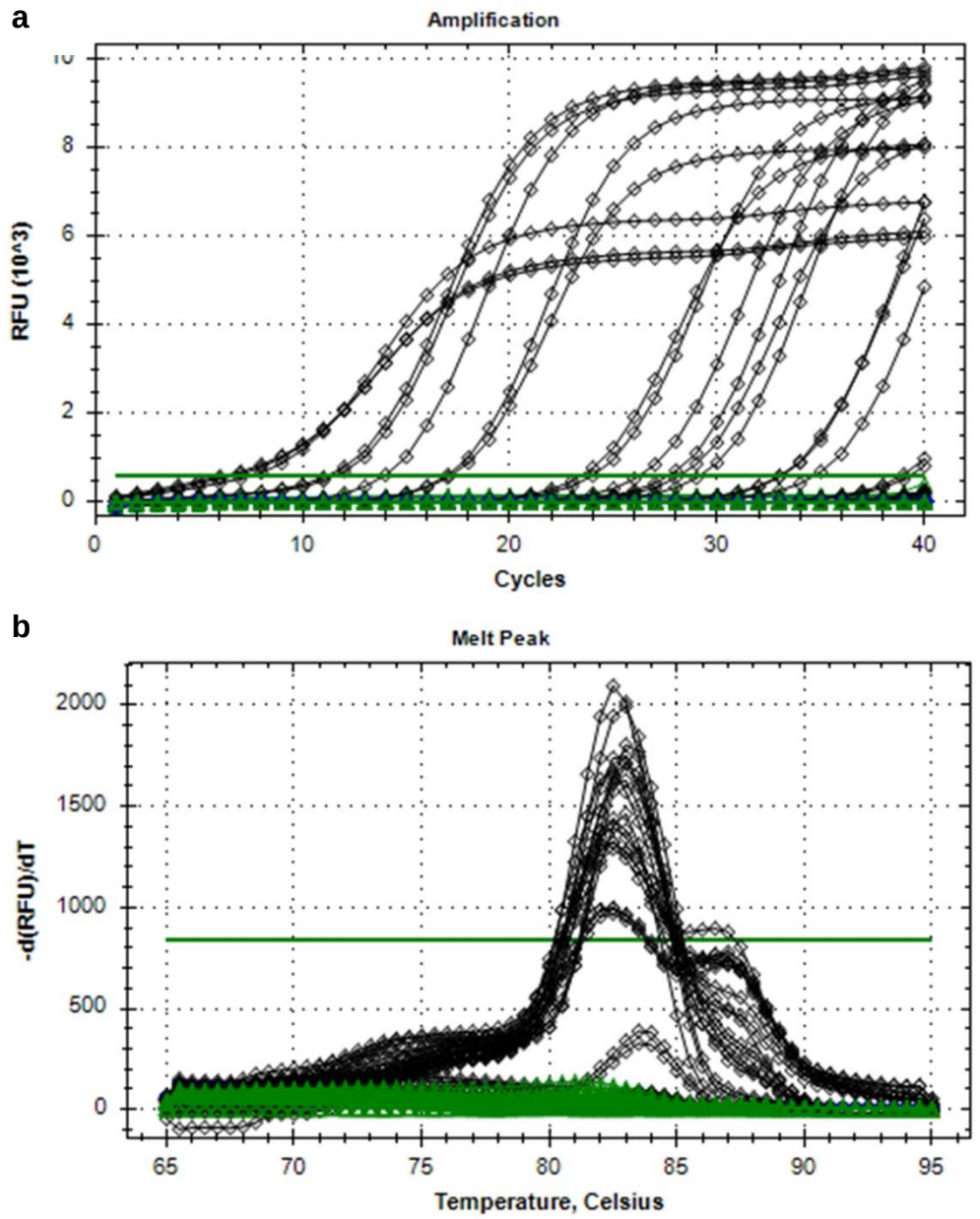

Figure 6.7.The *psbA* gene amplification on Bio-Rad CFX96 Real Time System. (a) The amplification plot shows 40 cycles of cDNA (sediment), DNA from phototrophs (positive control) and NTC samples. (b) The first negative differential of fluorescence with respect to temperature ploted against temperature. Traces of samples are green (triangle), positive controls are black (diamond), no template control are blue (circle).

