# Environmental controls on bacteriohopanepolyol signatures in estuarine sediments

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# Declaration

I hereby certify that the work presented in this thesis is my own, except where otherwise acknowledged, and has not been submitted previously for a degree at this, or any other University.

Kate A. Osborne

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## Abstract

To date, research on the fate of methane in marine settings has mainly focused on anaerobic microbial processes. An alternative fate for methane is aerobic methane oxidation (AMO) by methanotrophic bacteria which takes place in aerobic surface sediments and the overlying water column. Tracing methanotroph activity in past environments can be achieved via analysis of a distinctive suite of biomarkers called bacteriohopanepolyols (BHPs). BHPs are membrane lipids produced by many prokaryotes comprising a pentacyclic triterpenoid structure with an extended polyfunctionalised side chain. Although, there is much debate about the role of BHPs, studies suggest they regulate cell membrane fluidity, however, the factors controlling their expression are poorly constrained. They have a wide range of structural variation which varies between bacterial phyla and species. The major BHPs produced by methanotrophs are collectively known as the 35-aminoBHPs, most commonly including 35-aminobacteriohopane-32,33,34-triol (aminotriol), 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol) and 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol), with aminopentol seen as a diagnostic marker for Type I methanotrophs from the phylum *Gammaproteobacteria.*

The changes in methanotroph community composition in estuarine sediments under a range of environmental perturbations and the effect this had on BHP composition, namely the 35-aminoBHPs, was assessed. Aerobic microcosms inoculated with River Tyne (UK) estuarine sediment with a 5% methane amended headspace (unless otherwise stated), were subjected to a range of environmental perturbations; methane concentration (0.1-5%), temperature (4-60°C), pH (4-9) and salinity (1-150 g/L NaCl). Methane oxidation rates were monitored and methanotroph diversity was determined by targeting the particulate methane monooxygenase gene (*pmoA*). Methane oxidation was observed between 4 and 50°C, at all tested pH values and up to salinities of 70 g/L; however, methanotroph community composition varied with temperature, pH and salinity and these changes were reflected in the 35-aminoBHP signatures quantified by LC-MS analysis. For example, aminopentol was not enriched at pH 9 when the unusual Type I *Methylomicrobium* spp. were dominant, whilst the maximal production of C-3 methylated aminopentol was witnessed at 50°C when a *Methylocaldum* sp. was enriched. The *hpnR* gene, required for the methylation of BHPs at the C-3 position, was also identified in sediments at the aforementioned temperature. Novel

compounds, identified after the analysis of six previously untested Type I marine methanotrophs within this study, were also found in microcosm sediments in varying abundances.

The effect that of growth stage on 35-aminoBHP abundance was determined by analysing aerobic microcosms inoculated with River Tyne estuarine sediment over a 28 day period at times before and after methane oxidation. It revealed the continued production of aminopentol at mesophilic temperatures after methane oxidation was complete. This may have implications for the interpretation of the sedimentary record where aminopentol witnessed in marine settings may not represent periods of significant methane oxidation but rather a response to methane limiting conditions.

Anaerobic producers of BHPs were investigated and the preservation and/or degradation of individual compounds was assessed in long-term studies. Microcosms inoculated with anoxic River Tyne estuarine sediment were subjected to sulphate-reducing and methanogenic conditions over a period of 706 and 665 days respectively. Changes in BHP composition over time were quantified by LC-MS with compounds including bacteriohopane-32,33,34,35-tetrol (BHT) and adenosylhopane found to be more resistant to degradation over the course of the study compared to bacteriohopane-32,33,34,35-tetrol cyclitol ether (BHT cyclitol ether). This indicates that some compounds are more resistant to degradation over time compared with others.

## Table of contents

## List of figures

## List of tables

## List of abbreviations

| | |
|---|---|
| **ANOVA** | Analysis of variance |
| **BES** | 2-bromoethanesulfonate |
| **BHP** | Bacteriohopanepolyol |
| **$CH_3COONa$ (NaOAc)** | Sodium acetate |
| **$CH_4$** | Methane |
| **$CHCl_3$** | Chloroform |
| **DCM** | Dichloromethane |
| **DGGE** | Denaturing gradient gel electrophoresis |
| **DNA** | Deoxyribonucleic acid |
| **GC-FID** | Gas Chromatography-Flame Ionisation Detection |
| **GC-MS** | Gas Chromatography-Mass Spectrometry |
| **HCl** | Hydrochloric acid |
| ***hpnR*** | C-3 methylase gene |
| **MeOH** | Methanol |
| **MMO** | Methane monooxygenase |
| **NaCl** | Sodium chloride |

| | |
|---|---|
| **NaOH** | Sodium hydroxide |
| **$Na_2S$** | Sodium sulfide |
| **$Na_2SO_4$** | Sodium sulphate |
| **NMS** | Nitrate mineral salts |
| **‘35-carbamylmethylester-bacteriohopane-32,33,34-triol’** | Compound related to aminotriol but with a peracetylated $[M+H]^+$ 16 Da higher |
| **‘35-carbamylmethylester-bacteriohopane-31,32,33,34-tetrol’** | Compound related to aminotetrol but with a peracetylated $[M+H]^+$ 16 Da higher |
| **‘35-carbamylmethylester-bacteriohopane-30,31,32,33,34-pentol’** | Compound related to aminopentol but with a peracetylated $[M+H]^+$ 16 Da higher |
| **‘Desmethylaminotriol’** | Compound related to aminotriol but with a peracetylated $[M+H]^+$ 14 Da lower |
| **‘Desmethylaminotetrol’** | Compound related to aminotetrol but with a peracetylated $[M+H]^+$ 14 Da lower |
| **‘Desmethylaminopentol’** | Novel compound related to aminopentol but with a peracetylated $[M+H]^+$ 14 Da lower |
| **PCR** | Polymerase chain reaction |
| **pMMO** | Particulate methane monooxygenase |
| ***pmoA*** | Particulate methane monooxygenase gene |
| **PTFE** | Polytetrafluoroethylene |

| | |
|---|---|
| **rRNA** | Ribosomal ribonucleic acid |
| **sMMO** | Soluble methane monooxygenase |
| ***sqhC*** | Squalene hopene cyclase gene |
| **TLE** | Total lipid extract |
| **TOC** | Total organic carbon |

## Preface to thesis

Bacteriohopanepolyols (BHPs) are membrane lipids produced by a wide range of bacteria, including methanotrophs, methylotrophs, cyanobacteria, nitrogen-fixing bacteria and purple non-sulfur bacteria. The structural and functional variability of BHP structures produced offers great potential for their use as molecular biomarkers for bacterial processes and populations in both modern and ancient environments.

The thesis commences with a broad introduction into sources of methane and methanotrophs (Chapter 1). This is followed by an introduction into the biological sources of BHPs, their function and use as biomarkers particularly for methanotrophic bacteria (Chapter 2). This is followed by a methods section detailing a microcosm-based experimental approach using River Tyne estuarine sediment as a source of bacterial inoculum; as well as methods for microbial community analysis and BHP signature analysis (Chapter 3). The results and discussion of the research are provided in Chapters 4-9. Each chapter introduces current knowledge and the gaps in understanding that has motivated the research.

Chapter 4: The BHP signatures of marine methanotrophs - their composition and the relative abundance of different compounds between genera and species. This chapter introduces some novel BHP compounds identified for the first time and discusses their potential as biomarkers for methanotrophy.

Chapter 5: The effect of methane concentration and temperature on methanotroph diversity and BHP composition, focusing on methanotroph-derived signatures, in controlled aerobic microcosm experiments inoculated with River Tyne estuarine sediment is assessed.

Chapter 6: The effect of pH and salinity on methanotroph diversity and BHP composition, focusing on methanotroph-derived signatures, in controlled aerobic microcosm experiments inoculated with River Tyne estuarine sediment is assessed.

Chapter 7: The effect that methanotroph growth stage has on 35-aminoBHP composition at mesophilic and thermophilic temperatures is investigated in controlled aerobic microcosm experiments inoculated with River Tyne estuarine sediment.

Chapter 8: A long-term study to investigate the types of BHP compounds produced in anaerobic systems and the degradation/preservation of compounds, in controlled microcosm experiments inoculated with anoxic River Tyne estuarine sediment is assessed.

Chapter 9: This chapter concludes the major results and findings from the research project and details additional questions that have arisen from the work and future avenues of research resulting from this.

# 1. Introduction

## 1.1. Methane - role as a climate driver

Methane is a potent greenhouse gas and is a significant climate driver. It is currently present in the atmosphere at lower concentrations than carbon dioxide but has a global warming potential 72 times stronger over a 20 year period (IPCC, 2007). Atmospheric methane concentrations are continuing to rise (Figure 1-1) and concentrations have tripled since pre-industrial times.

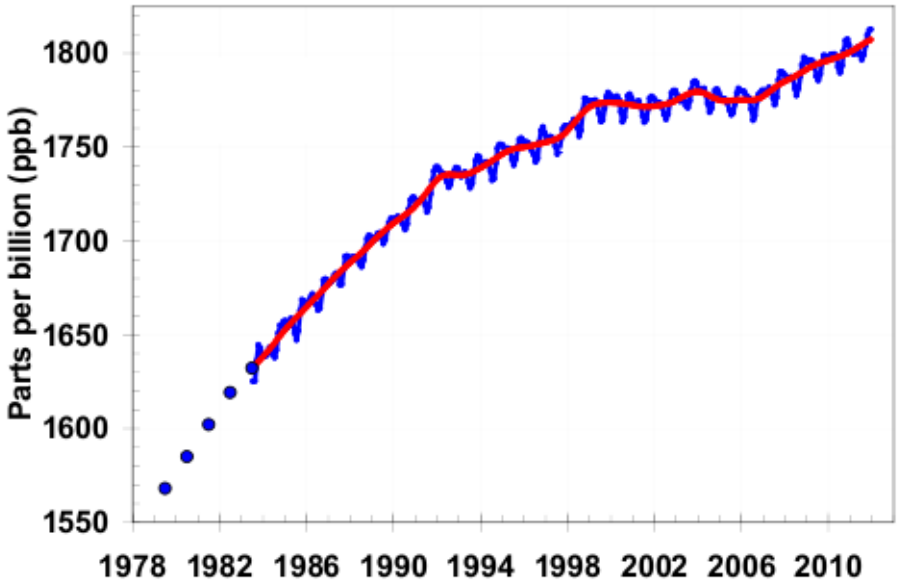



**Figure 1-1:** Global average atmospheric methane concentrations from 1979 to 2010- the National Oceanic and Atmospheric Administration (NOAA) global air sampling network (Butler, 2010).

Increasing methane concentrations could have major implications for climate and atmospheric chemistry. Therefore, it is important to understand the processes and feedbacks of methane cycling.

## 1.2. Sources of atmospheric methane

Methane is produced by two fundamentally different processes:

1) Biogenic - the biological production of methane. It is the final step in the decay of organic matter and is carried out by anaerobic archaea, methanogens, by a process known as methanogenesis. Key pathways include the reduction of carbon dioxide with

hydrogen (hydrogenotrophic methanogenesis), but methane can also be produced from methyl compounds and acetate (acetoclastic methanogenesis) (Madigan et al., 2009). It takes place in anoxic environments, for example, freshwater and marine sediments, wetlands, paddy fields, landfill sites and the intestinal tracts of ruminants and termites (Thauer et al., 2008).

2) Thermogenic - the breakdown of organic matter found in deep petroleum reservoirs and source rocks and chemical cracking processes (Judd, 2004).

### 1.2.1. Natural and anthropogenic sources of atmospheric methane

Total atmospheric methane emissions have been estimated at ~600 Tg $y^{-1}$ (IPCC, 2013 and references therein). Anthropogenic sources account for up to 70% (410 Tg $y^{-1}$) of these emissions whereas natural sources only account for ~30% of total atmospheric methane (Lelieveld et al., 1998; Wuebbles and Hayhoe, 2002). However, there are large uncertainties in these exact figures as data sets are often too small to make accurate global assumptions and calculations.

Natural emissions arise from wetlands, termites and other wild ruminants, oceans and freshwaters, and gas hydrates (Wuebbles and Hayhoe, 2002). The greatest contributor to natural emissions is wetlands at ~72%, with tropical wetlands accounting for a large proportion of this despite occupying less than 20% of the global wetland area (Lelieveld et al., 1998; Wuebbles and Hayhoe, 2002). Oceans and freshwaters account for ~8% of total natural emissions. Although large quantities of methane are stored in gas hydrates in marine and continental sediments, only a small fraction reaches the atmosphere at present, ~3-5% (Lelieveld et al., 1998; Lowe, 2006).

Anthropogenic emissions arise from biogenic sources related to agriculture and waste disposal, domestic ruminants, animal and human wastes, paddy fields, biomass burning and landfills. Methane is also emitted by non-biogenic sources such as during the extraction of fossil fuels such as natural gas, coal, and petroleum (Wuebbles and Hayhoe, 2002). Agricultural sources account for ~56% of total anthropogenic emissions and the use of fossil fuels ~25% of emissions (Lelieveld et al., 1998).

A minor sink for atmospheric methane is dry soil oxidation ~7% (~30 Tg $y^{-1}$) and the reaction with the hydroxyl radical in the troposphere a major sink, almost 90%, the remainder is transported to the stratosphere (Wuebbles and Hayhoe, 2002).

#### 1.2.1.1. Natural sources - wetlands

Tropical wetlands account for a large proportion of natural methane emissions despite occupying less than 20% of the global wetland area. The majority of non-tropical wetland methane emissions are from boreal wetlands. The short duration of the thaw season means Arctic tundra methane emissions are comparatively low. Temperate wetlands are less important as the area occupied is less than 5% of total wetlands (Lelieveld et al., 1998). Emissions from wetlands are influenced by a range of environmental factors, most importantly, soil temperature and water table depth. Changes in precipitation patterns and projected future temperature increases could increase emissions from these environments (Wuebbles and Hayhoe, 2002).

#### 1.2.1.2. Natural sources - oceanic

Oceans contribute less to natural emissions than wetlands, with one study suggesting a figure of ~10 Tg $y^{-1}$ which is ~5% of total natural methane emissions (Lelieveld et al., 1998). However, studies quantifying this have given a wide range of estimations from 0.4 to 48 Tg $y^{-1}$ (Judd, 2004 and references therein) with a figure of 20 Tg $y^{-1}$ (Judd, 2004) being suggested as a realistic value for methane release from the seabed to the atmosphere. Large quantities of methane are stored in gas hydrates in marine sediments. However, only a very small fraction of this methane reaches the atmosphere as it is oxidised in anoxic and oxic settings to carbon dioxide (Thauer et al., 2008).

### 1.3. Sources of methane emissions in the marine environment

Methane in marine sediments may originate from deep thermogenic sources or result from methanogenesis in shallower sediments. There are several possible sources of methane emissions in marine environments such as estuaries, deep-sea sedimentary fans, mud volcanos, cold seeps, pockmarks and gas hydrates, which have been a focus of research during recent times.

#### 1.3.1. Gas hydrates

Gas hydrates are mainly located along coastal margins and are ice-like crystalline mixtures of frozen water and gas molecules formed under cold and high pressure conditions. Estimates of the quantity of hydrate-bound methane in the ocean vary

widely with some figures suggesting up to ~10,000 Gt of carbon but current thinking suggests a figure of between ~500-3000 Gt (Buffett and Archer, 2004; Milkov, 2004). They are stable in a narrow range of temperatures and pressure; consequently, any increases in ocean temperature may have significant implications for hydrate stability (Reeburgh, 2007 and references therein; Westbrook et al., 2009).

### 1.3.2. Estuaries and sedimentary fans

Methane production in estuaries originates from *in situ* methanogenesis within anoxic sediments (Upstill-Goddard et al., 2000). It has been suggested that whilst the majority of methane, ~90% in some locations, is removed from estuaries before reaching the atmosphere, 0.1-0.4 Tg $y^{-1}$ may be lost globally to the atmosphere from estuaries (Upstill-Goddard et al., 2000). Estuaries and shelf areas only represent a small fraction of the world's oceanic area but have been estimated to produce ~75% of the total marine methane emissions to atmosphere (Bange et al., 1994). A more recent study indicated that methane is mainly formed in shallow coastal regions with maximum concentrations being observed in estuarine/fjord systems (Bange, 2006).

Sedimentary fans are also sources of methane emissions from the ocean. These sediment bodies build oceanward of the continental shelf and receive sediment from river mouths or via submarine canyons. The Congo Fan has one of the world’s largest canyon structures which cuts landward into the mouth of the river. It hosts gas hydrates and an extensive network of pockmarks (Uenzelmann-Neben et al., 1997).

### 1.3.3. Pockmarks

Pockmarks are cone-shaped circular or elliptical depressions ranging from a few metres to ≥ 300 m in diameter and from 1 m to 80 m in depth (Gay et al., 2006). They are observed worldwide along the continental margins at depths ranging from 30 m to over 3000 m (Hovland et al., 2005; Gay et al., 2006; Chand et al., 2009). It is hypothesised that pockmarks form by sudden local gas/porewater/sediment eruption, with periodic short outbursts followed by long periods of inactivity or microseepage (Hovland et al., 2005). The source of the gases and waters are poorly constrained, but there is a link with gas hydrate destabilisation (Hovland et al., 2005). They have also been attributed to the expulsion of water by the melting of deep lying permafrost, freshwater seepage through artesian aquifers or escape of hydrocarbon fluids from

underlying petrogenic source rocks (Chand et al., 2009). Pockmarks generally appear in fine-grained sediments so it seems their occurrence is more closely related to the type of seabed sediment than the source path of fluid venting (Chand et al., 2009).

### 1.3.4. Mud volcanoes and cold seeps

Mud volcanoes and cold methane seeps (sometimes called cold vents) are areas of the ocean floor where hydrogen sulfide, methane and other hydrocarbon-rich fluid seepage occurs. They exhibit high methane fluxes to the hydrosphere and potentially to the atmosphere. The number of active submarine mud volcanoes has been found to be higher than previously estimated (Niemann et al., 2006 and references therein). The Haakon Mosby mud volcano (HMMV) is a well-studied cold methane seep situated at the Norwegian continental margin where there is active fluid and gas seepage (Niemann et al., 2006; Lösekann et al., 2007). The volcano has a central zone with gas saturated sediments which is surrounded by an area with shallow gas hydrates overlain by microbial mats (e.g. Krüger et al., 2005; Pape et al., 2011).

## 1.4. Fate of methane in marine systems

To date, research on marine methane has mainly focused on anaerobic microbial processes (Valentine, 2002; Boetius and Suess, 2004; Krüger et al., 2005). However, an alternative fate for methane is aerobic methane oxidation by methanotrophic bacteria which takes place in aerobic surface sediments and the overlying water column.

### 1.4.1. Anaerobic oxidation of methane (AOM)

Anaerobic oxidation of methane (AOM) is an important sink for methane emissions from the marine environment and is the main sink for methane from gas hydrate reservoirs. Methane from various sources (Section 1.3) diffuses through anoxic sediment and is oxidised in a process involving a syntrophic consortium of anaerobic methanotrophic archaea (ANME), three ANME have been identified to date, and sulphate-reducing bacteria (Valentine, 2002; Niemann et al., 2006; Valentine, 2011). In the lower anoxic sulphate-depleted sediment horizons, methane is produced, while in the upper anoxic sulphate-containing horizons the methane is oxidised by anaerobic methanotrophs (Whiticar, 2002).

Studies have estimated that AOM in marine sediments removes as much methane as is oxidised aerobically in terrestrial environments or in the atmosphere (Boetius and Suess, 2004 and references therein). Therefore, it plays an important role in methane cycling particularly in ocean settings. The net rate of AOM in marine sediments has been estimated from 70 Tg $y^{-1}$ (Reeburgh et al., 1993) to 300 Tg $y^{-1}$ (Hinrichs and Boetius, 2002). It has also been estimated that methane oxidation in anoxic zones of sediments reduces the emission of methane from the ocean by perhaps more than 80% (Krüger et al., 2005 and references therein). Some estimates have even suggested that > 90% of methane produced by marine systems is consumed by this process (Bridgham et al., 2013 and references therein). AOM has been shown to be the main methane sink in sediments surrounding the active centre of the HMMV (Knittel et al., 2005; Niemann et al., 2006; Lösekann et al., 2007).

### 1.4.2. Aerobic methane oxidation (AMO) - an alternative fate

Aerobic methane oxidation (AMO) is an alternative fate for methane emissions from ocean sediments if it has bypassed the anaerobic sink. Methanotrophic bacteria consume methane produced in the methanogenic zones of sediments or from thermogenic breakdown of kerogen in the deep subsurface, oxidising it to carbon dioxide (Hanson and Hanson, 1996). AMO takes place in the uppermost oxic surface sediments and the overlying water column.

There have been relatively few studies to determine the role of methanotrophs in ocean settings and their potential impact on methane cycling in this environment. One study measured high methane oxidation rates in the lower part of the water column in the Eel River basin, the site of present day gas hydrate dissociation (e.g. Valentine et al., 2001). The active centre of the HMMV had a high abundance of aerobic methanotrophs (Type I) and was the first cold seep in which a high *in situ* abundance of aerobic methanotrophs was witnessed (Lösekann et al., 2007).

This study will focus on aerobic methanotrophs in marine systems, namely estuarine systems, but the findings from this research will also have implications in wider marine systems and also terrestrial environments, which have been the main focus of previous AMO studies. The capacity for both methanogenesis and aerobic methane oxidation has been shown in sediments of the River Tyne estuary, Newcastle, UK (Jones et al., 2008; Blake, 2010). Therefore, sediment from the River Tyne estuary was used to

investigate the effect of environmental factors on the occurrence of aerobic methane oxidation.

### 1.5. Aerobic methanotrophs - a biological methane sink

Aerobic methanotrophic bacteria use methane as their sole energy and carbon source. They are widespread in nature and are found in a variety of environments such as soils, sediments, fresh and marine waters, lakes and peat bogs. They act as a biofilter, reducing methane release to the atmosphere and therefore play an important role in carbon cycling (Hanson and Hanson, 1996).

#### 1.5.1. Phylogeny

Methanotrophs belong to two bacterial phyla the *Proteobacteria* and *Verrucomicrobia*. Methanotrophs within the phylum *Proteobacteria* have been widely studied (Hanson and Hanson, 1996) whereas those of the phylum *Verrucomicrobia* have only recently been described (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008; Op den Camp et al., 2009; van Teeseling et al., 2014). Methanotrophs within the *Proteobacteria* can be divided into two taxonomic groups, Type I within the *Gammaproteobacteria* and Type II within the *Alphaproteobacteria*, based on differences such as their cellular ultrastructure, carbon assimilation pathways and fatty acid composition.

Prior to 2011, there were fourteen recognised genera of methanotrophs from three families *Methylococcaceae, Methylocystaceae and Beijerinckiaceae* (Figure 1-2; adapted from Dedysh, 2009). Since this date, eight novel methanotrophs have been isolated with six of these belonging to the Type I group. The mesophilic Type I methanotroph, *Methylogaea,* was isolated from a rice paddy field (Geymonat et al., 2011), whereas the Type I genus *Methylovulum* was isolated from forest soil (Iguchi et al., 2011); both belonging to the *Methylococcaceae* family (Figure 1-2). Three novel methanotrophs were recently isolated from marine environments, *Methylomarinum* and *Methylomarinovum* from shallow submarine hydrothermal systems in Japan (Hirayama et al., 2014), and *Methyloprofundus* from marine sediment in Monterey Canyon off the coast of California (Tavormina et al., 2015). A novel methanotroph isolated from pond water in Japan and South Africa was classified as *Methyloparacoccus* (Hoefman et al., 2014).

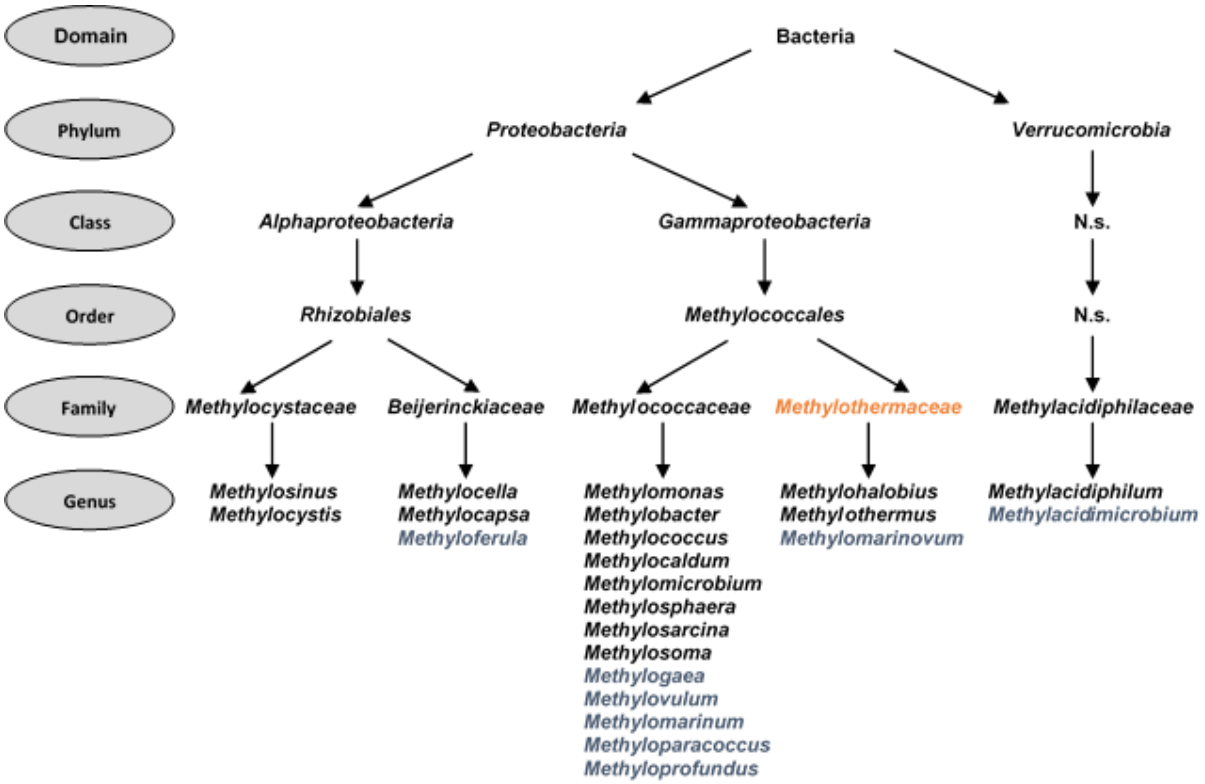



**Figure 1-2:** Aerobic methanotrophic bacterial diversity (adapted from Dedysh, 2009). N.s. not specified. Assigned new family (orange) and novel genera isolated (blue) since 2009.

### 1.5.2. Physiology

Methanotrophs of the *Proteobacteria* are separated into Type I and Type II groups on the basis of their carbon assimilation pathways. The former, using the ribulose monophosphate pathway (RuMP), are members of the *Gammaproteobacteria* and the latter using the serine pathway are members of the *Alphaproteobacteria* (Figure 1-3; Hanson and Hanson, 1996). The RuMP pathway is more efficient than the serine pathway as all the carbon for cell material is derived from formaldehyde whereas, one molecule of formaldehyde and one of carbon dioxide is used for the latter pathway (Madigan et al., 2009). There is a sub-group of Type I methanotrophs (Bowman et al., 1993), previously known as Type X, which includes *Methylococcus* spp. and *Methylocaldum* spp. (Semrau et al., 2010). These organisms use the RuMP pathway but also assimilate carbon via ribulose bisphosphate (Summons et al., 1994).

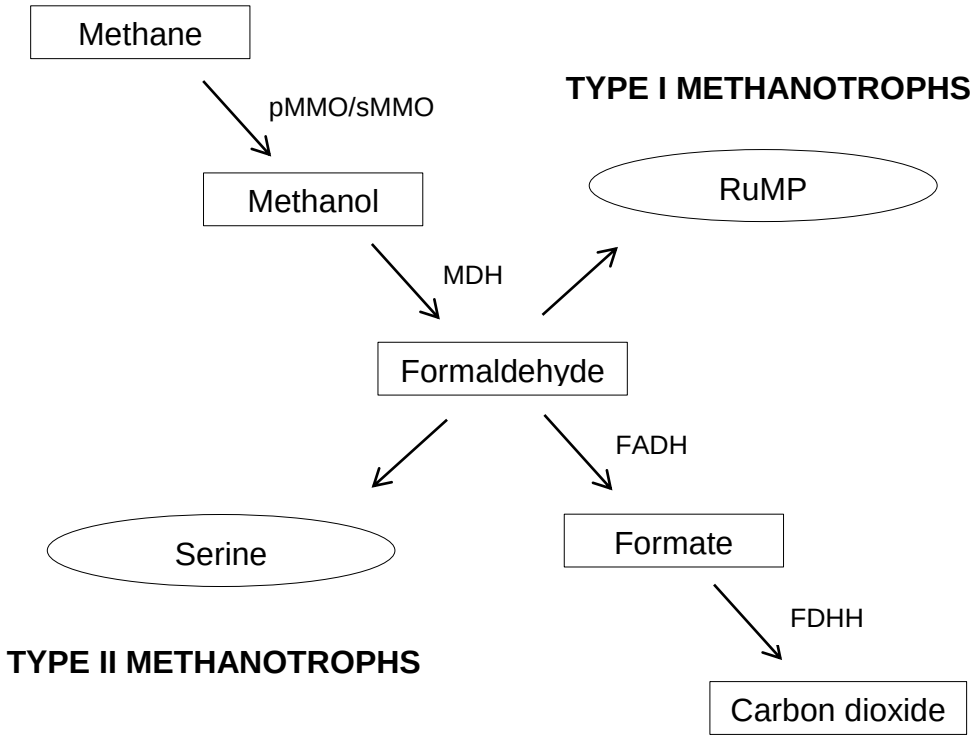



**Figure 1-3:** Type I and Type II methanotrophs use different pathways for carbon assimilation (Hanson and Hanson, 1996).

Methanotrophic bacteria are obligate aerobes requiring oxygen to oxidise methane to methanol with methane monoxygenase (MMO) enzymes catalysing this process. With the exception of *Methylocella* spp., which are facultative methanotrophs (Dedysh et al., 2005) having the ability to use multicarbon compounds such as acetate, they are unable to grow on substrates containing carbon-carbon bonds (Hanson and Hanson, 1996). There are two forms of the MMO enzyme, a particulate membrane bound form (pMMO) and a soluble cytoplasmic (sMMO) form. All methanotrophs contain the particulate form of the MMO enzyme with the exception of *Methylocella* spp. and *Methyloferula stellata* (Dedysh et al., 2000; Dunfield et al., 2003). They lack the intracytoplasmic membrane system to which pMMO is bound in other methanotroph species thus, only the soluble form of the enzyme appears to be present. A few methanotroph genera such as *Methylococcus, Methylosinus* and *Methylocystis* contain both forms of the MMO enzyme. The expression of the different forms of enzyme is controlled by the concentration of copper ions present with the soluble form being expressed when copper ions become limiting (Murrell et al., 1998; Murrell et al., 2000; Hakemian and Rosenzweig, 2007).

### 1.6. Methanotrophs in the environment - role in the global methane cycle

Methanotrophs are widespread in nature and are mostly mesophilic, non-halophilic neutrophiles (Hanson and Hanson, 1996; Sharp et al., 2014). Generally, Type I methanotrophs are characteristic of aquatic environments whilst Type II methanotrophs are most widely found in terrestrial environments (e.g. McDonald et al., 1996; McDonald et al., 2005b; Moussard et al., 2009). Previous reports have suggested Type I methanotrophs are better adapted to lower methane concentrations whilst Type II methanotrophs appear to favour higher methane concentrations (Hanson and Hanson, 1996; Kolb, 2009; Graef et al., 2011).

Methanotrophs were shown to be an important sink for methane in the Deepwater Horizon oil spill in the Gulf of Mexico in 2010 with a methanotroph bloom respiring the majority of the released methane and leaving behind a residual population (Kessler et al., 2011). It was postulated that any future large-scale release of methane from hydrates in the deep ocean would result in a similar rapid methanotrophic response and prevent methane release to the atmosphere (Kessler et al., 2011). Another study, however, suggested caution when drawing comparisons between this anthropogenic event and a methane release from gas hydrates (Solomon, 2014).

Methanotrophs play an important role in methane cycling in wetlands with some estimates suggesting that between 40 and 70% of wetland methane emissions are oxidised (Bridgham et al., 2013 and references therein). They could become increasingly important with the predicted increase in methanogenic activity resulting from projected increases in global temperatures. Two thirds of wetland methane emissions are from tropical wetlands with the majority of non-tropical emissions result from boreal wetlands, for example, in northern Canada and Siberia (Hanson and Hanson, 1996 and references therein). Soil moisture content plays a significant role in emissions in these environments with wetter conditions resulting in increased methane release. Arctic tundras and temperate wetlands only account for a small proportion for total emissions from wetlands (Lelieveld et al., 1998) but climate change and projected increases in temperature, could result in melting permafrost and greater methane emissions. Measurements suggest that up to 80% of the methane produced in flooded paddy fields is oxidised at the soil surface by methanotrophs (Conrad and Rothfuss,

1991) indicating that they play a significant role in perturbing methane release from these environments.

### 1.6.1. Extremophilic/tolerant methanotrophs

Methanotrophs have been found in a variety of extreme environments such as those with high and low pH or temperature and at high salinities (e.g. Trotsenko and Khmelenina, 2002b; Trotsenko et al., 2009).

#### 1.6.1.1. Acidophilic methanotrophs

Methanotrophs adapted to acidic environments have been found in a variety of environments including acidic forests, peatlands, *Sphagnum* mosses and mud volcanoes (Dunfield et al., 2003; Dedysh et al., 2004; Pol et al., 2007; Kip et al., 2011).

A moderately acidophilic methanotroph was isolated from acidic *Sphagnum* peat bogs in West Siberia and European North Russia. This microorganism, *Methylocapsa acidiphila,* showed growth between pH 4.2-7.2 (Dedysh et al., 2002). Three further moderately acidophilic methanotroph strains were isolated from these locations. These belonged to the *Methylocella* genus and were classified as *Methylocella palustris* (Dedysh et al., 2000). Two additional species from this genus were isolated from acidic forest cambisol in North Germany and *Sphagnum* tundra peatlands in North Russia (Dunfield et al., 2003; Dedysh et al., 2004). Two strains of *Methylocystis heyeri* were isolated from an acidic *Sphagum* peat bog lake in Germany and an acidic tropical forest soil in Thailand (Dedysh et al., 2007).

Acidophilic methanotrophs are not limited to those found in the *Alphaproteobacteria* class. *Sphagum* mosses originating from Dutch peatlands have provided isolates of methanotrophic bacteria belonging to the *Gammaproteobacteria* class. One strain was closely related to the *Methylomonas* genus and the other to *Methylosoma* and *Methylovulum* (Kip et al., 2011). In 2007-2008 three independent research groups described the isolation of methanotrophs found in geothermal environments characterised by high temperatures and low pH. These isolates represented the first methanotrophs to be found outside the *Proteobacteri*a phylum and belonged to that of the *Verrucomicrobia* phylum. One of these isolates from the Solfatara volcano mudpot, Italy and the surrounding bare soil, showed growth down to pH 0.8 (Pol et al., 2007).

Thermoacidophilic methanotrophs capable of methane oxidation at high temperature and low pH were isolated from a geothermal area in Tikitere, New Zealand (Dunfield et al., 2007) and an acidic hot spring in Russia (Islam et al., 2008). These isolates were all assigned different names at the time of discovery but were later classified as *Methylacidiphilum* (Op den Camp et al., 2009). Recently a methanotroph from the *Verrucomicrobia* has been isolated and shown to represent a novel genus, *Methylacidimicrobium*. This isolate grew at mesophilic temperatures ranging from 35-44°C (van Teeseling et al., 2014).

#### 1.6.1.2. Halotolerant, halophilic and alkaliphilic methanotrophs

Methanotrophs adapted to high salinity and pH have been reported from marine environments, saline lakes, soda lakes and coal mines (Khmelenina et al., 1997; Kalyuzhnaya et al., 2001; Han et al., 2009).

*Methylomicrobium pelagicum* was isolated from the Sargasso Sea and was the first methanotroph reported that required NaCl for growth (Sieburth et al., 1987). *Methylobacter marinus* (Lidstrom, 1988; Bowman et al., 1993) and *Methylomicrobium japanense* (Fuse et al., 1998; Kalyuzhnaya et al., 2008) have also been isolated from marine settings. More recently, methanotrophs have been isolated from marine environments in Japan, *Methylomarinum vadi* (Hirayama et al., 2013), *Methylomarinovum caldicuralii* (Hirayama et al., 2014), *Methylocaldum marinum* (Takeuchi et al., 2014) and off the coast of California, *Methyloprofundus sedimenti* (Tavormina et al., 2015).

Halotolerant and alkaliphilic methanotrophic bacteria were first isolated from moderately saline lakes in Tuva, Central Asia. Two strains referred to as *Methylobacter alcaliphilus* required a sodium source to grow in the alkaline medium and grew in the range of pH 7.0-10.5 and 0.15-10% NaCl (Khmelenina et al., 1997). The microorganisms were later assigned to the *Methylomicrobium* genus (Kalyuzhnaya et al., 2008). *Methylomicrobium* spp. have been isolated from highly alkaline soda lakes in Kenya with optimal growth being witnessed at pH 9.0-10.0 (Sorokin et al., 2000). Methanotrophic isolates, classified as *Methylomicrobium buryatense*, were also found in soda lakes in the southeastern Transbaikal region of Russia (Kalyuzhnaya et al., 2001). The most halotolerant methanotroph isolated to date is *Methylohalobius*

*crimeensis* isolated from hypersaline lakes in the Crimean Peninsula, Ukraine with growth still witnessed at 15% NaCl (Heyer et al., 2005).

High methanotroph diversity was also found in an alkaline soil from a Chinese coal mine with both Type I and II methanotrophs actively involved in methane oxidation (Han et al., 2009). However, the predominantly active methanotrophs in saline and alkaline soda lakes were found to be Type I (Trotsenko and Khmelenina, 2002a; Lin et al., 2004).

#### 1.6.1.3. Psychrotolerant and psychrophilic methanotrophs

Psychrophilic and psychrotolerant methanotrophs have been isolated from tundra soils, polar lakes and permafrost environments (Omelchenko et al., 1996; Bowman et al., 1997; Khmelenina et al., 2002).

*Methylobacter psychrophilus* was the first described psychrophilic methanotroph. It was isolated from tundra in northern regions of Russia and showed an optimum growth temperature of 6-10°C (Omelchenko et al., 1996). A psychrophilic strain of methanotroph was isolated from a saline Antarctic lake and classified as *Methylosphaera hansonii* with optimum growth of 10-13°C (Bowman et al., 1997). The psychrotolerant *Methylomonas scandinavica* was isolated from a deep igneous rock environment and had optimal growth at 17°C close to its *in situ* temperature (Kalyuzhnaya et al., 1999).

Psychrotolerant methanotrophs are not limited to those from the *Gammaproteobacteria* class. The *Methylocella and Methylocapsa* genera of the *Alphaproteobacteria* class contain species that have psychrotolerant characteristics. The moderately acidophilic *Methylocella palustris, Methylocella silvestris* and *Methylocella tundra* were isolated from an acidic cambisol forest in North Germany and peat bogs and tundra in West Siberia and European North Russia. They grew in the temperature range of 4-30°C (Dedysh et al., 2000; Dunfield et al., 2003; Dedysh et al., 2004). *Methylocapsa acidiphila* also isolated from peat bogs showed growth at temperatures as low as 10°C (Dedysh et al., 2002).

Psychrophilic/tolerant and acidophilic methanotrophs in tundra soils and permafrost regions may play a greater role in perturbing methane emissions to the atmosphere in the future if methanogenic activity increases with postulated global temperature rises.

#### 1.6.1.4. Thermotolerant and thermophilic methanotrophs

Thermophilic and thermotolerant methanotrophs have been isolated from environments such as hot springs and agricultural soil (Bodrossy et al., 1997; Bodrossy et al., 1999; Tsubota et al., 2005). They belong to the Type I methanotrophs and consist of the genera *Methylocaldum*, *Methylococcus*, *Methylothermus* and *Methylomarinovum* (Bodrossy et al., 1997; Tsubota et al., 2005; Hirayama et al., 2011; Hirayama et al., 2014).

The extensively studied *Methylococcus capsulatus* strains Texas and Bath grow in the range 30-50°C with an optimum of 42°C (Trotsenko et al., 2009 and references therein). The moderately thermophilic Type I methanotroph *Methylocaldum szegediense* was isolated from the effluent of an underground hot spring and had optimal growth at 55°C (Bodrossy et al., 1997). In this study, two other members of the genus showed moderately thermophilic characteristics, *Methylocaldum tepidum* and *Methylocaldum gracile* with a growth range of 20-47°C. A 'truly thermophilic' methanotroph, *Methylothermus* strain HB, was isolated from underground hot springs in Hungary and had the ability to grow up to 72°C which was 10°C higher than any other known methanotrophs at this time (Bodrossy et al., 1999). *Methylothermus thermalis* was isolated from a hot spring in Japan and was moderately thermophilic growing up to 67°C; it also showed halotolerant characteristics (Tsubota et al., 2005). *Methylothermus subterraneous*, also moderately thermophilic, was isolated from a subsurface hot aquifer. This genus represents the most thermophilic methanotrophs isolated to date (Hirayama et al., 2011).

#### 1.6.1.5. Bioremediation

Methanotrophic bacteria are of interest for bioremediation of sites contaminated with trichloroethylene (TCE) because they are able to oxidise TCE by the MMO enzyme pathway (Smith et al., 1997). It has since been discovered that methanotrophs can degrade a large range of halogenated hydrocarbons (Semrau et al., 2010). The soluble form is responsible for higher rates of oxidation of TCE compared to the particulate form of the enzyme (pMMO) (Auman and Lidstrom, 2002 and references therein). As previously discussed (Section 1.5.2), when the concentration of copper ions becomes limiting, the level of sMMO expression increases. Copper ions are not limiting in most

natural environments so the role of sMMO in natural populations of methanotrophs is not known but for bioremediation purposes this could be engineered (Semrau et al., 2010).