6.4.0 Discussion

The determination of *isoA* gene expression was not successful because out of the 48 samples analyzed by RT-qPCR, amplification was observed in only three (Table 6.1) and non-specific products were observed with the *isoA* primer pair used (Figure 6.5b). Consequently, the data obtained lacked biological significance. Nevertheless, lessons were learnt having conducted the experiment.

The most important lessons learned after conducting the experiment is that the current *isoA* primer pair require refinement before any subsequent RT-qPCR. McGenity et al. (2018) shared a similar opinion. The number of genome sequences and diversity of isoprene degrading species has increased since the previous primer pair were designed (Johnston et al., 2017, McGenity et al., 2018, Murphy, 2017). Therefore, the new primer pair will have improved coverage. The *isoA* primers used for this study were shown to have limited coverage by the McGenity Group at University of Essex and the Murrell Group at the University of East Anglia (personal communications). These primer sets are too specific to *Actinobacteria* and therefore not suitable to amplify a broad range of sequence types. They amplify *isoA* genes mainly from enrichments (Figure 6.5 a). They have limited coverage because they were designed from the few sequences of isoprene degrading strains available at the time most of which were isolated by Acuña Alvarez et al. (2009). The study also confirmed that *16S rRNA* gene is a good reference gene for similar experiments with environmental samples because it was amplified in all samples. That is why rRNA genes were recommended for normalization in RT-qPCR experiments (Wong and Medrano, 2005).

The positive control *psbA* gene (coding for protein D1 of photosystem II reaction centre) was not detected in any sample (Figure 6.7). This may be because the gene was below detection limit in the environment or the messenger RNA was too labile to be recovered. Where possible, it will be advisable to use more than one gene, as positive control.

In conclusion, this experiment has provided a detailed protocol for conducting diel gene expression experiments that can be used for similar studies.

Chapter 7. Concluding Remarks

The cycling of volatile hydrocarbons in estuarine environment is not fully understood. The aim of this project was to investigate the aerobic and anaerobic degradation of ethane, propane, benzene, toluene and isoprene in the Colne estuary, UK, particularly to identify key bacterial and archaeal communities involved with the biodegradation. The overarching hypothesis was that bacterial and archaeal communities with the ability to aerobically or anaerobically degrade these volatile organic compounds (VOCs) will be found in estuarine environments.

The study has shown that benzene, toluene, isoprene, ethane and propane are degraded rapidly by aerobic bacterial communities from the Colne estuary. The putative VOC-degrading phylotypes identified were affiliated with *Proteobacteria* and *Actinobacteria*. These populations evolved to use specific types of VOCs as follows: *Pseudomonas* was the main benzene degrading taxa; *Amphritea* the main toluene degrading taxa; and *Rhodococcus* was the dominant isoprene taxa. Ethane or propane degrading bacterial communities were partition into low-NaCl loving and marine populations identified from the head (Hythe) and middle (Wivenhoe) of the estuary, respectively. *Thauera* and *Azoarcus* were the ethane and propane degrading taxa from the Hythe. Whereas *Amphritea* was the main propane degrading taxa from Wivenhoe. Ethane degraders from Wivenhoe were dominated by *Cycloclasticus* and included *Pseudoruegeria*, *Roseibacterium*, and *Marinobacterium* taxa. The *Rhodococcus wratislaviensis* strain I and *Mycobacterium hodleri* strain B isolated from sediment slurries were metabolically versatile. They contained the *prmABCDE* gene cluster (Figures 5.7 – 5.8) that encodes propane monooxygenase for ethane and propane

degradation, emphasizing their role in the cycling of volatile hydrocarbons in the estuary.

The anaerobic degradation of benzene, toluene, propane and isoprene was also observed but not the one of ethane. Anaerobic degradation of benzene involved phylotypes affiliated with *Deltaproteobacteria (eg Pelobacter)*, *Epsilonproteobacteria (eg Sulfurimonas)* and methanogens identified in the same enrichment (section 4.4.1). The putative anaerobic bacteria identified from enrichments were affiliated with *Deltaproteobacteria*, *Epsilonproteobacteria, Firmicutes, Verrucomicrobia, Actinobacteria* and *Chloroflexi,* while the archaeal population were from *Euryarchaeota* and *Crenarchaeota* (Figures 4.15 – 4.16; Tables 4.2 – 4.3).

In aerobic enrichments, chemical structure was the key driver of the change in the VOC-degrading bacterial community (Figures 3.7, 3.9 – 3.10 c - d). The sampling location had an additional effect on the bacterial taxa identified in aerobic ethane enrichments with marine genera identified in Wivenhoe (Figure 3.10 a – b). In anaerobic enrichments, however, sampling location appeared to be the main driver of the bacterial community composition rather than the VOC degraded (Figure 4.5 c - d). Aerobic and anaerobic degradation was faster at the head of the estuary (the Hythe) than any other location along the Colne estuary as observed previously by Acuña Alvarez et al. (2009).

Each VOC studied was used by aerobic bacterial populations suggesting that aerobic degradation is a more ecologically common phenomenon than the anaerobic process. It also demonstrates that hydrocarbon degrading capacity is readily available in estuarine environments which is of ecological and biotechnological significance. This knowledge could be applied in bioremediation efforts during accidental oil spillage.

Estuaries are prone to pollution whenever there is an oil spill at sea or ocean because of tidal action. The sudden introduction of tonnes of petroleum products (or crude oil) in coastal ecosystems has devastating effects including mortalities and bioaccumulation (McGenity et al., 2012). Approximately 600,000 tonnes of crude oil were spilled in the Gulf of Mexico during the BT deep water horizon event in 2010, resulting in the pollution of the coastal environment (McGenity et al., 2012, Atlas and Hazen, 2011, Michel et al., 2013). Other chronic sources of hydrocarbons in estuaries include boats and their fuels; riverine run-off, leakage from storage tanks and pipes etc.

The fate of the oil (including VOCs) depends on the location and prevailing environmental conditions at the time of spillage such as temperature and wind speed. For example, coastal environments were oiled heavily after the Exxon Valdez spill in 1989 because a major wind storm (50 mph) was reported at the location 2 days after the accident (Atlas and Hazen, 2011). In generally, most VOCs will evaporate. The more soluble but toxic fractions like benzene will dissolve in water causing mortality amongst aquatic life while some fractions will be degraded by microbial populations in the location etc (Head et al., 2006).

This study has shown that estuaries are a potential sink for diverse VOCs (alkanes, alkenes and monoaromatic hydrocarbons). The biodegradation and concomitant enrichment of bacterial phylotypes is an indication they are food to these populations. That is most likely one of the reasons why VOCs like benzene and toluene are usually below detection limit in UK estuaries located near industries (Dawes and Waldock, 1994). Most VOCs produced by microalgae and methanogens are also consumed *in situ* (Steinke et al., 2011). Interestingly, *Cycloclasticus*, a putative polyaromatic-

hydrocarbon-degrading bacterium was enriched with ethane confirming that petroleum hydrocarbon degraders are present in estuaries. McKew et al. (2007) found several hydrocarbonoclastic bacteria including *Alcanivorax* in the Thames estuary. Estuaries may play a role as nurseries for hydrocarbon-degrading species (since they have more nutrients compared to the oceans) before been transported by tidal action to the oceans.

Biodegradation is the most environmentally friendly option for environmental restoration after oil spillage. Initially, physical and chemical containment approaches are used to remove some of the oil and curtail spread. Booms and skimmers would be used as barriers to prevent spread and for oil removal from water surfaces, respectively. *In-situ* burning of oil is done if wind speed is low or absent. Chemical approaches such as the spread of chemical dispersants by aeroplanes to promote dispersion and bioavailability of oil for biodegradation have been used (New Jersey Sea Grant Consortium, 2010). Nevertheless, microbial consumption of hydrocarbons is the main process that completes the environmental remediation process (Head et al., 2006, Atlas and Hazen, 2011).