Extremophilic/tolerant methanotrophs are important for bioremediation as they can degrade different environmental pollutants under extreme conditions. Methanotrophs play a role in perturbing methane release from Canadian oilsands tailing ponds and degrade residual hydrocarbons present in the waters. The ponds are primarily anoxic and produce methane but have an aerobic surface layer. High methane oxidation rates were witnessed in Fort McMurray tailings ponds with the high salinity and alkalinity favouring *Gammaproteobacteria* particularly *Methylocaldum*, a genus that can survive high-temperature process waters (Saidi-Mehrabad et al., 2013).

### 1.7. Summary

Methane is a potent greenhouse gas and a significant climate driver with both natural and anthropogenic sources. Therefore, it is important to understand the processes and feedbacks of methane cycling. There are many sources of methane in the marine environment and anaerobic oxidation of methane (AOM) is an important sink for these methane emissions. This process has been estimated to consume > 90% of methane produced by marine systems (Bridgham et al., 2013 and references therein).

Aerobic methanotrophs are widespread in nature and use methane as their sole carbon and energy source. They are mostly mesophilic, non-halophilic neutrophiles, but have also been found in a wide range of extreme environments. They play an important role in methane cycling in a range of environmental settings and also have bioremediation applications. Methanotrophs and methane oxidation have also been studied extensively in terrestrial environments but marine systems have received little attention. To date, only a few methanotrophs have been isolated from marine environments and they have all been Type I in nature (e.g. Sieburth et al., 1987; Lidstrom, 1988; Fuse et al., 1998; Hirayama et al., 2013; Hirayama et al., 2014; Takeuchi et al., 2014; Tavormina et al., 2015) though the presence of Type II methanotrophs in these environments has been suggested. Therefore, aerobic methane oxidation (AMO) remains an unappreciated and relatively unstudied process taking place in the uppermost oxic surface sediments and the overlying water column.

# 2. Identifying methanotroph lipid signatures in modern and paleoenvironment

Methane cycling has been an important component of the global carbon cycle since the Archaean and is postulated to have perturbed global climate in the past, especially when released in large quantities over short time scales (e.g. Wagner et al., 2008). Ice-core records have also shown fluctuations in atmospheric methane concentrations with higher concentrations during interglacials (e.g. Loulergue et al., 2008). Therefore, it is important to understand methane cycling and the role methanotrophs have played in this process in the past and with differing methane concentrations.

The labile nature of DNA and its susceptibility to oxidation, hydrolysis and bacterial enzymes, limits its application in paleoenvironments whereas the carbon backbones of certain types of lipids may be preserved for billions of years (Briggs and Summons, 2014). These biological markers or biomarkers are molecular fossils, originating formerly from living microorganisms. They are complex organic compounds composed of carbon, hydrogen, and other elements. They are found in sediments, rocks and crude oils and show little or no change in structure from their parent organic molecules in living microorganisms (Peters et al., 2005). Biomarkers provide evidence of microbial processes in modern and ancient sediments.

We discuss the use of a specific group of biomarkers in parallel with microbial community analyses to identify methanotrophs and their lipid signatures in a modern sedimentary system. This will allow us to identify and develop diagnostic markers that can be applied to the sedimentary record to trace methanotrophy and ultimately methane cycling in paleoenvironments.

## 2.1. Identification of aerobic methanotrophs

### 2.1.1. A molecular microbiology approach

A variety of molecular tools have been employed to study the ecology and diversity of methanotrophs in the environment (McDonald et al., 2008). Early molecular studies of methanotroph diversity used 16S rRNA gene probes. The first primers designed targeted methylotrophs using either the RuMP or serine carbon assimilation pathways (Tsien et al., 1990). Since these early examples, methanotroph-specific primers have

been developed. Primers targeting individual genera have been developed along with those that are specific for either Type I or Type II methanotrophs (McDonald et al., 1996; Bodelier et al., 2005; Chen et al., 2007).

#### 2.1.1.1. Functional genes

Genes that encode for enzymes unique to methanotrophs have also been targeted to investigate methanotroph diversity. The first example of functional genes being targeted were for those encoding the methanol dehydrogenase (MDH) enzyme (McDonald and Murrell, 1997a) which catalyses the oxidation of methanol to formaldehyde (Chapter 1; Figure 1-2). Primers targeting the *mxaF* gene, encoding a subunit of this enzyme, were used to study methanotroph communities in a blanket peat bog. However, this enzyme is found in all Gram-negative methylotrophic bacteria and not methanotrophs alone, so have limitations when using a targeted approach to study methanotroph diversity (McDonald and Murrell, 1997a).

The genes encoding for the soluble form of the methane monooxygenase (sMMO) enzyme have been targeted to study methanotroph diversity in environmental samples. Initially, different primers were synthesised to target the five different genes in the enzyme complex (McDonald et al., 1995). Later studies focussed on primers to amplify the *mmoX* gene within the cluster (Miguez et al., 1997; Horz et al., 2001; Auman and Lidstrom, 2002). However, a significant limitation of these primers is that sMMO is only found in a small number of methanotroph genera (Chapter 1, Section 1.5.2).

As previously discussed (Chapter 1, Section 1.5.2), particulate methane monooxygenase (pMMO) is universally found in all genera of methanotrophs with the exception of *Methylocella* sp. and *Methyloferula stellata*. This property allows it to be used as a functional marker for most methanotrophs. Primers have been designed targeting the *pmoA* gene which encodes for a subunit of the pMMO enzyme (Holmes et al., 1995; McDonald and Murrell, 1997b; Bourne et al., 2001; Horz et al., 2001; McDonald et al., 2005a; Martineau et al., 2010). This has allowed fragments of the gene to be amplified and used in conjunction with denaturing gradient gel electrophoresis (DGGE), *pmoA* gene diversity and thus methanotroph diversity in environmental samples can be elucidated. Primers targeting this gene can be used alongside those for the *mmoX* gene to allow identification of methanotrophs belonging to all known genera.

#### 2.1.1.2. Detecting methanotroph DNA in the sedimentary record

Analysis of ancient DNA in the sedimentary record can provide insights into the methanotrophic populations present in the past. The remains of methanotrophic bacteria were discovered in lacustrine sediments > 9,400 years old in Ace Lake, Antarctica. Type I and Type II methanotrophic bacteria, *Methylomonas* and *Methylocystis* respectively, were present. This is the oldest example of methanotroph characterisation from ancient DNA (Coolen et al., 2004; Coolen et al., 2008). The labile nature of DNA limits its potential to trace methanotrophy in ancient systems therefore, we propose a biomarker approach.

### 2.1.2. A lipid biomarker approach

Methanotrophs produce a number of characteristic lipids such as phospholipid fatty acids (PLFAs) which have been used as biomarkers for tracing methane cycling in modern environments (e.g. Bowman et al., 1993; Bull et al., 2000). Type I methanotrophs possess high levels of PLFAs with 16-carbon fatty acid lengths (Hanson and Hanson, 1996). In the majority of genera, the unsaturated equivalent of this 16-carbon fatty acid dominates. However, there are exceptions to this with species from the genera *Methylococcus*, *Methylocaldum*, *Methylothermus*, *Methylogaea, Methylovulum* and *Methylomarinovum* being abundant in the saturated fatty acid (Bowman et al., 1993; Bodrossy et al., 1997; Tsubota et al., 2005; Geymonat et al., 2011; Iguchi et al., 2011; Hirayama et al., 2014). *Methylomonas* and *Methylovulum* also contain saturated 14-carbon fatty acid chains (Bowman et al., 1993; Iguchi et al., 2011). A common feature in all Type II methanotrophs is the high abundance of unsaturated PLFAs of 18-carbon fatty acid lengths. The unusual nature of two PLFAs, 18:1ω8c and 18:1ω7c, led to their use as specific environmental biomarkers for Type II methanotrophs (Hanson and Hanson, 1996). However, 18:1ω8c and 18:1ω7c have since been found in abundance in Type I methanotrophs, *Methylohalobius* (Heyer et al., 2005) and *Methylomarinovum* (Hirayama et al., 2014), respectively.

PLFAs extracted from pure cultures have provided useful fingerprints for taxonomy and identification of methanotrophs. These fingerprints have been used to give an insight into methanotroph communities in the environment. However, PLFAs are not preserved in the geological record with the structure being very labile. Therefore, they

are limited to modern settings when being used to probe methanotroph community structure.

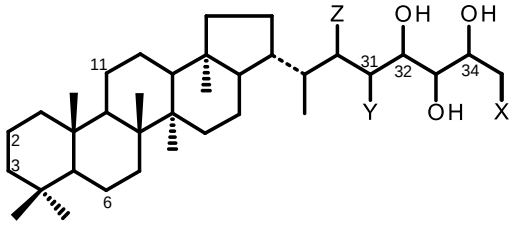
Some Type I methanotrophs, *Methylococcus capsulatus* and *Methylosphaera hansonii* (Jahnke et al., 1992; Schouten et al., 2000), have been shown to produce 4-methylsterols. This sterol has been used to identify methanotroph activity in modern sediments in Ace Lake, Antarctica (Schouten et al., 2001; Coolen et al., 2004) and Haakon Mosby mud volcano (HMMV) (Elvert and Niemann, 2008). Nevertheless, the presence of this sterol in only a small number of Type I methanotrophs and its rare occurrence in environmental settings limits their scope as markers for methanotrophy.

The labile nature of both DNA and the lipids mentioned above limits their ability to trace methanotrophy and ultimately investigate methane cycling in paleoenvironments. Therefore, we propose a different group of lipid biomarkers to allow us to identify and trace methanotrophy in both modern and ancient settings.

## 2.2. Bacteriohopanepolyols (BHPs)

### 2.2.1. Structural variation of BHPs

Bacteriohopanepolyols (BHPs, see appendix 1 for structures) are membrane lipids found in prokaryotes comprising of a pentacyclic triterpenoid structure with an extended polyfunctionalised side chain (Figure 2-1). BHPs have a wide range of structural variation which varies between bacterial groups and species.




**Tetrafunctionalised**
Z=H, Y=H, X=OH or $NH_2$ or composite group
**Pentafunctionalised**
Z=H, Y=OH, X=OH or $NH_2$ or composite group
Z=OH, Y=H, X=OH
**Hexafunctionalised**
Z=OH, Y=OH, X=OH or $NH_2$ or composite group

**Figure 2-1:** Generalised structure of bacteriohopanepolyols (BHPs).

The pentacyclic ring system, which is common to all compounds, can have methylation at C-2 or C-3 and unsaturation at C-6 and/or C-11 (Talbot and Farrimond, 2007 and references therein). In addition to the ring system, there can be a variation in the number and nature of the functional groups in the side chain (tetra-, penta- and hexafunctionalised structures are known). The most commonly occurring BHPs

contain four functional groups, typically three hydroxyl groups (OH) at C-32, 33 and 34 with the C-35 position occupied by either another OH, bacteriohopane-32,33,34,35-tetrol (**III**; BHT herein), an amine group $NH_2$, 35-aminobacteriohopane-32,33,34-triol (**V**; aminotriol herein) or a complex amino sugar group, bacteriohopane-32,33,34,35-tetrol cyclitol ether (**XII**; BHT cyclitol ether herein). Other side chain variants include a methyl group at C-31, a ketone group at C-32, -$OCONH_2$ at C-34, and structures with the side chain condensed into a cyclic ether form (**VIII**; adenosylhopane).

### 2.2.2. Biosynthesis of BHPs and biological sources

The initial step in the biosynthetic pathway of BHPs is the cyclisation of squalene, catalysed by the enzyme squalene hopene cyclase (SHC) via a carbocation, a positively charged carbon atom (Woodward and Bloch, 1953; Ochs et al., 1992; Kannenberg and Poralla, 1999). This results in the $C_{30}$ hopanoids diploptene (**I**) (80%) and diplopterol (**II**) (20%) (Rajamani and Gao, 2003), which are precursors of a wide variety of BHPs. Either diploptene (**I**) or diplopterol (**II**) or both are found in a wide range of bacteria (Rohmer et al., 1984; Berry et al., 1993; Cvejic et al., 2000a). Over 20 years ago, adenosylhopane (**VIII**) was suggested as a key intermediate in the formation of the BHP side chain (Neunlist et al., 1988) and more recently a mechanism has been proposed for the biosynthesis of polyfunctionalised side chains from diploptene (**I**). The pathway involves the addition of an adenosyl moiety from S-adenosyl methionine (SAM) resulting in adenosylhopane (**VIII**) (Bradley et al., 2010). Adenine is then cleaved from adenosylhopane (**VIII**) and further downstream processes result in the development of the side chain. Mutant strains of *Methylobacterium* deficient in the *hpnG* gene, which encodes the enzyme involved in adenine cleavage, accumulated adenosylhopane (**VIII**) and did not produce BHPs with side chains (Bradley et al., 2010). It was also historically hypothesised that ribosylhopane, resulting from the cleavage of adenine from adenosylhopane (**VIII**), was a key intermediate in biosynthesis of the side chains. Ribosylhopane was recently confirmed as the precursor to aminotriol (**V**) in *Streptomyces coelicolor* with all genes required for aminotriol (**V**) biosynthesis from diploptene (**I**) being characterised (Figure 2-2; adapted from Liu et al., 2014).

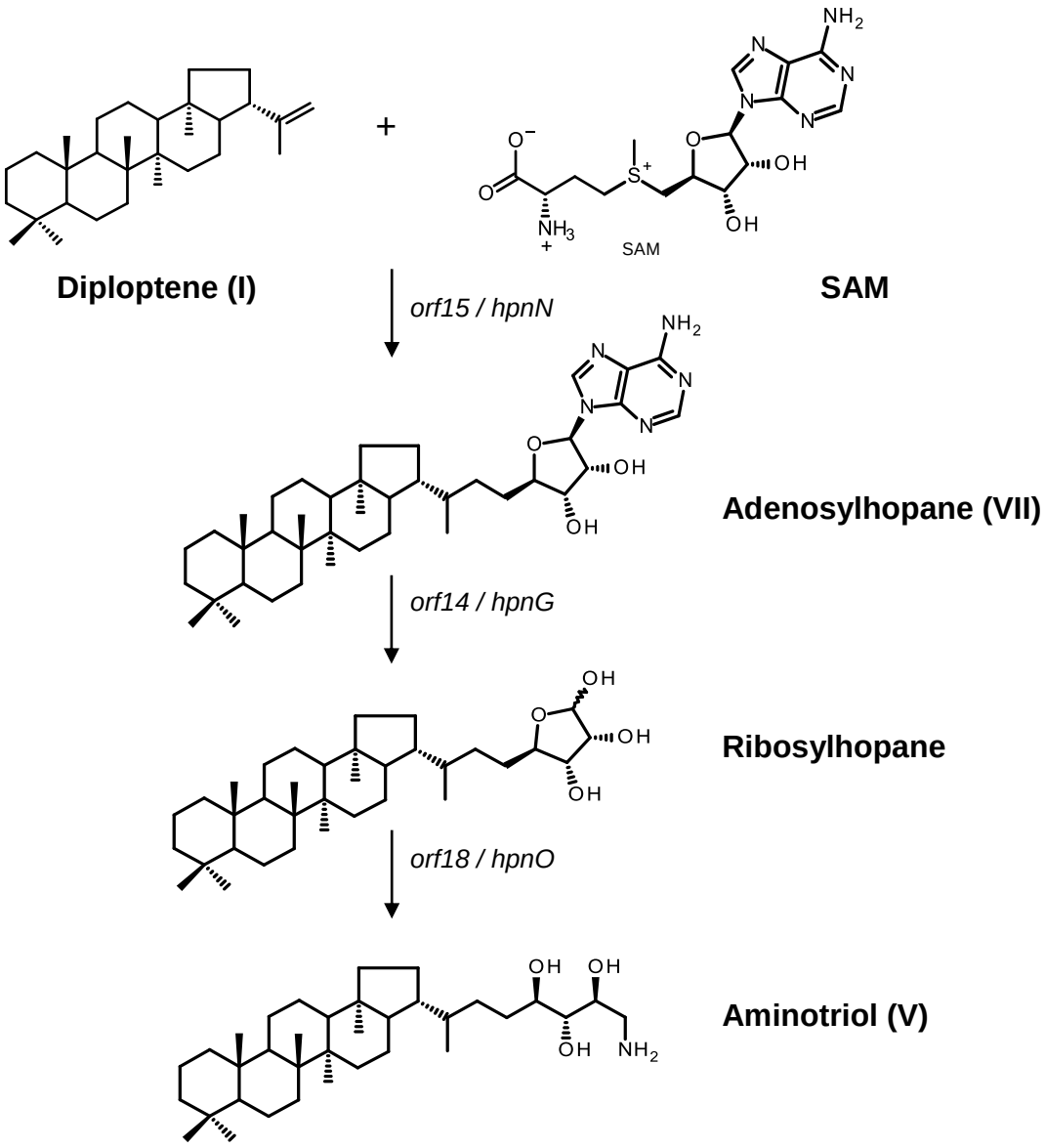



**Figure 2-2:** Hypothetical biosynthesis of aminotriol (**V**) in *Streptomyces coelicolor* (adapted from Liu et al., 2014).

Early studies of bacterial species in culture suggested that BHPs were biosynthesised by up to 50% of bacteria (Rohmer et al., 1984). However, culture-independent genetic studies have disputed this figure (Pearson et al., 2007; Pearson et al., 2009). The squalene hopene cyclase (*sqhC*) gene encodes the enzyme (SHC) that catalyses cyclisation of hopanoids from their acyclic precursor, squalene (Ochs et al., 1992). It was recently estimated that fewer than one in 10 bacterial cells in soils and fewer than one in 20 bacterial cells in the ocean contains the *sqhC* gene (Pearson et al., 2007; Pearson and Rusch, 2009).

BHPs have been found in a wide range of aerobic bacteria including cyanobacteria, acetic acid bacteria, nitrogen-fixing bacteria, methylotrophs and methanotrophs (e.g. Rohmer et al., 1984; Berry et al., 1993; Cvejic et al., 2000a; Talbot et al., 2001; Talbot et al., 2007; Talbot et al., 2008). They are also produced by some facultatively anaerobic organisms such as purple non-sulfur bacteria (Neunlist et al., 1985). Several years later the production of BHPs in microbial mats and deposits that formed in anoxic environments suggested production by anaerobic bacteria and they have since been discovered in obligate anaerobes such as anammox bacteria (Sinninghe Damsté et al., 2004), sulphate-reducing bacteria (Blumenberg et al., 2006; Blumenberg et al., 2009a; Blumenberg et al., 2012) and *Geobacter* (Fischer et al., 2005; Eickhoff et al., 2013). BHPs remain undetected in archaea.

### 2.2.3. Function and role of BHPs

Although, there is much debate about the role of BHPs, studies suggest they regulate cell membrane fluidity, however, the factors controlling their synthesis are poorly constrained (Ourisson et al., 1987; Kannenberg and Poralla, 1999). They are postulated to be sterol surrogates and it has been shown that diplopterol (**II**) has the ability to order saturated lipids and form a liquid-ordered phase in models akin to those of eukaryotic membranes (Sáenz et al., 2012). Several studies involving Gram-negative bacteria have shown that BHPs localise to outer membranes in the cell (Jahnke et al., 1992; Jurgens et al., 1992; Simonin et al., 1996; Doughty et al., 2011).

There have been several lab studies involving pure cultures of bacteria with results suggesting that BHPs may play a role in protecting the cell from environmental stress (Kannenberg and Poralla, 1999). It has been shown that total hopanoid content increased when bacteria were subjected to different environmental stimuli. For example, the high hopanoid content of the ethanol-producing bacteria *Zymomonas mobilis* is thought to allow ethanol tolerance. An increase in total hopanoid content with increasing ethanol concentration as well as temperature was also reported for this microorganism (Schmidt et al., 1986). Conversely, a later study reported no increase in hopanoid content with increasing ethanol concentration (Hermans et al., 1991). Another study supported this latter finding with no significant increase in hopanoid content seen with increasing ethanol concentration thus suggesting that a high

constitutive level of hopanoids in the microorganism allow ethanol tolerance (Moreau et al., 1997).

In *Alicyclobacillus acidocaldarius*, a microorganism adapted to living at high temperature and low pH, the content of hopanoids with an extended polyfunctionalised side chain, increased with temperature and with decreasing pH (Poralla et al., 1984). In *Frankia aurantia*, total hopanoid content also increased with temperature with more composite structures observed too (Joyeux et al., 2004). Conversely, studies in mutant strains of *Streptomyces scabies*, which no longer have the ability to make hopanoids, showed that hopanoids were not required for normal growth or for tolerance of ethanol, osmotic and oxidative stress and high temperature or low pH stresses (Seipke and Loria, 2009). A similar mutant of *Burkholderia cenocepacia* in which the genes encoding squalene-hopene cyclase enzymes were deleted, has also been investigated. This mutant strain showed similar growth to the wild type at pH 7 and pH 8, albeit with a slight delay in reaching the exponential growth phase in the latter, however, growth was significantly affected by low pH conditions (Schmerk et al., 2011). This suggests that hopanoid function varies significantly with taxonomy and physiology.

The deletion of the *sqhC* gene in the Gram-negative bacteria *Rhodopseudomonas palustris* TIE-1, resulted in increased sensitivity to acidic and alkaline conditions relative to the wild type (Welander et al., 2009). This was particularly noticeable in stationary phase when grown chemoheterotrophically with the mutant strain deviating from the behaviour of the wild type, displaying retarded growth. Total hopanoid production remained constant but an increase in methylation at C-2 in response to pH stress was observed. These findings suggest hopanoids, particularly those with methylation, have a role in maintaining cell homeostasis and especially those in stationary phase (Welander et al., 2009). Another study of *Methylococcus capsulatus* Bath also supported the previous findings indicating that methylated hopanoids, this time at the C-3 position, play a role in cell survival in stationary phase (Welander and Summons, 2012). The removal of the *hpnR* gene, responsible for methylation at the C-3 position, resulted in similar exponential cell growth to that of the wild type. However, the number of colony forming units dropped by six orders of magnitude in late stationary phase in the mutant strain but was maintained throughout this phase in the wild type strain.

Growth stage can also have an effect on hopanoid production. *Streptomyces coelicolor* A3(2) does not produce hopanoids in liquid culture but produces them on solid medium when sporulating (Poralla et al., 2000). Their production seems to occur during aerial mycelium formation and is hypothesised to protect the cell by decreasing the water permeability of the cell membrane (Poralla et al., 2000). The vegetative cells of the cyanobacteria *Nostoc punctiforme* may undergo differentiation into akinetes when insufficient light is available or when phosphorus is limiting ATP synthesis. Akinetes are survival structures that protect the cell from cold and desiccation. In one study, the cells that had differentiated into akinetes exhibited ten-fold higher concentrations of 2-methylhopanoids in comparison to vegetative cells (Doughty et al., 2009). Furthermore, it was witnessed that the increase in hopanoid levels in akinetes was due to a 34-fold increase in hopanoid content located in the outermost membranes relative to vegetative cells. This suggests hopanoids play a role in protecting the cell from environmental stress in this 'dormant' state (Doughty et al., 2009). This work was continued by investigating hopanoid transport within the cell and its localisation to the outer membrane of *Rhodopseudomonas palustris* TIE-1 (Doughty et al., 2011). The deletion of genes involved in transport of hopanoids within the cell prevented movement from cytoplasmic to outer membranes. Mutant cells lacking the ability to transport hopanoids also displayed retarded cell growth at higher temperatures (Doughty et al., 2011).

In another study, hopanoids seemed to provide a physical barrier preventing oxygen from diffusing into the cell. In a nitrogen-fixing *Frankia* sp. the vesicle in which nitrogen fixation occurs, an anaerobic process, has a lipid envelope. High levels of hopanoids are found in these lipid envelopes and they make up a large proportion of total lipids, 20-87% (Berry et al., 1991; Berry et al., 1993; Kleemann et al., 1994; Nalin et al., 2000). More recently, a study probing *sqhC* diversity in environmental samples noted that in general, nitrogen fixation is a common but not universal feature amongst bacteria with *sqhC* genes (Pearson et al., 2007).

Finally, hopanoids also seem to play a role in bacterial antibiotic resistance by affecting membrane permeability. In *Burkholderia cenocepacia*, the *sqhC* gene was deleted removing its ability to produce hopanoids. The mutant strain showed increased sensitivity to antibiotics such as polymyxin B and erythromycin suggesting that hopanoids play a role in membrane permeability (Schmerk et al., 2011). The swimming

motility of the bacteria was also severely affected following the deletion of both genes. In another study using a bacterium of the same genus, *B. multivorans*, it was once again suggested that hopanoids play a role in membrane permeability and subsequent polymyxin B resistance (Malott et al., 2012) with mutant strains unable to produce hopanoids, showing increased membrane permeability in the presence of polymyxin.

### 2.2.4. BHPs as molecular biomarkers

BHPs are found in a wide range of environmental samples such as lacustrine environments (Talbot et al., 2003c; Talbot and Farrimond, 2007), marine (Cooke et al., 2008b; Blumenberg et al., 2009b; Taylor and Harvey, 2011; Blumenberg et al., 2013; Kharbush et al., 2013; Talbot et al., 2014), riverine sediments (Selver et al., 2012), soils (Cooke et al., 2008a; Xu et al., 2009), peats (van Winden et al., 2012b; van Winden et al., 2012c) and extreme environments (Talbot et al., 2005; Zhang et al., 2007; Gibson et al., 2008).

The tetrafunctionalised BHT (**III**), aminotriol (**V**) and BHT cyclitol ether (**XII**) are the most commonly occurring BHPs and are produced by a wide range of bacteria (e.g. Sinninghe Damsté et al., 2004; Talbot and Farrimond, 2007; Talbot et al., 2008; Blumenberg et al., 2012; Eickhoff et al., 2013) and consequently are not indicative for any particular bacterial group. Adenosylhopane (**VIII**), the precursor to all side-chain extended BHPs (Bradley et al., 2010), is produced by all organisms but has only been observed to accumulate in a small number of organisms, some purple non-sulfur bacteria, a nitrogen-fixing bacterium, an ammonium-oxidising bacterium (e.g. Talbot and Farrimond, 2007) and a single Type II methanotroph (van Winden et al., 2012c). It is therefore not diagnostic for any particular bacterial group, but high levels of adenosylhopane (**VIII**) have been found in terrestrial soils (Cooke et al., 2008a) and it has been postulated that it, and related structures, may be indicators of terrestrial inputs into aquatic environments (Cooke et al., 2008b; Cooke et al., 2009).

The hexafunctionalised BHhexol (**IV**) is more source-specific with only two known bacterial sources, the thermophilic *Alicyclobacillus acidoterrestris* (Rezanka et al., 2011) and *Methylomirabilis* spp. (Kool et al., 2014), an unusual anaerobic, intra-aerobic methane oxidiser.

Methylation at C-2 was seen as marker for cyanobacteria and until recently, 2-methylhopanes were seen as a biomarker proxy for cyanobacteria and oxygenic photosynthesis (Summons et al., 1999). However, they have now been shown to have non-cyanobacterial sources (e.g. Rashby et al., 2007) and more recently it has been shown that only a small number of cyanobacteria, 19% of all cyanobacterial genera, (Ricci et al., 2014) contain the *hpnP* gene that encodes the enzyme required for methylation at the C-2 position (Welander et al., 2010). It was suggested that C-2 methylation may be indicators of an environmental niche instead (Ricci et al., 2014).

Some Type I methanotrophs and some acetic acid bacteria have the ability to methylate hopanoids at the C-3 position (Neunlist and Rohmer, 1985b; Cvejic et al., 2000a) via the *hpnR* gene (Welander and Summons, 2012). However, in the latter case, this is usually accompanied by unsaturation in the ring system at C-6 and/or C-11 (e.g. Simonin et al., 1994).

### 2.2.5. BHPs as biomarkers for aerobic methane oxidation (AMO)

Methanotrophs produce a group of distinctive BHPs, collectively known as the 35-aminoBHPs, with an amine functionality at the terminal C-35 position and 3, 4 or 5 additional hydroxyl groups (Cvejic et al., 2000a; van Winden et al., 2012c).

The hexafunctionalised 35-aminobacteriohopane-30,31,32,33,34-pentol (**VII**; aminopentol herein) is a specific biomarker for Type I aerobic methane oxidising bacteria based on past culture studies (Cvejic et al., 2000a; Talbot et al., 2001; van Winden et al., 2012c). Two other 35-aminoBHPs are commonly found in methanotrophs. The pentafunctionalised 35-aminobacteriohopane-31,32,33,34-tetrol (**VI**; aminotetrol herein) is found in almost all Type I and Type II methanotrophs (Neunlist and Rohmer, 1985a, b; Cvejic et al., 2000a; Talbot et al., 2001). The exception to this observation is the Type II genus *Methylocella* (van Winden et al., 2012c). The tetrafunctionalised aminotriol (**V**) is found in all Type II methanotrophs and some Type I (Neunlist and Rohmer, 1985a; Cvejic et al., 2000a; van Winden et al., 2012c) and also has non-methanotrophic bacterial sources as discussed in Section 2.2.4 (e.g. Talbot and Farrimond, 2007; Talbot et al., 2008).

Some Type I methanotrophs also produce 35-aminoBHPs methylated at the C-3 position including 3β-methyl-35-aminobacteriohopane-30,31,32,33,34-pentol (**VIIa**; 3-

methylaminopentol herein) and the pentafunctionalised pseudohomologue 3β-methyl-35-aminobacteriohopane-31,32,33,34-tetrol (**VIa**; 3-methylaminotetrol herein). The former has been found in both *Methylococcus* spp. and *Methylocaldum* spp. and the latter only in *Methylocaldum* spp. (Neunlist and Rohmer, 1985b; Cvejic et al., 2000a). The tetrafunctionalised aminobacteriohopane-32,33,34-triol (**Va**; 3-methylaminotriol herein) has not been detected in pure cultures to date but is hypothesised to occur. The presence of 3β-methyl-35-aminobacteriohop-11-ene-30,31,32,33,34-pentol (**VIIc;** unsaturated $\Delta^{11}$ 3-methylaminopentol herein) was reported for the first time in *Methylocaldum szegediense* and to date has not been reported elsewhere in pure culture or environmental samples. Since this discovery, the non-methylated equivalent of unsaturated $\Delta^{11}$ aminopentol (**VIIb**) has been observed in the *Methylovulum*-like strain M200 and the *Methylomonas*-like strain M5 (van Winden et al., 2012c). The *Methylovulum*-like strain M200 also contained unsaturated $\Delta^{11}$ aminotetrol (**VIb**), which is the only example of this compound (van Winden et al., 2012c).

The *hpnR* gene was present in three of the nine methanotroph genomes screened including the Type I methanotroph *Methylococcus capsulatus*, which is supported by evidence of the presence of 3-methylaminotetrol (**VIa**) and -pentol (**VIIa**) in culture studies (Neunlist and Rohmer, 1985b; Jahnke et al., 1999). Two species of *Methylomicrobium* were also identified as containing the *hpnR* gene and one of these species previously tested for BHP production did not contain any C-3 methylated BHPs. Moreover, aminopentol (**VII**) was also absent (Talbot et al., 2001) which is unusual for a Type I methanotroph and perhaps suggests that aminopentol (**VII**) is not universally present in Type I methanotrophs. Although not listed as one of the three methanotroph genomes to contain the gene, the presence of the *hpnR* gene is inferred for the *Methylocaldum* genus due to supporting evidence from culture studies showing the presence of C-3 methylated aminotetrol (**VIa**) and aminopentol (**VIIa**) (Cvejic et al., 2000a). No Type II methanotrophs have been found to make methylated BHPs (Neunlist and Rohmer, 1985a; Cvejic et al., 2000a; Talbot et al., 2001; van Winden et al., 2012c) and have not been reported as containing the *hpnR* gene (Welander and Summons, 2012).

As previously mentioned, aminotriol (**V**) has a wide range of non-methanotroph sources and low levels of aminotetrol (**VI**) have been found in some sulphate-reducing bacteria of the genus *Desulfovibrio* (aminotriol:aminotetrol 20-100:1) (Blumenberg et

al., 2006; Blumenberg et al., 2009a; Blumenberg et al., 2012). Furthermore, trace levels of aminopentol (**VII**) were reported in one species, *Desulfovibrio salexigens* (aminotriol:aminopentol 1352:1) (Blumenberg et al., 2012). The same authors stated in a subsequent publication that aminopentol (**VII**) remains an excellent biomarker for Type I methanotrophic bacteria (Berndmeyer et al., 2013).

There are entire methanotroph genera (Chapter 1, Figure 1.2) that remain untested for their BHP inventory and it remains to be seen if they follow the same BHP distributions as those already seen for Type I and II methanotrophs or perhaps contain other diagnostic structures.

### 2.2.6. Aminopentol as a biomarker for AMO in the sedimentary record

Aminopentol (**VII**) is seen as a diagnostic marker for Type I methanotrophy in the environment and can be used to infer aerobic methane oxidation in the sedimentary record. It has been observed in Congo sedimentary fan sediments at 1.2 Ma years old (Talbot et al., 2014). Aminopentol (**VII**) has been witnessed in a wide range of environments such as soils (Cooke et al., 2008a; Xu et al., 2009); peats (van Winden et al., 2012c); geothermal environments (Gibson et al., 2008); water column particulates (Blumenberg et al., 2007; Berndmeyer et al., 2013); lake sediments from a range of climate regions (Talbot and Farrimond, 2007; Coolen et al., 2008; Talbot et al., 2008); Arctic rivers and estuaries (Cooke et al., 2009), the Yangtze river, estuary and shelf (Zhu et al., 2011); the Congo estuary and sedimentary fan (Talbot et al., 2014) and Amazon wetlands, continental shelf and sedimentary fan (Wagner et al., 2014).

There have been limited reports of the methylated equivalent of aminopentol, 3-methylaminopentol (**VIIa**), in modern settings and has only been observed in one freshwater lake sediment (Talbot and Farrimond, 2007), three alkaline lakes (Farrimond et al., 2004) and a geothermal site (Gibson et al., 2008) which is at odds with the reports of 3-methylBHP degradation products, the C-3 methylated hopanes, in the sedimentary record (e.g. Collister et al., 1992; Farrimond et al., 2004; Eigenbrode et al., 2008). Maybe environmental conditions at the time of deposition, such as hypersaline conditions, favoured 3-methylBHP producing methanotrophs.

### 2.2.7. Geohopanoids and diagenesis of BHPs

BHPs are the biological precursors of geohopanoids (hopanols, hopanoic acids, hopanes and hopenes) and these diagenetic products have been described as the most abundant class of biomarkers on earth (Ourisson and Albrechet, 1992). The oldest reported geohopanoid is debateable due to contamination issues but intact polyfunctionalised BHPs, namely BHT (**III**), have been reported in environmental samples up to ~50 Ma years old (van Dongen et al., 2006). However, the side-chain remains a labile component and has been shown to be degraded readily on deposition (Sinninghe Damsté et al., 1995; Schaeffer et al., 2008; Schaeffer et al., 2010).

Whilst the geohopanoids are the ultimate degradation products of BHPs, an early degradation product has been shown to be 32,35-anhydrobacteriohopanetetrol (**XIV**; anhydroBHT herein). It has been found in a range of sediments and a hydrothermal vent (Watson, 2002; Bednarczyk et al., 2005; Talbot et al., 2005; Cooke et al., 2008b). AnhydroBHT (**XIV**) is formed via acid-catalysed cyclisation of the BHT (**III**) side chain (Schaeffer et al., 2008) but also composite BHPs such as BHT cyclitol ether (**XII**) and bacteriohopane-32,33,34,35-tetrol cyclitol ether (**XIII**; BHT glucosamine herein) (Schaeffer et al., 2010). AnhydroBHT (**XIV**) was a product of degradation of BHT in *Rhodopseudomonas palustris* when exposed to elevated temperature and pressure (Eickhoff et al., 2014). The loss of an adenine moiety from adenosylhopane is also another potential source of anhydroBHT (**XIV**) but has yet to be demonstrated conclusively (Costantino et al., 2001; Cooke et al., 2008b; Eickhoff et al., 2014).

#### 2.2.7.1. $C_{30}$ hopanoids and geohopanoid markers of AMO

The hopanoid biomarkers diploptene (**I**) and diplopterol (**II**) have been used to infer methane cycling (Talbot et al., 2014 and references therein). However, these biomarkers are not specific to methanotrophs (Rohmer et al., 1984) and are produced by a variety of other organisms but in combination with highly depleted carbon isotope signatures can be used to infer aerobic methane oxidation (Jahnke et al., 1995; Naeher et al., 2014).

C-3 methylated hopanes are the diagenetic products of C-3 methylated BHPs. These diagenetic products, in combination with depleted carbon isotope values, have been used as evidence for aerobic methane oxidation (e.g. Peckmann and Thiel, 2004;

Birgel and Peckmann, 2008). However, as noted above, 3-methyl BHPs are relatively uncommon in modern systems and therefore may be absent in some ancient methane impacted systems (Birgel et al., 2006; Birgel et al., 2011).

### 2.3. Summary

The role of methane and methanotrophy in the global carbon cycle in modern and paleoenvironments is of great importance. Aerobic methane oxidation (AMO) by aerobic methanotrophic bacteria, a relatively unstudied and unappreciated process, is an alternative fate for methane emissions. BHPs have great potential as biomarkers for methanotrophy and aerobic methane oxidation but have received little attention to date. A better understanding of the factors controlling the expression of BHPs in methanotrophs is essential to realise their full potential as biomarkers and allow proxies to be developed. Previous studies investigating BHP signatures of methanotrophs have involved pure cultures. To give a better approximation to natural systems, a sediment microcosm approach under controlled conditions, was undertaken. The addition of methane as a carbon and energy source resulted in the enrichment of methanotroph communities which were subsequently used to study methanotroph BHP signatures under a range of environmental conditions.

### 2.4. Aims of the thesis

The main aims of the thesis are as follows:

(1) to investigate changes in methanotroph community composition in River Tyne estuarine sediment microcosm experiments under a range of environmental perturbations.

(2) to determine the effect these environmental perturbations have on BHP composition, focusing on the methanotroph-derived 35-aminoBHPs.

(3) to determine the effect different stages of growth have on the abundance of 35-aminoBHPs.

(4) to investigate anaerobic BHP producers in long-term River Tyne estuarine sediment microcosm studies and the preservation and/or degradation of individual compounds.

# 3. Methods and method development

## 3.1. A microcosm-based approach

Traditionally, studies investigating BHP composition in bacteria, subjected to a range of environmental stresses, have employed culture-dependent methods (e.g. Moreau et al., 1997; Poralla et al., 2000; Joyeux et al., 2004; Schmerk et al., 2011). Furthermore, there has only been one such study looking at BHP composition in a methanotroph, i.e. composition with increasing growth temperature (Jahnke et al., 1999). This culture-dependent approach does provide essential insight into bacterial BHP biosynthesis and resulting signatures but does not necessarily reflect the response that would be seen in the environment. Moreover, the culturable organisms used in such studies may not represent major *in situ* organisms.

Model systems are simplified representations of more complex systems (Jessup et al., 2004). Microcosms are commonly used model systems and are defined as 'small, contained ecological systems' (Srivastava et al., 2004). These 'model systems' allow us to study the effect of multiple environmental variables on a specific process. This study uses an aerobic microcosm-based approach to investigate the effect different environmental variables have on BHP composition with the focus being methanotroph-derived BHPs. Coupled with microbial community analysis, a link can be made between methanotroph diversity in response to environmental variables and the effect this has on BHP composition.

The advantage of microcosms is that they offer a high degree of experimental control, and the small size and short generation times allows replicated experiments across a wide range of spatial and temporal scales (Jessup et al., 2004). The major limitation of this approach is that they may not provide a true representation of the 'real' environmental response to a perturbation (Srivastava et al., 2004).

Despite the limitations, this approach is an effective way to test whether a hypothesised effect occurs even if it doesn't fully represent the complexity of the natural environment.

### 3.2. Site description and sampling

#### 3.2.1. Site description - River Tyne estuary

The River Tyne flows through the densely populated city of Newcastle upon Tyne, UK, and its surrounding suburbs (population ~1,100,000). It has a catchment area of 2935 $km^2$ and a mean freshwater discharge of ~45 $m^3s^{-1}$ (Spencer et al., 2007b). There are two main tributaries: the North Tyne which rises on the Scottish border, north of Kielder Water and flows through Kielder Forest receiving humic-rich waters from blanket peat areas and the South Tyne which rises on Alston Moor, Cumbria which drains relatively pristine moorland (Spencer et al., 2007b; Ahad et al., 2008). The combination of these two tributaries at Acomb, Northumberland, provides ~90% of total freshwater discharge into the River Tyne with the remaining ~10% coming from three polluted urban rivers; the Derwent, the Team and the Ouseburn (Spencer et al., 2007a). The River Tyne flows in an easterly direction towards Wylam; the last ~33 km of the river is tidal with a freshwater residence time of ~5-20 days (Ahad et al., 2006; Spencer et al., 2007b).

The geology of the River Tyne consists of Carboniferous limestones and the Namurian Millstone Grit Series in the upper section of the Tyne. This is characterised by thick, coarse and fine-grained sandstones, together with silt and mudstones. The lower Tyne basin consists of Lower and Middle Coal Measures where the dominant rock types are shales, mudstones and sandstones (Ahad et al., 2008).