The 16S-rRNA-gene-based method employed in this study has revealed the identity of potential aerobic and anaerobic VOC degraders in estuarine environments. Since specific populations were shown to degrade specific groups of VOCs, such populations could be targeted for biostimulation by nutrient addition in the environment. It is worth noting, however, biodegradation of pollutants observed in the laboratory do not always translate to similar results in the field. Nevertheless, biodegradation observed in laboratory microcosms has also been observed on the field (Head et al., 2006).

Bioaugmentation and biostimulation are the main bioremediation approaches. The environment is supplemented with hydrocarbonoclastic (hydrocarbon-degrading) microbes in bioaugumentation to enhance biodegradation. However, McGenity et al. (2012) summarized the many limitations of this approach to include the use of single strains which may not be adapted to the polluted environment; inadequate dispersion/ access to the pollutant; absence of protection from grazers; etc. On the other hand, biostimulation provides limiting nutrients (nitrogen and phosphorous) through inorganic fertilizers addition, thereby encouraging indigenous hydrocarbonoclastic populations to flourish and degrade the pollutant (Head et al., 2006). The so-called bioremediation accelerators are employed to release nutrients slowly to prevent eutrophication. Biostimulation, when used professionally is a powerful tool.

This thesis has contributed to our understanding of the cycling of volatile hydrocarbons in estuarine environments by revealing the identity of novel microbial phylotypes not previously known to be involved with the biodegradation of benzene, toluene, isoprene, ethane and propane. The findings suggest that aerobic bacterial communities in estuaries play an ecological role as carbon sinks through biodegradation of volatile hydrocarbons, thereby, reducing the quantity of VOCs emitted to the atmosphere. It also assumes that syntrophy plays a key role in the cycling of volatile hydrocarbons (especially benzene) in anoxic estuarine sediments.

The findings of this study have raised questions that will merit further investigation. The following are suggestion for further investigation:

- Anaerobic oxidation of isoprene has remained a big question to be resolved. Further attempts to demonstrate anaerobic degradation of isoprene are needed.
- Metabolic interactions of bacterial and archaeal communities associated with anaerobic VOC degradation (especially benzene and propane). Such studies for estuarine environments are few.
- Isolation and characterization of the *Cycloclasticus* enriched with ethane. This study will determine whether it has polyaromatic hydrocarbons (PAH) degrading capability.
- *In situ* measurement of biogenic ethane and propane. The enrichment and isolation of ethane and propane degrading bacteria is an indication of a possible source of these VOCs in the Colne estuary. One possible source could be biogenesis by methanogens.
- Determine the influence of anaerobic VOC degradation on sulfate loss, hydrogen sulphide and methane production.
- Investigate the diel expression of *isoA* gene in estuarine sediment with new primer sets (Chapter 6). Both absolute quantification and relative gene quantification approaches will be tested.

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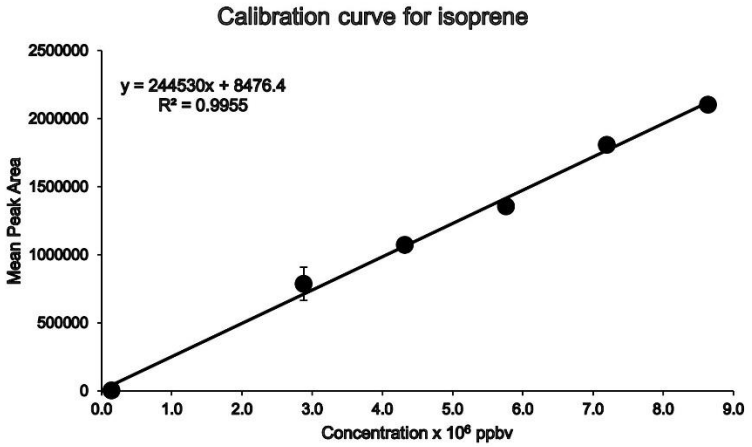
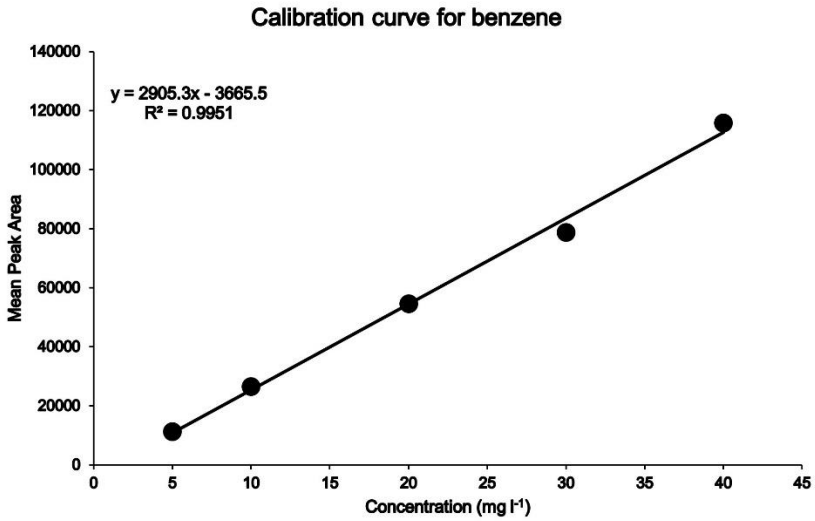
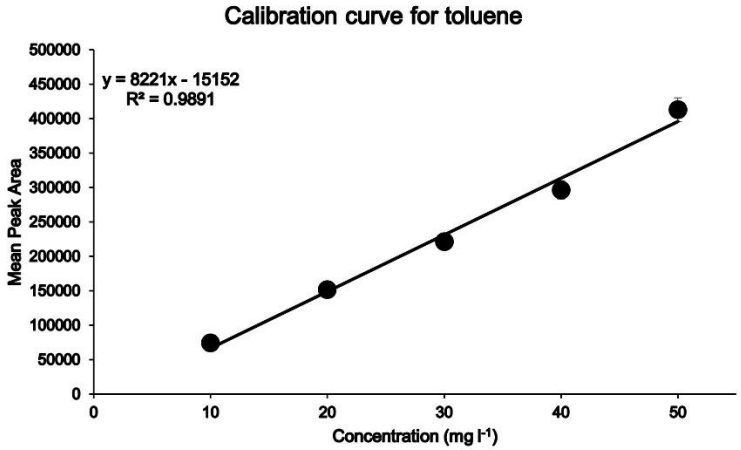
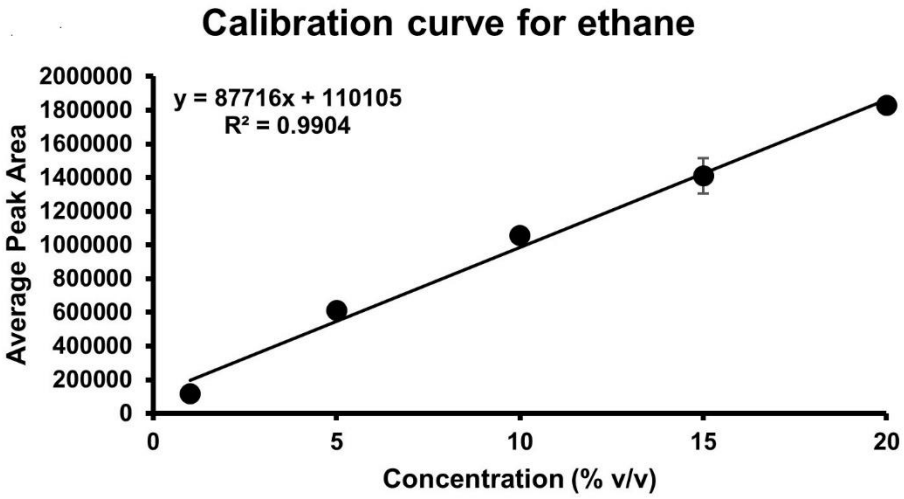
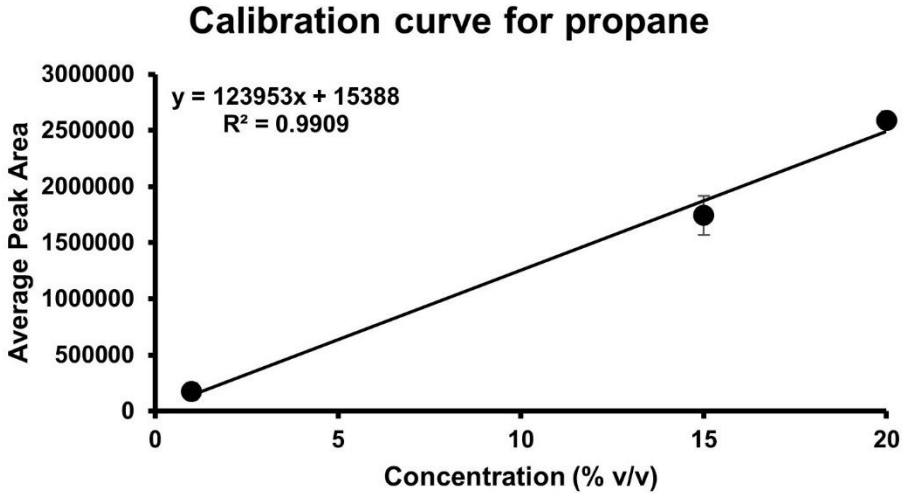
Appendix 1: Representative GC-FID calibration curves for isoprene, benzene, toluene, ethane and propane