#### 3.2.2. Sampling

Sediment samples were collected at low tide from undisturbed sediment from the Scotswood area of the River Tyne, UK (Figure 3-1 and Figure 3-2; Latitude 54°57'50.87"N, Longitude 1°40'56.84"W) and were transported back to the lab at ambient temperature and then stored at 4°C before being processed. Scotswood is situated ~13 miles from the River mouth and ~6.5 miles below the upper tidal limit of the Tyne at Wylam, and can therefore be considered a true estuarine environment. The salinity at the point of sampling was 7 g/L NaCl and the pH 6-8. A study from the same site recorded average water/surface sediment temperatures in spring/summer were 15.8°C and autumn/winter were 12.6°C (Blake, 2010).


**Figure 3-1:** Position of Newcastle upon Tyne (left) and an aerial view of the River Tyne highlighting Scotswood sampling location (red) on the South bank of the River Tyne (right) and the tidal limit highlighted in white (map adapted from Google earth image).

**Figure 3-2:** Scotswood sampling location at low tide.

For short-term aerobic microcosm experiments, the top ~2 cm layer of light brown surface sediment was collected (Figure 3-3). Below the 2 cm level the sediment is dark grey/black and anoxic.

**Figure 3-3:** Aerobic surface sediment layer has been removed revealing the anoxic sediment.

For long-term anaerobic sulphate-reducing and methanogenic microcosm experiments, dark grey/black anoxic sediment was collected from ~30 cm below the surface sediments.

### 3.3. Short-term aerobic studies: materials and methods

Microcosms were used to investigate the effect of variations in methane concentration, temperature, pH, and salinity on BHP composition and methanotroph communities in River Tyne estuarine sediment.

#### 3.3.1. Nutrient medium for enrichment of methanotrophs in aerobic microcosms

Nutrient growth medium from (Widdel and Bak, 1992), (see appendix 2), was used for aerobic short-term microcosm studies. It was modified from that of the original recipe by excluding the oxygen scavenger, sodium sulphide ($Na_2S$), and not degassing the medium with nitrogen before use. The growth medium has previously been shown to support the growth of methanotrophs in methane oxidation potential experiments prepared with sediment from the same site on the River Tyne (Blake, 2010).

The medium was adjusted to pH 7.5 ± 0.7 and the salt concentration was matched to the *in situ* brackish salinity (7 g/L NaCl), unless otherwise stated, of the River Tyne estuary at the sampling location of the sediment. For the pH study, sediment was added to the nutrient medium to create a slurry which was then adjusted to the desired

pH using HCl or NaOH (1N). Slurry (25 mL), containing sediment (~4 g), was dispensed into serum bottles.

### 3.3.2. Microcosm set-up

Prior to assembling microcosms, glass serum bottles (60 mL, Wheaton via VWR) and butyl rubber stoppers were sterilised by autoclaving (20 min at 121°C). Each microcosm contained nutrient growth medium (22 mL, Section 3.3.1) and River Tyne estuarine surface sediment (~3-4 g). This resulted in 35 mL of headspace. The serum bottles were closed with butyl rubber stoppers and sealed with aluminium crimps (Sigma Aldrich, UK) (Figure 3-4).

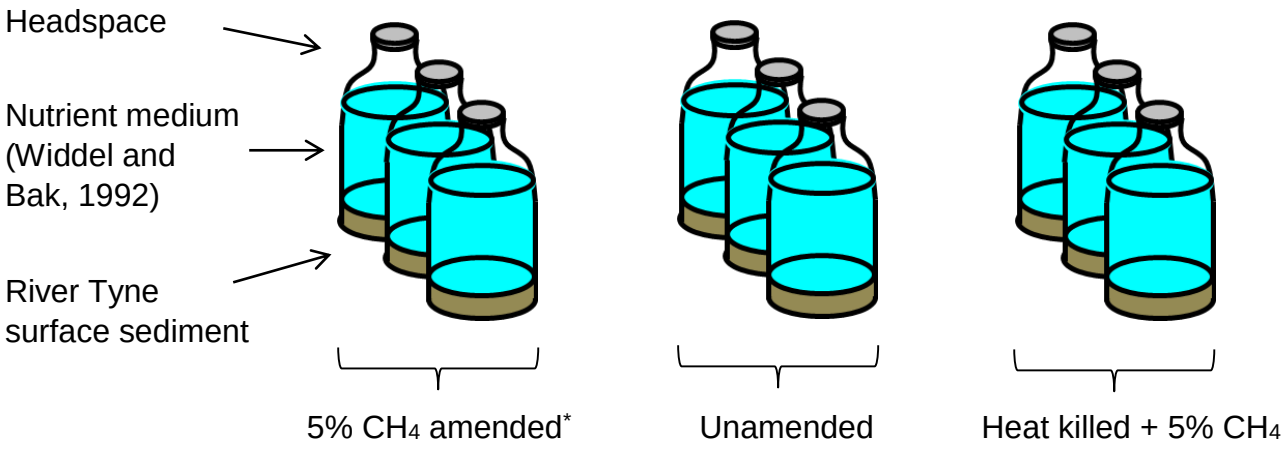



**Figure 3-4:** Preparation of short-term aerobic microcosm experiments.

* For methane concentration experiments, the amount of methane the microcosms were amended with varied according to experimental conditions (Table 3-1).

Microcosms were prepared in triplicate (Figure 3-4) with 5% $CH_4$ (v/v) (BOC Ltd, UK) amended headspace, unless otherwise stated, unamended (to measure background levels of methane in the sediment) and heat killed controls with 5% methane, unless otherwise stated (abiotic controls). A triplicate set of microcosms representing the initial bacterial communities and BHP composition of the River Tyne sediments were prepared and sacrificially sampled (Section 3.3.3); referred to as Time 0 herein. Surface sediments (top ~2 cm of sediment) were used in the preparation of all microcosms subjected to the conditions shown below (Table 3-1).

The heat killed control microcosms were autoclaved (20 min at 121°C) and allowed to cool to ambient room temperature. The microcosms were amended with 5% methane by injecting through the butyl rubber stopper into the headspace of the amended and heat killed sets. Headspace methane concentrations of amended, unamended and heat killed controls were measured at regular intervals using gas chromatography with flame ionisation detector (GC-FID) (Section 3.3.6). When all methane in the amended sets had been oxidised, the microcosms, along with their unamended and heat killed counterparts, were sacrificially sampled (Section 3.3.3).

### 3.3.3. Sacrificial sampling of microcosms

To sacrifice, microcosms were shaken to ensure homogeneity of the sediment and medium, sediment slurry (5 mL) was removed through the stopper using a sterile syringe and stored in a sterile universal tube for microbiological analysis (Section 3.6). BHP composition was determined from the remaining sediment slurry (~20 mL) in the microcosm; both aliquots were stored at -20°C prior to analysis.

### 3.3.4. Freeze-drying of microcosm contents

All microcosms were defrosted overnight at 4°C. Sediment slurry was poured into a Teflon centrifuge tube (50 mL) and centrifuged (15 min, 12,000 rpm). This was repeated twice more with additions of Milli-Q water (~20 mL) to the serum bottles to ensure all slurry was transferred. The sediment pellet in the tube was frozen (-20ºC) and subsequently freeze-dried (ModulyoD, Thermo Scientific, UK). The resulting sediment (~1 g) was manually ground to a fine powder using a mortar and pestle.

### 3.3.5. Microcosm experimental conditions

The microcosm set-up described (Section 3.3.2) was followed for the experimental conditions in Table 3-1. A wide range of parameters were tested including extreme conditions to enrich a diverse methanotroph community. The microcosms were incubated at 21°C, unless otherwise stated.

**Table 3-1:** Aerobic microcosm experimental conditions.

| **Temperature (°C)** | 4 | 8* | 15* | 21 | 30 | 40 | 50 | 60 |
|---|---|---|---|---|---|---|---|---|
| **pH** | 4 | 5 | 6 | 7 | 8 | 9 | | |
| **Salinity (g/L)** | 1 | 15 | 35 | 70 | 120 | 150 | | |
| **Methane concentration (%)** | 0.1 | 0.5 | 1 | 5 | | | | |

* 8 and 15°C microcosms and corresponding controls were prepared at a later date using a different surface sediment batch to those of the original temperature experiment (4°C, 21-60°C).

#### 3.3.5.1. Growth stage study

The experimental design was based on previous data from a pilot study which used River Tyne sediment from anoxic depths (~30cm), which was subsequently incubated under aerobic conditions, with a 5% methane amended headspace at 4, 30 and 60°C. In this study, the microcosms were incubated for a period of 20 days rather than being sacrificially sampled when 5% methane had been oxidised. At 30°C, ~10 times the concentration of aminopentol (**VII**) was witnessed after 20 days compared with the original temperature study (Chapter 5, Section 5.4.5.3). This suggests continued production of 35-aminoBHPs in stationary phase. This experiment was adapted, this time using aerobic surface sediments, to test the hypothesis that 35-aminoBHPs continue to be produced in stationary phase. Additional time points were added to determine 35-aminoBHP composition in exponential and stationary phases. The experiment was performed at 30°C to replicate the previous study and also at 50°C to test the hypothesis that 3-methylaminopentol (**VIIa**) production increases during stationary phase; the original temperature study indicated production of 3-methylaminopentol (**VIIa**) in methanotrophs at this temperature (Chapter 5, Section 5.4.5.3).

Microcosms were prepared as described (Section 3.3.2) with the following alterations. Six sets of 5% methane amended microcosms were prepared in triplicate to be sacrificed during different stages of growth. Unamended (to measure background levels of methane in the sediment) and heat killed controls + 5% $CH_4$ (abiotic controls)

were prepared for both temperatures (30 and 50°C). Microcosms were monitored for methane for the duration of the experiment and sacrificed (Section 3.3.3) on completion of the experiment at 28 days. A triplicate set of microcosms representing the initial BHP compositions present in the River Tyne surface sediment (Time 0) were also prepared and sacrificed.

The heat killed control set of microcosms were autoclaved as described above (Section 3.3.2). The microcosms were amended with 5% methane by injecting through the butyl stopper into the headspace of the amended and heat killed sets. Methane concentrations of amended, unamended and heat killed controls were measured at regular intervals using GC-FID (Section 3.3.6). Microcosm sets were sacrificed according to Table 3-2.

**Table 3-2:** Growth stage sacrificial time points.

| **Growth stage** | **30°C** | **50°C** |
|---|---|---|
| **Exponential phase** | 5 days | 4 days |
| **5% $CH_4$ consumed** | 7 days | 8 days |
| **Stationary phase** | 11 days | 11 days |
| **Stationary phase** | 15 days | 15 days |
| **Stationary phase** | 20 days | 20 days |
| **Stationary phase** | 28 days<br>Unamended<br>Heat killed + 5% $CH_4$ | 28 days<br>Unamended<br>Heat killed + 5% $CH_4$ |

### 3.3.6. Analysis of microcosm headspace gasses, using GC-FID

Methane consumption in the headspace of microcosms was analysed using a Carlo-Erba HRGC 5160 gas chromatograph in split mode with the injector at 150°C and FID at 300°C. Separation was performed on a HP-PLOT-Q capillary column with a length

of 30 m and internal diameter 0.32 mm packed with 20 µm Q phase. The GC was held isothermally at 35°C for 90 min and heated to 250°C at 10°C/min and held at final temperature for 10 min with hydrogen as the carrier gas (flow 1 mL/min, pressure of 50 kPa, split at 100 mL/min. The acquisition was stored on a Thermo LabSystems Atlas chromatography data system.

A nitrogen-flushed, gas-tight, push-lock syringe (SGE, Australia) was used to manually inject headspace gas samples (100 µL) from microcosms. A calibration curve was determined using a series of known volumes (10, 20, 40, 60, 80, and 100 µL) of 1% or 10% methane standards (Scientific & Technical Gases Ltd, Newcastle under Lyme, UK). Methane was detected by single ion monitoring (*m/z* = 15) and quantified on the basis of peak area, using the external methane standards. Rates of methane consumption in the replicated (x3) microcosm experiments were calculated from the linear decrease in methane with time at the phase of most rapid methane consumption and reported in µmol $CH_4$ $day^{-1}$ $g^{-1}$ sediment. Rates were compared statistically using one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) (Minitab 17 Statistical Software).

### 3.4. Long-term anaerobic studies: materials and methods

Sulphate-reducing and methanogenic microcosms were used to investigate anaerobic BHP producers and BHP degradation pathways in River Tyne estuarine sediment.

#### 3.4.1. Nutrient medium for enrichment of sulphate-reducing bacteria and methanogens in anaerobic microcosms

Anaerobic nutrient growth medium (Widdel and Bak, 1992) was used for long-term microcosm studies (see appendix 2). The growth medium was designed to enrich mesophilic sulphate-reducing bacteria and sulphate-reducing conditions were established by the addition of sodium sulphate ($Na_2SO_4$, 28 mM), to the medium. By omitting the addition of $Na_2SO_4$, the medium was also used in the preparation of methanogenic microcosms as it has previously been shown to successfully enrich fermentative bacteria and methanogens (Jones et al., 2008). The medium was adjusted to pH 7.5 ± 0.7 and the salt concentration was matched to the *in-situ* brackish salinity (7 g/L NaCl) of the River Tyne estuary at the sampling location of the sediment.

### 3.4.2. General anaerobic microcosm set-up

Prior to assembling of microcosms, serum bottles (120 mL, Wheaton via VWR) and butyl rubber stoppers were sterilised by autoclaving (20 min, 121°C). Each microcosm contained anaerobic nutrient growth medium (90 mL) (see appendix 2) and anoxic River Tyne sediment (~10 g), prepared under a constant stream of nitrogen (Figure 3-5). This resulted in a 20 mL headspace. The serum bottles were closed with butyl rubber stoppers and sealed with aluminium crimps (Sigma Aldrich, UK). Microcosms were stored stopper down at ambient room temperature, ~20°C, in the dark.

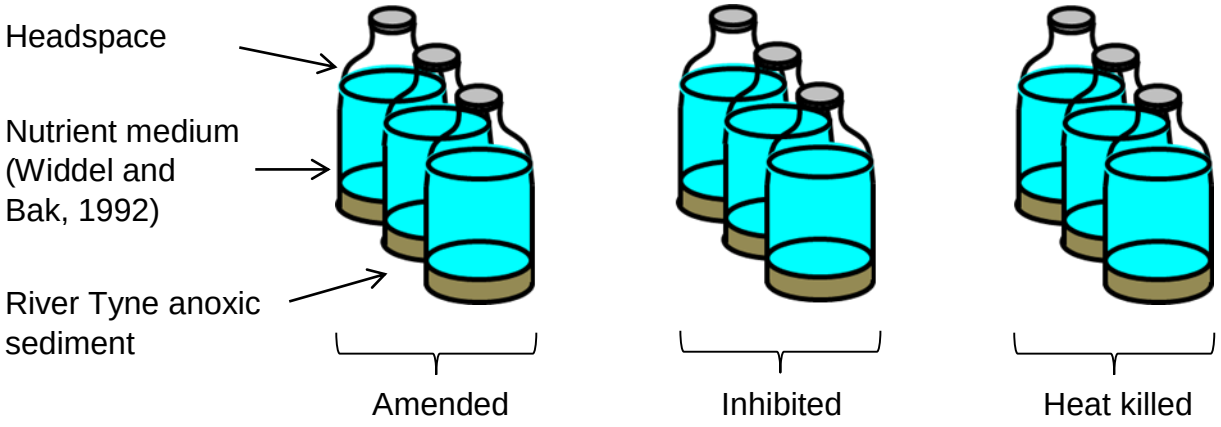



**Figure 3-5:** Preparation of long-term anaerobic microcosm experiments.

#### 3.4.2.1. Sulphate-reducing microcosms

Sodium acetate ($CH_3COONa$, 100 mM), was added as a carbon source, to microcosms through the butyl rubber stopper after the serum bottles had been closed and crimp sealed. Sodium sulphate ($Na_2SO_4$, 28 mM), as the sulphate source, was also added through the stopper. Heat killed control microcosms (abiotic controls) were autoclaved (20 min at 121°C). Inhibited microcosms were prepared with sodium molybdate ($Na_2MoO_4$, 20 mM), an inhibitor of sulphate-reduction, to monitor the BHP compounds resulting from other bacterial communities enriched in the sediment. Sulphate levels were monitored at regular intervals thereafter, using Ion Chromatography (Section 3.4.6). Microcosms, including heat killed and inhibited control counterparts, were sacrificed (Section 3.4.3) immediately after analysis (Time 0) and subsequently at 28, 56, 94, 433, 706 days. At day 56, sulphate levels were depleted (0 mM) and two sets were allowed to turn methanogenic; subsequently these were sacrificed at 465 and

619 days. Sodium sulphate ($Na_2SO_4$, 84 mM), was added through the rubber stopper at day 58 to replenish sulphate in all remaining microcosms, apart from those left to turn methanogenic. Sodium acetate ($CH_3COONa$, 100 mM), was added through the rubber stopper at day 231, to all remaining microcosms, after sulphate-reduction plateaued. At ~273 days, sulphate-reduction levels once again plateaued; microcosms were left in this starved state.

#### 3.4.2.2. Methanogenic microcosms

Cellulose (0.32 g) was added to the sediment slurry in serum bottles. The serum bottles were closed with butyl rubber stoppers and crimp sealed. Heat killed control microcosms (abiotic controls) were autoclaved (20 min at 121°C), with overnight incubation at 40°C to germinate endospores, followed by a second round of autoclaving to kill any vegetative cells arising from incubation. An inhibitor of methanogenesis, 2-bromoethanesulfonate (BES, 10 mM), was added to the basal medium of one set of microcosms. BES is a structural analogue of Coenzyme M (CoM) and inhibits the final stage of methanogenesis (Ungerfeld et al., 2004). Methane gas accumulation in the headspace of microcosms was monitored at regular intervals using GC-FID (Section 3.4.7). Microcosms, including heat killed and inhibited control counterparts, were sacrificed (Section 3.4.3) immediately after analysis (Time 0) and subsequently at 30, 106, 225, 505 and 665 days. Methane accumulation slowed and seemed to be plateauing therefore, on day 76, cellulose (0.32 g) was added after removing stopper. This resulted in methane levels being reset to zero. Heat killed controls were autoclaved once again as above.

### 3.4.3. Sacrificial sampling of microcosms

To sacrifice, microcosms were shaken to ensure homogeneity of the sediment and medium; sediment slurry (15 mL) was removed and stored in a sterile universal tube for future microbiological analysis if required. BHP composition was determined from the remaining sediment slurry (~80 mL) in the microcosm; both aliquots were stored at -20°C prior to analysis.

### 3.4.5. Freeze-drying of microcosm contents

All microcosms were defrosted overnight at 4°C. Sediment slurry was poured into a Teflon centrifuge tube (50 mL) and centrifuged (15 min, 12,000 rpm). This was repeated twice more with additions of Milli-Q water (~20 mL) to the serum bottles to ensure all slurry was transferred. The sediment pellet in the tube was frozen (-20ºC) and subsequently freeze-dried (ModulyoD, Thermo Scientific, UK). The resulting sediment (~3-4 g) was ground to a fine powder using a mortar and pestle.

### 3.4.6. Analysis of sulphate in microcosms using Ion Chromatography

Sulphate was measured by Ion Chromatography using a Dionex ICS-1000 fitted with an IonPac AS14A column, a conductivity detector and an AS40 autosampler. The eluent was 8.0 mM $Na_2CO_3$/1.0 mM $NaHCO_3$ solution, flow rate of 1 mL/min and the injection loop was 25 µL. The detection limit was 0.1 µM. Prior to analysis, microcosms were stood upright for 2 hrs to allow sediment to settle. Prior to measurement, headspace overpressure was removed using a nitrogen-flushed gas tight syringe. A nitrogen-flushed syringe (1 mL) was used to remove an aliquot (50 µL) of medium and was dispensed into a vial. Samples were diluted (1 in 200) and filtered through a 0.20 µm PTFE filter (VWR, UK).

### 3.4.7. Analysis of methanogenic microcosm headspace gasses, using GC-FID

Headspace methane in microcosms was analysed using a Carlo-Erba HRGC 5160 gas chromatograph, as described (Section 3.3.6). Immediately prior to measurement, headspace overpressure was removed using a nitrogen-flushed gas tight syringe. A helium flushed gas-tight, push-lock syringe (SGE, Australia) was used to manually inject headspace gas samples (100 µL) from microcosms. A calibration curve was determined using a series of known volumes (10, 20, 40, 60, 80 and 100 µL) of 1%, 10% or 100% methane standards, depending on methane concentrations present in headspace (Scientific & Technical Gases Ltd, Newcastle under Lyme, UK). Methane was detected by single ion monitoring (*m/z* = 15) and quantified on the basis of peak area, using the external methane standards. Methane production in the replicated (x3) microcosm experiments was calculated and reported in mmol methane. The volume of gas removed prior to measurement and the concentration of methane was also calculated. This allowed cumulative methane production with time to be calculated.

### 3.5. Methanotroph pure cultures

#### 3.5.1. Isolation and enrichment of *Methylobacter* spp.

*Methylobacter marinus* A45 was isolated from marine samples off the coast of California (Lidstrom, 1988; Bowman et al., 1993). Briefly, isolates were grown on nitrate mineral salts (NMS) medium to which 0.5% (w/v) NaCl was added under a (5:4:1) methane:air:$CO_2$ atmosphere at 37°C (Bowman et al., 1993). *Methylobacter* sp. BB5.1 was isolated from estuarine sediments in Newport Bay, California (Smith et al., 1997). Briefly, enrichments were prepared by diluting the sediment 10-fold with nitrate mineral salts (NMS) medium to which 1.5% (w/v) NaCl was added under a 20% (v/v) methane atmosphere at room temperature with shaking (Smith et al., 1997). *Methylobacter* sp. BBA5.1 was isolated from an unspecified marine environment (isolated by M. Kalyuzhnaya pers. comm. with D. Birgel). All isolates provided as bacterial cell pellets via D. Birgel.

#### 3.5.2. Isolation and enrichment of *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp.

*Methylomarinum vadi* IT-4 was isolated from a microbial mat sample collected at a shallow submarine hydrothermal system (depth, ~23 m) off Taketomi Island, Okinawa, Japan. The mats were found near the vent with the temperature at the mats being approximately 30-40°C whereas that of the vent fluid was 52°C (Hirayama et al., 2013). *Methylomarinovum caldicuralii* IT-9 was isolated from the hot vent fluid collected at a main vent site (depth, 23 m) in a shallow submarine hydrothermal system in a coral reef off Taketomi Island, Okinawa, Japan (Hirayama et al., 2014). For both genera, cultivation was conducted using MJmet medium; a detailed site description and procedures for enrichment and isolation have been previously reported (Hirayama et al., 2007). *Methylomarinovum* sp. IN45 is a new isolate from a deep-sea hydrothermal field (H. Hirayama, pers. comm.). All isolates provided as bacterial cell pellets by H. Hirayama.

### 3.5.3. *Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b culturing conditions

*Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b (Whittenbury et al., 1970) were obtained from the NCIMB culture collection (Strains #11130 and #11131 respectively, NCIMB, Aberdeen, Scotland) and resuscitated on nitrate mineral salts (NMS) medium with a 1:1 (v/v) mix of methane in air at 30°C with shaking at 150 rpm, according to the NCIMB guidelines.

### 3.5.4. Freeze-drying of bacterial cells

*Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b cultures were defrosted at 4°C. The culture medium was poured into a Teflon centrifuge tube (50 mL) and centrifuged (15 min, 12,000 rpm). This was repeated twice more with additions of Milli-Q water (~20 mL) to the serum bottles to ensure all culture medium was transferred.

All bacterial cell pellets, including *Methylobacter* spp., *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp. were frozen (-20ºC) and subsequently freeze-dried (ModulyoD, Thermo Scientific, UK). The resulting bacterial cell pellets were ground to a fine powder using a mortar and pestle.

## 3.6. Microbial community analyses

Microbial community analysis was performed on short-term aerobic microcosms to assess methanotroph community composition in response to different experimental conditions.

### 3.6.1. DNA extraction

DNA was extracted from 2 mL of microcosm sediment slurry (aerobic studies) using PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., USA) in conjunction with FastPrep-24 Ribolyser (MP Biomedicals, USA). For *Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b an UltraClean® Microbial Isolation Kit (MO BIO Laboratories Inc., USA) was used. For each set of DNA extractions a procedural blank was included to ensure extracts remained contamination-free throughout the extraction procedure. DNA extractions were carried out according to the manufacturers

instructions with the following minor modifications, The FastPrep-24 Ribolyser was used at a speed of 6 $ms^{-1}$ for 40 s for homogenisation and cell lysis. DNA extracts were stored at -20°C until further use. DNA was diluted ($10^{-1}$) to reduce the levels of inhibitory contaminants to prevent PCR inhibition (Head, 1999).

### 3.6.2. Polymerase chain reaction (PCR) amplification of genes

In aerobic microcosms, functional genes, the particulate methane monoxygenase gene (*pmoA*) and the C-3 methylase gene (*hpnR*), were targeted with specific primer sets to amplify methanotroph communities and C-3 methylated hopanoid producers, respectively.

All PCR amplifications were performed in sterile PCR tubes (200 µL, Starlab, UK) in a total reaction volume of 50 µL. Each reaction contained 1 µL of DNA template, oligonucleotide primers (1 µL, 10 pmol/µL), deoxynucleoside triphosphates (1 µL, 10 mM), $MgCl_2$ (1.5 µL, 50 mM), $NH_4$ reaction buffer (5 µL, 10x solution) and Taq polymerase (0.2 µL, 5U/µL Bioline, London, UK) and molecular grade water (39.3 µL, Sigma-Aldrich, UK). All amplification reactions were carried out in an automated thermal cycler (Techne TC-512, Bibby Scientific, UK). For each PCR, a negative control (no template DNA, PCR reaction mix only), control (procedural blank from DNA extraction), and a positive control (genomic DNA extracted from a pure culture, i.e. *Methylococcus capsulatus* for the *pmoA* gene) were included. PCR products were stored at -20°C prior to further analysis.

#### 3.6.2.1. PCR amplification of the *pmoA* gene

The *pmoA* gene (470 bp product) was amplified with the A189f (5'-GGiGACTGGGACTTCTGG-3') (Holmes et al., 1995) and Mb661r (5'-CCGGiGCAACGTCiTTACC-3') (Costello and Lidstrom, 1999) primer set. For DGGE (Section 3.6.4), the A189f primer was modified at the 5' termini with a GC-clamp (Sheffield et al., 1989) and degenerate bases within the Mb661r reverse primer were replaced with inosine residues (Jugnia et al., 2009). PCR amplification was carried out using touchdown PCR conditions as follows: 4 min initial denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 60-50°C for 1 min (decreased by 0.33°C every cycle) and 72°C for 3 min. This was followed by 10 additional cycles at the lowest annealing temperature (50°C), with a final extension at 72°C for 5 min.

#### 3.6.2.2. PCR amplification of the *hpnR* gene

The *hpnR* gene (535 bp product) was amplified with the MCA27 (5'-CCGCAGCTATCGTGTAATGA-3') and MCA28R (5'-GATGTCGAACAGGCGGTAGT-3') primer set (Welander and Summons, 2012). PCR amplification was carried out as follows: 3 min initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min primer annealing at 58°C, 1 min extension at 72°C with a final extension step for 10 min at 72°C. The PCR positive control was *Methylococcus capsulatus* gDNA. PCR amplicons were purified prior to DNA sequencing using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions. Briefly, PCR product (5µL) was mixed with ExoSAP-IT (2 µL) at 37°C for 15 min, followed by inactivation of ExoSAP-IT at 80°C for 15 min.

### 3.6.3. Agarose gel electrophoresis

Products of PCR amplification were visualised using agarose gel electrophoresis to confirm gene fragments were the correct size and to confirm the DNA dilution to use for further downstream applications. PCR products were analysed in 1% (w/v) agarose gels in 1 x TAE buffer (Tris-acetate-EDTA buffer; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) and stained with ethidium bromide (1.6 µL). For each PCR product, 5 µL of product plus 2 µL of 6 x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in filter sterile water; Sigma, UK) was run on the gel at 100 V for 45 min. Hyperladder II DNA size marker (2 µL, Bioline, UK), ranging from 50 bp to 2 kb, was ran alongside samples to determine PCR fragment size. Gels were visualised with a BioSpectrum Imaging System (UVP, Cambridge, UK) with VisionWorksLS Software.

### 3.6.4. Denaturing gradient gel electrophoresis (DGGE) of *pmoA* gene

For each sample, *pmoA* gene amplicons (11 µL) were loaded onto a 10% (w/v) acrylamide gel containing a 20 to 55% denaturing gradient, where 100% denaturant consists of 7 M urea and 40% formamide. The gels were run at 60°C for 16 h at 100 V using an INGENYphorU system (Ingeny International BV, Goes, The Netherlands). The gels were stained with SYBR gold (Invitrogen, Paisley, UK) and visualised with a BioSpectrum Imaging System (UVP, Cambridge, UK). Dominant DGGE bands were excised from the gels and eluted in 100 µL of sterile water at 4°C overnight. DNA eluate

(2µL) was reamplified with A189f (Holmes et al., 1995) and Mb661r (Costello and Lidstrom, 1999), without a GC clamp or inosine residues according to the following PCR conditions: 5 min initial denaturation at 94°C; 30 cycles, where 1 cycle consists of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final elongation at 72°C for 30 min (Martineau et al., 2010). PCR amplicons were purified prior to DNA sequencing using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions, previously briefly described (Section 3.6.2.2).

### 3.6.5. DNA sequencing of *pmoA* fragments

Sequencing reactions were performed by GeneVision (Newcastle upon Tyne, UK) using an ABI Prism 3730xl DNA sequencer with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Sequence data was compared to the EMBL Nucleotide Sequence Database at the European Bioinformatics Institute (EBI) using Fasta3 (Pearson and Lipman, 1988) to identify nearest neighbours.

### 3.6.6. Phylogenetic analysis of *pmoA* gene fragments

All *pmoA* gene sequences (34 sequences, length ~289-425 bp) were deposited in the Genbank database with accession numbers (KF958142-KF958175). Partial *pmoA* nucleotide sequences were aligned to their nearest neighbours using ClustalW (Thompson et al., 1994) subsequently a neighbour-joining phylogenetic tree was constructed using the p-distance method (Nei and Kumar, 2000). Bootstrap analysis was performed using 1000 replicates to determine the degree of confidence in the topology of the phylogenetic tree (Felsenstein, 1985). All analyses were conducted in MEGA5 (Tamura et al., 2011). A *pmoA* distance of 7% has previously been shown to correspond to the 3% 16S rRNA distance level (Degelmann et al., 2010) thus broadly represents differentiation at the species level.

## 3.7. Bulk geochemistry

### 3.7.1. Total organic carbon

Total organic carbon (TOC) values were obtained from triplicate aliquots of initial sediments (Time 0) used to assemble microcosms. Freeze-dried sample (~0.1 g) was weighed into a porous crucible and HCl (1 mL, 4 mM) was added to remove any inorganic carbon from the sediment. After 24 h at 65°C samples were analysed using

a Leco CS230 Carbon-Sulphur analyser. A standard sample was analysed after every 10 samples to check accuracy.

### 3.8. Bacteriohopanepolyol (BHP) analysis

The following section describes the final protocol used to extract sediment lipids from all of the above experiments. This protocol was the result of method development performed on the original two day extraction protocol see below (Section 3.9).

#### 3.8.1. Modified Bligh and Dyer lipid extraction

Each set of extractions consisted of 15 sediment samples and a procedural blank of only the solvents used in the extractions. The samples were extracted using a methodology adapted from Summons et al., 1994 and is based on the Kates modification (Kates, 1975) of the original Bligh and Dyer extraction (Bligh and Dyer, 1959). Freeze-dried sediment from microcosm experiments (0.5-1 g) and bacterial cell material (~50 mg) were extracted in a 50 mL Teflon centrifuge tube using a monophasic mixture of Milli-Q water/methanol (MeOH)/chloroform ($CHCl_3$) (4 mL: 10 mL: 5 mL) (MeOH distilled in-house, $CHCl_3$, distol grade, Fisher Scientific, UK). The water was added first and the sample shaken by hand to ensure that the sample was fully dispersed before the other solvents were added. The mixture was sonicated for 15 min at 40°C and was then centrifuged for 15 min at 12,000 rpm (Sorvall RC 5B Plus). The supernatant was transferred to a second centrifuge tube. The sample was then extracted a second time this time using a pre-mixed solvent mixture; it was sonicated and centrifuged again as previously described. The supernatant was transferred to a third centrifuge tube. The sample was then extracted for a third time as previously described using a pre-mixed solvent mixture and was sonicated and centrifuged as previously described. The supernatant was transferred to a fourth tube.

Phase separation was performed by adding 5 mL $CHCl_3$ to tubes 2, 3 and 4 which were then shaken. 5 mL water was then added to all the tubes and was gently mixed. The tubes were centrifuged for 5 min at 12,000 rpm to aid complete separation of the $CHCl_3$ and MeOH/water phase. The $CHCl_3$ fraction from tube 2 was then transferred to a 100 mL round bottom flask. The $CHCl_3$ fraction from tube 3 was transferred to tube 2 and was centrifuged for 2 min at 12,000 rpm. The $CHCl_3$ fraction was added to the round bottom flask. The $CHCl_3$ fraction from tube 4 was then transferred to tube 2 and was

once again centrifuged for 2 min at 12,000 rpm; the $CHCl_3$ fraction was added to the round bottom flask. The three $CHCl_3$ fractions in the round bottom flask is the total lipid extract (TLE) of the sample. These were then rotary evaporated to dryness, transferred to a vial using a warm solution of $CHCl_3$/MeOH (2:1 v/v) then evaporated to dryness under a stream of nitrogen at 50°C.

### 3.8.2. Solid phase extraction

The extract was re-dissolved in 1500 µL $CHCl_3$/MeOH (2:1 v/v) heated for ~10 min at 50°C and split into three aliquots 250 µL, 500 µL and 750 µL. Aliquots were then dried under a stream of nitrogen at 50°C.

An aliquot of TLE (500 µL) was dissolved in $CHCl_3$ (200 µL) and was loaded onto an ISOLUTE (Biotage) $NH_2$ 1 mg/6 mL column which had been conditioned with hexane (6 mL). The TLE was loaded onto the column, the vial was rinsed with diethyl ether/acetic acid (98:2 v/v; 6 mL) and this was used to elute the non-polar fraction. The vial was rinsed again with $CHCl_3$/MeOH (2:1 v/v; 250 µL) and was loaded onto the column. The vial was rinsed with MeOH (2 x 6 mL) and this was used to elute the polar fraction. Fractions were dried under a stream of nitrogen at 50°C.

This method was recently developed in Newcastle (Bischoff and Rush, unpublished data) and is similar to methods described by other workers which produce three fractions (neutral, acid and polar) (e.g. Kaur et al., 2015). However, initial investigation revealed that some BHPs, particularly adenosylhopane (**VIII**) and related compounds eluted in the first fraction with the remainder eluting in the polar fraction. By excluding the generation of the non-polar fraction, all BHPs were then found to elute in the polar fraction as described above.

### 3.8.3. Addition of standard and derivatisation

An internal standard, 5α-pregnane-3β,20β-diol (Sigma Aldrich, UK), was added to each polar fraction resulting from SPE and bacterial cell TLE, which was not subjected to SPE. It was then dried under a stream of nitrogen at 50°C. Samples were derivatised prior to analysis. Pyridine/acetic anhydride (1:1 v/v; 500 µL) (Fisher Scientific, UK, and Sigma Aldrich, UK respectively) was added to an aliquot of extract and heated in a closed vial for 1 h at 50°C before being left overnight at room temperature. The

samples were then blown down to dryness under a stream of nitrogen at 50°C. The extract was dissolved in MeOH/2-propanol (60:40 v/v; 500 µL) (2-propanol, HiPerSolv grade, VWR, UK) sonicated and heated briefly and transferred to an LC-MS vial through a 0.20 µm PTFE filter (VWR, UK). This was repeated with an additional 500 µL of MeOH/2-propanol (60:40 v/v) to ensure all extract was transferred. The extract in the LC-MS vial was blown down under nitrogen and re-dissolved in MeOH/2-propanol (60:40 v/v; 500 µL) ready for analysis. Acetylation results in the addition of an acetyl group to hydroxyl and amine groups, but not all groups, in the BHP structure which makes them amenable to LC-MS analysis.

### 3.8.4. LC-MS analysis

Reversed-phase HPLC analysis of the acetylated BHPs was carried out using a slightly modified method to that described previously (Cooke et al., 2008a) using a Surveyor HPLC system (Thermo Finnigan, Hemel Hempstead, UK) fitted with a Phenomenex (Macclesfield, UK) Gemini $C_{18}$ 5 µm column (150 mm x 3.0 mm i.d.) and a Phenomenex security guard column of the same material. Separation was achieved at a flow rate of 0.5 mL/min at 30°C with the following gradient profile: 90% A and 10% B (0 min); 59% A, 1% B and 40% C (at 25 min), then isocratic (to 40 min) and returning to the starting conditions over 5 min and finally stabilising for 15 min before the next injection (A = MeOH, B = water and C = 2-propanol; HiPerSolv, HPLC grade, VWR, UK). A Thermo Finnigan LCQ ion trap mass spectrometer equipped with an atmospheric pressure chemical ionisation (APCI) source operated in positive ion mode was used to perform HPLC-MS. Settings were: capillary temperature 155°C, APCI vaporiser temperature 400°C, corona discharge current 8 µA, sheath gas flow 40 and auxiliary gas 10 (arbitrary units). Analysis was carried out in data-dependent mode with two scan events: SCAN 1 - full mass spectrum (*m/z* 300-1300) and SCAN 2 - data-dependent $MS^2$ spectrum of most abundant ion from SCAN 1. A semi-quantitative estimate of BHP concentrations was calculated from the characteristic base peak ion peak areas of individual BHPs in mass chromatograms (from SCAN 1), ($[M + H]^+$) for N containing BHPs and ($[M + H\text{-}CH_3COOH]^+$) for BHPs which do not contain a N atom, in comparison to the *m/z* 345 ($[M + H\text{-}CH_3COOH]^+$) base peak area response of the acetylated internal standard 5α-pregnane-3β,20β-diol. Relative response factors were determined from the average response of five acetylated BHP standards and were

used to adjust the peak areas of the BHPs relative to that of the internal standard: BHPs containing one or more N atoms give an averaged response ~12 times that of the standard and compounds with no N atoms give a response ~8 times that of the standard (van Winden et al., 2012c).

### 3.8.5. Statistical analysis

BHP concentrations resulting from microcosm treatments were compared statistically using one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) (Minitab 17 Statistical Software). Analysis was only performed when residuals were shown to be normally distributed using the Anderson-Darling test and equal variances were assumed from Levene's test.

## 3.9. Method development

The aim of method development was two-fold; to increase the concentration of BHPs extracted from the sediment and to shorten the existing method to allow greater throughput of samples without compromising extraction efficiency. Prior to method development, the modified Bligh and Dyer extraction method described in Section 3.8.1 was a longer two day method with the following modifications: for the first extraction, the samples were sonicated for 1 h at 40°C instead of 15 min and rotated on a wheel for 4 h, the second extraction the samples were sonicated for 1 h and then rotated on a wheel overnight, the third extraction the samples were sonicated for 1 h at 40°C rotated on a wheel for 4 h. There was no pre-mixing of the monophasic solvents. The mixture in the centrifuge tubes were sonicated for 1 h instead of 15 min.

### 3.9.1. Modified Bligh and Dyer extraction - improving extraction efficiency

The modified Bligh and Dyer extraction method often uses phosphate buffer in place of Milli-Q water and dichloromethane (DCM) (distilled in-house) in place of $CHCl_3$ (Sturt et al., 2004; Sáenz et al., 2011; Lengger et al., 2012). Modified Bligh and Dyer methods using different solvents (Table 3-3,b-d) were compared to the existing Bligh and Dyer method used (Table 3-3a). All extractions were performed using ~2 g of River Tyne estuarine sediment and were carried out in triplicate. The different treatments were carried out using the original two day extraction method.

**Table 3-3:** Sediment extractions performed using the modified Bligh and Dyer extraction method with different combinations of solvents.

| a | b | c | d |
|---|---|---|---|
| 4 mL Milli-Q $H_2O$<br>10 mL MeOH<br>5 mL $CHCl_3$ | 4 mL PB Milli-Q $H_2O$<br>10 mL MeOH<br>5 mL $CHCl_3$ | 4 mL Milli-Q $H_2O$<br>10 mL MeOH<br>5 mL DCM | 4 mL PB Milli-Q $H_2O$<br>10 mL MeOH<br>5 mL DCM |

PB = Phosphate buffered Milli-Q water (0.05 M $KH_2PO_4$ solution adjusted to pH 7.4 with NaOH pellets and then extracted with 3 x 50 mL DCM).

Total* BHP concentrations extracted from sediment when using different solvent treatments were compared (Figure 3-6).

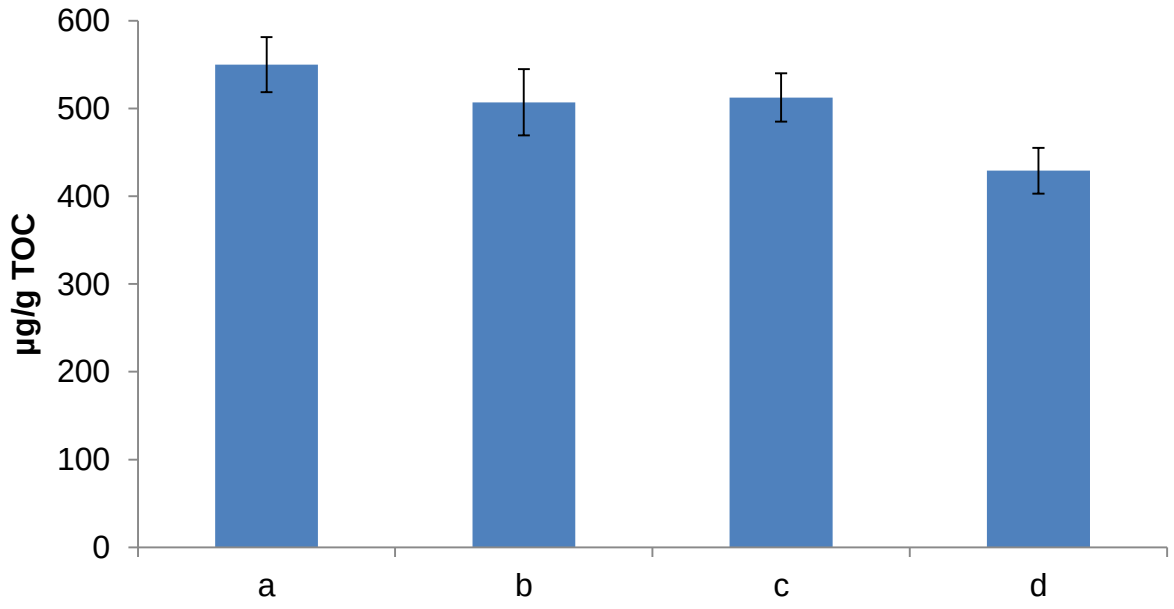



**Figure 3-6:** Total* concentration of BHP compounds (µg/g TOC) extracted from River Tyne estuarine sediment using different extractions treatments. x axis labels (a-d) refer to solvent treatments in Table 3-3. Error bars represent 1 x S.E. (N = 3).

* Total concentration of seven BHP compounds, BHT (**III**), 2-methylBHT (**IIIa**), aminotriol (**V**), aminopentol (**VII**), adenosylhopane (**VIII**), 'adenosylhopane-type 2' (**IX**), BHT cyclitol ether (**XII**) These seven compounds represent the majority of BHPs in all samples investigated (~80-85%).

BHP concentrations resulting from different solvent treatments were statistically compared using one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD).

The solvent treatments tested (Table 3-3,b-d) did not result in an increase in total BHPs extracted from the sediment (Figure 3-6) compared with the original Bligh and Dyer method (Table 3-3a). No significant difference was seen in the total BHP concentrations extracted for treatment 'a' compared with treatments 'b' ($p = 0.356$) and 'c' ($p = 0.418$). Treatment 'd' resulted in a significantly lower ($p = 0.025$) total BHP concentration compared with treatment 'a'.

BHT cyclitol ether (**XII**) concentrations extracted from sediment when using different solvent treatments were compared (Figure 3-7).

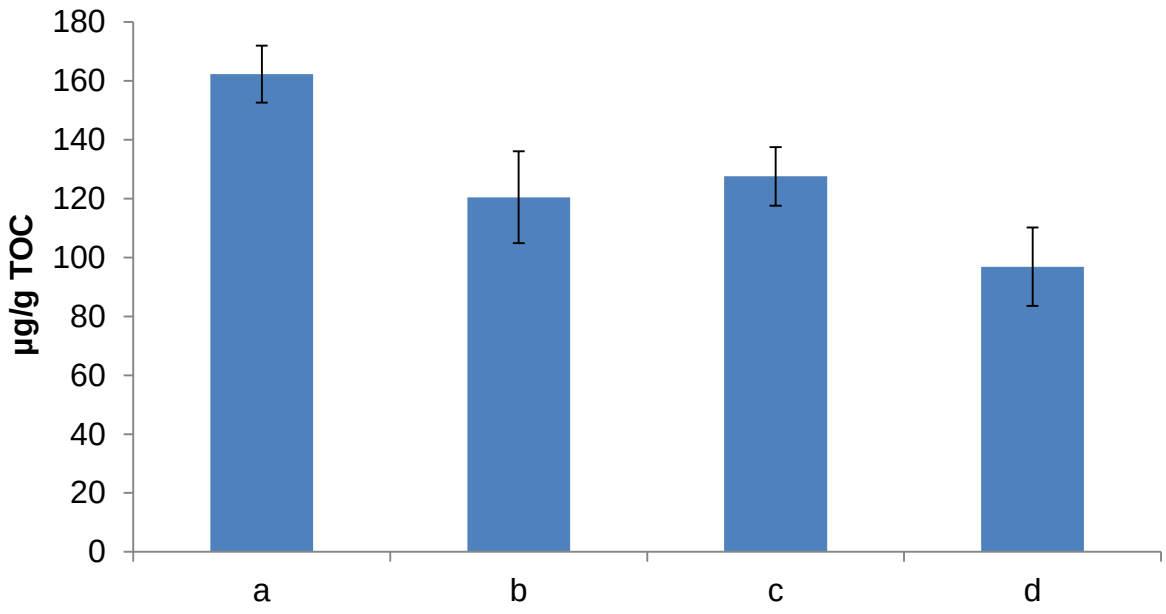



**Figure 3-7:** BHT cyclitol ether (**XII**) concentration (µg/g TOC) in River Tyne estuarine sediment using different extractions treatments. x axis labels (a-b) refer to solvent treatments in Table 3-3. Error bars represent 1 x S.E. (N = 3).

A significant difference was seen in BHT cyclitol ether (**XII**) concentrations extracted from sediment for treatment 'a' compared with treatments 'b' ($p = 0.044$) and 'd' ($p = 0.006$). Therefore, it suggests that phosphate buffered water and the combination of phosphate buffered water and DCM results in a lower concentration of BHT cyclitol ether (**XII**) extracted from sediment.

The preparation of phosphate buffered water was an additional time-consuming step and did not result in an increase in total BHPs extracted for treatment ‘b’ and ‘c’ and resulted in a reduction in total BHPs extracted for treatment ‘d’. Coupled with the effect phosphate buffered water had specifically on BHT cyclitol ether (**XII**), the existing Bligh and Dyer method (Table 3-3a) used for extractions continued to be used.

### 3.9.2. Reducing extraction time - increasing sample throughput

As previously mentioned (Section 3.9.1) the extraction method was a two day procedure. To increase throughput, the method was shortened (Table 3-4) and the effect this had on total BHP concentrations extracted from sediment was determined.

**Table 3-4:** Shortening modified Bligh and Dyer extraction method.

| a | b | c | d | e |
|---|---|---|---|---|
| 3 x 15 min sonication | 3 x 30 min sonication | 3 x 1 h sonication | 3 x 1 h sonication & overnight rotation | Original 2 day method (Section 3.8.1) |

The concentrations of total BHPs extracted from sediment using shortened methods was compared to that of the orignal two day method (Figure 3-8).

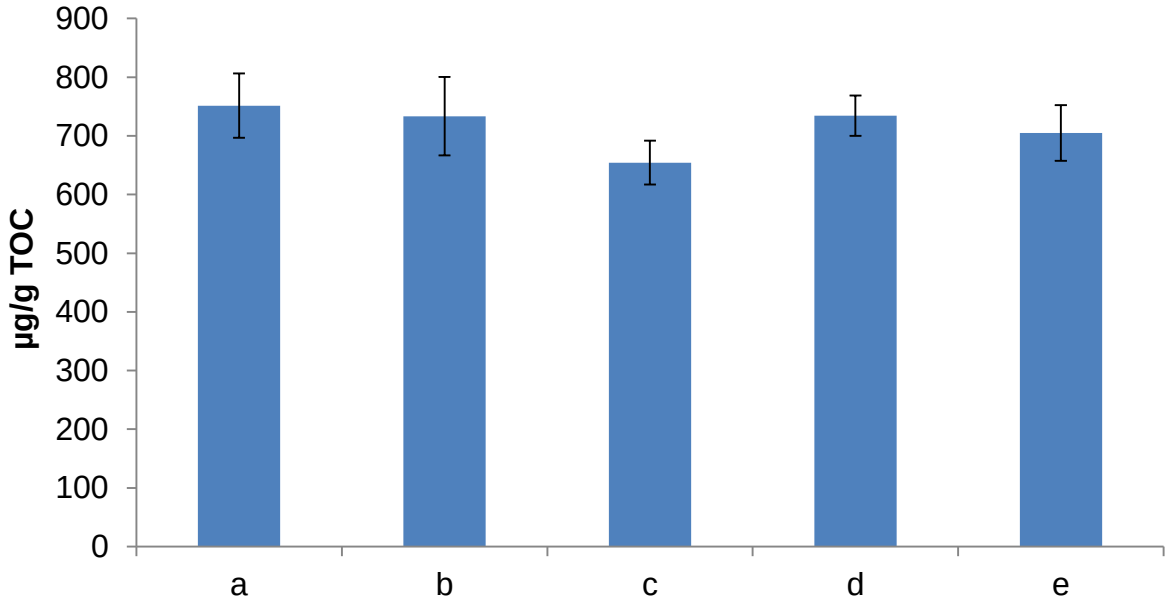



**Figure 3-8:** Total* concentration of BHP compounds (µg/g TOC) extracted from River Tyne estuarine sediment after different time periods. x axis labels (a-e) refer to solvent treatments in Table 3-4. Error bars represent 1 x S.E. (N = 3).

* Total concentration of seven BHP compounds, BHT (**III**), 2-methylBHT (**IIIa**), aminotriol (**V**), aminopentol (**VII**), adenosylhopane (**VIII**), 'adenosylhopane-type 2' (**IX**), BHT cyclitol ether (**XII**). These seven compounds represent the majority of BHPs in all samples investigated (~80-85%).

No significant difference ($p \geq 0.488$) was seen between the total BHP concentrations extracted (Figure 3-8) with the shortened methods (Table 3-4,a-d) compared with the original two day method (Table 3-4e). Based on this evidence, the shortest extraction method (Table 3-4a) was adopted for all microcosm sediment extractions as this increased sample throughput whilst having no effect on BHP extraction efficiency.

# 4. BHP signatures of marine methanotroph cultures

## 4.1. Introduction

The BHP compositions of a number of methanotroph cultures have been reported and there are marked differences in abundances of compounds between Type I and Type II methanotrophs and different genera within these groups (e.g. Talbot et al., 2001). To date, less than a third of known Type I methanotroph genera have had their BHPs compositions elucidated for any member species (Chapter 2, Figure 1-2). The number is greater for Type II methanotrophs but the number of different genera is significantly lower. The hexafunctionalised 'aminopentols', which include the methylated and/or unsaturated equivalents, are the most abundant BHPs in the Type I genera *Methylococcus*, *Methylomonas* and *Methylocaldum* (Neunlist and Rohmer, 1985b; Cvejic et al., 2000a; Talbot et al., 2001; van Winden et al., 2012c). The most abundant BHP in the *Methylovulum*-like strain M200, also a Type I, is aminotriol (**V**) and this was the first confirmed example where aminopentol (**VII**) was not the most abundant BHP in Type I methanotrophs (van Winden et al., 2012c). An earlier reported example of a Type I methanotroph where aminopentol (**VII**) was not the most abundant BHP, and actually entirely absent, was *Methylomicrobium album* (Talbot et al., 2001). At the time, this was presumed to be a contaminated culture but recent evidence seems to confirm its absence (Birgel et al., unpublished data). Type I methanotrophs also produce varying amounts of aminotetrol (**VI**) depending on the genus (Neunlist and Rohmer, 1985b; Cvejic et al., 2000a; Talbot et al., 2003a; van Winden et al., 2012c). Aminotriol (**V**) is the most abundant BHP in the majority of screened species of the Type II methanotrophs, *Methylosinus*, *Methylocella* and *Methylocystis* (Neunlist and Rohmer, 1985a; Talbot et al., 2001; van Winden et al., 2012c), but in a few species aminotetrol (**VI**) is the most abundant BHP; this compound is present in all tested Type II genera apart from *Methylocella* (Neunlist and Rohmer, 1985a; Cvejic et al., 2000a; van Winden et al., 2012c).

The majority of Type I methanotrophs have not had their BHPs elucidated and until recently, methanotrophs isolated from truly marine environments had not been examined. As previously alluded to, aminopentol (**VII**) was absent in marine *Methylomicrobium* spp. (Talbot et al., 2001; Birgel et al., unpublished data), a genus of Type I methanotrophs frequently found in a range of marine environments (Sieburth

et al., 1987; Lidstrom, 1988; Bowman et al., 1993; Kalyuzhnaya et al., 2008). Therefore, it is important to determine whether aminopentol (**VII**), seen as a diagnostic marker for Type I methanotrophs, is absent from all methanotrophs isolated from marine environments or is specific to *Methylomicrobium* spp. This knowledge will have implications for the use of aminopentol (**VII**) as a biomarker to trace aerobic methane oxidation (AMO) in modern and paleoenvironments in marine environments as *Methylomicrobium* spp.

## 4.2. Aims and hypotheses

### 4.2.1. Aims

This chapter describes the distribution and composition of BHPs in six strains of previously unscreened methanotrophic bacteria isolated from marine environments. The six strains investigated belong to three Type I genera, *Methylobacter* (*Methylobacter marinus* A45, *Methylobacter* sp. BBA5.1 and *Methylobacter* sp. BB5.1), *Methylomarinum* (*Methylomarinum vadi* IT-4) and *Methylomarinovum* (*Methylomarinovum caldicuralii* IT-9 and *Methylomarinovum* sp. IN45).

This chapter has three aims:

(1) to ascertain the composition of BHPs present in marine methanotroph cultures.

(2) to determine the relative abundances of different BHPs within the suite of methanotrophs.

(3) to determine differences between species of the same genus.

### 4.2.2. Hypotheses

- *Aminopentol will be the most abundant BHP in the suite of Type I methanotrophs.*

Generally, the most abundant BHP in Type I methanotrophs is aminopentol (**VII**) (e.g. Rohmer et al., 1984; Neunlist and Rohmer, 1985b; Jahnke et al., 1999; Cvejic et al., 2000; Talbot et al., 2001; van Winden et al., 2012c). The three untested genera of methanotrophs belong to the Type I group, therefore, it is expected that this will be the most abundant compound.

- *Species of the same genus will have similar BHP compositions.*

Data from the literature generally shows that the relative abundance of tetra-, penta- and hexafunctionalised compounds present in different species of the same genera are similar (e.g. Rohmer et al., 1984; Neunlist and Rohmer, 1985b; Talbot et al., 2001; van Winden et al., 2012c).

## 4.3. Materials and methods

### 4.3.1. Isolation and enrichment of *Methylobacter* spp., *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp.

The isolation and enrichment of the six strains examined, *Methylobacter marinus* A45, *Methylobacter* sp. BBA5.1, *Methylobacter* sp. BB5.1, *Methylomarinum vadi* IT-4, *Methylomarinovum caldicuralii* IT-9 and *Methylomarinovum* sp. IN45 are described (Chapter 3, Section 3.5.1 and Section 3.5.2).

Bacterial cell material was freeze-dried (Chapter 3, Section 3.5.4) and BHPs were extracted from the cell material using a modified Bligh and Dyer extraction method (Chapter 3, Section 3.8.1). Total lipid extracts were prepared and then analysed using LC-MS (Chapter 3, Section 3.8.2 and Section 3.8.4).

## 4.4. Results

### 4.4.1. Marine methanotrophs - a suite of novel compounds

In addition to aminotriol (**V**) aminotetrol (**VI**) and aminopentol (**VII**), the six strains of marine methanotrophs analysed contained a suite of novel compounds. These compounds were related to the 35-aminoBHPs, aminotriol (**V**, *m/z* = 714), aminotetrol (**VI**, *m/z* = 772) and aminopentol (**VII**, *m/z* = 830), but with one suite having a different C-35 terminal group with a peracetylated $[M+H]^+$ 16 Da higher and the other suite with a peracetylated $[M+H]^+$ 14 Da lower compared to the aforementioned compounds (summarised in Table 4-1). They were found in all six strains of methanotroph analysed although not all members of the suite were present in every strain (Figure 4-7 and Figure 4-9).

#### 4.4.1.1. 'Carbamylmethylestertriol', 'carbamylmethylestertetrol' and 'carbamylmethylesterpentol'

Tentatively named '35-carbamylmethylester-bacteriohopane-32,33,34-triol' (**V**$^{\mathbf{MeCarb}}$; carbamylmethylestertriol herein), found in all six strains, has a base peak ion $[M+H]^+$ of *m/z* 730 which indicates the presence of an odd number of N atoms in the molecule. The loss of three acetylated hydroxyl groups, $CH_3COOH$ (= 60 Da) to give ion fragments of *m/z* 670, 610, 550 (Figure 4-1b) was observed in the $MS^2$ spectrum of this compound as also observed for aminotriol (**V**) (Figure 4-1a; cf. Talbot et al., 2003). Further structural information was obtained from the $MS^3$ spectrum from fragmentation of the *m/z* 670 from $MS^2$ (Figure 4-3c). The ion fragments of *m/z* 568 and 628 (fragments labelled in pink) indicate the loss of $COCH_2$ from partial loss of ketene from the two remaining acetylated hydroxyls (Figure 4-1c). The ion fragment of *m/z* 638 (fragment labelled green) indicates neutral loss of $CH_3OH$ (= 32 Da) and is interpreted as indicating part of the terminal group. This also confirms that the fourth functional group present in this molecule is not a regular acetate derivative (either -OH or $-NH_2$) as this mass loss has not been observed in previously investigated peracetylated BHPs. The ion *m/*z 475 (fragment labelled blue) indicates the neutral loss of 75 Da (after loss of all three regular acetylated hydroxyls) and indicates the full terminal group with the proposed structure "$-NHCOOCH_3$" which allows for the initial loss of 32 Da (Figure 4-1c). In comparison, the neutral loss of the acetylated terminal group in aminotriol (**V**) ($NH_2COCH_3$, 59 Da) results in an ion *m/z* 475, not visible in the spectrum (Figure 4-1a), but has been previously observed in the $MS^3$ spectrum. The ion fragment of *m/z* 191 is a common ion in BHP mass spectra indicating the A+B rings. An additional suite of ions resulting from neutral loss of the A+B ring fragment are indicated in purple (Figure 4-3c), each representing loss of different numbers of full or partial functional groups from the side chain.

Tentatively named '35-carbamylmethylester-bacteriohopane-31,32,33,34-tetrol' (**VI**$^{\mathbf{MeCarb}}$; carbamylmethylestertetrol herein), found in all six strains, has a base peak ion of *m/z* 788. The loss of four acetylated hydroxyl groups, $CH_3COOH$, akin to that of aminotetrol (**VI**) (Figure 4-2a), gives the ion fragments of *m/z* 728, 668, 608, 548 (Figure 4-2b). The ion fragment of *m/z* 686 indicates the loss of $COCH_2$ from partial loss of ketene from the three remaining acetylated hydroxyls (Figure 4-2c). There is also a fragment of *m/z* 696 which indicates the partial loss of the terminal group,

$CH_3OH$, although this is significantly less intense than the equivalent ion in 'carbamylmethylestertriol'(**V**$^{\mathbf{MeCarb}}$). The ion fragment of *m/*z 473 indicates the neutral loss of 75 Da, the full terminal group "$NH_2COOCH_3$".

Tentatively named '35-carbamylmethylester-bacteriohopane-30,31,32,33,34-pentol' (**VII**$^{\mathbf{MeCarb}}$; carbamylmethylesterpentol herein), found in all six strains, has a base peak ion of *m/z* 846. The loss of five acetylated hydroxyl groups, $CH_3COOH$, akin to that of aminopentol (**VII**) (Figure 4-3a), gives ion fragments of *m/z* 786, 726, 666, 606, 546 (Figure 4-3b). The ion fragment of *m/z* 744 indicates the loss $COCH_2$ (Figure 4-3c). There is also a fragment at *m/z* 754, visible after zooming in (blue box), which indicates the partial loss of the terminal group, $CH_3OH$. The ion fragment of *m/*z 471 indicates the neutral loss of 75 Da which is the full terminal group "$NH_2COOCH_3$".

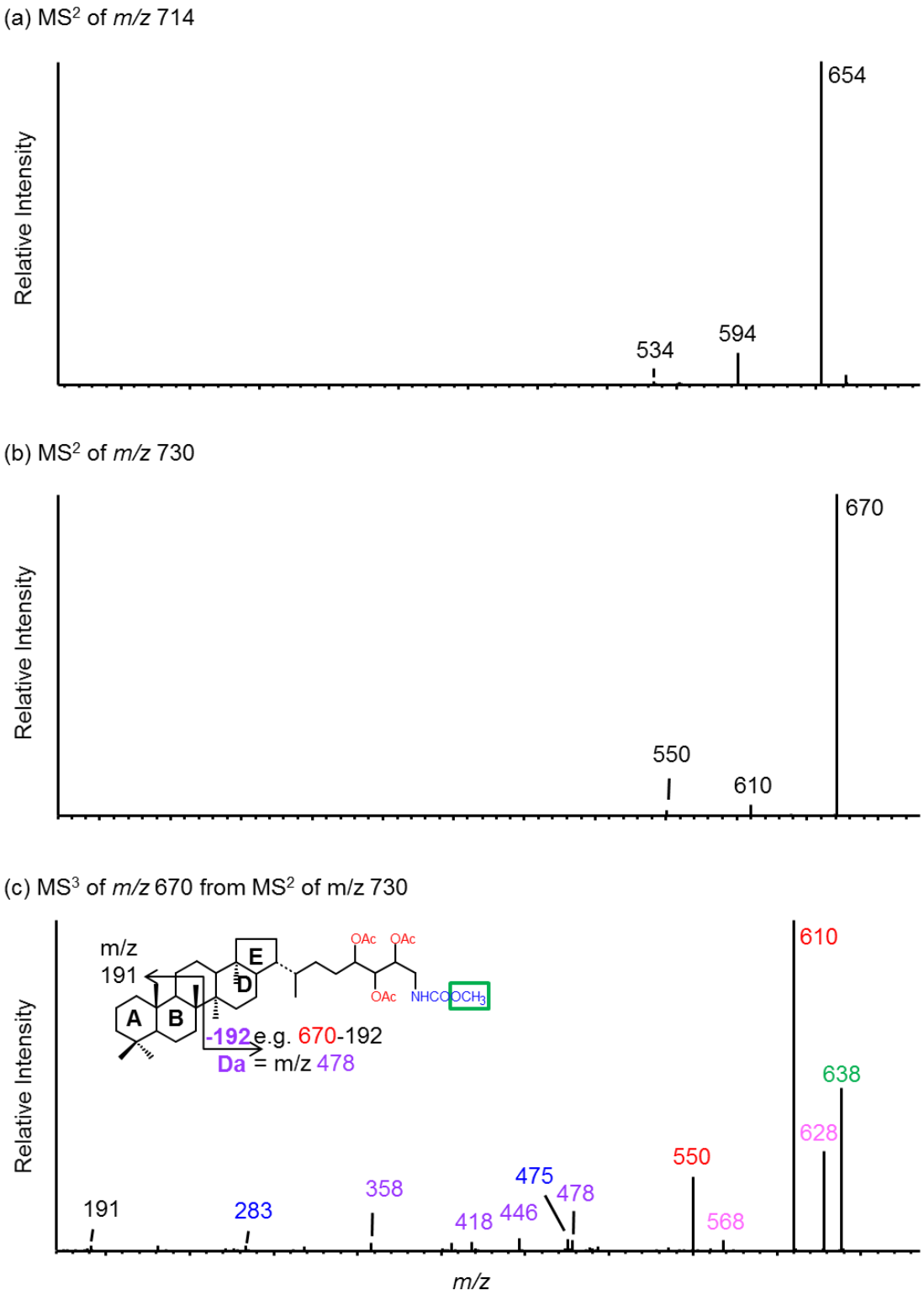



**Figure 4-1:** APCI $MS^2$ spectra from acetylated total extract of *Methylomarinum vadi* IT-4 of (a) aminotriol (**V**), *m/z* 714 (b) 'carbamylmethylestertriol' ($\mathbf{V}^{\mathbf{MeCarb}}$), *m/z* 730 and the $MS^3$ spectrum of *m/z* 670 (c) 'carbamylmethylestertriol' ($\mathbf{V}^{\mathbf{MeCarb}}$), (Ac=$COCH_3$).

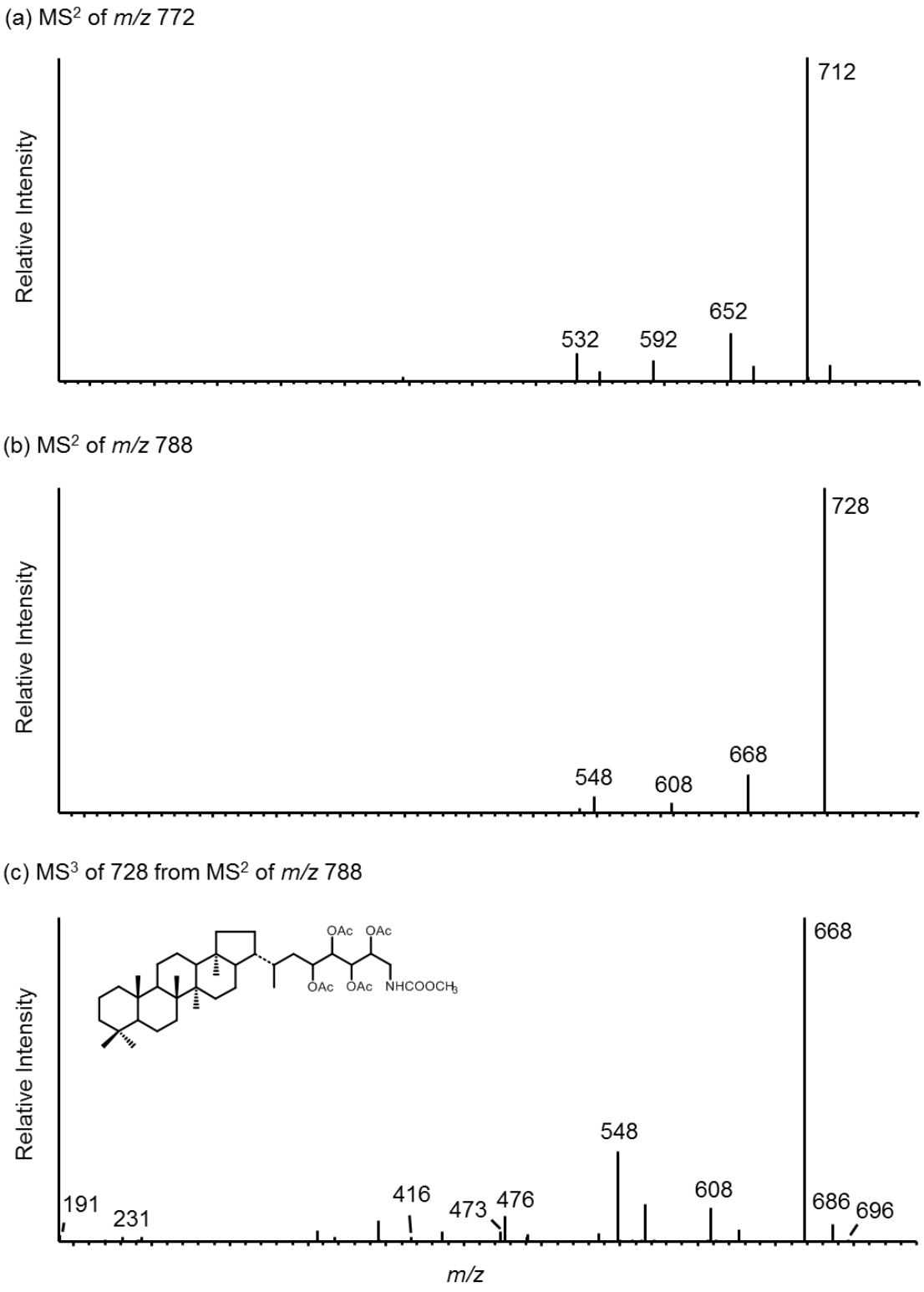



**Figure 4-2:** APCI $MS^2$ spectra of the acetylated total extract of *Methylomarinovum* sp. IN45 (a) aminotetrol (**VI**), *m/z* 772 (b) 'carbamylmethylestertetrol' (**VI$^{MeCarb}$**), *m/z* 788 and the $MS^3$ spectrum of (c) 'carbamylmethylestertetrol' (**VI$^{MeCarb}$**), (Ac=$COCH_3$).

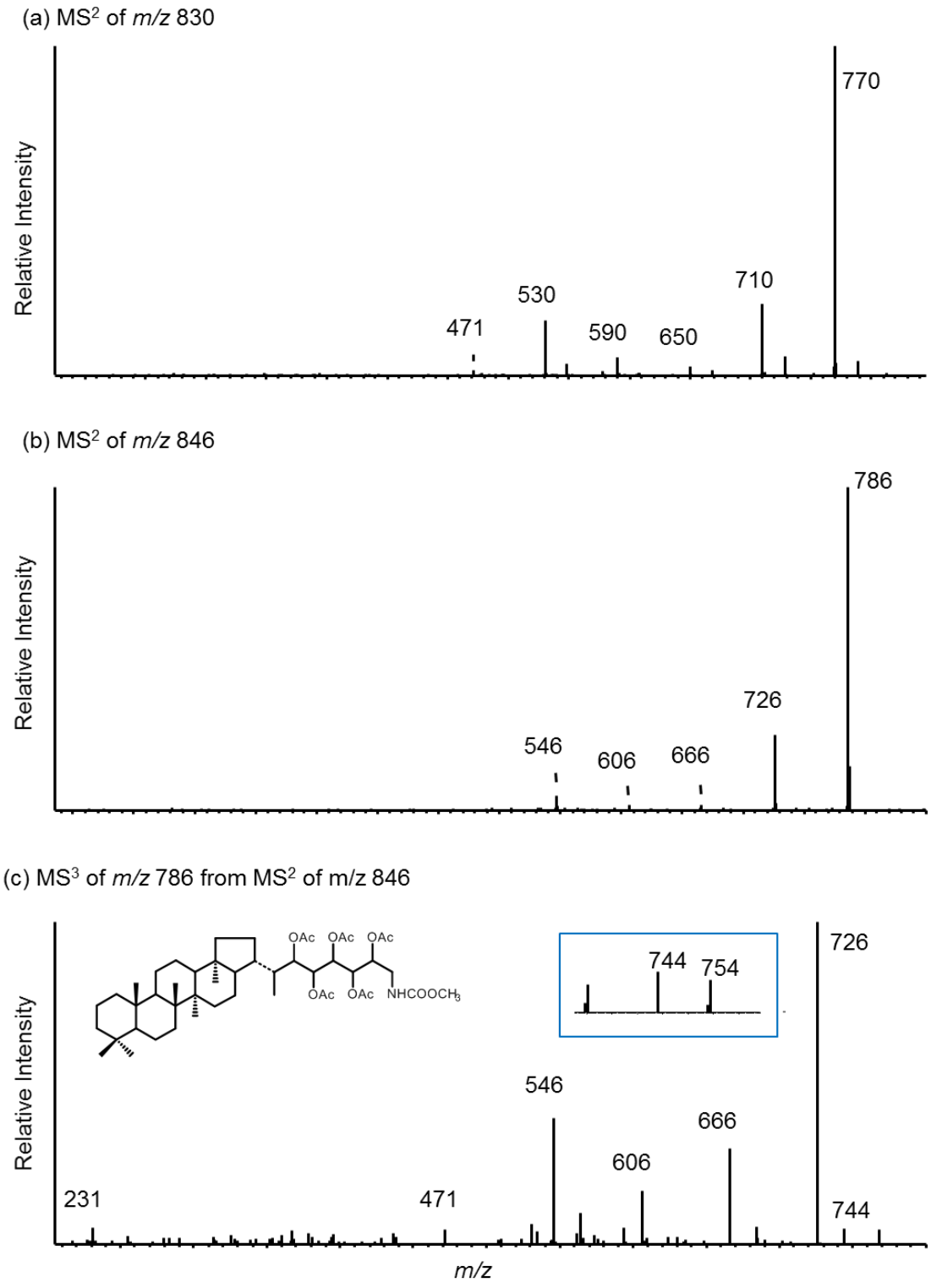



**Figure 4-3:** APCI $MS^2$ spectra of the acetylated total extract of *Methylobacter marinus* A45 of (a) aminopentol (**VII**), *m/z* 830 (b) 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**), *m/z* 846 and the $MS^3$ spectrum of (c) 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**), (Ac=$COCH_3$).

The compound related to ‘aminopentol isomer’ (**VII**$^{iso}$) (related to aminopentol (**VII**) but structure unknown, see Section 4.5.1.2) but with a peracetylated $[M+H]^+$ 16 Da higher, ‘carbamylmethylesterpentol isomer’ (**VII**$^{iso,MeCarb}$) herein, has a base peak ion of *m/z* 804 with the loss of five acetylated hydroxyl groups akin to that of ‘aminopentol isomer’ (**VII**$^{iso}$) (Figure 4-4a) to give ion fragments of *m/z* 786, 744, 726, 564, 546 (Figure 4-4b).

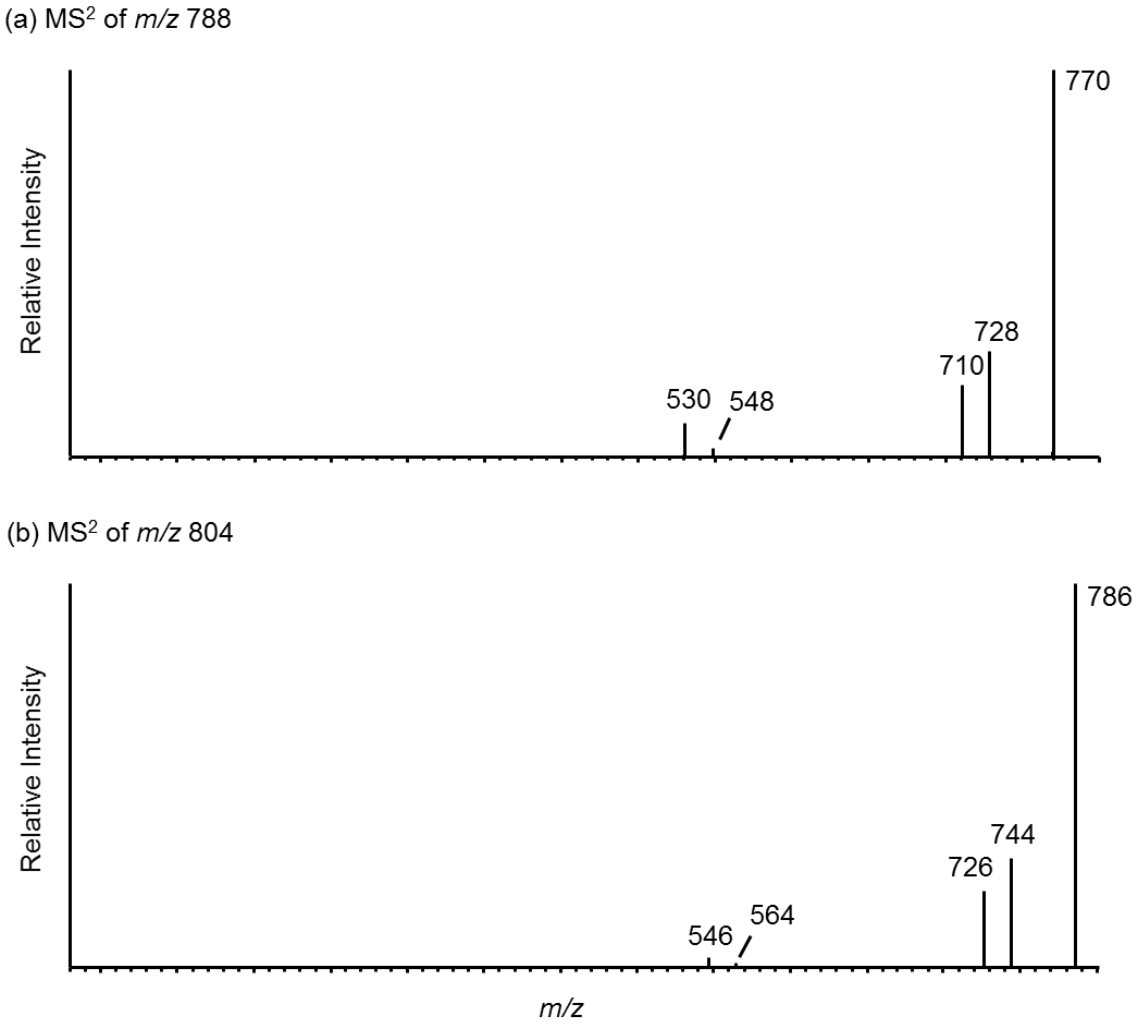



**Figure 4-4:** APCI $MS^2$ spectra of the acetylated total extract of *Methylomarinum vadi* IT-4 (a) ‘aminopentol isomer’ (**VII**$^{iso}$), *m/z* 770 and (b) ‘carbamylmethylesterpentol isomer’ (**VII**$^{iso,MeCarb}$), *m/z* 804.

#### 4.4.1.2. ‘Desmethylaminotriol’, ‘desmethylaminotetrol’ and ‘desmethylaminopentol’

Tentatively named ‘desmethylaminotriol’ (**V**$^{\mathbf{des}}$), found in all strains apart from *Methylobacter* sp. BB5.1 and *Methylomarinovum caldicuralii* IT-9, has a base peak ion of *m/z* 700 with the loss of acetylated hydroxyl groups as observed for aminotriol (**V**) to give ion fragments of *m/z* 640, 580, 520 (Figure 4-5b). The *m/z* of 700 suggests a compound with a structure similar to that of aminotriol (**V**) but with one of the methyl groups on the ring system being absent. The ion fragment of *m/z* 478 (indicated in purple) results from the regular neutral loss of the A+B ring fragment (i.e. loss of 192 Da; Figure 4-5c). This suggests the methyl group is absent from somewhere other than the A+B ring.

Tentatively named ‘desmethylaminotetrol’ (**VI**$^{\mathbf{des}}$), found in all six strains, was identified based on relative retention times witnessed for other members of the suite i.e. retention time differences between aminotriol (**V**) and ‘desmethylaminotriol’ (**V**$^{\mathbf{des}}$) and aminopentol (**VII**) and ‘desmethylaminopentol’ (**VII**$^{\mathbf{des}}$).

Tentatively named ‘desmethylaminopentol’ (**VII**$^{\mathbf{des}}$), found in all six strains, has a base peak ion *m/z* 816 with the loss of acetylated hydroxyl groups (AcOH) akin to that aminopentol (**VII**) (Figure 4-6a) to give ion fragments of *m/z* 756, 696, 636, 576, 516 (Figure 4-6b). The *m/z* of 816 suggests a compound with a structure similar to that of aminopentol (**VII**) but with one of the methyl groups on the ring system being absent. The ion fragment of *m/z* 564 results from the neutral loss of the A+B ring fragment. Once again, this suggests the methyl group is absent from somewhere other than the A+B ring.

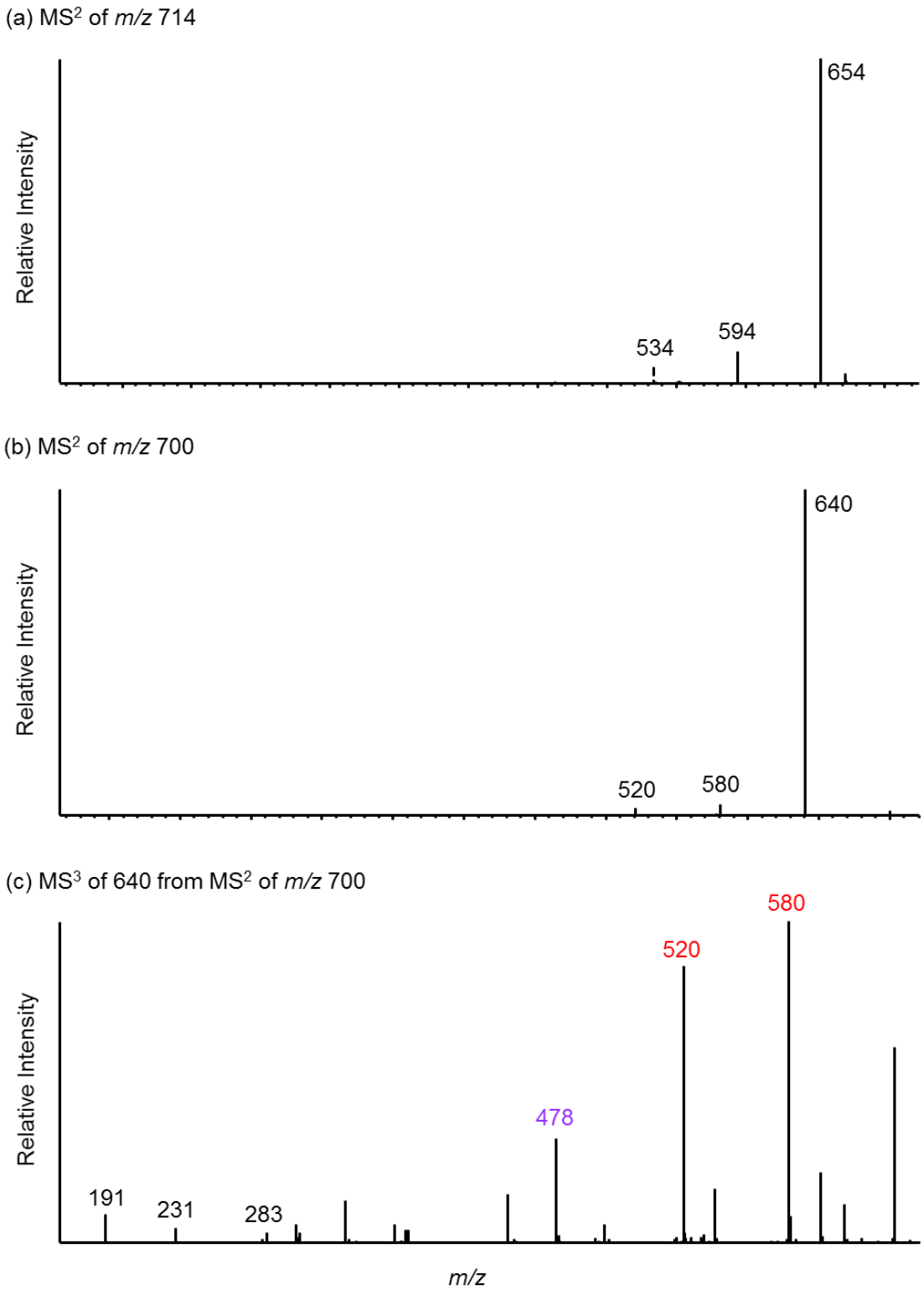



**Figure 4-5:** APCI $MS^2$ spectra of the acetylated total extract of *Methylomarinum vadi* IT-4 of (a) aminotriol (**V**), *m/z* 714 (b) 'desmethylaminotriol' ($\mathbf{V^{des}}$), *m/z* 700 and the $MS^3$ spectrum of (c) 'desmethylaminotriol' ($\mathbf{V^{des}}$).