NB:

Values are mean of duplicate ± SE represented by bar.

0.5 mM benzene = 39.1 mg l^{-1}; 0.5 mM Toluene = 46.1 mg l^{-1}

Appendix 2: Number of bacterial and archaeal sequences identified after quality control of Illumina MiSeq data obtained for Chapter 3 and Chapter 4

Table 1: Total number of bacterial *16S rRNA* gene sequences obtained

S/N	Sample code	Sample Description	Bacterial Sequences identified	Experiment (Chapter)
1	Bat301_AnO_Main	Brightlingsea No-VOC Day 301	4640	Main Anaerobic Expt. (Chapter 4)
2	Bbt301_AnO_Main		4471	
3	BaBz_t301_AnO_Main	Brightlingsea + Benzene Day 301	3417	
4	BbBz_t301_AnO_Main		2870	
5	BcBz_t301_AnO_Main		2651	
6	Hat213_AnO_Main	Hythe No-VOC Day 213	3967	
7	Hbt213_AnO_Main		4689	
8	Hct213_AnO_Main		4122	
9	HbBz_t213_AnO_Main	Hythe + Benzene Day 213	4158	
10	HcBz_t213_AnO_Main		5446	
11	Hat316_AnO_Main	Hythe No-VOC Day 316	4439	
12	Hbt316_AnO_Main		3767	
13	Hct316_AnO_Main		4531	
14	HaPr_t316_AnO_Main	Hythe + Propane Day 316	2629	
15	HbPr_t316_AnO_Main		1912	