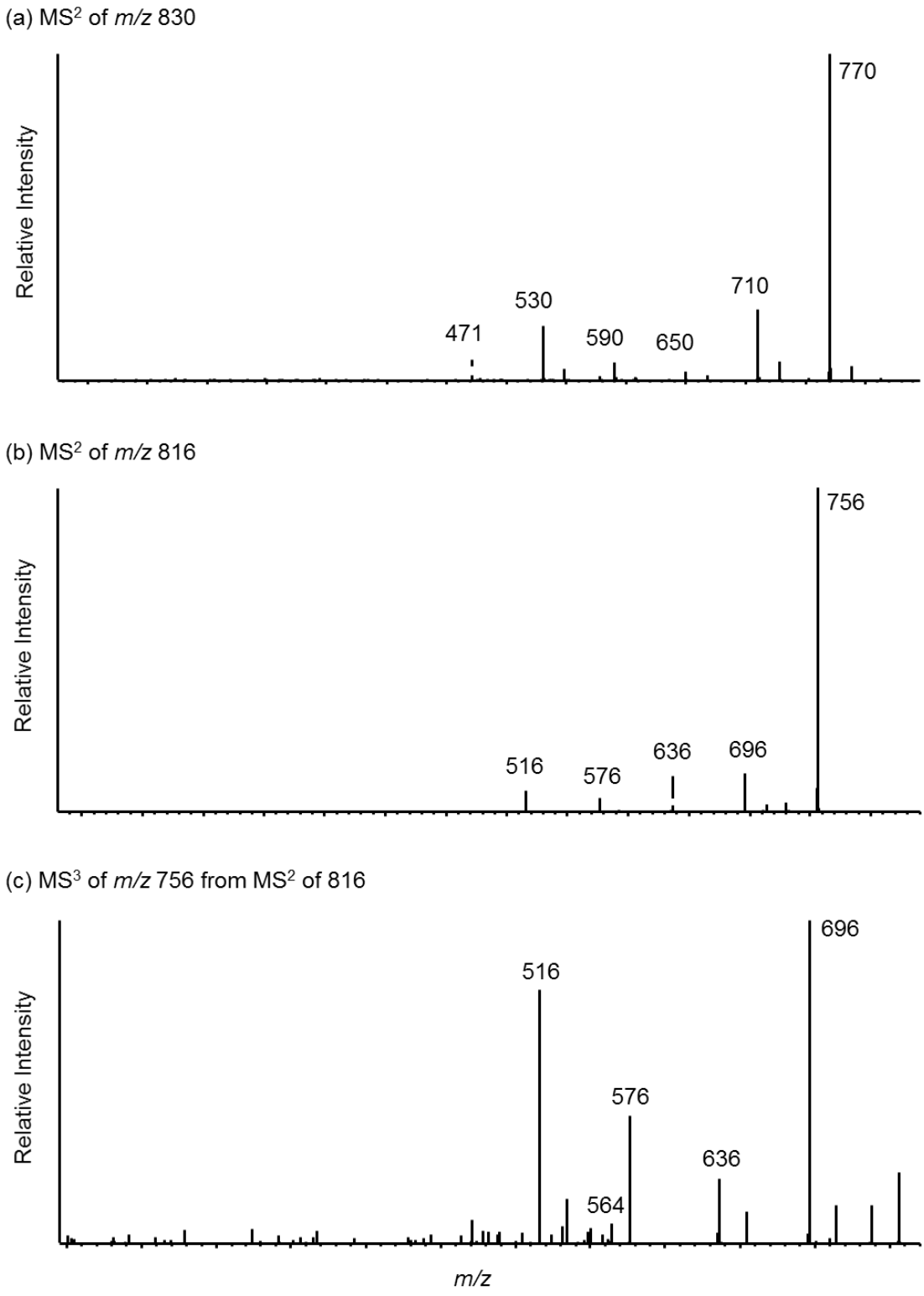



**Figure 4-6:** APCI $MS^2$ spectra of the acetylated total extract of *Methylomarinum vadi* IT-4 of (a) aminopentol (**VII**), *m/z* 830 (b) 'desmethylaminopentol' (**VII$^{des}$**), *m/z* 816 and the $MS^3$ spectrum of (c) 'desmethylaminopentol' (**VII$^{des}$**).

**Table 4-1:** Summary of the fragmentation patterns of 'carbamylmethylester' and 'desmethyl' compounds related to aminotriol (**V**), aminotetrol (**VI**) and aminopentol (**VII**).

| Ion | 'Aminotriols' | | | 'Aminotetrols' | | | 'Aminopentols' | | |
|---|---|---|---|---|---|---|---|---|---|
| | **$V^{des}$** | **V** | **$V^{MeCarb}$** | **$VI^{des}$** | **VI** | **$VI^{MeCarb}$** | **$VII^{des}$** | **VII** | **$VII^{MeCarb}$** |
| $[M+H]^+$ | **700** | **714** | **730** | 758 | **772** | **788** | **816** | **830** | **846** |
| $[M+H-COCH_2]^+$ | | *672* | | | *730* | | | **788** | |
| $[M+H-CH_3COOH]^+$ | **640** | **654** | **670** | 698 | **712** | **728** | **756** | **770** | **786** |
| $[M+H-CH_3COOH-CH_3OH]^+$ | | | **638** | | | **696** | | | **754** |
| $[M+H-CH_3COOH-COCH_2]^+$ | | *612* | **628** | | *670* | **686** | | *728* | **744** |
| $[M+H-2CH_3COOH]^+$ | **580** | **594** | **610** | 638 | **652** | **668** | **696** | **710** | **726** |
| $[M+H-2CH_3COOH-COCH_2]^+$ | | *552* | **568** | | *610* | | | *668* | |
| $[M+H-3CH_3COOH]^+$ | **520** | **534** | **550** | 578 | **592** | **628** | | **650** | **666** |
| $[M+H-3CH_3COOH-COCH_2]^+$ | | *492* | | | *550* | | | *608* | |
| $[M+2H-3CH_3COOH-COCH_2]^+$ | | *493* | | | | | | | |
| $[M+H-3CH_3COOH-CH_3CONH_2]^+$ | | *475* | | | | | | | |
| $[M+H-3CH_3COOH-CH_3COONH_2]^+$ | | | **475** | | | | | | |
| $[M+H-4CH_3COOH]^+$ | | | | | **532** | **548** | | **590** | **606** |
| $[M+H-4CH_3COOH-COCH_2]^+$ | | | | | *490* | | | *548* | |
| $[M+2H-4CH_3COOH-COCH_2]^+$ | | | | | *491* | | | | |
| $[M+H-4CH_3COOH-CH_3CONH_2]^+$ | | | | | *473* | | | | |
| $[M+H-4CH_3COOH-CH_3COONH_2]^+$ | | | | | | **473** | | | |
| $[M+H-5CH_3COOH]^+$ | | | | | | | | **530** | **546** |
| $[M+2H-5CH_3COOH-COCH_2]^+$ | | | | | | | | *489* | |
| $[M+H-5CH_3COOH-CH_3CONH_2]^+$ | | | | | | | | **471** | |
| $[M+H-5CH_3COOH-CH_3COONH_2]^+$ | | | | | | | | | **471** |

Bold text = observed ions; italics = expected ion, not observed (cf. Talbot et al., 2003b); normal text = predicted ions.

### 4.4.2. BHP inventory of *Methylobacter* spp., *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp.

Six previously untested methanotrophs isolated from marine environments and belonging to the genera *Methylobacter*, *Methylomarinum* and *Methylomarinovum* were analysed for their BHP composition. The suite of 'carbamylmethylester' and 'desmethyl' compounds (Section 4.4.1.1 and Section 4.4.1.2) and their elution relative to the 'regular' 35-aminoBHPs are shown on partial mass chromatograms of the acetylated total extracts (Figure 4-7 and Figure 4-9). The relative abundances of BHPs are indicated as the % of total BHPs in acetylated extracts are also shown (Figure 4-8 and Figure 4-10) with associated quantitative data, where available, for individual BHPs (appendix 3a).

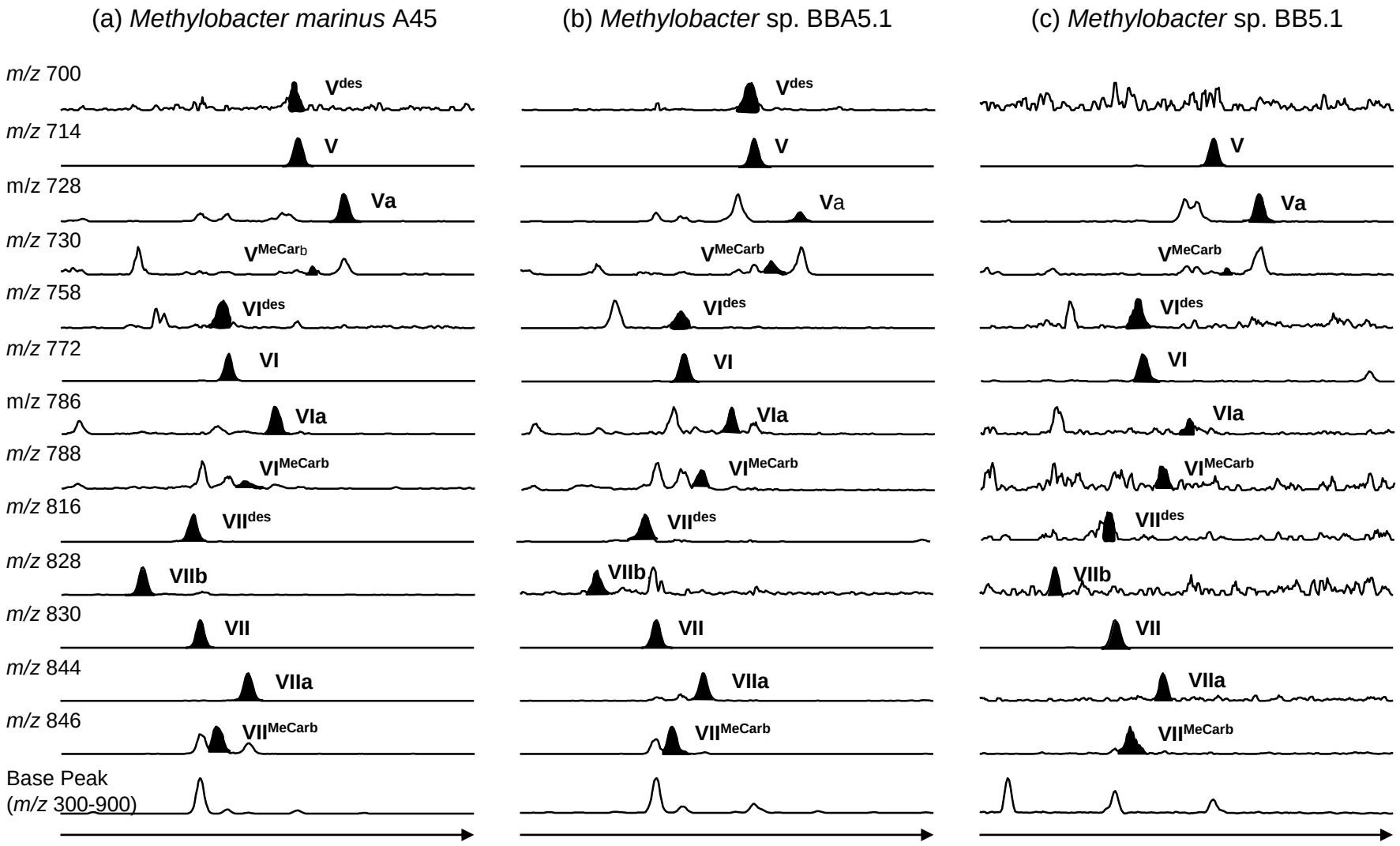



**Figure 4-7:** Partial mass chromatograms (16-28 min) showing BHPs in the acetylated total extracts of *Methylobacter* spp.

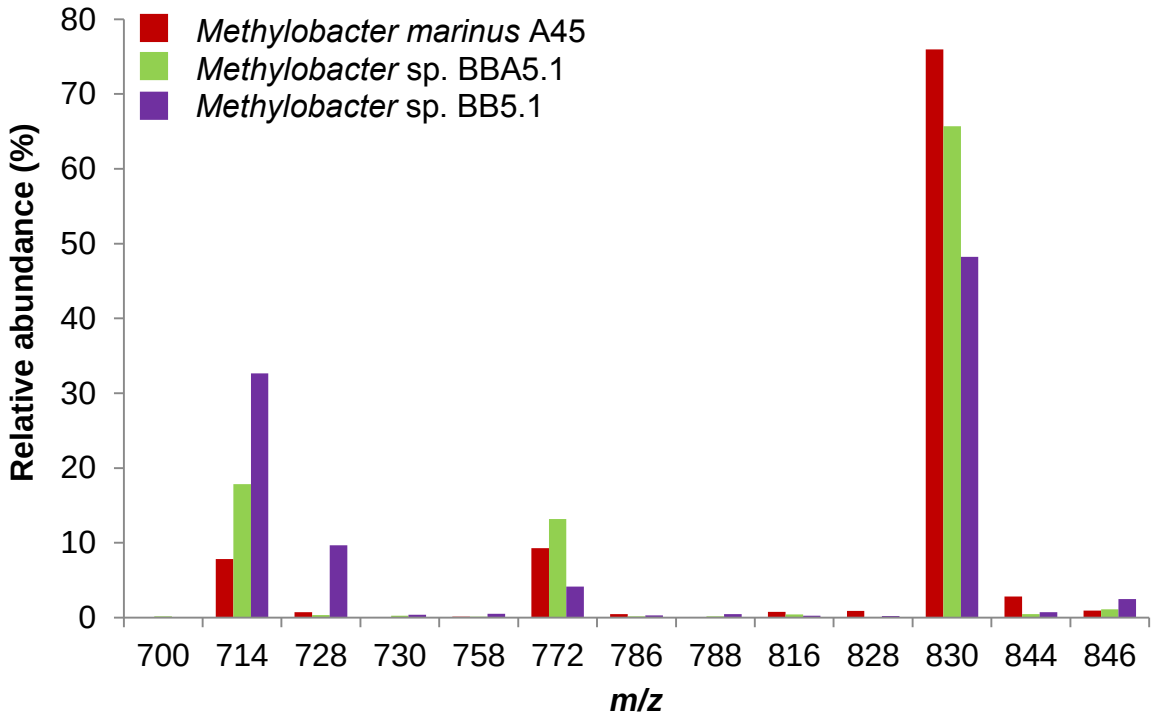



**Figure 4-8:** Relative abundances of BHPs as % of total in the acetylated total extracts of *Methylobacter* spp.

For all three *Methylobacter* strains, aminopentol (**VII**, *m/z* = 830) was the most abundant BHP; *Methylobacter marinus* A45 - 76%, *Methylobacter* sp. BBA5.1 - 66% and *Methylobacter* sp. BB5.1 - 48%. This was accompanied by lower abundances of aminotetrol (**VI**, *m/z* = 772), 9.3%, 13% and 4.2% respectively and aminotriol (**V**, *m/z* = 714), 7.8%, 18% and 33% respectively (Figure 4-8). C-3 methylated equivalents of aminotriol (**Va**, *m/z* = 728), aminotetrol (**VIa**, *m/z* = 786) and aminopentol (**VIIa**, *m/z* = 844) were present in all three species albeit in trace amounts apart from 3-methylaminotriol (**Va**) in *Methylobacter* sp. BB5.1 which was in greater abundance (9.7%). Trace levels of unsaturated $\Delta^{11}$ aminopentol (**VIIb**, *m/z* = 828) were found in all three species. The 'carbamylmethylester' compounds related to aminotriol (**V**, *m/z* = 714), aminotetrol (**VI**, *m/z* = 772) and aminopentol (**VII**, *m/z* = 830) but with a peracetylated $[M+H]^+$ 16 Da higher (*m/z* = 730, 788 and 846 respectively) were detected in all three *Methylobacter* strains in low amounts. They elute just after their 'regular' compounds due to the less polar nature of the suggested terminal group (Figure 4-7). These 'carbamylmethylester' compounds fragment in the same manner to give the same ion fragment losses but $[M+H]^+$ 16 Da higher (Figure 4-1 to Figure 4-4). An additional suite of 'desmethyl' compounds related to aminotriol (**V**, *m/z* = 714),

aminotetrol (**VI**, *m/z* = 772) and aminopentol (**VII**, *m/z* = 830) but with a peracetylated $[M+H]^+$ 14 Da lower (*m/z* = 700, 758 and 816 respectively) were present in trace amounts.

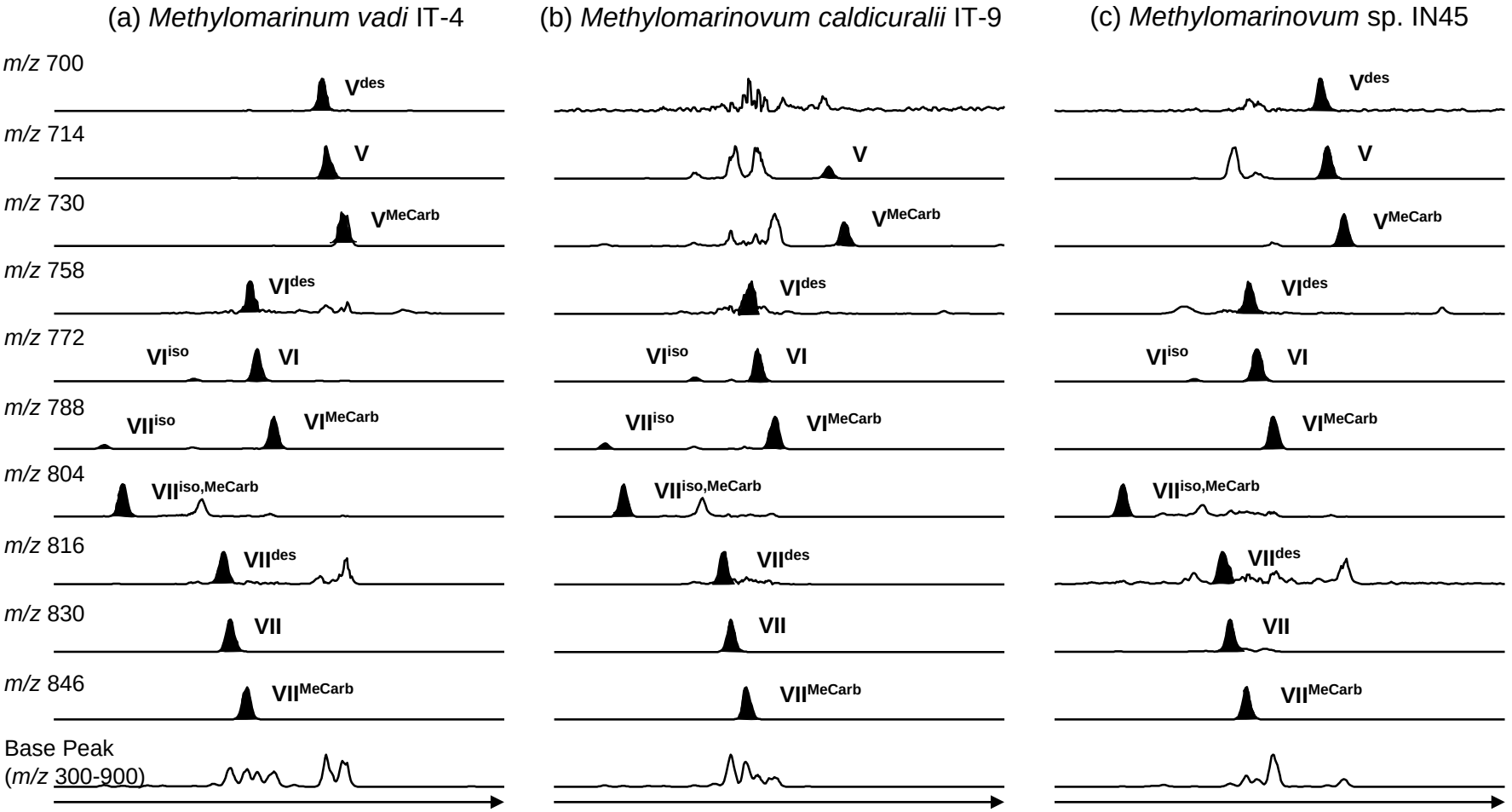



**Figure 4-9:** Partial mass chromatograms (15-28 min) showing BHPs in the acetylated total extracts of (a) *Methylomarinum vadi* IT-4, (b) *Methylomarinovum caldicuralii* IT-9 and (c) *Methylomarinovum* sp. IN45.

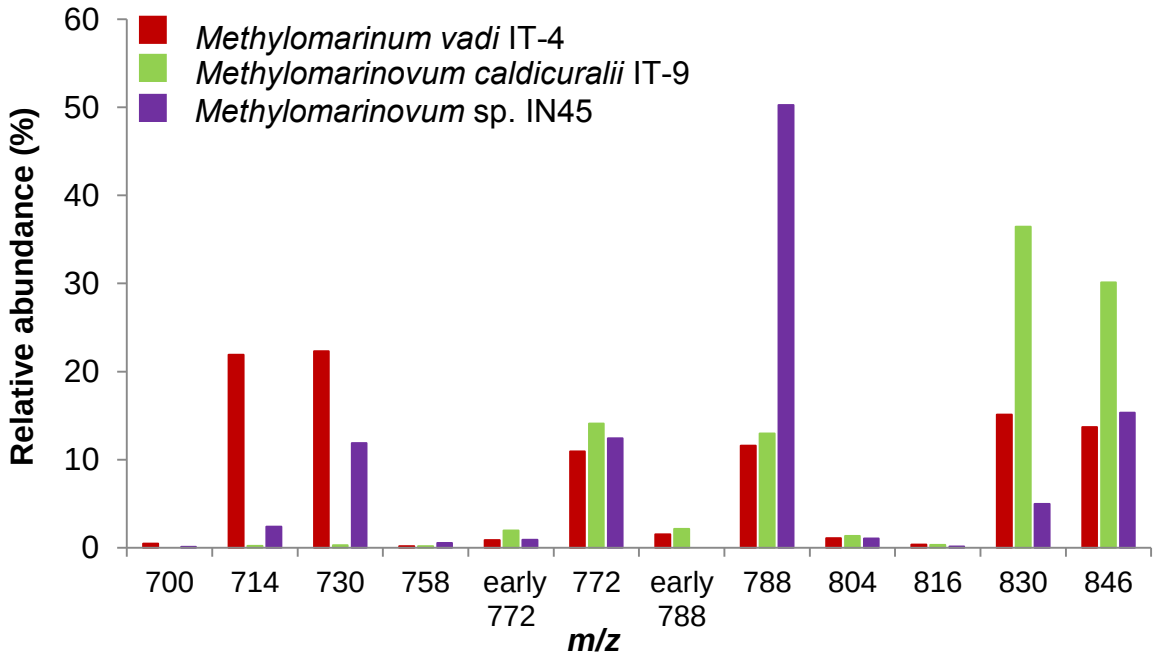



**Figure 4-10:** Relative abundances of BHPs as % of total in the acetylated total extracts of *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp.

The most abundant BHPs in *Methylomarinum vadi* IT-4 were aminotriol (**V**, *m/z* = 714), 22% and 'carbamylmethylestertriol' (**V**$^{\mathbf{MeCarb}}$, *m/z* = 730), 22% which were in almost equal amounts, for *Methylomarinovum caldicuralii* IT-9 it was aminopentol (**VII**, *m/z* = 830), 37% and for *Methylomarinovum* sp. IN45 'carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$, *m/z* = 788), 50% was dominant (Figure 4-10). No C-3 methylated or unsaturated equivalents were found in *Methylomarinum vadi* IT-4 or the *Methylomarinovum* strains. 'Early eluting aminotetrol' (**VI**$^{\mathbf{iso}}$, *m/z* = early 772), which elutes a couple of minutes earlier than aminotetrol (**VI**, *m/z* = 772), was present in all three strains (Figure 4-9). It had a mass spectrum identical to that of 'regular' aminotetrol (**VI**, *m/z* = 772). An 'aminopentol isomer' (**VII**$^{\mathbf{iso}}$, *m/z* = early 788), which elutes several minutes earlier than aminopentol (**VII**, *m/z* = 830), was present in *Methylomarinum vadi* IT-4 and *Methylomarinovum caldicuralii* IT-9 and the related compound 'carbamylmethylesterpentol isomer' (**VII**$^{\mathbf{iso,MeCarb}}$, *m/z* = 804), was detected in all three strains examined. The additional suite of 'desmethyl' compounds related to aminotriol (**V**, *m/z* = 714), aminotetrol (**VI**, *m/z* = 772) and aminopentol (**VII**, *m/z* = 830) but with $[M+H]^+$ 14 Da lower (*m/z* = 700, 758 and 816 respectively) were present in trace amounts.

## 4.5. Discussion

### 4.5.1. BHP signatures of marine methanotrophs

#### 4.5.1.1. *Methylobacter* spp.

The most abundant BHP in all three strains of *Methylobacter* isolated from marine environments, *Methylobacter marinus* A45, *Methylobacter* sp. BBA5.1 and *Methylobacter* sp. BB5.1, was aminopentol (**VII**) (Figure 4-8). This is in agreement with other Type I methanotroph cultures, *Methylococcus capsulatus* (Neunlist and Rohmer, 1985b; Talbot et al., 2001), *Methylomonas* sp. (Jahnke et al., 1999; Talbot et al., 2001; van Winden et al., 2012c) and *Methylocaldum tepidum* (Cvejic et al., 2000a). However, there was significant variation in the relative abundance of compounds between species. Trace levels of unsaturated $\Delta^{11}$ aminopentol (**VIIb**) were found in all three species. This compound has only been observed in two Type I methanotrophs to date, the *Methylovulum*-like strain M200 strain and *Methylomonas*-like strain M5 (van Winden et al., 2012c). This was accompanied by lower abundances of aminotetrol (**VI**) and aminotriol (**V**). The high abundance of aminotriol (**V**) in *Methylobacter* sp. BB5.1 is unusual for a Type I methanotroph but has been seen in the *Methylovulum*-like strain M200 (van Winden et al., 2012c) and *Methylomicrobium album* where aminotriol (**V**) and aminotetrol (**VI**) were in equal abundance (Talbot et al., 2001).

C-3 methylated equivalents of aminotriol (**Va**), aminotetrol (**VIa**) and aminopentol (**VIIa**) were present in all three *Methylobacter* species albeit in trace amounts with the exception of a greater abundance of 3-methylaminotriol (**Va**) in *Methylobacter* sp. BB5.1. 3-methylaminopentol (**VIIa**) has only been observed in *Methylococcus capsulatus* (Neunlist and Rohmer, 1985b) and *Methylocaldum* spp. (Cvejic et al., 2000a) and 3-methylaminotetrol (**VIa**) only in *Methylocaldum* spp. (Cvejic et al., 2000a). Recently, 3-methylaminotriol (**Va**) has been observed in *Methylomicrobium* spp., the first report in methanotroph pure cultures, accompanied by trace amounts of 3-methylaminopentol (**VIIa**) (Birgel et al., unpublished data).

The suite of ‘carbamylmethylester’ compounds, ‘carbamylmethylestertriol’ (**V$^{MeCarb}$**), ‘carbamylmethylestertetrol’ (**VI$^{MeCarb}$**) and ‘carbamylmethylesterpentol’ (**VII$^{MeCarb}$**), detected in low abundance in all three species, have not been reported in other methanotrophs to date (Chapter 2, Figure 1-2). In light of this new evidence, data from

previous analyses of *Methylococcus capsulatus* and the *Methylovulum*-like strain M200, have been re-examined and 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**) is absent; it is also absent in the recently analysed *Methylomicrobium* spp. (Talbot et al., 2001; van Winden et al., 2012c ; Birgel et al., unpublished data). It is present, however, in *Methylomonas methanica* but absent in a different species of this genus *Methylomonas*-like strain M5 (van Winden et al., 2012c) so does not appear to be universally present when aminopentol (**VII**) is detected. 'Carbamylmethylestertriol' (**V$^{MeCarb}$**) has been retrospectively identified in *Methylomonas methanica* too.

#### 4.5.1.2. *Methylomarinum vadi* IT-4

The most abundant BHPs in *Methylomarinum vadi* IT-4 were aminotriol (**V**) and 'carbamylmethylestertriol' (**V$^{MeCarb}$**) which were in equal amounts (Figure 4-10). This is unusual for a Type I methanotroph but has been seen in the *Methylovulum*-like strain M200 (van Winden et al., 2012c). Aminopentol (**VII**) was the next most abundant BHP and was accompanied by similar amounts of 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**). This was also seen with aminotetrol (**VI**) and its related 'carbamylmethylestertetrol' (**VI$^{MeCarb}$**) compound (Figure 4-10). The levels of these 'carbamylmethylester' compounds were considerably higher than those seen in *Methylobacter* spp. where only trace levels were reported, and also occur at higher levels than in cultures which were retrospectively examined.

'Early eluting aminotetrol' (**VI$^{iso}$**), which suggests a higher polarity, was present. It has only been seen in *Methylocella palustris* prior to this work (van Winden et al., 2012c). 'Aminopentol isomer' (**VII$^{iso}$**) was also present. It has been reported in one methanotroph culture prior to this, a *Methylovulum*-like strain M200 (van Winden et al., 2012c) but after re-examining previously analysed cultures, it has been identified in *Methylomonas methanica* and *Methylococcus capsulatus*. It has also been found in specific environmental settings where aminopentol (**VII**) is abundant relative to other BHPs (Talbot et al., 2014). It was postulated that this compound results from one of the alcohol groups not being acetylated which is perhaps due to steric hindrance e.g. two alcohol groups are attached to the same C atom (van Winden et al., 2012c). This early eluting isomer also has a related compound with a peracetylated $[M+H]^+$ 16 Da higher, 'carbamylmethylesterpentol isomer' (**VII$^{iso,MeCarb}$**). This is the first observation of this compound in a methanotroph strain.

No C-3 methylated equivalents of aminotriol (**Va**), aminotetrol (**VIa**) and aminopentol (**VIIa**) were present in *Methylomarinum vadi* IT-4 and this represents an additional example of a Type I methanotroph where aminopentol (**VII**) is present without its C-3 methylated equivalent (**VIIa**) (Talbot et al., 2001; van Winden et al., 2012c).

#### 4.5.1.3. *Methylomarinovum* spp.

Significant variation was observed between the BHP composition of the two species of this genera, *Methylomarinovum caldicuralii* IT-9 and *Methylomarinovum* sp. IN45 (Figure 4-10). The most abundant BHP in *Methylomarinovum caldicuralii* IT-9 was aminopentol (**VII**) and this is in agreement with other Type I methanotrophs, *Methylococcus capsulatus* (Neunlist and Rohmer, 1985b; Talbot et al., 2001), a *Methylomonas* sp. (Jahnke et al., 1999; Talbot et al., 2001; van Winden et al., 2012c) and *Methylocaldum tepidum* (Cvejic et al., 2000a). The second most abundant BHP was aminotetrol (**VI**). Once again, aminotetrol (**VI**) and aminopentol (**VII**) were accompanied by similar abundances of 'carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$) and 'carbamylmethylesterpentol' (**VII**$^{\mathbf{MeCarb}}$). The most abundant BHP in *Methylomarinovum* sp. IN45 was 'carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$). Although in lower abundances, 'carbamylmethylestertriol' (**V**$^{\mathbf{MeCarb}}$) and 'carbamylmethylesterpentol' (**VII**$^{\mathbf{MeCarb}}$) were also higher in comparison to their 'regular' homologues. *Methylomarinovum* sp. IN45 was isolated from a deep-sea hydrothermal field and perhaps the high levels of 'carbamylmethylester' compounds witnessed are the result of a physiological adaptation to high pressure and temperature in this environment. This may explain why the relative abundances of compounds in *Methylomarinovum caldicuralii* IT-9, the same genus but isolated from a shallow submarine hydrothermal environment. Perhaps the terminal group of the 'carbamylmethylester' compounds is more effective at stabilising the cell membrane under these conditions.

'Early eluting aminotetrol' (**VI**$^{\mathbf{iso}}$) was present in both strains and 'aminopentol isomer' (**VII**$^{\mathbf{iso}}$) was present in *Methylomarinovum caldicuralii* IT-9. 'Carbamylmethylesterpentol isomer' (**VII**$^{\mathbf{iso,MeCarb}}$) was present in both strains even when 'aminopentol isomer' (**VII**$^{\mathbf{iso}}$) was absent from *Methylomarinovum* sp. IN45. This is again an example of where the 'carbamylmethylester' compounds were in greater abundance than the

‘regular’ 35-aminoBHPs. No C-3 methylated equivalents of aminotriol (**Va**), aminotetrol (**VIa**) and aminopentol (**VIIa**) were present in either strains.

### 4.5.2. Distributions of BHPs in methanotrophic bacteria

BHP distributions from the recently analysed *Methylobacter* spp., *Methylomarinum vadi* IT-4, *Methylomarinovum* spp. and *Methylomicrobium* spp. (Birgel et al., unpublished data) were compared to methanotroph data from the literature (
Figure 4-11). To allow for a more accurate comparison with data from the literature, which was analysed before the novel compounds had been identified, ‘carbamylmethylester’ compounds or early eluting isomers were not included when producing the ternary plot and only aminotriol (**V**), aminotetrol (**VI**), aminopentol (**VII**) and their methylated equivalents were considered.

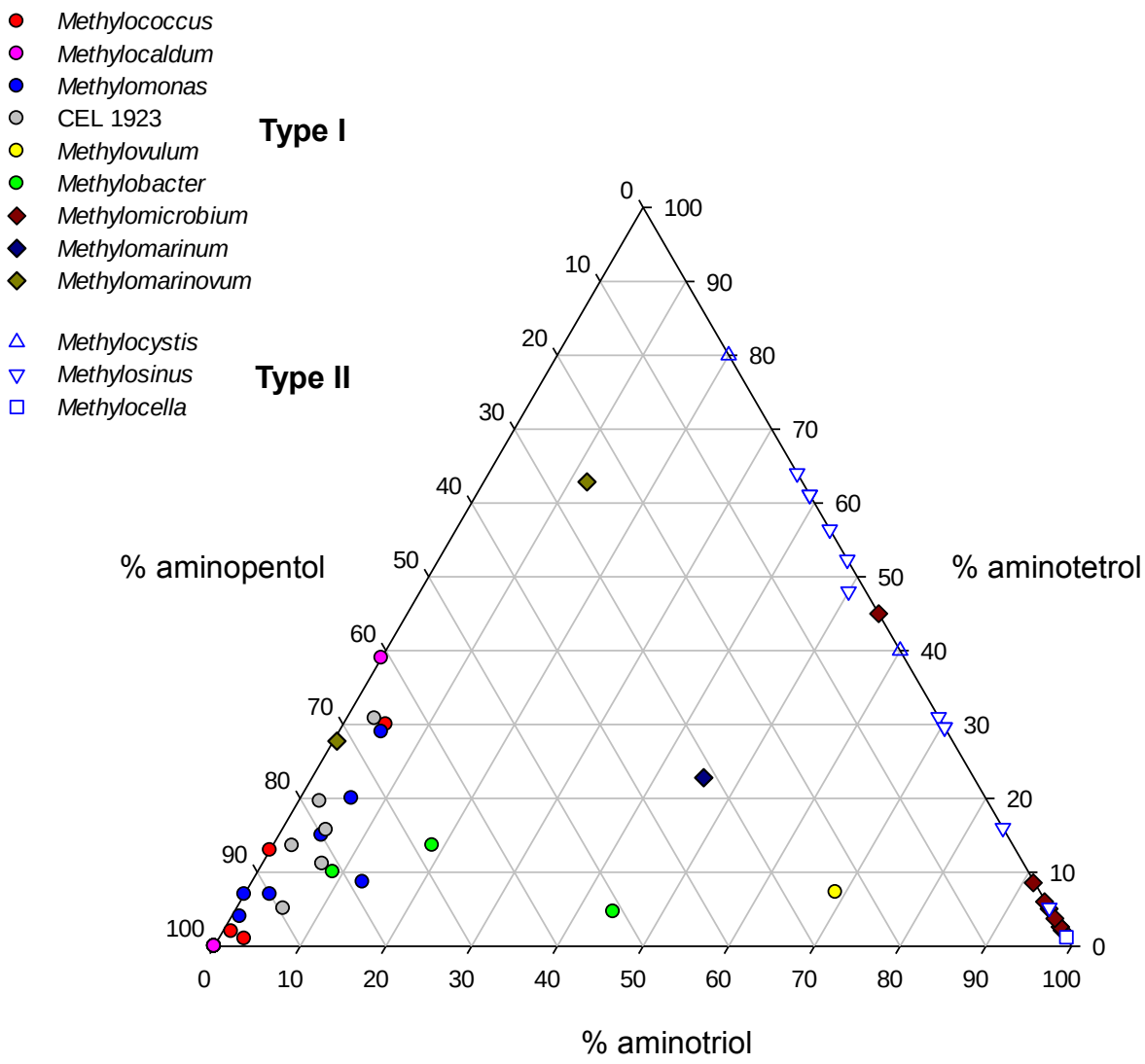



**Figure 4-11:** Ternary plot of the BHP distributions of analysed methanotroph pure cultures and existing data from the literature (Rohmer et al., 1984; Neunlist and Rohmer, 1985b; Jahnke et al., 1999; Cvejic et al., 2000a; Talbot et al., 2001; van Winden et al., 2012c).

Prior to the investigation of BHPs in the *Methylovulum*-like strain M200 (van Winden et al., 2012c), all Type I methanotrophs clustered in the left-hand corner of the plot (cf. Farrimond et al., 2000) with a high percentage of aminopentol (**VII**) and lower contributions from aminotriol (**V**) and aminotetrol (**VI**) (Figure 4-11). The high relative abundance of aminotriol (**V**) observed in *Methylovulum*-like strain M200 was therefore seen as an outlier. All of the *Methylobacter* spp. recently analysed cluster with the Type I methanotrophs in the left-hand corner of the plot but *Methylobacter* sp. BB5.1 increased the spread of the cluster with almost 40% aminotriol (**V**) content. *Methylomarinovum caldicuralii* IT-9 clusters in the left-hand corner with the Type I

methanotrophs but *Methylomarinovum* sp. IN45 and *Methylomarinum vadi* IT-4 are remote from this cluster akin to the *Methylovulum*-like strain M200. This suggests a greater variance in BHP compositions in Type I methanotrophs than previously thought.

All Type II methanotrophs cluster along the right-hand side of the plot with high relative abundances of aminotriol (**V**) and aminotetrol (**VI**). The recent analysis of *Methylomicrobium* spp. (Birgel et al., unpublished data) supports a tentative earlier observation from a single pure culture (Talbot et al., 2001) that this genus does not contain aminopentol (**VII**) and these strains cluster with the Type II methanotrophs in the right-hand corner of the plot.

The absence of aminopentol (**VII**) in certain methanotrophs, widely found in saline environments, may explain its absence in several settings were Type I methanotrophs have been observed. For example, Type I methanotrophs were detected in all three units of Ace Lake sediments but aminopentol (**VII**) was only detected in sediments deposited under freshwater conditions (unit III) (Coolen et al., 2008). No aminopentol (**VII**) was detected in the methanotrophic symbionts in the gill tissue of a cold-seep mussel despite other lipid evidence suggesting a Type I methanotroph (Jahnke et al., 1995)**.** Another study reported the absence of aminopentol (**VII**) in carbonate samples from the Alaminos Canyon, northern Gulf of Mexico (Birgel et al., 2011). Conversely, aminopentol (**VII**) was detected in the water column of the Baltic Sea with supporting evidence for the presence of Type I methanotrophs from $^{13}$C-depleted PLFAs (Berndmeyer et al., 2013). Aminopentol (**VII**) was also detected in the water column of the Black Sea in the oxic-anoxic water transition (Blumenberg et al., 2007). Therefore, the absence of aminopentol (**VII**) is not evidence of the absence of methanotrophs or aerobic methane oxidation.

### 4.6. Conclusions

Marine-isolated methanotrophs from previously unexamined genera and/or species displayed marked differences in the relative abundances of BHPs. Aminopentol (**VII**) was the most abundant BHP in all *Methylobacter* spp. and *Methylomarinovum caldicuralii* IT-9 which is typically seen for Type I methanotrophs other than those from the *Methylomicrobium* genus (Birgel et al., unpublished data) and the *Methylovulum*-

like strain M200 (van Winden et al., 2012c). *Methylomarinum vadi* IT-4 and *Methylomarinovum* sp. IN45 disproves the hypothesis that aminopentol (**VII**) would be the most abundant BHP and are additional examples where this compound is not the most abundant BHP in a Type I methanotroph. Furthermore, *Methylomarinum vadi* IT-4 and the *Methylomarinovum* spp. are also examples of Type I methanotrophs where aminopentol (**VII**) is present but its C-3 methylated equivalent (**VIIa**) is absent. It was hypothesised that species of the same genus would have similar BHP compositions but this was disproved with significant differences in BHP composition seen between the three *Methylobacter* strains and the two *Methylomarinovum* strains. These findings indicate that aminopentol (**VII**) is not absent from all marine methanotrophs, however, several previous studies of marine/saline systems have not identified aminopentol (**VII**) in the sedimentary BHP distribution (Jahnke et al., 1995; Coolen et al., 2008; Birgel et al., 2011). This has significant implications for the development of a proxy using aminopentol (**VII**) to trace aerobic methane oxidation (AMO) in marine settings as this compound is absent in methanotrophs commonly found in these settings.