S/N	Sample code	Sample Description	Bacterial Sequences identified	Experiment (Chapter)
16	Hat56_AnO_Main	Hythe No-VOC Day 56	5020	Main Anaerobic Expt. (Chapter 4)
17	Hbt56_AnO_Main		3961	
18	HaTl_t56_AnO_Main	Hythe + toluene Day 56	1311	
19	HcTl_t56_AnO_Main		4879	
20	Wat210_AnO_Main	Wivenhoe No-VOC Day 210	2751	
21	Wbt210_AnO_Main		2677	
22	Wct210_AnO_Main		3634	
23	WaBz_t210_AnO_Main	Wivenhoe + Benzene Day 210	4857	
24	WbBz_t210_AnO_Main		3266	
25	WcBz_t210_AnO_Main		2814	
26	Hat124_AnO_Trial	Hythe No-VOC Day 124	4653	Anaerobic Trial Expt. (Chapter 4)
27	Hbt124_AnO_Trial		2506	
28	Hct124_AnO_Trial		3666	
29	HaBz_E2_AnO_Trial	Hythe + Benzene 2nd enrichment (E2)	5734	
30	HbBz_E2_AnO_Trial		4857	
31	HcBz_E2_AnO_Trial		3041	
32	HaIs_E1_t124_AnO_Trial	Hythe + Isoprene First Enrichment (E1)	2793	
33	HbIs_E1_t124_AnO_Trial		1815	
34	HcIs_E1_t124_AnO_Trial		3117	
35	Hat0_O_Main	Hythe Day 0	6789	Main Aerobic Deg Expt. (Chapter 3)
36	Hbt0_O_Main		8439	
37	Hct0_O_Main		6023	

S/N	Sample code	Sample Description	Bacterial Sequences identified	Experiment (Chapter)
38	Hat37_O_Main	Hythe No-VOC Day 37	4598	Main Aerobic Deg Expt. (Chapter 3)
39	Hbt37_O_Main		5132	
40	Hct37_O_Main		5424	
41	HaBz_E3_O_Main	Hythe + Benzene, 3rd enrichment	3451	
42	HbBz_E3_O_Main		1528	
43	HcBz_E3_O_Main		7844	
44	HaEt_E3_O_Main	Hythe + ethane, 3rd enrichment	6515	
45	HbEt_E3_O_Main		9434	
46	HcEt_E3_O_Main		12686	
47	HaIs_E3_O_Main	Hythe + Isoprene, 3rd enrichment	10495	
48	HbIs_E3_O_Main		11791	
49	HcIs_E3_O_Main		11904	
50	HaPr_E3_O_Main	Hythe + propane, 3rd enrichment	8485	
51	HbPr_E3_O_Main		13429	
52	HcPr_E3_O_Main		4302	
53	HaTl_E3_O_Main	Hythe + Toluene, 3rd enrichment (E3)	14024	
54	HbTl_E3_O_Main		1787	
55	HcTl_E3_O_Main		1996	
56	Wat0_O_Main	Wivenhoe Day 0	7944	
57	Wct0_O_Main		5310	
58	Wat37_O_Main	Wivenhoe No-VOC Day 37	3911	
59	Wbt37_O_Main		3365	
60	Wct37_O_Main		7490	

S/N	Sample code	Sample Description	Bacterial Sequences identified	Experiment (Chapter)
61	WaBz_E3_O_Main	Wivenhoe + Benzene 3rd enrichment	10232	Main Aerobic Deg Expt. (Chapter 3)
62	WbBz_E3_O_Main		13566	
63	WcBz_E3_O_Main		11006	
64	WaEt_E3_O_Main	Wivenhoe + ethane, 3rd enrichment	554	
65	WbEt_E3_O_Main		5307	
66	WcEt_E3_O_Main		7718	
67	WaIs_E3_O_Main	Wivenhoe + Isoprene, 3rd enrichment	16180	
68	WbIs_E3_O_Main		6433	
69	WcIs_E3_O_Main		9441	
70	WaPr_E3_O_Main	Wivenhoe + propane 3rd enrichment	7633	
71	WbPr_E3_O_Main		6744	
72	WcPr_E3_O_Main		6992	
73	WaTl_E3_O_Main	Wivenhoe + Toluene 3rd enrichment	6554	
74	WbTl_E3_O_Main		10551	
75	WcTl_E3_O_Main		10284	
76	Hat0	Hythe Day 0	4882	Simple Nitrogen Flushing Expt (Chapter 4)
77	Hbt0		6014	
78	Hct0		7109	
79	Hat22_O	Hythe No-VOC day 22 aerobic	5585	
80	Hbt22_O		10231	
81	Hct22_O		7791	
82	HaIs_t22_O	Hythe + isoprene day 22 aerobic	6355	
83	HcIs_t22_O		7920	