A suite of compounds related to aminotriol (**V**), aminotetrol (**VI**) and aminopentol (**VII**) but with a peracetylated $[M+H]^+$ 16 Da higher, the 'carbamylmethylesters', were detected for the first time in methanotrophs and were present in all six bacterial strains tested. They were present in trace amounts in three *Methylobacter* spp. but higher abundances were seen in *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp. The most abundant BHP in *Methylomarinum vadi* IT-4 was 'carbamylmethylestertriol' (**V$^{MeCarb}$**) accompanied by almost equal amounts of aminotriol (**V**). The 'carbamylmethylesters' were in higher abundance than the 'regular' 35-aminoBHP compounds in *Methylomarinovum* sp. IN45 with 'carbamylmethylestertetrol' (**VI$^{MeCarb}$**) being the most abundant compound. Perhaps the unusually high abundances of 'carbamylmethylesters' in this species may allow it to be identified in natural settings.

# 5. Influence of methane concentration and temperature on BHP signatures of River Tyne estuarine sediment aerobic microcosms

## 5.1. Introduction

Methanotrophs are widespread in nature and are mostly mesophilic, non-halophilic neutrophiles (Hanson and Hanson, 1996). Type I methanotrophs are generally characteristic of aquatic environments whilst Type II methanotrophs are most widely found in terrestrial environments (e.g. McDonald et al., 1996; McDonald et al., 2005b; Moussard et al., 2009). Methanotrophs isolated from marine environments to date have been classified as Type I only (Sieburth et al., 1987; Lidstrom, 1988; Fuse et al., 1998; Hirayama et al., 2013; Hirayama et al., 2014; Takeuchi et al., 2014; Tavormina et al., 2015) but Type II methanotrophs have been found in Colne Estuary surface sediments but were not actively consuming methane based on $^{13}CH_4$ SIP incubations (Moussard et al., 2009). Previous studies have suggested that Type I methanotrophs are better adapted to lower methane concentrations whilst Type II methanotrophs appear to favour higher methane concentrations (Hanson and Hanson, 1996; Kolb, 2009; Graef et al., 2011). Although most methanotrophs are mesophiles some have been isolated from environments with a wide range of temperatures for example, tundra soils and permafrost environments (Omelchenko et al., 1996; Bowman et al., 1997; Khmelenina et al., 2002), hot springs and agricultural soil environments (Bodrossy et al., 1997; Bodrossy et al., 1999; Tsubota et al., 2005).

There have been few studies investigating how the BHP composition in methanotrophs may be influenced by growth temperature (Jahnke et al., 1999; van Winden et al., 2012a). A pure culture study investigated the growth of a psychrotolerant strain of *Methylomonas methanica* 'CEL 1923' at temperatures between 10-35°C with 5°C incremental increases (Jahnke et al., 1999). It showed that the concentration of aminopentol (**VII**) decreased with increasing temperature whereas the concentration of aminotetrol (**VI**) did not show any relationship to an increase in temperature. In a separate study of a natural system, *Sphagnum* mosses were incubated at different temperatures (5, 10, 15, 20, and 25°C) in mesocosm experiments to investigate the temperature effect on methane flux within peat bogs (van Winden et al., 2012a). The BHP composition of methanotrophs symbiotically associated with the *Sphagnum* plants were investigated, where aminotetrol (**VI**) and aminopentol (**VII**) showed a

strong non-linear response to temperature with high concentrations of these compounds detected at only the highest temperature tested (25°C), despite the fact that the methane consumption rate actually decreased at this temperature (van Winden, 2011).

Estuaries typically have high current speeds with regions of high turbidity resulting in frequent sediment suspensions, and are often characterised by the presence of large tidal flats. High turbulence in these environments favours methane emission (Middelburg and Nieuwenhuize, 2001) and thus methane oxidation is significant in the low salinity regions (de Angelis and Scranton, 1993). Tidal flats which are uniquely exposed during tidal fluctuations are also major methane sources (Kelley et al., 1995; Middelburg and Nieuwenhuize, 2001). The dynamic and heterogeneous nature of the River Tyne estuary would be expected to give rise to a genetically and functionally varied methanotroph community, even at a single sampling location, due to exposure to multiple imposed gradients of key environmental parameters, such as salinity, pH, gas exchange and to some extent temperature. Estuaries offer a dynamic but constrained link between freshwater/terrestrial and marine environments and act as a sink for material from all of these sources including methanotrophs that may have been passively introduced from external sources. River Tyne estuarine sediment within the tidal reaches was used as the inoculum in the microcosms.

## 5.2. Aims and hypotheses

### 5.2.1. Aims

This chapter presents the BHP sedimentary signatures and methanotroph community composition from aerobic microcosm experiments inoculated with River Tyne estuarine sediment and incubated at a range of different methane concentrations and temperatures.

Furthermore, a Type I methanotroph *Methylomonas methanica* S1 and a Type II methanotroph *Methylosinus trichosporium* OB3b, were analysed for the presence of the *hpnR* gene indicative of the production of 3-methylhopanoids (Welander and Summons, 2012).

This chapter has four aims:

(1) to determine the effect of methane concentration on methanotroph community composition.

(2) to determine the effect of temperature on methanotroph community composition.

(3) to determine the effect of methane concentration and temperature on BHP composition, focusing on the methanotroph-derived 35-aminoBHPs.

(4) to analyse DNA extracts from microcosm sediments subjected to variations in temperature, and pure cultures of *Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b, for the presence of the *hpnR* gene.

Please note the results presented in section 5.4.1 and 5.4.4, methane oxidation rates (apart from 8 and 15°C) and methanotroph community composition, were generated by a colleague at Newcastle University, Angela Sherry (Sherry et al., 2015 in review).

### 5.2.2. Hypotheses

- *Type I methanotrophs will be the dominant group enriched in River Tyne estuarine sediments.*

The sediment inoculum used in the microcosm studies is from an estuarine environment and is expected to be dominated by Type I indigenous methanotroph populations as observed in other estuarine sediment studies, such as Newport Bay estuary, California, USA (McDonald et al., 2005b), the Blyth estuary, Northumberland, UK (Cunliffe et al., 2008) and the Colne estuary, Brightlingsea, UK (Moussard et al., 2009).

#### 5.2.2.1. Estuarine sediment microcosms to study the effect of methane concentration on methanotroph diversity and BHP composition

- *Methanotroph community composition changes with increasing methane concentration.*

Methanotrophs fall into two categories with respect to their affinity for methane. Low affinity methanotrophs (including most cultured species) maintain growth on the

relatively high methane concentrations. In contrast high affinity methanotrophs grow at atmospheric concentrations of methane (e.g. Kolb, 2009; Tate et al., 2012).

- *Aminopentol concentration increases with increasing methane concentration.*

Higher concentrations of methane in the microcosm headspace will result in a greater methanotroph population. Consequently, the greater number of methanotroph cells in the sediment should result in an increased concentration of aminopentol (**VII**).

#### 5.2.2.2. Estuarine sediment microcosms to study the effect of temperature on methanotroph diversity and BHP composition

- *Methanotroph community composition changes in response to temperature.*

Methanotroph genera have different optimal temperatures for growth (e.g. Trotsenko and Khmelenina, 2005; Trotsenko et al., 2009). Therefore, it is expected that different genera will be enriched across the temperature range of the study.

- *Changes in methanotroph communities with temperature are reflected in the 35-aminoBHP composition.*

In pure culture there is variation in the 35-aminoBHP compositions in different genera of methanotrophs (Chapter 2, Section 2.25 and Chapter 4, Section 4.4.2). Therefore, it is postulated that shifts in the methanotroph community composition will be reflected in the 35-aminoBHP signatures observed.

## 5.3. Materials and methods

### 5.3.1. Estuarine sediment microcosms to study the effect of methane concentration on methanotroph diversity and BHP composition

The microcosm experimental set-up is described in full in Chapter 3, Section 3.3.2. Briefly, microcosms were prepared in triplicate according to the experimental conditions (Table 5-1: $CH_4$ amended and heat killed controls + $CH_4$ (abiotic controls)), all incubations were carried out at 21°C and used River Tyne estuarine surface sediment as the inoculum. Triplicate unamended microcosms (without the addition of methane) were also prepared to determine background levels of methane in the sediment (Table 5-1).

**Table 5-1:** Methane concentration microcosm experimental conditions.

| **$CH_4$ amended** | 0.1% $CH_4$ | 0.5% $CH_4$ | 1% $CH_4$ | 5% $CH_4$ |
|---|---|---|---|---|
| **Heat killed** | 0.1% $CH_4$ | 0.5% $CH_4$ | 1% $CH_4$ | 5% $CH_4$ |
| **Unamended** | -* | | | |

* (-) denotes no methane addition.

Triplicate microcosms, representing the initial bacterial communities and BHP compositions in the River Tyne surface sediments, were prepared with fresh sediment and sacrificially sampled; referred to as Time 0. TOC measurements (Chapter 3, Section 3.7.1) were performed in triplicate on fresh sediment and all BHP concentrations were normalised to the average of these values, 7.47% (range 7.44-7.49%).

### 5.3.2. Estuarine sediment microcosms to study the effect of temperature on methanotroph diversity and BHP composition

The microcosm set-up is described in full in Chapter 3, Section 3.3.2. Briefly, microcosms were prepared in triplicate according to the experimental conditions (Table 5-2: 5% $CH_4$ amended, unamended and heat killed controls + 5% $CH_4$ (abiotic controls)) and used River Tyne estuarine surface sediment as the inoculum.

**Table 5-2:** Temperature microcosm experimental conditions.

| | **4°C** | **8°C**[a] | **15°C**[a] | **21°C** | **30°C** | **40°C** | **50°C** | **60°C** |
|---|---|---|---|---|---|---|---|---|
| **5% $CH_4$ amended** | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ |
| **Heat killed** | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ |
| **Unamended** | -* | - | - | - | - | - | - | - |

* (-) denotes no methane addition.

[a] Additional temperature experiments at 8 and 15°C were completed at a later date, more representative of *in-situ* surface sediment temperatures, using different initial sediment compared with the original study.

Triplicate microcosms, representing the initial bacterial communities and BHP compositions in the River Tyne surface sediment, were prepared and sacrificially sampled immediately; referred to as Time 0. TOC measurements (Chapter 3, Section 3.7.1) were performed on the triplicate of initial sediment and all BHP concentrations normalised to the average of these values, 7.37% (range 7.32-7.44%), additional temperature experiments, 7.71% (range 7.58-7.86%).

When all methane in the amended sets had been oxidised, the microcosms, along with their unamended and heat killed counterparts, were sacrificially sampled (Section 3.3.3). Microcosm sediments were freeze-dried (Chapter 3, Section 3.3.4) and BHPs were extracted from the sediment using a modified Bligh and Dyer extraction method (Chapter 3, Section 3.8.1). The polar fraction resulting from the SPE of total lipid extracts were prepared and analysed using LC-MS (Chapter 3, Section 3.8.2 to 3.8.4). Microbial community analysis (Chapter 3, Section 3.6) was performed on microcosm sediment DNA extracts to assess methanotroph community composition, in response to methane concentration and temperature, by targeting the *pmoA* gene. *hpnR* gene analysis was performed on DNA extracts from temperature-controlled microcosm sediments and the methanotroph pure cultures, *Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b (Chapter 3, Section 3.6.2.2).

## 5.4. Results

### 5.4.1. Estuarine sediment microcosms to study the effect of methane concentration on methanotroph diversity and BHP composition

#### 5.4.1.1. Methane oxidation rates and community analysis of methanotrophs in River Tyne estuarine sediment microcosms

Methane oxidation rates in response to increasing methane concentration (0.1-5% headspace methane concentration) were determined by monitoring the consumption of methane in microcosm headspace (Chapter 3, Section 3.3.6; Figure 5-1).

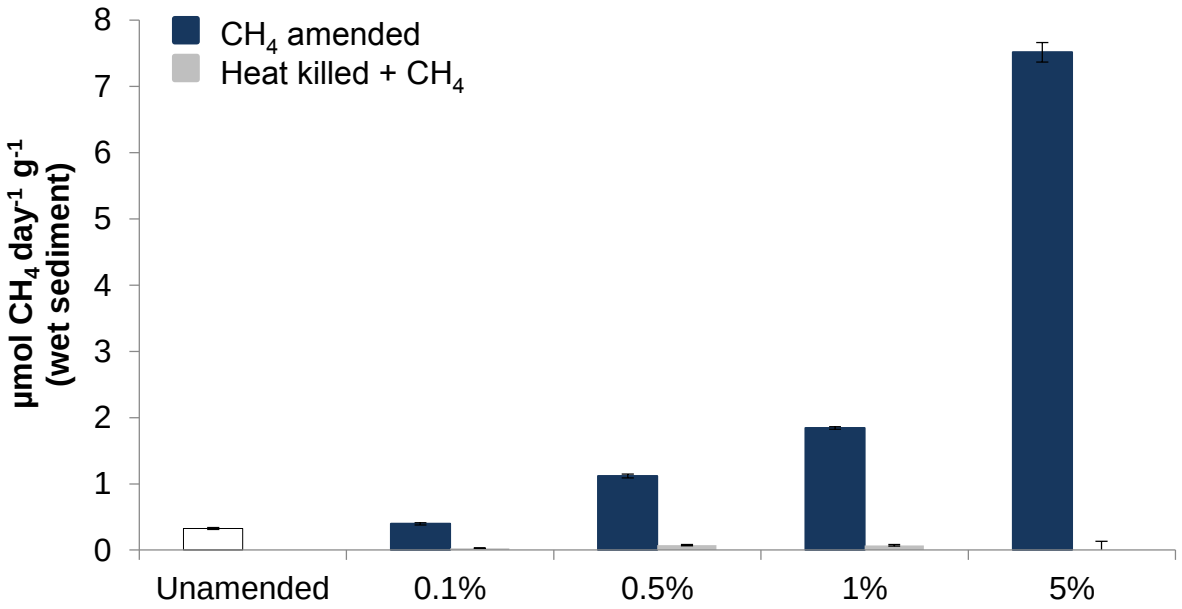



**Figure 5-1:** Methane oxidation rates in response to methane concentration in River Tyne estuarine sediment microcosms. $CH_4$ amended microcosms (blue bars) and heat killed controls (grey bars). Error bars represent 1 x S.E. (N = 3). Data from Sherry et al., 2015 in review.

Rates of methane oxidation (µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment) in methane amended microcosms were statistically compared to the unamended control and corresponding heat killed controls using one-way analysis of variance (ANOVA) with Fisher's LSD.

Methane oxidation rates increased with increasing methane concentration with the maximum rate determined in microcosms amended with 5% methane (7.52 ± 0.1 µmol $CH_4$ $day^{-1}$ $g^{-1}$, Figure 5-1). Oxidation rates in methane amended microcosms were significantly higher than in the corresponding heat killed controls at all concentrations ($p \leq 0.001$). Oxidation rates in response to all methane concentrations, except 0.1% methane, were significantly higher than initial background levels of methane in the sediment as determined in the unamended microcosms (0.5%, 1% and 5% $CH_4$ $p = 0.000$; 0.1% $CH_4$ $p = 0.466$).

The PCR-DGGE profiles of the *pmoA* gene across all methane concentrations showed the same dominant bands (Figure 5-2), no gene was detected following PCR amplification in Time 0 microcosms. In microcosms amended with 1% methane, band 1 shared 96% sequence identity with an environmental clone C69, a putative *Methylobacter* sp., from a wetland soil (HQ883363) (Yun et al., 2012) and its closest

cultured representative was *Methylovulum miyakonense* (84%, AB501288) (Iguchi et al., 2011). The banding pattern in the gel indicates the strong likelihood of the presence of additional species (*Methylobacter* spp.) which were also identified at 4-30°C in the temperature study (bands 2 and 3, Figure 5-8).

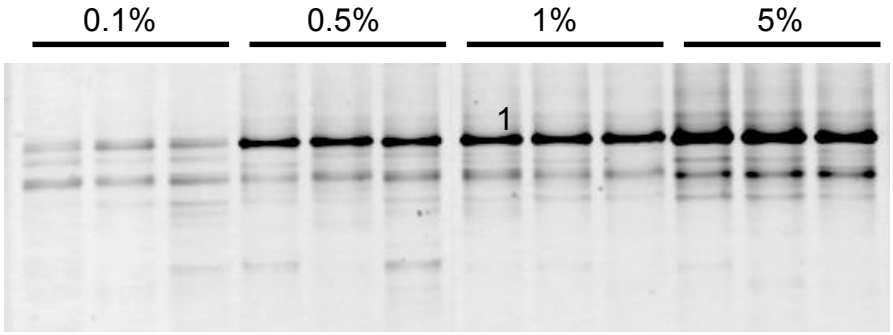



**Figure 5-2:** PCR-DGGE of the *pmoA* gene as an indicator of methanotroph community composition in aerobic River Tyne estuarine sediment microcosms in response to different methane concentrations.

### 5.4.2. 35-aminoBHP concentrations in River Tyne estuarine sediment microcosms subjected to variations in methane concentration

Aminotriol (**V**) and related 'carbamylmethylester' (**V$^{MeCarb}$**) and 'desmethyl' (**V$^{des}$**) compounds (Chapter 4, Section 4.4.1) were detected in microcosms at all concentrations of methane amendment, unamended controls, and apart from 'carbamylmethylestertriol' (**V$^{MeCarb}$**), in the initial sediment (Figure 5-3). Aminotetrol (**VI**) was detected in microcosm sediments at all concentrations of methane amendment, unamended controls and in the initial sediments (Figure 5-4). Aminopentol (**VII**) was detected in microcosms at all concentrations of methane amendment and in the initial sediments, but was not detected in unamended microcosms; 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**) was not detected in the initial sediments (Figure 5-5).

Aminotriol (**V**), aminotetrol (**VI**) and aminopentol (**VII**) concentrations in methane amended microcosm sediments were statistically compared to the initial sediments (Time 0) and unamended sediments using one-way analysis of variance (ANOVA) with Fisher's LSD. Aminotetrol (**VI**) concentrations in methane amended microcosm sediments were statistically compared to the unamended control and corresponding

heat killed controls also. 'Desmethylaminotriol' (**V**$^{des}$) concentrations in methane amended microcosm sediments were statistically compared to the initial sediment (Time 0) and corresponding heat killed controls and 'carbamylmethylesterpentol' (**VII**$^{MeCarb}$) to the unamended control only.

### 5.4.2.1. Aminotriol and related compounds

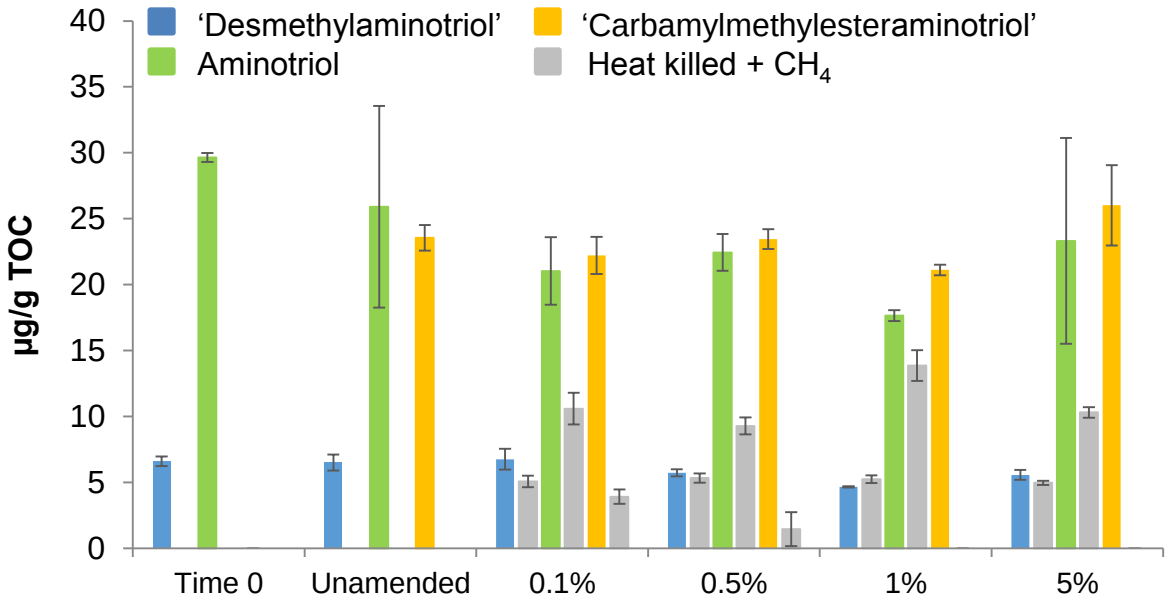



**Figure 5-3:** Concentration (µg/g TOC) of 'desmethylaminotriol' (**V**$^{des}$), aminotriol (**V**) and 'carbamylmethylestertriol' (**V**$^{MeCarb}$) in $CH_4$ amended River Tyne estuarine sediment microcosms in response to methane concentration (corresponding heat killed controls to the right, where compound present). Error bars represent 1 x S.E. (N = 3).

There were no significant differences in aminotriol (**V**) concentrations in microcosms across all methane concentrations compared with the initial sediment concentration ($p \geq 0.091$) and unamended sediments ($p \geq 0.229$) (Figure 5-3). The concentration of 'desmethylaminotriol' (**V**$^{des}$) was highest in sediments amended with 0.1% methane, 6.8 ± 0.8 µg/g TOC. The concentrations were lower than those of aminotriol (**V**) and 'carbamylmethylestertriol' (**V**$^{MeCarb}$). No significant differences were determined in 'desmethylaminotriol' (**V**$^{des}$) concentrations between the initial sediments (Time 0) and 0.1%, 0.5% and 5% methane amended microcosm sediments ($p \geq 0.081$), but 'desmethylaminotriol' (**V**$^{des}$) concentration was significantly lower in 1% methane amended sediments ($p = 0.003$). 'Desmethylaminotriol' (**V**$^{des}$) concentrations were significantly higher compared to the heat killed equivalents in microcosms amended

with 0.1% methane ($p = 0.007$), with no significant differences observed in microcosms amended with at 0.5%, 1% and 5% methane ($p \geq 0.302$). Concentrations in heat killed controls at all methane concentrations were significantly lower than those in the initial sediment ($p \leq 0.036$). 'Carbamylmethylestertriol' (**V**$^{\mathbf{MeCarb}}$) was not detected in the initial sediment but was observed in microcosms at all methane concentrations and in unamended controls, with the highest concentration, 26 ± 3.0 µg/g TOC, at 5% methane amended concentration (Figure 5-3).

#### 5.4.2.2. Aminotetrol

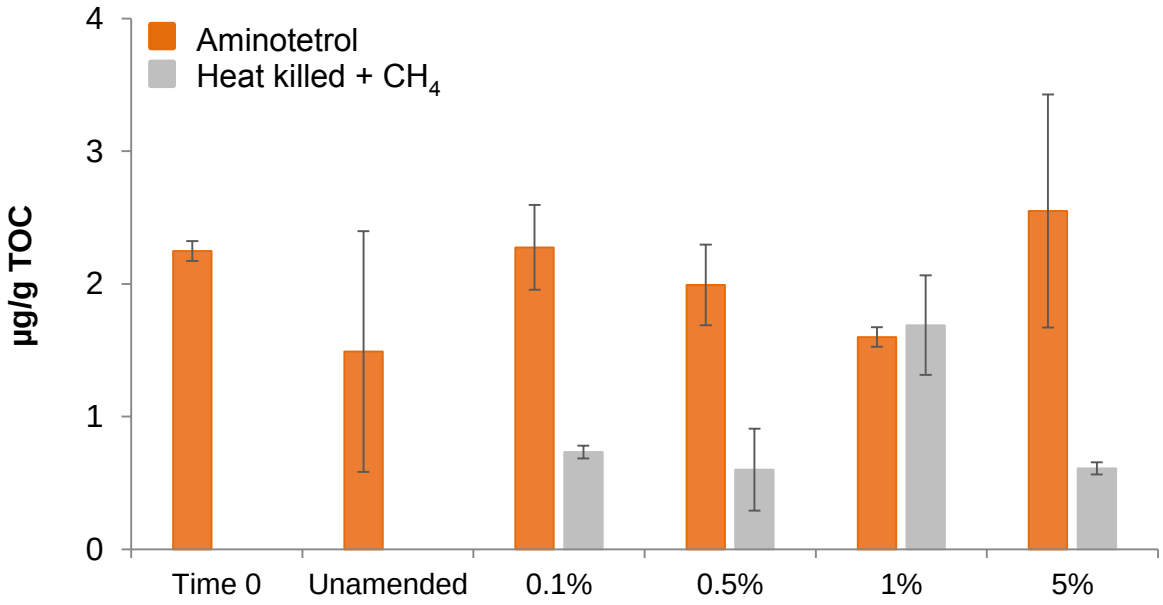



**Figure 5-4:** Concentration (µg/g TOC) of aminotetrol (**VI**) in $CH_4$ amended River Tyne estuarine sediment microcosms in response to methane concentration (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3).

Concentrations of aminotetrol (**VI**) were similar with increasing methane concentration (Figure 5-4) and lower than aminotriol (**V**) and related 'carbamylmethylester' compounds (Figure 5-3). No significant difference were apparent in aminotetrol (**VI**) concentrations between the initial sediment (Time 0) and methane amended microcosm sediments at all concentrations ($p \geq 0.322$). Aminotetrol (**VI**) concentrations were significantly higher in methane amended microcosms compared to their heat killed equivalents at 0.1%, 0.5% and 5% methane concentrations ($p \leq 0.041$), but were not significantly higher than the corresponding heat killed control microcosms at 1% methane concentration ($p = 0.890$). No significant differences were seen between

pairwise comparisons of all methane concentrations and the unamended control ($p \geq 0.113$).

#### 5.4.2.3. Aminopentol and related compounds

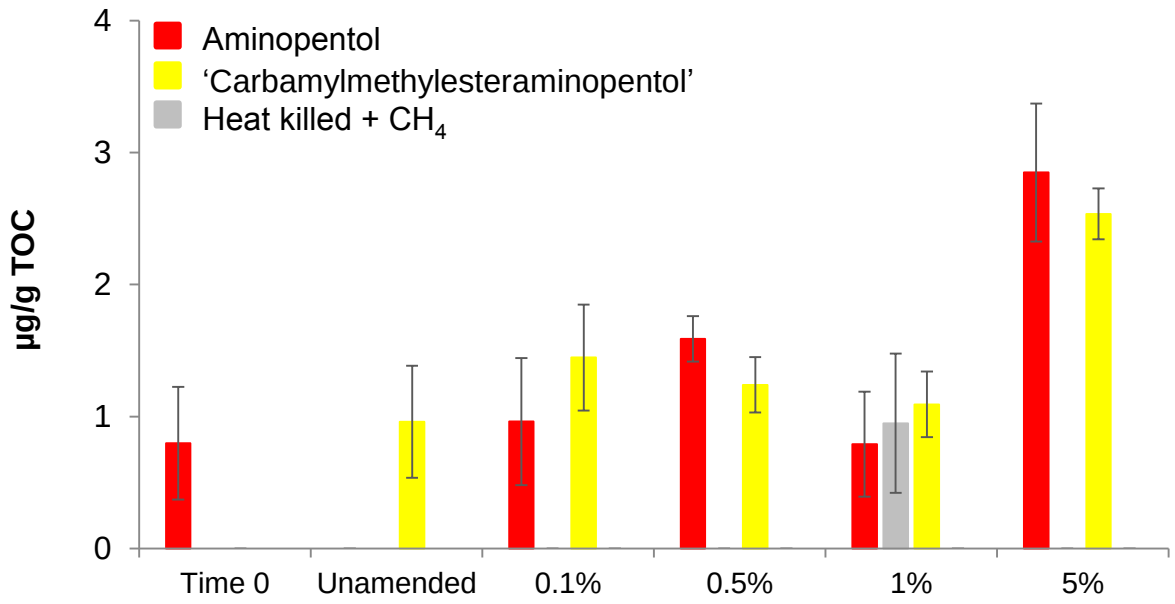



**Figure 5-5:** Concentration (µg/g TOC) of aminopentol (**VII**) and 'carbamylmethylesterpentol' (**VII**$^{\mathbf{MeCarb}}$) in $CH_4$ amended River Tyne estuarine sediment microcosms in response to methane concentration (corresponding heat killed controls to the right, where compound present). Error bars represent 1 x S.E. (N = 3).

Aminopentol (**VII**) concentration was significantly higher in microcosms amended with 5% methane relative to the initial sediment (Time 0) ($p = 0.006$) (Figure 5-5). There were no significant differences between the initial sediment and all other methane concentrations ($p \geq 0.211$). 'Carbamylmethylesterpentol' (**VII**$^{\mathbf{MeCarb}}$) was detected in microcosm sediments at all methane concentrations and was significantly higher in 5% methane amended sediments compared with unamended controls ($p = 0.022$), however it was not detected in the initial sediment (Figure 5-5).

### 5.4.3. Total BHP concentrations in River Tyne estuarine sediment microcosms subjected to variations in methane concentration

Total BHP concentrations in microcosm sediments in response to methane concentration were determined (Figure 5-6). The total BHP concentration of the initial

sediment used for the experiment (Time 0) is also shown. For individual compound concentrations and relative abundances see appendix 3b.

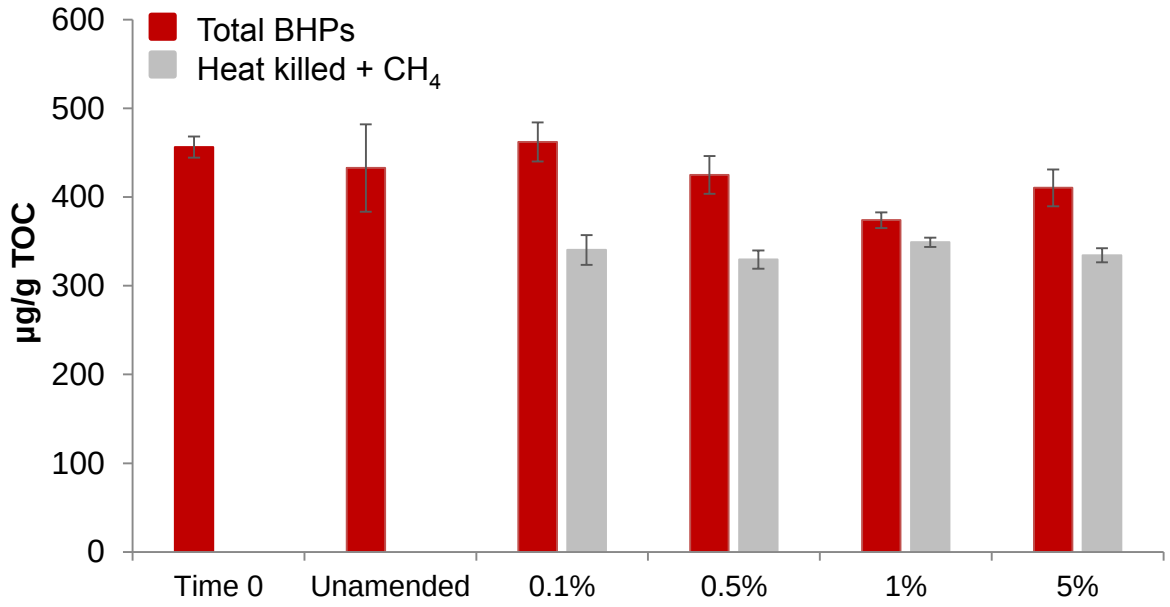



**Figure 5-6:** Total BHP concentrations (µg/g TOC) in $CH_4$ amended River Tyne estuarine sediment microcosms in response to methane concentration (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3).

BHP concentrations in methane amended microcosm sediments were statistically compared to the initial sediment (Time 0), unamended control and corresponding heat killed controls using one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD).

Total BHP concentrations were similar, apart from a slight decrease at 1% methane, with increasing methane concentration with the highest concentration, 462 ± 22 µg/g TOC, detected in sediments amended with 0.1% methane (Figure 5-6). No significant differences were determined in total BHP concentrations between the initial sediment (Time 0) and 0.1%, 0.5% and 5% methane amended microcosm sediments ($p \geq 0.141$). However, total BHPs in the initial sediment were significantly higher, than those in microcosms amended with 1% methane ($p = 0.013$). Total BHP concentrations, except those in 1% methane amended microcosms ($p = 0.415$), were significantly higher in microcosms with 0.1%, 0.5% and 5% methane compared to their corresponding heat killed controls (all $p \leq 0.020$). No significant difference was seen between all methane concentrations and the unamended control ($p \geq 0.064$). Total BHPs in heat killed

controls at all methane concentrations were significantly lower than the initial sediment (all $p \leq 0.002$).

### 5.4.4. Estuarine sediment microcosms to study the response of temperature on methanotroph diversity and BHP composition

#### 5.4.4.1. Methane oxidation rates and community analysis of methanotrophs in River Tyne estuarine sediment microcosms

Methane oxidation rates in response to increasing temperature (4-60°C) were determined by monitoring the consumption of 5% methane in microcosm headspace (Chapter 3, Section 3.3.6; Figure 5-7).

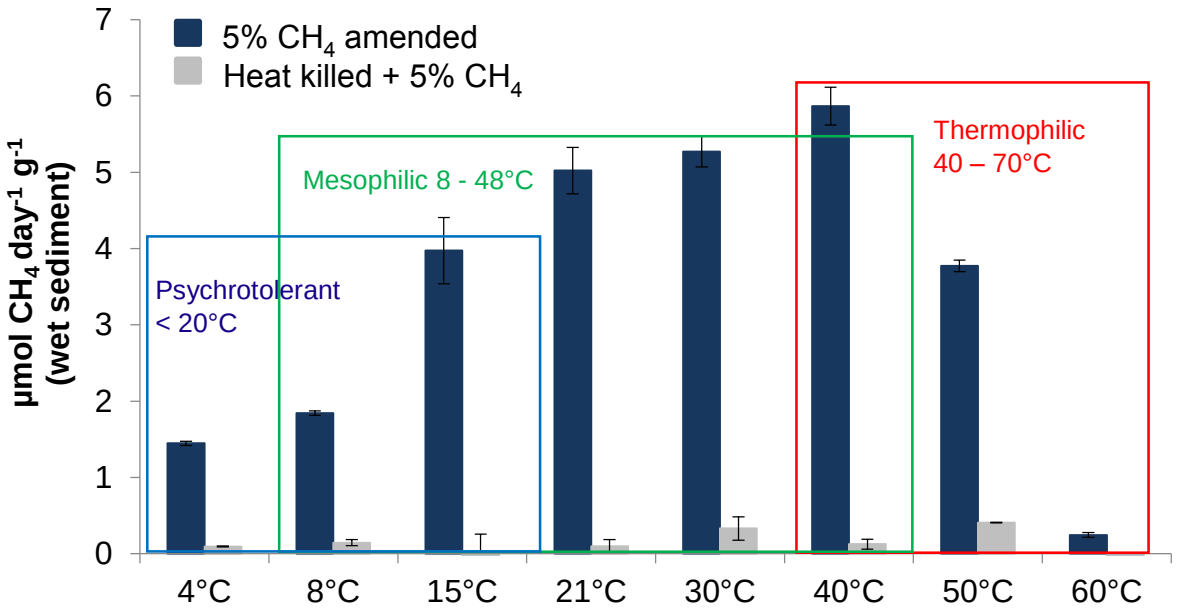



**Figure 5-7:** Methane oxidation rates in response to temperature in River Tyne estuarine sediment microcosms. 5% $CH_4$ amended microcosms (blue bars) and heat killed controls (grey bars). Error bars represent 1 x S.E. (N = 3). Data from Sherry et al., 2015 in review.

Methanotrophs were active over a wide range of temperatures from 4-50°C and rates of methane oxidation in 5% methane amended microcosms were significantly higher than corresponding heat killed controls ($p = 0.000$ for 4-50°C) (Figure 5-7). Rates increased in response to increasing temperature up to a maximum of 5.87 ± 0.2 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment at 40°C. The slowest rate of methane oxidation occurred in microcosms incubated at 60°C, which showed no significant difference to

corresponding heat killed controls (p = 0.096). An overlay of different classifications of microorganisms based on temperature profile (Figure 5-7), suggests that psychrotolerant - mesophilic methanotrophs may be putatively present in the River Tyne and active at low-ambient temperatures (< 4-20°C). Also mesophilic methanotrophs are likely present and active at mid-range temperatures (8-48°C) and mesophilic - thermophilic methanotroph populations may be present and active at mid-high range temperatures (40-60°C) (Madigan et al., 2009).

PCR-DGGE profiles of the *pmoA* gene showed shifts in the community composition of methanotrophs in response to temperature (Figure 5-8); amplification of the *pmoA* gene was not detected following PCR in Time 0 sediments. Microcosms incubated at 4 and 21°C showed the same profiles and the most dominant band (band 2) shared 96% sequence identity with an environmental clone C69, a putative *Methylobacter* sp., from a wetland soil (HQ883363) (Yun et al., 2012), consistent with band 1 in the methane concentration microcosms (1%) (Figure 5-2). The closest cultured representative was *Methylobacter psychrophilus* isolated from a moss-vegetated area on the tundra in the polar Ural (AY945762). Band 2 was also present in microcosms incubated at 30°C, with an additional band (band 3) (also present at lower temperatures 4 and 21°C, Figure 5-8) which was closely related to the *pmoA* sequence from *Methylobacter* sp. BB5.1 (AF016982) and methanotroph isolate 2 (93%, AF182482, isolated from estuarine sediments in Newport Bay, California (McDonald et al., 2005b). Band 4 identified in microcosms incubated at 30°C was closely related to an uncultured methanotrophic bacterium clone *pmoA*-24 from an unpublished study of methanotrophs in rice rhizosphere soil (97%, JQ671235). At 40°C, bands 2-4 identified as *Methylobacter* spp. were no longer present and a shift in methanotroph community composition was observed. Band 5 shared 100% sequence identity with the methanotrophic bacterium LK5 (AF533663) and 97% sequence identity with an uncultured *Methylobacter* sp. clone A9 (JN255559) identified in a laboratory reactor treating landfill leachate. At 50°C, the dominant methanotroph (band 6) shared 100% sequence identity to the cultured thermophilic methanotroph, *Methylocaldum* sp. T-025 (AB275418) (Bodrossy et al., 2003) and *Methylocaldum szegediense* OR2, and an uncultured bacterium DGGE band E20-A from an environmental study of different upland soils (AJ579666) (Knief et al., 2003).

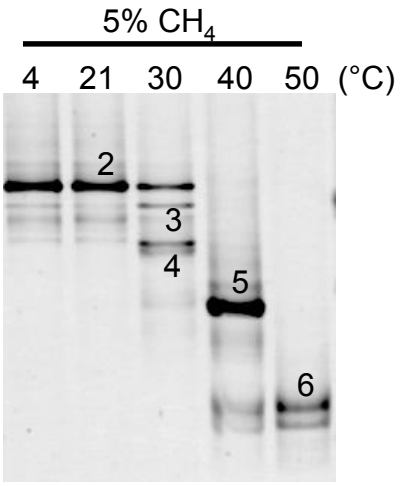



**Figure 5-8:** PCR-DGGE of the *pmoA* gene as an indicator of methanotroph community composition in aerobic River Tyne estuarine sediment microcosms in response to temperature.

### 5.4.5. 35-aminoBHP concentrations in River Tyne estuarine sediment microcosms subjected to variations in temperature

Aminotriol (**V**), 'carbamylmethylestertriol' (**V**$^{\mathbf{MeCarb}}$) and 'desmethylaminotriol' (**V**$^{\mathbf{des}}$) compounds (Chapter 4, Section 4.4.1) and aminotetrol (**VI**) and 'carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$) were detected in microcosms across all temperatures and in the initial sediment (Figure 5-9 and Figure 5-10). Aminopentol (**VII**) and 'carbamylmethylesterpentol' (**VII**$^{\mathbf{MeCarb}}$) were detected at all temperatures apart from 60°C whereas other related compounds were only detected at specific temperatures (Figure 5-11).

Aminotriol (**V**), aminotetrol (**VI**) and 'carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$) concentrations in 5% methane amended microcosm sediments were statistically compared to the initial sediment (Time 0), whereas aminopentol (**VII**) concentrations were compared across the temperature range. 'Desmethylaminotriol' (**V**$^{\mathbf{des}}$) concentrations in methane amended microcosm sediments were compared to the initial sediment (Time 0) and also to the corresponding heat killed controls using one-way analysis of variance (ANOVA) with Fisher's LSD.

### 5.4.5.1. Aminotriol and related compounds

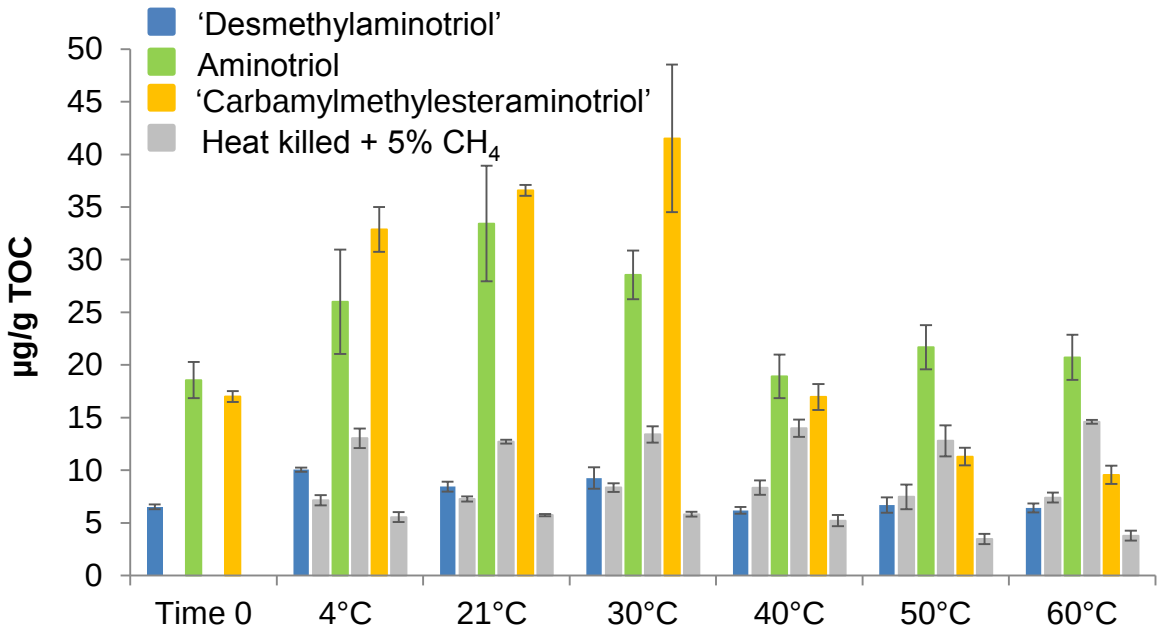



**Figure 5-9:** Concentration (µg/g TOC) of 'desmethylaminotriol' ($\mathbf{V}^{\mathbf{des}}$), aminotriol (**V**) and 'carbamylmethylestertriol' ($\mathbf{V}^{\mathbf{MeCarb}}$) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to temperature (corresponding heat killed controls to the right). Error bars represent 1 x S.E. (N = 3) except 50°C heat killed (N = 2)*.

* Herein, N = 2 for 50°C heat killed controls due to a total lipid extract (TLE) replicate being lost.

The concentration of aminotriol (**V**) varied with temperature (Figure 5-9) and was significantly higher in 5% methane amended sediments compared with the initial sediment (Time 0) at 21°C ($p = 0.007$), where the highest concentration was measured, and at 30°C ($p = 0.050$); there were no significant differences at temperatures greater than 30°C ($p \geq 0.133$). 'Desmethylaminotriol' ($\mathbf{V}^{\mathbf{des}}$) concentrations were significantly higher relative to the initial sediment in 5% methane amended sediments at 4°C, 21°C and 30°C (4°C, $p = 0.000$; 21°C, $p = 0.020$; 30°C, $p = 0.002$), but concentrations were not significantly higher at 40-60°C ($p \geq 0.674$). Concentrations in 5% methane amended sediments were only significantly higher than in corresponding heat killed controls at 4°C ($p = 0.001$). 'Carbamylmethylestertriol' ($\mathbf{V}^{\mathbf{MeCarb}}$) was enriched relative to the initial sediment and followed a similar trend to that of aminotriol (**V**) concentrations. The compound was enriched relative to unamended controls at 4-30°C

(data not shown on Figure 5-9, see appendix 3c) and was higher at all temperatures compared to corresponding heat killed controls (Figure 5-9).

### 5.4.5.2. Aminotetrol and related compounds

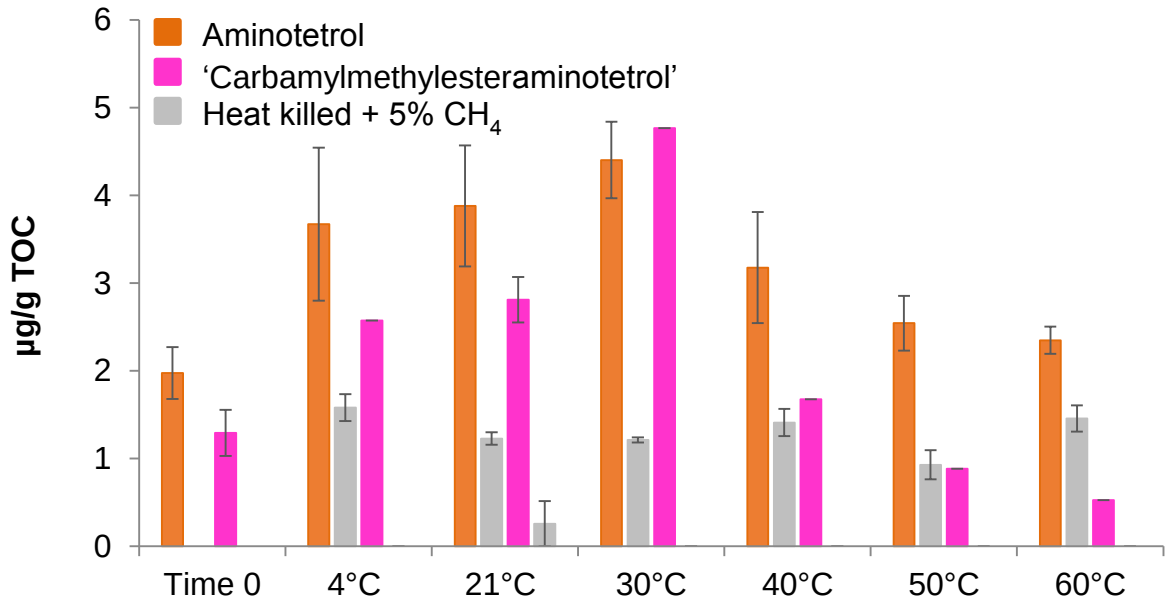



**Figure 5-10:** Concentration (µg/g TOC) of aminotetrol (**VI**) and 'carbamylmethylestertetrol' (**VI**$^{MeCarb}$) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to temperature (corresponding heat killed controls to the right, where compound present). Error bars represent 1 x S.E. (N = 3) except 50°C heat killed (N = 2).

Aminotetrol (**VI**) concentrations were significantly higher than the initial sediment (Time 0) in 5% methane amended sediments at 4-30°C ($p \leq 0.043$) with the highest concentration of 4.4 ± 0.4 µg/g TOC detected at 30°C (Figure 5-10). 'Carbamylmethylestertetrol' (**VI**$^{MeCarb}$) concentrations followed a similar trend to aminotetrol (**VI**) with the maximum concentration determined at 30°C, 4.8 ± 0.5 µg/g TOC (Figure 5-10). Concentrations were significantly higher than the initial sediment in 5% methane amended sediments ($p \leq 0.020$).

### 5.4.5.3. Aminopentol and related compounds

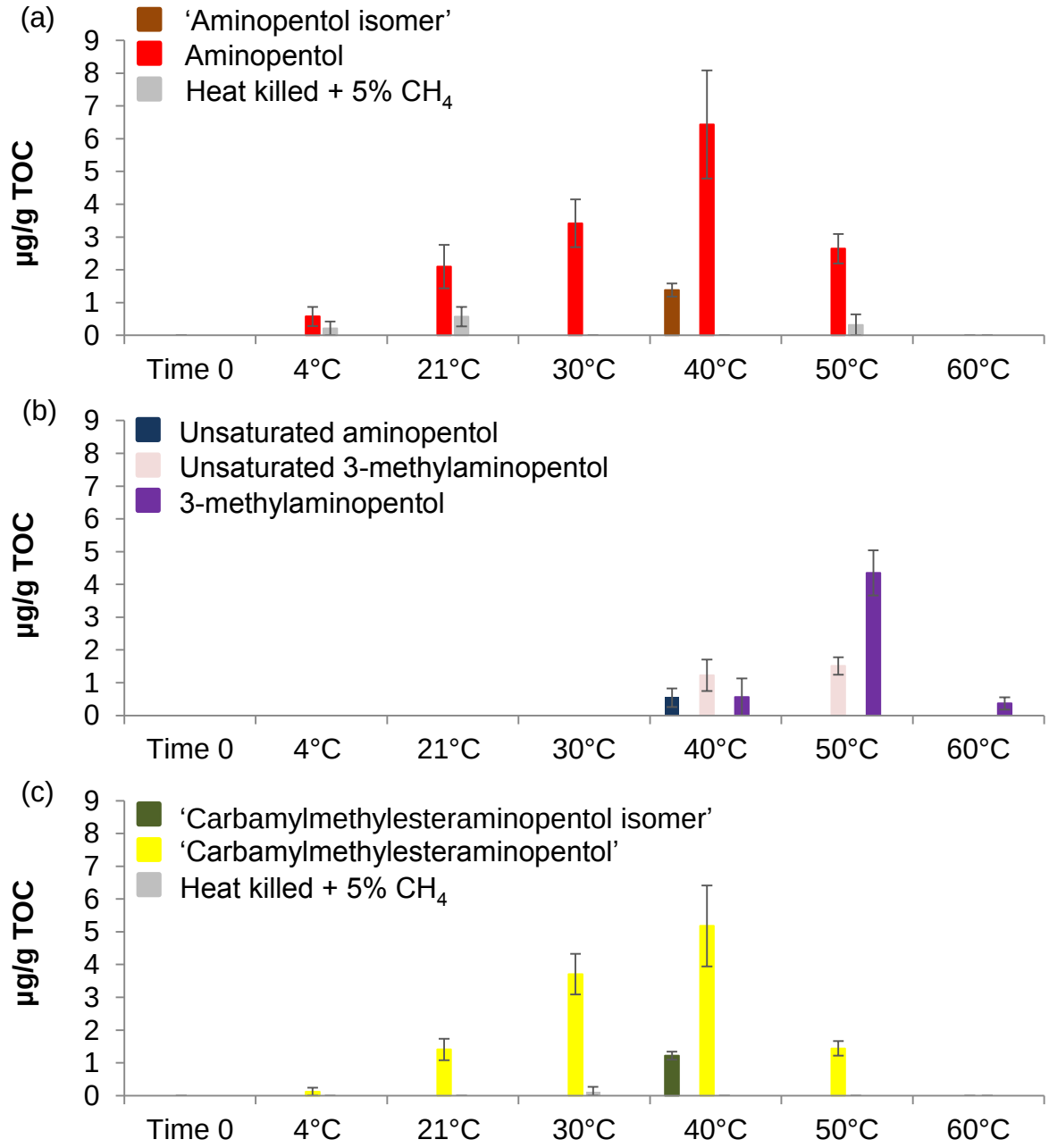



**Figure 5-11:** Concentration (µg/g TOC) of (a) 'aminopentol isomer' (**VII$^{iso}$**) and aminopentol (**VII**); (b) unsaturated $\Delta^{11}$ aminopentol (**VIIb**), unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**), and 3-methylaminopentol (**VIIa**); (c) 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**) and 'carbamylmethylesterpentol isomer' (**VII$^{iso,MeCarb}$**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to temperature (corresponding heat killed controls to the right, where compound present). Error bars represent 1 x S.E. (N = 3) except 50°C heat killed (N = 2).

Aminopentol (**VII**) concentrations increased over the temperature range from 4-50°C, with no compound detected in the initial sediment (Time 0) or in microcosms incubated at 60°C (Figure 5-11a). The concentration of aminopentol (**VII**) increased with temperature, with a maximum of 6.4 ± 1.7 µg/g TOC at 40°C (Figure 5-11a). Aminopentol (**VII**) concentrations were significantly higher in 5% methane amended sediments at 40°C compared with all other temperatures ($p \leq 0.038$). The concentrations were also significantly higher in sediments at 30°C compared with 4°C ($p = 0.048$). 'Carbamylmethylesterpentol' (**VII$^{MeCarb}$**) followed a similar trend to that of aminopentol (**VII**) with a maximum concentration of 5.2 ± 1.2 µg/g TOC in sediments incubated at 40°C (Figure 5-11c), which was significantly higher than at all other temperatures ($p \leq 0.002$), except sediments incubated at 30°C. Concentrations of 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**) were also significantly higher at 30°C compared with 4°C, 21°C, 50°C and 60°C ($p \leq 0.032$).

'Aminopentol isomer' (**VII$^{iso}$**) and 'carbamylmethylesterpentol isomer' (**VII$^{iso,MeCarb}$**) were only detected in microcosm sediments incubated at 40°C, where the regular isomer was at its highest concentration (Figure 5-11a and c). Neither were detected in the initial sediment, heat killed or unamended control microcosms (data not shown on Figure 5-11, see appendix 3c).

Unsaturated $\Delta^{11}$ aminopentol (**VIIb**) was only detected at 40°C and was not present in the initial sediments (Time 0), heat killed or unamended control microcosms (Figure 5-11b). 3-Methylaminopentol (**VIIa**) was present at 40-60°C with the greatest concentration, 4.4 ± 0.7 µg/g TOC, detected at 50°C which is higher than its non-methylated equivalent at the same temperature, 2.6 ± 0.4 µg/g TOC (Figure 5-11b). The compound was not detected in the heat killed or unamended control microcosms at any of the temperatures tested. Unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) was detected at 40 and 50°C in similar concentrations.

The presence of 3-methylaminopentol (**VIIa**) was confirmed from its spectrum and its relative retention time compared to aminopentol (**VII**). It has a base peak ion of *m/z* 844 with the loss of five acetylated hydroxyl groups to give ion fragments of *m/z* 784, 724, 664, 604, 544 (Figure 5-12). The ion fragment at *m/z* 485 indicates the loss of 59 Da which is the terminal amine group. The spectrum of unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) has the same ion fragmentation pattern as

3-methylaminopentol (**VIIa**) but each ion 2 Da lower. It has a base peak ion of *m/z* 842 and ion fragments of *m/z* 782, 722, 662, 602, 542 with the loss of the terminal amine group at *m/z* 483.

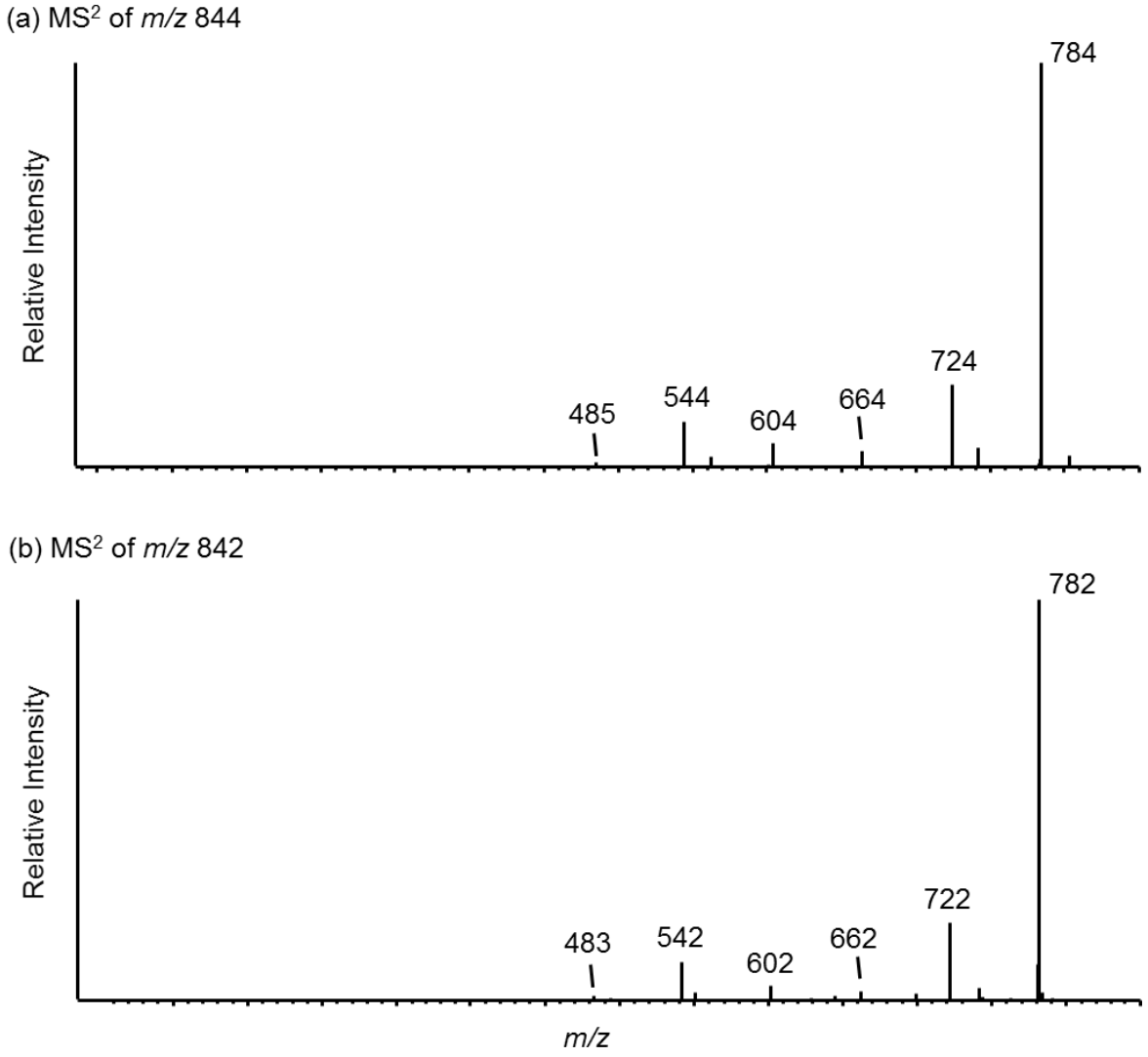



**Figure 5-12:** APCI $MS^2$ spectra of the acetylated total extract from River Tyne estuarine sediment in a microcosm incubated at 50°C (a) 3-methylaminopentol (**VIIa**), *m/z* 844 and (b) unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**), *m/z* 842.

Additional microcosm experiments were set-up at temperatures (8 and 15°C) more representative of *in situ* River Tyne conditions. However, as these experiments were prepared from a different batch of initial sediment it was not possible to directly compare concentrations (see appendix 3c for data). Therefore, the percentages of aminopentol (**VII**) and 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**) of the total BHPs from the original experiment (4 and 21-60°C) were compared to the additional temperature

experiments at 8 and 15°C (Figure 5-13). Neither of these compounds were detected in the two batches of sediments (Time 0) which were used in the preparation of the experimental microcosms.

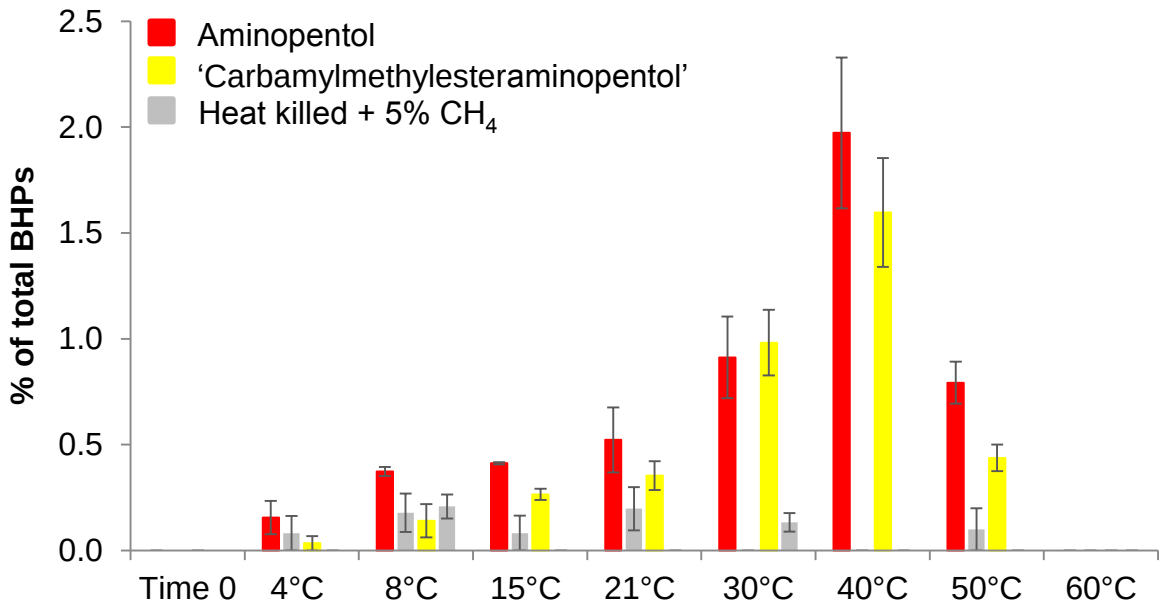



**Figure 5-13:** Relative % of aminopentol (**VII**) and 'carbamylmethylesterpentol' (**VII**$^{\mathbf{MeCarb}}$) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to temperature (corresponding heat killed controls to the right, where compound present). Error bars represent 1 x S.E. (N = 3) except 50°C heat killed (N = 2).

The relative percentage of aminopentol (**VII**) and 'carbamylmethylesterpentol' (**VII**$^{\mathbf{MeCarb}}$) increased in microcosm sediments with temperature with the greatest percentage evident at 40°C (Figure 5-13), where the absolute concentration of the compound was also greatest (Figure 5-11). Relative percentage of compounds in microcosms incubated at 8 and 15°C followed the trend of the original temperature study, with greater abundances than those incubated at 4°C and lower abundances than microcosms incubated at 21°C (Figure 5-13).

### 5.4.6. *hpnR* gene analysis in River Tyne estuarine sediment microcosms subjected to variations in temperature

DNA extracts from temperature-controlled microcosm sediments were analysed for the presence of the *hpnR* gene (Welander and Summons, 2012) which is required for the C-3 methylation of BHPs (Figure 5-14).

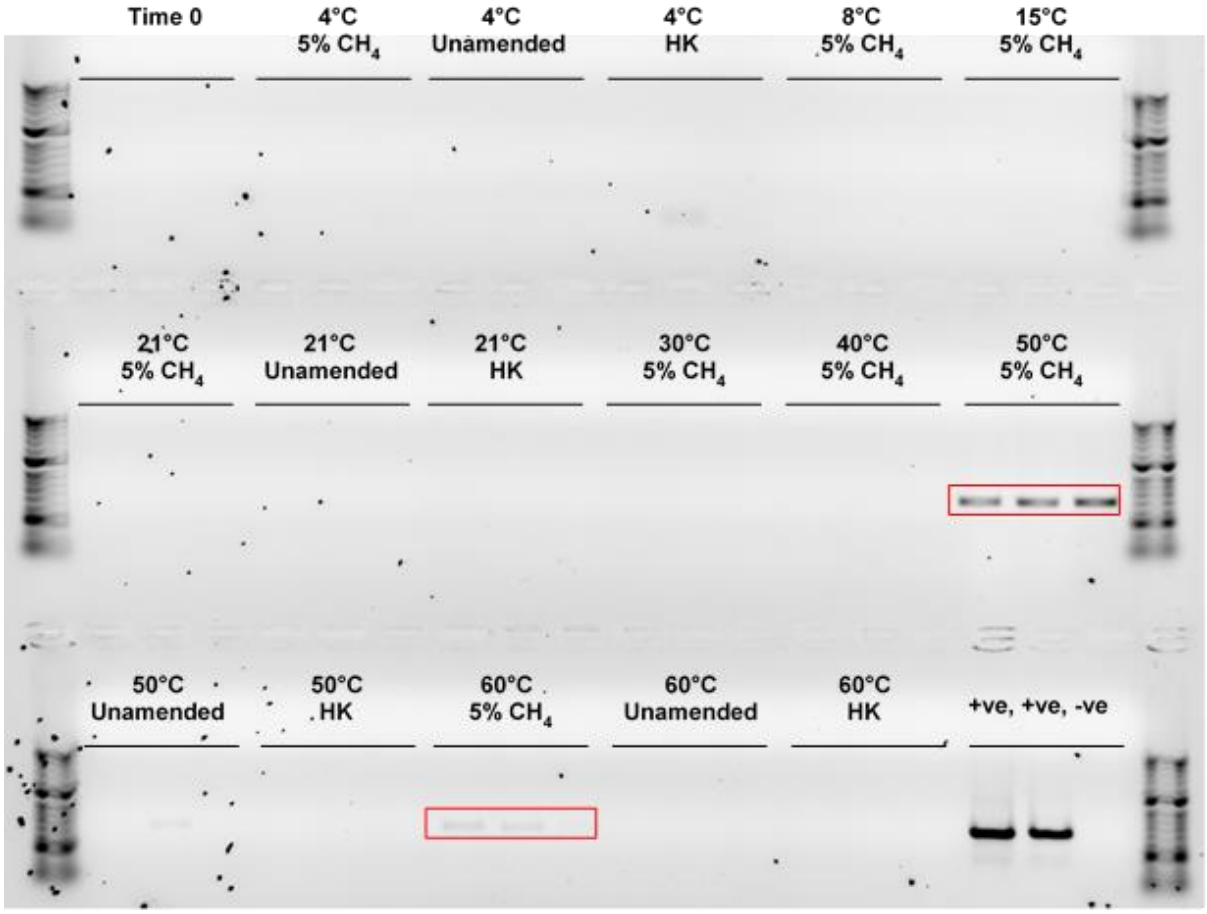



**Figure 5-14**: Agarose gel showing the *hpnR* gene (535 bp) in 5% methane amended microcosms and controls incubated under a range of temperatures (4-60°C) (first and last lane are Hyperladder II DNA size markers, HK = heat killed, +ve = PCR positive control, *Methylococcus capsulatus* gDNA, -ve = PCR negative control).

The *hpnR* gene was detected in microcosms incubated at 50°C (Figure 5-14), which were shown to be enriched with a *Methylocaldum* sp. (Figure 5-8). At 60°C, negligible methane oxidation was evident (Figure 5-7) and PCR amplification of the *pmoA* gene did not result in a product, suggesting methanotrophs cannot withstand this temperature. However, the *hpnR* gene was detected in 5% methane amended microcosms incubated at 60°C (Figure 5-14). Unexpectedly, the *hpnR* gene was not detected in sediments incubated at 40°C (5% $CH_4$), despite the detection of 3-methylaminopentol (**VIIa**) at 40°C (Figure 5-11b). Microcosms incubated at 40°C were shown to be enriched with *Methylobacter* spp. which differed from the *Methylobacter* spp. identified at 4-30°C (Figure 5-8). The *hpnR* gene was not detected in the initial sediment (Time 0) or sediments from microcosms incubated at 4-30°C with 5% methane. Furthermore, DNA extracts from unamended microcosms, without the

addition of methane and heat killed controls (HK) did not show the presence of the *hpnR* gene as no PCR product was visualised on the agarose gel (Figure 5-14).

#### 5.4.6.1. *hpnR* gene analysis in methanotroph pure cultures

DNA extracts from methanotroph pure cultures were analysed for the presence of the *hpnR* gene indicating the potential for 3-methylhopanoid production. The gene was not detected in the Type I methanotroph *Methylomonas methanica* S1 or the Type II *Methylosinus trichosporium* OB3b. PCR amplicons were not amplified in comparison to the positive control which contained gDNA from *Methylococcus capsulatus* (Type I) when visualised using agarose gel electrophoresis (data not shown).

### 5.4.7. Total BHP concentrations in River Tyne estuarine sediment microcosms subjected to variations in temperature

Total BHP concentrations in microcosm sediments in response to temperature were determined (Figure 5-15). The total BHP concentration of the initial sediment used for the experiment (Time 0) is also shown. For individual compound concentrations and relative abundances see appendix 3c.

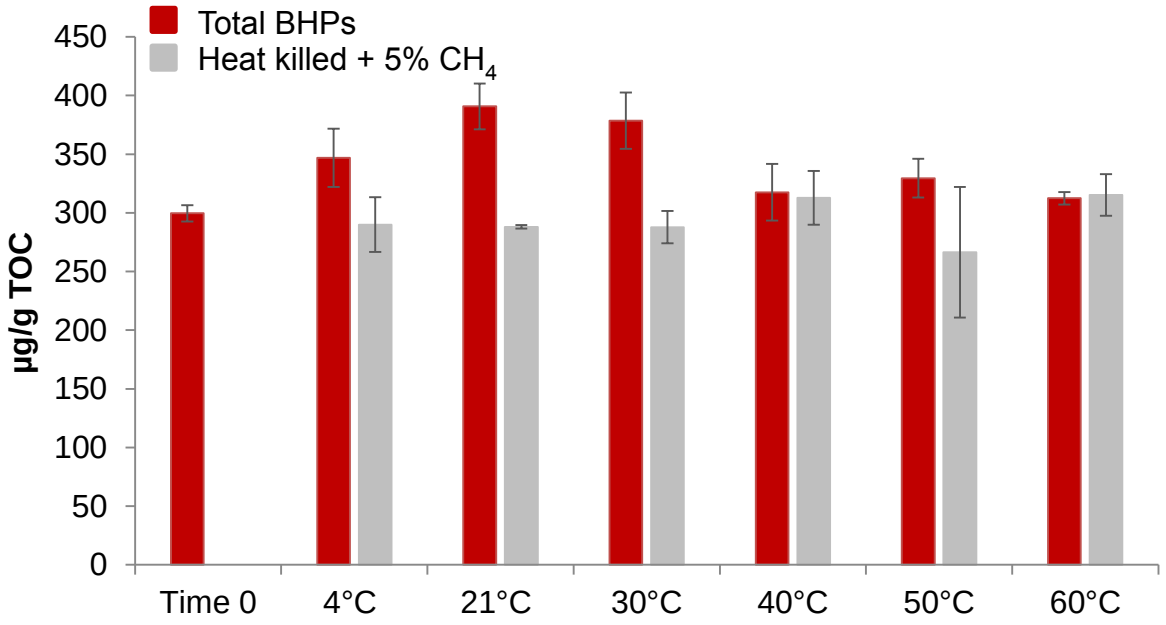



**Figure 5-15:** Total BHP concentrations (µg/g TOC) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to temperature (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3) except 50°C heat killed (N = 2).

Total BHP concentrations in 5% methane amended microcosm sediments were statistically compared to the initial sediment (Time 0), unamended controls (data not shown on Figure 5-15, see appendix 3c) and corresponding heat killed controls using one-way analysis of variance (ANOVA) with Fisher's LSD.

Total BHP concentrations were significantly higher relative to the initial sediment in 5% methane amended sediments at 21°C ($p = 0.001$) and 30°C ($p = 0.004$). No significant difference were seen in total BHP concentrations between pairwise comparisons of the initial sediment (Time 0) and 5% methane amended sediments at 4°C, 40°C, 50°C and 60°C ($p \geq 0.070$) (Figure 5-15). Total BHPs in 5% methane amended sediments were significantly higher than in corresponding heat killed controls at 4°C, 21°C, 30°C and 50°C ($p \leq 0.032$) but were not significantly different at 40 and 60°C ($p \geq 0.853$). Totals were also significantly higher in 5% methane amended sediments than in unamended controls at 21-30°C and 50°C ($p \leq 0.034$) (data not shown on Figure 5-15, see appendix 3c). Total BHPs in heat killed controls at all temperatures were not significantly different to the initial sediment concentrations ($p \geq 0.249$, Figure 5-15).

#### 5.4.7.1. Temperature effects on individual BHP compound concentrations

The effect of temperature on compounds other than the 35-aminoBHPs (~80% of total BHPs) was determined. BHT (**III**) and 2-methylBHT (**IIIa**) (see appendix 3c for data) showed no significant difference in concentrations in 5% methane amended sediments compared with initial sediment (Time 0) concentrations at all temperatures (BHT, $p \geq 0.185$; 2-methylBHT, $p \geq 0.118$ at all temperatures). 5% methane amended sediments showed no significant difference in concentration compared with their corresponding unamended controls at all temperatures (BHT, $p \geq 0.061$; 2-methylBHT, $p \geq 0.097$). No significant difference was seen between 5% methane amended sediments at 4-30°C and 50°C their corresponding heat killed controls (BHT, $p \geq 0.348$; 2-methylBHT, $p \geq 0.077$), but concentrations were significantly higher at 40 and 60°C (BHT, 40°C, $p = 0.006$ and 60°C, $p = 0.030$) (2-methylBHT, 40°C, $p = 0.001$ and 60°C, $p = 0.014$). Adenosylhopane (**XIII**) concentrations in 5% methane amended sediments showed no significant difference with the initial sediment at all temperatures ($p \geq 0.103$). No significant difference was observed between 5% methane amended sediments compared with their corresponding unamended controls at 4-40°C and 60°C ($p \geq 0.057$), but were significantly lower at 50°C ($p = 0.019$). No significant difference was seen with corresponding heat killed controls at all temperatures ($p \geq 0.062$), apart from 30°C ($p = 0.015$) or between initial sediment and heat killed controls ($p \geq 0.125$). 'Adenosylhopane-type 2' (**IX**) concentrations in 5% methane amended sediments showed no significant difference with the initial sediment or heat killed controls at all temperatures ($p \geq 0.068$), apart from concentrations detected in microcosms incubated at 40°C ($p = 0.005$).

### 5.4.8. BHT cyclitol ether concentrations in River Tyne estuarine sediment microcosms subjected to variations in temperature

BHT cyclitol ether (**XII**) and BHT glucosamine (**XIII**) concentrations in microcosm sediments in response to methane concentration (Figure 5-16 and Figure 5-18) and temperature (Figure 5-17 and Figure 5-19) were determined.

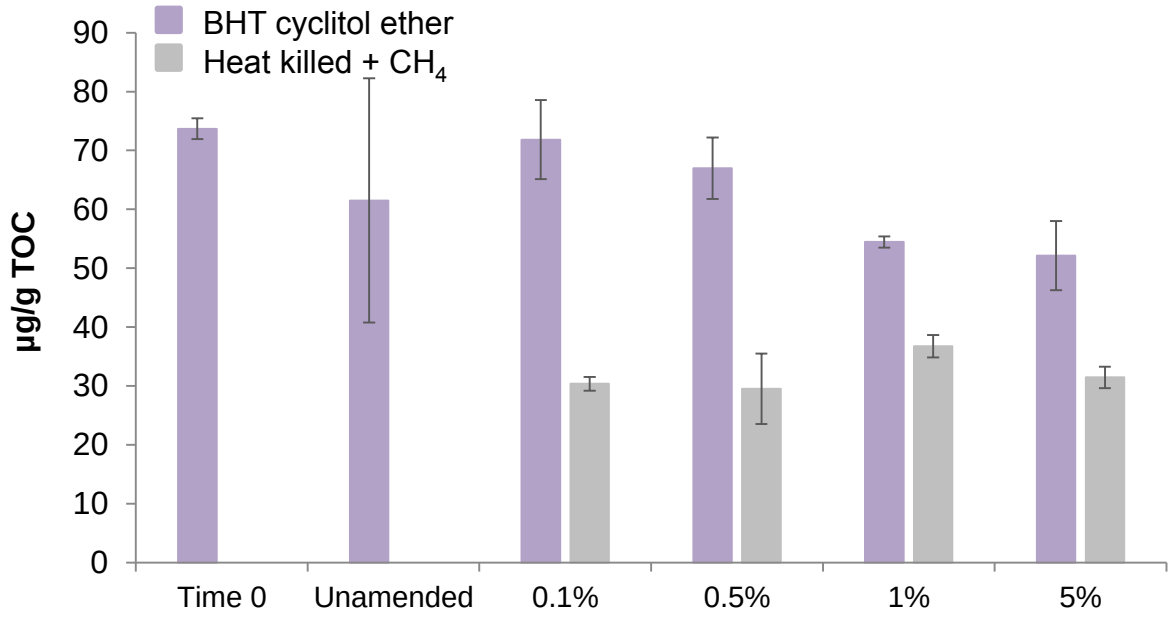



**Figure 5-16:** Concentration (µg/g TOC) of BHT cyclitol ether (**XII**) in $CH_4$ amended River Tyne estuarine sediment microcosms in response to methane concentration (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3).

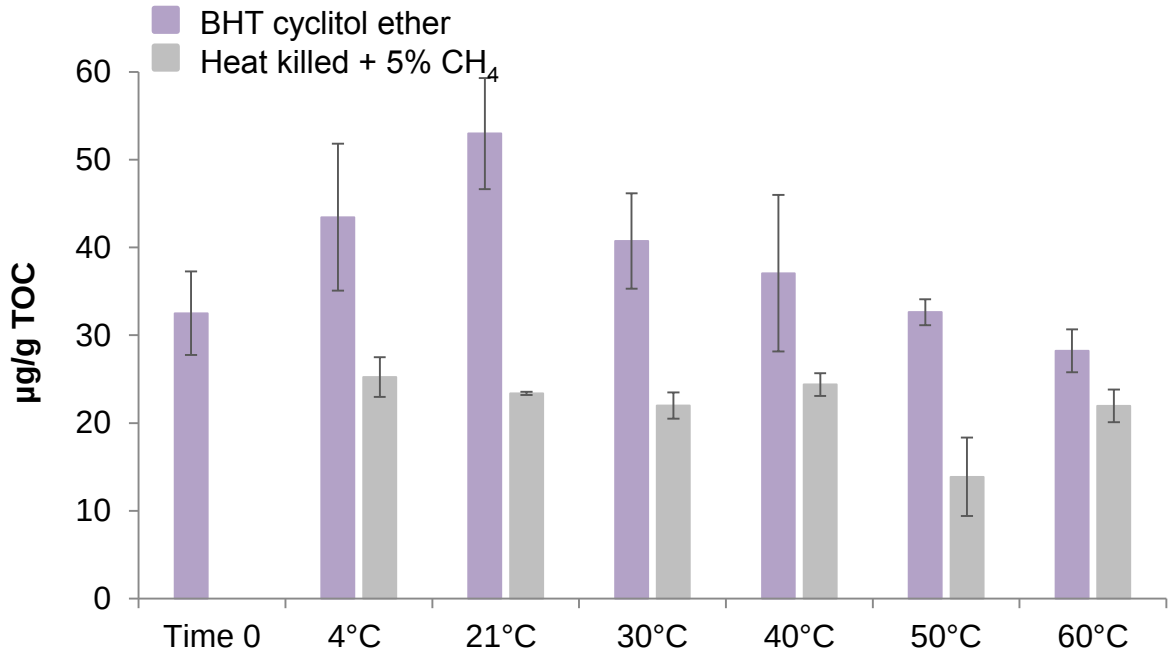



**Figure 5-17:** Concentration (µg/g TOC) of BHT cyclitol ether (**XII**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to temperature (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3) except 50°C heat killed (N = 2).

For the methane concentration study, BHT cyclitol ether (**XII**) was ~16% of total BHPs in initial sediment and 0.1% and 0.5% methane amended sediments but, in heat killed equivalents was only ~9% of total BHPs with absolute concentrations reduced by > 50% (Figure 5-16). In 1% and 5% methane amended sediments BHP concentrations were ~33% and ~40% lower, respectively, than heat killed equivalents.

In the temperature study, BHT cyclitol ether (**XII**) was ~11% of total BHPs in initial sediment and ~11-13% in 5% methane amended sediments between 4-40°C but in heat killed equivalents only ~8-9% of total BHPs (Figure 5-17). Absolute concentrations were ~42-56% lower in heat killed control sediments incubated at 4-30°C compared to 5% methane amended sediments but only ~35% less at 40°C. BHT cyclitol ether (**XII**) concentrations were ~58% lower in heat killed control sediments at 50°C compared to 5% methane amended sediments but only ~22% lower at 60°C.

In the temperature study, BHpentol cyclitol ether (**XIIa**) was also detected in the initial sediment (Time 0), 5% methane amended sediments and their unamended controls at 4-40°C but was absent at temperatures > 40°C and in all heat killed control sediments

at all temperatures (see appendix 3c for data). BHpentol cyclitol ether (**XIIa**) concentrations in heat killed control sediments were lower than the initial sediment, or the compound was not detected, at all methane concentrations (see appendix 3b for data). BHhexol cyclitol ether (**XIIb**) also showed lower concentrations in heat killed sediments compared with the initial sediment (Time 0).

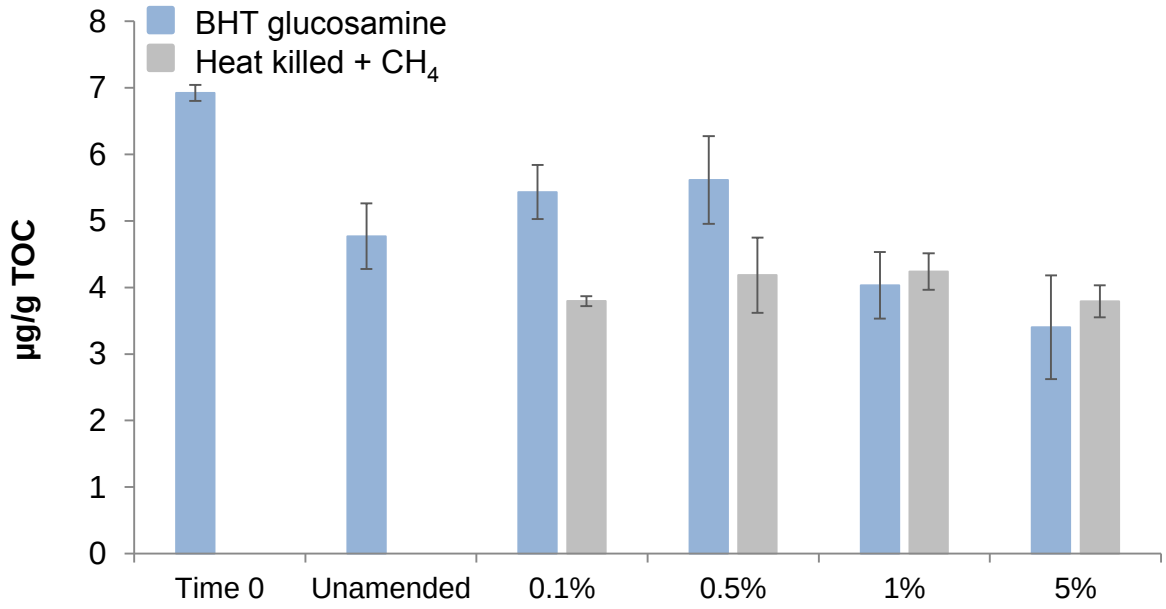



**Figure 5-18:** Concentration (µg/g TOC) of BHT glucosamine (**XIII**) in $CH_4$ amended River Tyne estuarine sediment microcosms in response to methane concentration (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3).