S/N	Sample code	Sample Description	Bacterial Sequences identified	Experiment (Chapter)
84	Hat280_N	Hythe No-VOC Control day 280 flushed with Nitrogen	8032	Simple Nitrogen Flushing Expt (Chapter 4)
85	Hct280_N		6935	
86	HcIs_t280_N	Hythe + isoprene flushed with Nitrogen day 280	7634	
Total bacterial sequences identified			**516840**	

Table 2: Total number of archaeal *16S rRNA* gene sequences obtained

S/N	Sample	Sample description	Number of archaeal sequences identified	Experiment
1	Bat301_AnO_Main	Brightlingsea No-VOC Day 301	27493	Main Anaerobic Deg Expt. (Chapter 4)
2	Bbt301_AnO_Main		27199	
3	BaBz_t301_AnO_Main	Brightlingsea + Benzene Day 301	50161	
4	BbBz_t301_AnO_Main		26677	
5	BcBz_t301_AnO_Main		25625	
6	Hat213_AnO_Main	Hythe No-VOC Day 213	20824	
7	Hbt213_AnO_Main		20197	
8	Hct213_AnO_Main		35938	
9	HaBz_t213_AnO_Main	Hythe + Benzene Day 213	19152	
10	HbBz_t213_AnO_Main		30263	
11	HcBz_t213_AnO_Main		29521	
12	Hat316_AnO_Main	Hythe No-VOC Day 316	38699	
13	Hbt316_AnO_Main		20691	
14	Hct316_AnO_Main		39580	
15	HaPr_t316_AnO_Main	Hythe + Propane Day 316	51347	
16	HbPr_t316_AnO_Main		49901	
17	HcPr_t316_AnO_Main		43774	
18	Hat56_AnO_Main	Hythe No-VOC Day 56	14784	
19	Hbt56_AnO_Main		16573	
20	Hct56_AnO_Main		23154	

S/N	Sample	Sample description	Number of archaeal sequences identified	Experiment
21	HaTl_t56_AnO_Main	Hythe + toluene Day 56	24032	Main Anaerobic Deg Expt (Chapter 4)
22	HbTl_t56_AnO_Main		26126	
23	HcTl_t56_AnO_Main		17036	
24	Wat210_AnO_Main	Wivenhoe No-VOC Day 210	20290	
25	Wct210_AnO_Main		21393	
26	WaBz_t210_AnO_Main	Wivenhoe + Benzene Day 210	36300	
27	WbBz_t210_AnO_Main		29995	
28	WcBz_t210_AnO_Main		28614	
29	Hat124_AnO_Trial	Hythe No-VOC Day 124	27541	Anaerobic Trial Expt (Chapter 4)
30	Hbt124_AnO_Trial		35960	
31	Hct124_AnO_Trial		30203	
32	HaIs_E1_t124_AnO_Trial	Hythe + Isoprene First Enrichment (E1) Day 124	21659	
33	HbIs_E1_t124_AnO_Trial		15552	
34	HcIs_E1_t124_AnO_Trial		12615	
35	HaBz_E2_AnO_Trial	Hythe + Benzene 2nd enrichment (E2)	7477	
36	HbBz_E2_AnO_Trial		32459	
37	HcBz_E2_AnO_Trial		29518	
38	Hat0	Hythe Day 0	2316	Simple Nitrogen Flushing Expt Chapter 4
39	Hbt0		5205	
40	Hct0		18081	

S/N	Sample	Sample description	Number of archaeal sequences identified	Experiment
41	Hat22_O	Hythe No-VOC day 22 aerobic	23026	
42	Hbt22_O		23508	
43	Hct22_O		25822	
44	HaIs_t22_O	Hythe + isoprene day 22 aerobic	17471	Simple Nitrogen Flushing Expt
45	Hat280_N	Hythe No-VOC Control day 280 flushed with Nitrogen	23595	
46	Hct280_N		18734	Chapter 4
47	HbIs_t280_N	Hythe + isoprene flushed with Nitrogen day 280	32992	
48	HcIs_t280_N		31869	
Total Archaeal sequences identified			**1250942**	