BHT cyclitol ether (**XII**) (Figure 5-16 and Figure 5-17) seems to be affected by heat kill treatment to a greater extent compared with the isomer BHT glucosamine (**XIII**) (Figure 5-18 and Figure 5-19). BHT glucosamine (**XIII**) concentrations were not so visibly reduced compared to heat killed equivalents when compared with BHT cyclitol ether (**XII**) concentrations.

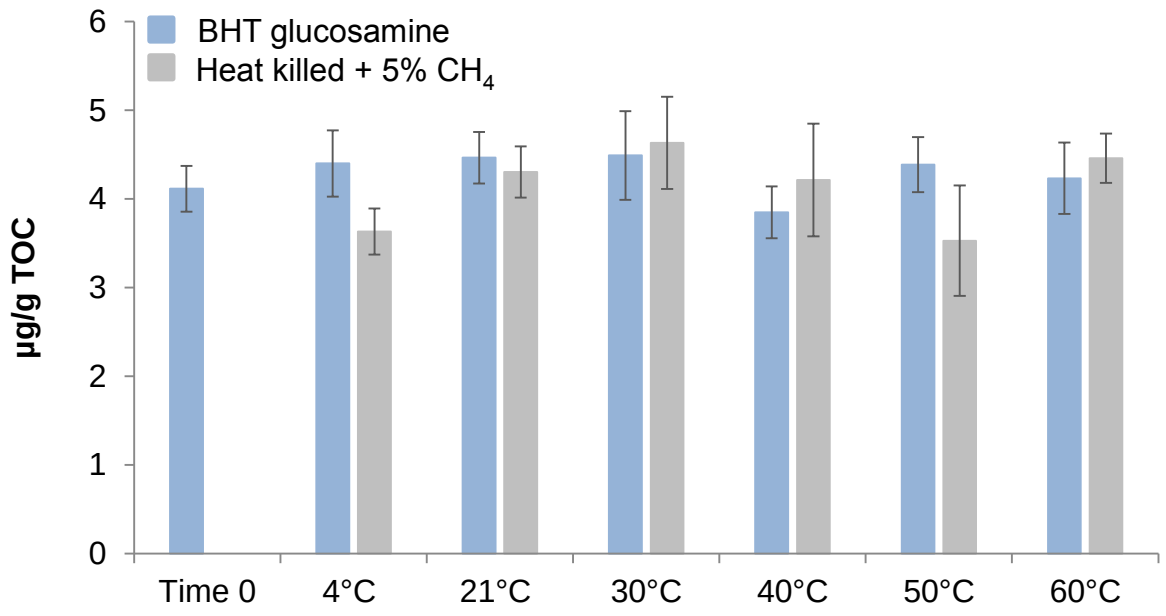



**Figure 5-19:** Concentration (µg/g TOC) of BHT glucosamine (**XIII**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to temperature (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3) except 50°C heat killed (N = 2).

## 5.5. Discussion

### 5.5.1. Methane oxidation rates in River Tyne estuarine sediment microcosms in response to methane concentration and temperature

The rate of methane oxidation increased with the concentration of methane in the headspace of the microcosm (Figure 5-1). Methane oxidation is an enzyme (MMO) catalysed reaction following Michaelis-Menten kinetics with the reaction velocity increasing with substrate (methane) concentration and the rates levelling off and approaching a maximum at higher substrate concentrations. An increase in methane oxidation rate was also witnessed in Arctic soil microcosms where rates were greater in microcosms incubated at 1000 ppm of methane compared with 15 ppm at all sampling depths and soil types (Martineau et al., 2014).

The rate of methane oxidation increased with temperature up to 40°C, then decreased at 50°C (Figure 5-7). Reduced methane oxidation rates at psychrophilic/tolerant temperatures (4-8°C) may be due to reduced enzyme activity and enzyme affinity at

lower temperatures, decreased membrane fluidity and altered transport of nutrients and waste products (D'Amico et al., 2006). An increase in enzyme activity would be expected as temperatures increase (15-30°C) and thus increased rates of reaction are witnessed (Madigan et al., 2009). The rate of reaction was fastest at 40°C suggesting this may be the optimum temperature for MMO enzyme activity, however at 50°C enzyme activity was reduced which may be due to the denaturing effects of high temperatures resulting in the decreased rates of methane oxidation. Negligible methane oxidation was seen at 60°C suggesting that methanotrophs in this study were unable to withstand temperatures ≥ 60°C. However, the *Methylocaldum* sp. enriched at 50°C, which shared 100% *pmoA* sequence identity with the cultured *Methylocaldum szegediense* OR2, has been shown to grow up to 62°C (Bodrossy et al., 1997). Other methanotrophs have also shown the ability to oxidise methane above the 50°C maximum witnessed in this microcosm study. *Methylothermus* strain HB, isolated from underground hot springs, grew up to 72°C (Bodrossy et al., 1999), *Methylothermus thermalis* isolated from a hot spring in Japan grew up to 67°C (Tsubota et al., 2005) and *Methylothermus subterraneus* also isolated from a hot spring in Japan grew up to 65°C (Hirayama et al., 2011).

Temperature has been shown to enhance bacterial methane oxidation in a peat moss study with rates being two times higher when incubated at 20°C compared with 10°C (Kip et al., 2010). An increase in methane oxidation rate in response to temperature was also witnessed in *Sphagnum* moss mesocosm experiments (5-25°C) with maximum rates seen at 20°C (van Winden et al., 2012a).

### 5.5.2. Methanotroph community composition in River Tyne estuarine sediment microcosms in response to methane concentration and temperature

Type I methanotrophs were the only group identified at all methane concentrations and temperatures, *pmoA* sequences related to Type II methanotrophs were not detected (Figure 5-2 and Figure 5-8). No change in methanotroph community composition was observed across all methane concentrations (Figure 5-2) contrary to the original hypothesis that communities would change. The relatively high methane concentrations tested in the microcosm study may have only enriched low affinity methanotrophs hence no shift in methanotroph community composition was witnessed.

Significant changes in methanotroph community composition were observed, however, in the temperature incubation study (Figure 5-7). In microcosm sediments incubated at 4-21°C, a *Methylobacter* sp. was enriched and at 30°C other *Methylobacter* species were identified (Figure 5-8), also present at the lower temperatures. One of these species was closely related to the *pmoA* sequence from *Methylobacter* sp. BB5.1, the most common *pmoA* sequence in estuarine sediments in Newport Bay, and a 'methanotroph isolate 2' also isolated from Newport Bay (Smith et al., 1997; McDonald et al., 2005b). At 40°C a *Methylobacter* sp. was also enriched but again with a different identity to either of those enriched at 30°C (Figure 5-8). Methanotroph diversity in estuarine sediments from Newport Bay, California, USA has been previously reported and the gene sequences identified grouped with *Methylomonas, Methylomicrobium* and *Methylobacter* genera, all Type I species. In the surface sediment examined from the Colne Estuary, UK, *Methylomonas* spp. and *Methylobacter* spp. were detected alongside *Methylomicrobium* spp. and *Methylococcus* spp. but after incubation with $^{13}$C-labelled methane, populations were mainly affiliated with *Methylomonas* spp., *Methylobacter* spp. and an uncultivated *Methylococcus* sp. (Moussard et al., 2009). The occurrence of *Methylobacter* spp. is a common feature in estuarine environments.

At 50°C the most dominant methanotroph in the microcosm sediments was identified as a *Methylocaldum* sp., which shared 100% *pmoA* sequence identity with the cultured *Methylocaldum szegediense* OR2, originally isolated from the effluent of an underground hot spring with optimum growth at 55°C and a range of 37-62°C (Bodrossy et al., 1997). The River Tyne estuary *in situ* conditions would not support the activity and growth of thermophilic methanotrophs, therefore, they are likely to have been passively dispersed into the sediments from terrestrial sources e.g. agricultural environments. Interestingly, *Methylocaldum szegediense* has also been detected in the temperate sediments of the Colne Estuary (Moussard et al., 2009), from marine sediments in Japan, growth up to 47°C (Takeuchi et al., 2014), and in environmental settings such as Canadian geothermal spring sediments with *in situ* temperatures of 45°C (Sharp et al., 2014).

Type II methanotrophs were not detected in any microcosm sediments which may be because the concentration of methane used in microcosms selected for Type I methanotrophs. Type II methanotrophs have previously been detected in estuarine

sediments from Newport Bay namely *Methylosinus* (McDonald et al., 2005b) but *Methylocystis* and *Methylosinus* were only detected in the original sediment samples of the Colne Estuary where methane concentrations in the top surface sediment ranged between 0.5 to 1 µM, lower than microcosm methane concentrations, and not after incubation with methane (Moussard et al., 2009).

### 5.5.3. The effect of methane concentration on 35-aminoBHP signatures in River Tyne estuarine sediment microcosms

Aminotriol (**V**) (Figure 5-3) and aminotetrol (**VI**) (Figure 5-4) concentrations were similar in all methane amended sediments with no significant differences compared with concentrations in the initial sediment. Aminotriol (**V**) is produced by a wide range of bacterial sources (e.g. Talbot and Farrimond, 2007; Talbot et al., 2008; Blumenberg et al., 2012) and was also found in varying abundances in *Methylobacter* pure cultures (Chapter 4, Section 4.4.2) which belong to the genus which was identified in microcosms at all methane concentrations in this study. Aminotetrol (**VI**) is more diagnostic being found in almost all Type I methanotrophs, including cultures of the genus enriched in this study, *Methylobacter* (Neunlist and Rohmer, 1985a; Cvejic et al., 2000a; Talbot et al., 2001).

Aminopentol (**VII**) is significantly more abundant in 5% methane amended sediments compared with initial sediment concentrations. The greater methane concentration may enrich a greater number of methanotroph cells resulting in the increases in aminopentol (**VII**) detected. No unsaturated compounds were detected in sediments across the methane concentration range. This is perhaps expected as only trace levels of unsaturated aminopentol (**VIIb**) have been observed in *Methylobacter* spp. examined (Chapter 4, Section 4.4.2, Figure 4-9) where methanotroph cell numbers would be much higher than those enriched in sediment microcosms. The unsaturated compounds may have been below detection limits in microcosm sediments, as concentrations of aminopentol (**VII**) were low even when significantly enriched relative to the initial sediment at 5% methane concentration. Conversely, the species of *Methylobacter* enriched may not synthesise unsaturated compounds which are only reported in a limited number of Type I methanotroph studies (Cvejic et al., 2000a; van Winden et al., 2012c). C-3 methylated compounds were not detected in the sediments across all methane concentrations whereas they have been identified in recently

examined *Methylobacter* spp. cultures (Chapter 4, Section 4.4.2). Once again, this may be because concentrations were below detection limits or differences between the species enriched in the microcosm sediments and the cultured organisms.

### 5.5.4. The effect of temperature on 35-aminoBHP signatures in River Tyne estuarine sediment microcosms

The concentrations of 35-aminoBHPs varied with estuarine sediment incubation temperature (Figure 5-9 to Figure 5-11). The compositions appear to be a reflection of the methanotrophs that were enriched at specific temperatures and do not directly correlate with methane oxidation rates and methanotrophic activity especially for aminotriol (**V**) and aminotetrol (**VI**). Aminotriol (**V**) is enriched in sediment microcosms at 4-30°C compared with the initial sediment (significantly at 21 and 30°C); a single *Methylobacter* sp. was identified at 4 and 21°C and at 30°C two additional species were identified (these were also present at 4 and 21°C as fainter bands). At 40°C *Methylobacter* sp. was also enriched but again with a different identity to that enriched at 30°C. Aminotriol (**V**) concentrations at this temperature were similar to those of the initial sediment which suggests that the species enriched has lower abundances of this compound. Differences in BHP abundances were seen in *Methylobacter* spp. recently analysed (Chapter 4, Section 4.4.2) with *Methylobacter* sp. BB5.1 having a greater abundance of aminotriol (**V**) compared with the two other marine *Methylobacter* spp. examined. Interestingly, a species identified at 30°C (also present at 4 and 21°C) was closely related to *Methylobacter* sp. BB5.1 and this may be the reason aminotriol (**V**) concentrations were higher at these temperatures compared with 40°C. 'Carbamylmethylestertriol' ($\mathbf{V^{MeCarb}}$) also had the same trend with increasing temperature as aminotriol (**V**) and suggests the synthesis of the 'regular' and 'carbamylmethylester' compounds may be related (Figure 5-9).

Aminotetrol (**VI**) was significantly enriched in sediment microcosms at 4-30°C compared with the initial sediment; a single *Methylobacter* sp. was identified at 4 and 21°C and at 30°C two additional species were identified. This seems to suggest that the *Methylobacter* spp. enriched at these temperatures synthesise more of this compound compared with those enriched at 40°C which showed no significant increase relative to the initial sediment. Once again, the 'carbamylmethylester' pseudohomologue of this compound showed a similar trend suggesting a relationship

between the synthesis of the two compounds (Figure 5-10). Quantitative PCR (qPCR) would confirm whether higher concentrations of the aforementioned compounds at lower temperatures was the result of more methanotroph cells and consequently more BHPs, or more BHPs per methanotroph cell.

Aminopentol (**VII**) was enriched relative to the initial sediment at all temperatures tested, except 60°C (Figure 5-11). The differences in aminopentol (**VII**) concentrations observed when *Methylobacter* spp. were enriched (4-40°C) suggests variation in BHP abundances at the species level. The greatest concentration was detected at 40°C where different *Methylobacter* spp. (band 5, Figure 5-8) were enriched compared to those enriched at the lower temperatures. Moreover, a small population of a *Methylocaldum* sp. may be present at this temperature (faint bands in the DGGE profile) and this may also result in greater concentrations of aminopentol (**VII**) overall. 'Aminopentol isomer' (**VII$^{iso}$**), was only present in estuarine sediments at one temperature tested (40°C) and this compound has previously been found in other environmental settings where aminopentol (**VII**) is abundant relative to other BHPs (Talbot et al., 2014). It has been reported in one methanotroph culture prior to this, the *Methylovulum*-like strain M200 (van Winden et al., 2012c) but it was not detected in the *Methylobacter* pure cultures examined during this study (Chapter 4, Section 4.4.2). 'Carbamylmethylesterpentol isomer' (**VII$^{iso,MeCarb}$**) was also detected in sediments incubated at a temperature of 40°C. Low levels of unsaturated $\Delta^{11}$ aminopentol (**VIIb**) were only detected in sediments at 40°C, with trace levels of this also detected in *Methylobacter* pure cultures in this study (Chapter 4, Section 4.4.2). However, this compound was not detected in sediments at 4-30°C despite the enrichment of *Methylobacter* spp. in these microcosms. Possibly, unsaturated $\Delta^{11}$ aminopentol (**VIIb**) is not found in all species of *Methylobacter* or, the trace quantities detected here in pure cultures would render it difficult to detect in natural settings. 'Carbamylmethylesterpentol' (**VII$^{MeCarb}$**) concentrations were similar to those of aminopentol (**VII**) but were actually greater than the 'regular' compound at 30°C. In the pure methanotroph cultures investigated in this study, high levels of 'carbamylmethylester' compounds were observed in *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp. with lower levels detected in *Methylobacter* spp. (Chapter 4, Section 4.4.2). Perhaps some of the species of *Methylobacter* enriched in the

microcosm sediments synthesise higher concentrations of ‘carbamylmethylester’ compounds than pure cultures examined in this study.

3-Methylaminopentol (**VIIa**) was present at 40-60°C with the concentration being greater than its non-methylated equivalent at 50°C. At this temperature, the dominant methanotroph identified shared 100% *pmoA* sequence identity with the cultured *Methylocaldum szegediense* OR2. Interestingly, a pure culture of *Methylocaldum szegediense* OR2 exhibited a greater abundance of the methylated equivalent compared with its non-methylated equivalent (Cvejic et al., 2000a). This suggests that relative BHP abundances observed in culture are reflected in sediment signatures from methanotrophs enriched in ‘natural’ settings. Trace amounts were detected in the sediment of one replicate microcosm incubated at 40°C which was enriched with a *Methylobacter* sp. with members of this genus recently shown to produce methylated compounds (Chapter 4, Section 4.4.2). Trace amounts were also detected at 60°C, however, methane oxidation was not observed at this temperature (Figure 5-7) and the *pmoA* gene could not be amplified from DNA extracts. This may simply be because the detection limits for the *pmoA* gene and BHPs are different. However, it could imply that a small number of thermophilic methanotrophs are surviving at this temperature, in a stationary or vegetative state unable to oxidise methane, but continue to synthesise 3-methylaminopentol (**VIIa**). It has been suggested 3-methylhopanoid production may be stimulated by environmental stresses the cell is exposed to during stationary phase (Welander and Summons, 2012). Another study reported that vegetative cells of *Nostoc punctiforme* which had differentiated into akinetes, due to insufficient light or when phosphorus is limiting, continued to produce BHPs (Doughty et al., 2009). Unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) was detected in sediments incubated at 40 and 50°C and has only previously been witnessed in a *Methylocaldum szegediense* OR2 culture (Cvejic et al., 2000a). Although a *Methylobacter* sp. dominated at 40°C, *Methylocaldum* spp. may also be present at this temperature, faint bands witnessed in the DGGE profile (Figure 5-8), and may be the source of the unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) as this compound was not detected in the *Methylobacter* pure cultures screened during this study (Chapter 4, Section 4.4.2).

#### 5.5.4.1. Distributions of BHPs in River Tyne estuarine sediments incubated at different temperatures - a comparison with methanotroph cultures

BHP distributions from the analysis of six marine methanotrophs (Chapter 4, Section 4.4.2), *Methylomicrobium* spp. (Birgel et al., unpublished data) and methanotroph data from the literature were compared to the BHP distributions detected in estuarine sediments incubated at different temperatures (Figure 5-20). The concentrations of aminotriol (**V**), aminotetrol (**VI**), aminopentol (**VII**) and their methylated equivalents were subtracted from the concentrations present in the initial sediments (Time 0) to determine abundances resulting from enrichment at different temperatures. To allow for a more accurate comparison with data from the literature, no 'carbamylmethylester' compounds or early eluting isomers were included when producing the ternary plot.

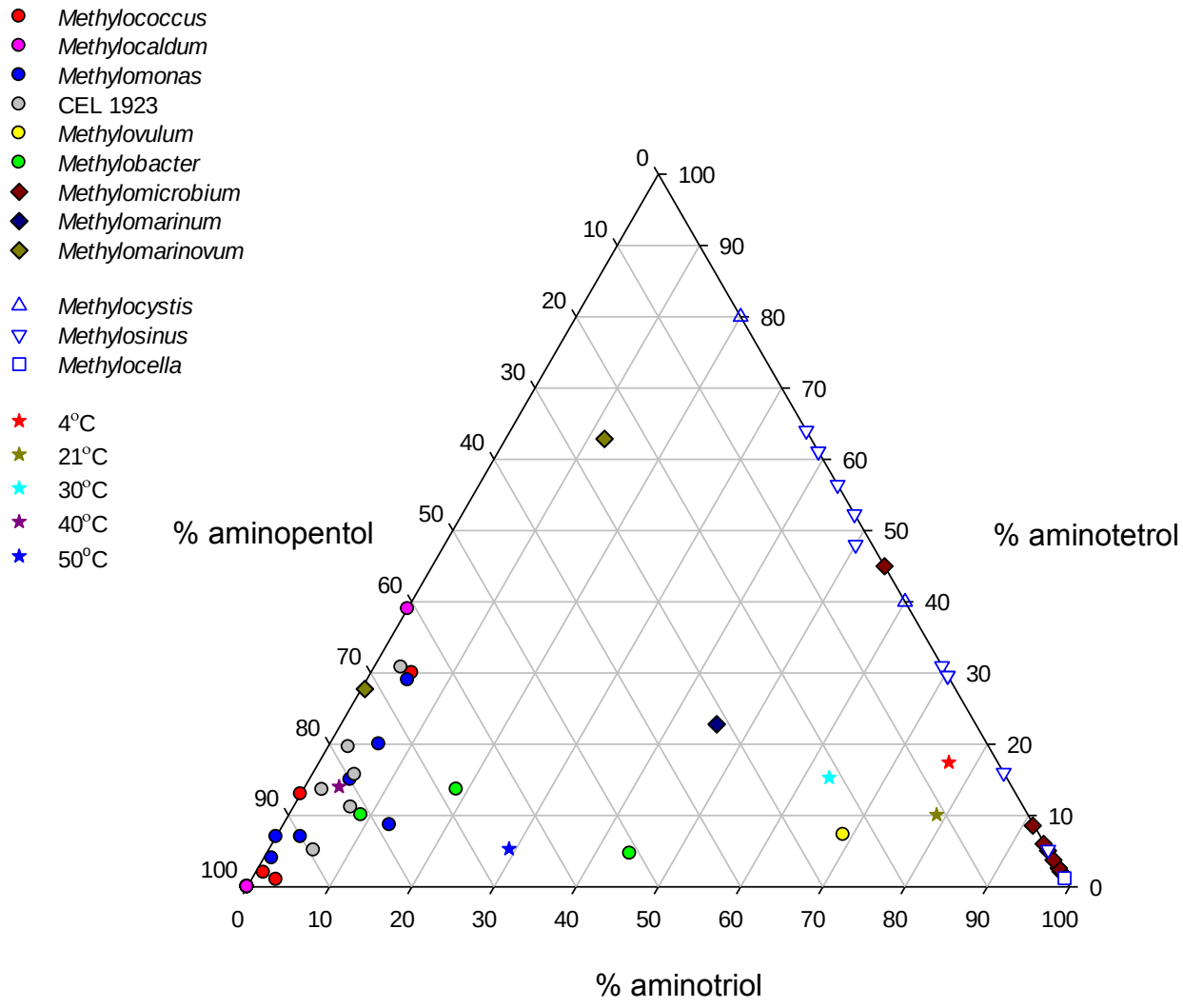



**Figure 5-20:** Ternary plot of the BHP distributions of estuarine sediments incubated at different temperatures, analysed methanotroph pure cultures and existing data from the literature (Rohmer et al., 1984; Neunlist and Rohmer, 1985b; Jahnke et al., 1999; Cvejic et al., 2000a; Talbot et al., 2001; van Winden et al., 2012c).

BHP distributions of River Tyne estuarine sediments incubated at 4-21°C, where *Methylobacter* spp. were enriched, clustered in the region near the *Methylovulum*-like strain M200 (Kip et al., 2011) where aminotriol (**V**) dominates (van Winden et al., 2012c) (Figure 5-20). The 16S rRNA and *pmoA* gene sequences of the *Methylovulum*-like strain M200 had the closest identity to *Methylovulum miyakonense* but also had a close relationship to *Methylosoma difficile* LC 2$^{T}$ and *Methylobacter* spp. (Kip et al., 2011). This close relationship with *Methylobacter* spp. may explain why the estuarine sediments incubated at these temperatures cluster near this pure culture. Moreover,

the closest cultured representatives of the methanotrophs enriched at 4-21°C were *Methylovulum miyakonense*.

The DGGE profile at 30°C was similar to the profiles at 4 and 21°C but also included additional bands which would perhaps explain the lower percentage of aminotriol (**V**) compared with 4 and 21°C. BHP distributions in sediments incubated at 40 and 50°C were very distinct from those at 4-30°C and clustered with the majority of Type I methanotroph genera on the ternary plot. A more temperature-resistant *Methylobacter* sp. was enriched at 40°C and the 35-aminoBHP composition was dominated by aminopentol (**VII**). This clustered close to the marine *Methylobacter* cultures recently analysed particularly that of *Methylobacter marinus* A45 (Chapter 4, Section 4.4.2). At 50°C a *Methylocaldum* sp. was enriched in the sediments and the 35-aminoBHPs were dominated by aminopentol (**VII**). This result did not closely cluster with the BHP distributions detected in *Methylocaldum* spp. (pink circles, Figure 5-20), due to higher levels of aminotriol (**V**). However, the two cultured species (pink circles) also showed different BHP distributions to one another suggesting species within this genus may possess different BHP compositions. Moreover, other undetected taxa may be present in the sediment, either methanotrophs or non-methanotrophs.

### 5.5.5. Detection of the *hpnR* gene in River Tyne estuarine sediment microcosms incubated at different temperatures

Culture-based investigations examining the BHP compositions of Type I methanotrophs have revealed the frequent absence of 3-methylaminopentol (**VIIa**) when aminopentol (**VII**) is present. For example, members of the Type I genera *Methylomonas* do not contain 3-methylaminopentol (**VIIa**) (Neunlist and Rohmer, 1985b; Jahnke et al., 1999; Talbot et al., 2001; van Winden et al., 2012c), or the *Methylovulum*-like strain M200 (van Winden et al., 2012c) and during this study 3-methylaminopentol (**VIIa**) was not detected in *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp. (Chapter 4, Section 4.4.2).

The *hpnR* gene required for C-3 methylation of BHPs (Welander and Summons, 2012) was targeted in DNA extracts from estuarine sediment microcosms. The gene was absent in sediments incubated at 4-30°C which were enriched with *Methylobacter* spp. (Figure 5-8) and concomitantly, 3-methyl compounds were also not detected in the sediments (Figure 5-9 to Figure 5-11). *Methylobacter* was not identified as one of the

three methanotrophs to contain the *hpnR* gene from the nine methanotroph genomes probed (Welander and Summons, 2012). However, the presence of the gene is inferred for some species of *Methylobacter* as C-3 methylated compounds were detected in three marine *Methylobacter* spp. analysed, for the first time during this study (Chapter 4, Section 4.4.2). Genetic studies have also shown that the *hpnR* gene is only present in a subset of some species of certain genera such as *Burkholderia* (Welander and Summons, 2012) which suggests that this could also be the case for methanotrophs. The *hpnR* gene was observed in sediments incubated at 40°C, as very faint bands in an agarose gel, which were enriched with a thermotolerant *Methylobacter* sp. compared to that identified at 4-30°C. 3-Methylaminopentol (**VIIa)** was present in the sediment of one replicate microcosm and unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) was present in all replicates (Figure 5-11) suggesting the presence of the gene but perhaps in low abundance. However, faint bands in the PCR-DGGE (Figure 5-8) at 40°C, which was identified as a *Methylocaldum* sp. 50°C, may be the source of the *hpnR* gene.

The *hpnR* gene was detected in microcosms incubated at 50°C which were enriched with a *Methylocaldum* sp. (Figure 5-8). Concomitantly, 3-methylaminopentol (**VIIa**) and unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) were detected in microcosm sediments at this temperature (Figure 5-11). This is in agreement with *Methylocaldum* spp. culture studies (Cvejic et al., 2000a) which demonstrated the production of 3-methylaminopentol (**VIIa**), unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) and also 3-methylaminotetrol (**VIa**), which was not detected in the current study. Although *Methylocaldum* was not identified as one of the three methanotrophs to contain the *hpnR* gene in a previous study (Welander and Summons, 2012), evidence from this study and a pure culture study (Cvejic et al., 2000a) infers its presence. The *hpnR* gene was also detected in microcosms incubated at 60°C and concomitantly trace amounts of 3-methylaminopentol (**VIIa**) were detected in two of the replicate microcosms sediments (Figure 5-11).

#### 5.5.5.1. Detection of the *hpnR* gene in methanotroph pure cultures

The *hpnR* gene was not detected in the Type I methanotroph *Methylomonas methanica* S1 which was expected as C-3 methylated BHPs have not been detected in this genus (Neunlist and Rohmer, 1985b; Jahnke et al., 1999; Talbot et al., 2001;

van Winden et al., 2012c) and it was not identified as one of the three methanotroph genomes to contain the gene (Welander et al., 2012). The gene was absent in *Methylosinus trichosporium* OB3b which was expected as Type II methanotrophs have been tested and found not to synthesise C-3 methylated BHPs (Neunlist and Rohmer, 1985a; Cvejic et al., 2000; Talbot et al., 2001; van Winden et al., 2012b) and were also reported not to contain the *hpnR* gene (Welander and Summons, 2012). This indicates that the absence of 3-methylhopanoids (bio or geo) is not evidence for the absence of methanotrophs in an environment and a more thorough investigation is required, such as isotope analysis.

### 5.5.6. Total BHP concentrations in River Tyne estuarine sediment microcosms in response to methane concentration and temperature

Total BHP concentrations showed no significant increase with increasing methane concentration in comparison with initial sediment concentrations (Time 0) (Figure 5-6). An increase in methane concentration did not result in large increases in 35-aminoBHP concentrations (Figure 5-3 to Figure 5-5) therefore the effect on total BHP concentrations would be minimal. The most abundant compound in all methane amended, unamended and heat killed controls was BHT (**III**) (see appendix 3b for data). This is one of the most commonly occurring BHPs and is produced by a wide range of bacteria (e.g. Sinninghe Damsté et al., 2004; Talbot and Farrimond, 2007; Talbot et al., 2008; Blumenberg et al., 2012; Eickhoff et al., 2013). Therefore, it is not diagnostic for any particular bacterial group. The second most abundant BHP was adenosylhopane (**VIII**); a compound that is produced by all microorganisms (Bradley et al., 2010), but has only been shown to accumulate in a small number of microorganisms such as some purple non-sulfur bacteria, a nitrogen-fixing bacterium, an ammonium-oxidising bacteria (e.g. Talbot and Farrimond, 2007) and at low levels in a single Type II methanotroph (van Winden et al., 2012c).

Total BHP concentrations showed a significant increase relative to initial sediment concentrations (Time 0) in sediments incubated at 21 and 30°C (Figure 5-15) but no differences were observed at the other temperatures, suggesting the majority of BHP producers were mesophiles. The most abundant compound in all methane amended and heat killed controls was BHT (**III**) with the second most abundant BHP being adenosylhopane (**VIII**) (see appendix 3b for data). BHT (**III**) abundances, and total

BHPs, have been shown to increase with temperature in *Sphagum* moss mesocosms experiments with the greatest concentrations witnessed at 25°C (van Winden, 2011). An increase in BHT (**III**) was not detected in this study and other common compounds such 2-methylBHT (**IIIa**), adenosylhopane (**XIII**) and 'adenosylhopane-type 2' (**IX**) were also not enriched in 5% methane amended sediments relative to the initial sediments at any temperature. The concentration of aminotriol (**V**), aminotetrol (**VII**) and related 'carbamylmethylester' compounds showed marked increases in concentration at these temperatures which would contribute to higher total BHP concentrations. The addition of methane to the microcosms enriched methanotrophs in the sediment but other aerobic bacterial groups were likely enriched using residual organic matter in the sediment and nutrients in the medium. The majority of bacteria in nature are mesophilic in nature and at 21 and 30°C would be the major *in situ* microbial communities in the sediment. Therefore, greater numbers of bacteria may be enriched in microcosm sediments at these temperatures resulting in greater BHP concentrations. Significant increases in BHPs may not have been witnessed in this study outside the mesophilic temperature range, as only a small *in situ* bacterial population would be present in the sediment to withstand higher temperatures.

### 5.5.7. Degradation of BHPs in River Tyne estuarine sediment microcosms subjected to heat kill treatment

Total BHP concentrations were significantly lower in all heat killed (autoclaved) controls in the methane concentration study relative to the initial sediment for all methane concentrations suggesting that some BHPs were degraded during the heat kill process. However, no significant difference was seen in total BHPs in heat killed controls in the temperature study relative to the initial sediment for all temperatures, although some differences were seen in individual compounds (Figure 5-15). Compounds such as BHT (**III**), 2-methylBHT (**IIIa**), adenosylhopane (**XIII**) and 'adenosylhopane-type 2' (**IX**) seemed to be more resistant to autoclaving compared with other compounds (see appendix 3b and 3c). BHT (**III**) degradation could be counterbalanced by production from the degradation of cyclitol ethers (Schaeffer et al., 2010), however, results from methane concentration and temperature studies do not seem to suggest this. The apparent recalcitrant nature of BHT (**III**) is supported by evidence in the sedimentary record where BHT (**III**) has been extracted from sediments

up to ~50 Ma years old, the oldest extracted intact polyfunctionalised BHP to date (van Dongen et al., 2006).

Aminotriol (**V**) and aminotetrol (**VI**) seemed to show more susceptibility to heat degradation when compared to the initial sediment than the aforementioned compounds (Figure 5-3 to Figure 5-4 and Figure 5-9 to Figure 5-10). This suggests that the C-35 terminal group is more readily cleaved compared to that of BHT (**III**) and adenosylhopane (**XIII**). Abiotic studies have shown the degradation of BHPs such as BHT (**III**) and aminotriol (**V**) in *Rhodopseudomonas palustris* TIE-1 when exposed to high temperatures and pressure (Eickhoff et al., 2014) and *Zymomonas mobilis* (Schaeffer et al., 2010). This suggests that BHT (**III**) may be resistant to the heat and pressure applied during the autoclaving process in the preparation of heat killed control microcosms (20 min at 121°C, with elevated pressure; Chapter 3; Section 3.3.2). However, at higher temperatures (170 and 250°C) and pressure (120 MPa), as seen in the *Rhodopseudomonas palustris* TIE-1 study (Eickhoff et al., 2014), and application of conditions for several days in both abiotic studies (Schaeffer et al., 2010; Eickhoff et al., 2014), BHT (**III**) was susceptible to degradation.

Evidence from this study suggests ether linked composite BHPs are susceptible to degradation from heat treatment with the concentration of BHT cyclitol ether (**XII**) in heat killed sediments consistently lower when compared with the initial sediment (Figure 5-16 and Figure 5-17). BHpentol cyclitol ether (**XIIa**) and BHhexol cyclitol (**XIIb**) concentrations were either lower, or the compound absent, from sediments that had been subjected to heat kill treatment (see appendix 3b and 3c). This may be the result of the autoclave process cleaving the composite moiety from the side chain. However, no increase in BHT (**III**), which can result from the cleavage of this moiety, was witnessed.

### 5.6. Conclusions

Only Type I methanotrophs were enriched in the River Tyne estuarine sediment, as hypothesised, with *Methylobacter* spp. dominating at all methane concentrations, 0.1-5%, and 4-40°C and a *Methylocaldum* sp. at 50°C. The dominance of Type I methanotrophs was hypothesised and has been observed in other studies of this type of setting (e.g. McDonald et al., 2005b; Cunliffe et al., 2008; Moussard et al., 2009). In the methane concentration study, aminopentol (**VII**) was only significantly enriched in

5% methane amended sediments. Moreover, no change in methanotroph community composition was observed with increasing methane concentration. This disproved the original hypothesis that community composition would change with increasing concentration and suggests that the relatively high methane concentrations tested in the microcosm study enriched only low affinity methanotrophs.

Methanotroph community composition varied with temperature with *Methylobacter* spp. dominating at 4-40°C, although with different temperature tolerances, i.e. different species of *Methylobacter* were enriched at 40°C compared to the lower temperatures. A *Methylocaldum* sp. was enriched at 50°C and methane oxidation was not observed or methanotrophs enriched above this temperature. This supports the hypothesis that methanotroph community composition changes in response to temperature. Shifts in methanotroph community composition were reflected in the 35-amino-BHP compositions, which supports the original hypothesis, with greater concentrations of aminotriol (**V**) and aminotetrol (**VI**) being detected at 4-30°C compared with 40°C. This does not correlate with methane oxidation rates and methanotrophic activity and suggests variation in BHP compositions in sediments is controlled by changes in the species of *Methylobacter* enriched. Evidence to support this was shown by the variance in BHP composition between species of the same genus in *Methylobacter* pure cultures (Chapter 4, Section 4.4.2). Aminopentol (**VII**) concentrations increased with temperature with the maximum seen at 40°C. This also supports the hypothesis that suggests BHP compositions vary at species level within the *Methylobacter* genus as a different, more tolerant, species of *Methylobacter* was enriched at this temperature. The dominant methanotroph at 50°C was a *Methylocaldum* sp., which shared 100% *pmoA* sequence identity with the cultured *Methylocaldum szegediense* OR2. The greater abundance of 3-methylaminopentol (**VIIa**) compared with its non-methylated equivalent witnessed in this culture (Cvejic et al., 2000a) was also seen in sediments enriched with this methanotroph. This indicates that relative BHP abundances observed in pure cultures may be reflected in sediment signatures in 'natural' settings.

The apparent recalcitrant nature of BHT (**III**) in this study is supported by evidence from the sedimentary record where this compound has been extracted from ~50 Ma year old sediments (van Dongen et al., 2006). Evidence from this microcosm study also

suggests that ether linked composite BHPs are susceptible to degradation from heat treatment.

# 6. Influence of pH and salinity on BHP signatures of River Tyne estuarine sediment aerobic microcosms

## 6.1. Introduction

Although most methanotrophs are neutrophiles, some have been isolated from environments with a wide range pH values and salinities. Methanotrophs belonging to *Methylocella* and *Methylocapsa* genera (Type II), have been isolated from acidic *Sphagnum* peat bogs, tropical forest soils and tundra peatlands (Dedysh et al., 2002; Dunfield et al., 2003; Dedysh et al., 2004; Dedysh et al., 2007). Acidophilic methanotrophs, belonging to the Type I group, have been isolated from Dutch peatlands with one strain closely related to the *Methylomonas* genus and the other to *Methylosoma* and *Methylovulum* (Kip et al., 2011). *Methylacidiphilum* spp. belonging to the phylum *Verrucomicrobia* have shown the ability to oxidise methane below pH 1 (Pol et al., 2007; Op den Camp et al., 2009).

There have been few studies investigating how BHP composition in cultured bacteria is influenced by pH and no specific studies for methanotrophs. In *Alicyclobacillus acidocaldarius*, an organism adapted to living at high temperature and low pH, the content of extended hopanoids increased with decreasing pH (Poralla et al., 1984). The deletion of the *sqhC* gene in *Rhodopseudomonas palustris* TIE-1, resulted in increased sensitivity to acidic and alkaline conditions relative to the wild type which was particularly noticeable in stationary phase (Welander et al., 2009). A mutant strain of *Burkholderia cenocepacia* was also affected by low pH conditions (Schmerk et al., 2011).

The first reported methanotroph that required NaCl for growth was *Methylomicrobium pelagicum* and was isolated from the Sargasso Sea (Sieburth et al., 1987). Since this discovery, *Methylobacter marinus* (Lidstrom, 1988; Bowman et al., 1993) and *Methylomicrobium japanense* (Fuse et al., 1998; Kalyuzhnaya et al., 2008) have also been isolated from marine settings. More recently, methanotrophs have been isolated from marine environments in Japan, *Methylomarinum vadi* (Hirayama et al., 2013), *Methylomarinovum caldicuralii* (Hirayama et al., 2014), *Methylocaldum marinum* (Takeuchi et al., 2014) and off the coast of California, *Methyloprofundus sedimenti* (Tavormina et al., 2015). *Methylomicrobium* spp. have been isolated from moderately

saline lakes growing in the range of pH 7.0-10.5 and 0.15-10% NaCl (Khmelenina et al., 1997) and highly alkaline soda lakes with optimal growth being witnessed at pH 9.0-10.0 (Sorokin et al., 2000). The most halotolerant methanotroph isolated to date is *Methylohalobius crimeensis* isolated from hypersaline lakes in the Crimean Peninsula, Ukraine with growth still witnessed at 15% NaCl (Heyer et al., 2005).

There have been no studies to date investigating how BHP composition in bacteria is affected by varying salinity. However, one study suggested a role in regulating osmosis, which would also be important in highly saline environments, with the production of hopanoids in *Streptomyces coelicolor* A3(2) appearing to occur during aerial mycelium formation thus reducing the water permeability of the cell membrane (Poralla et al., 2000).

## 6.2. Aims and hypotheses

### 6.2.1. Aims

This chapter presents the BHP sedimentary signatures and methanotroph community composition from aerobic microcosm experiments inoculated with River Tyne estuarine sediment and incubated at a range of different pH values and salinities.

This chapter has three aims:

(1) to determine the effect of pH on methanotroph community composition.

(2) to determine the effect of salinity on methanotroph community composition.

(3) to determine the effect of pH and salinity on BHP composition, focusing on the methanotroph-derived 35-aminoBHPs.

Please note the results presented in section 6.4.1 and 6.4.4, methane oxidation rates and methanotroph community composition, were generated by a colleague at Newcastle University, Angela Sherry (Sherry et al., 2015 in review).

### 6.2.2. Hypotheses

#### 6.2.2.1. Estuarine sediment microcosms to study the effect of pH on methanotroph diversity and BHP composition

- *Methanotroph community composition changes with pH.*

Methanotrophs have different pH optima for growth (Kalyuzhnaya et al., 2001; Dunfield et al., 2003; Dedysh et al., 2004; Pol et al., 2007; Han et al., 2009; Kip et al., 2011). Therefore, it is expected that different genera will be enriched across the pH range of the experiment.

#### 6.2.2.2. Estuarine sediment microcosms to study the effect of salinity on methanotroph diversity and BHP composition

- *Methanotroph community composition changes with salinity.*

Methanotroph genera have different optimal salinities for growth with *Methylomicrobium* spp. favouring high salinity conditions (Sorokin et al., 2000; Kalyuzhnaya et al., 2001; Han et al., 2009). Therefore, it is expected that different genera will be enriched across the salinity range of the experiment.

- *Changes in methanotroph communities with pH and salinity are reflected in 35-aminoBHP composition.*

In pure culture there is a variation in the 35-aminoBHP composition in different genera of methanotrophs (Chapter 2, Section 2.2.5 and Chapter 4, Section 4.4.2). Therefore, it is postulated that shifts in the methanotroph community composition will be reflected in the 35-aminoBHP signatures observed.

## 6.3. Materials and methods

### 6.3.1. Estuarine sediment microcosms to study the effect of pH on methanotroph diversity and BHP composition

The microcosm set-up is described in full in Chapter 3, Section 3.3.2. Briefly, microcosms were prepared in triplicate according to the experimental conditions (Table 5-2: 5% $CH_4$ amended, unamended and heat killed controls + 5% $CH_4$ (abiotic

controls)) and all incubations were carried out at 21°C and used River Tyne estuarine surface sediment as the inoculum.

**Table 6-1:** pH microcosm experimental conditions.

| | **pH 4** | **pH 5** | **pH 6** | **pH 7** | **pH 8** | **pH 9** |
|---|---|---|---|---|---|---|
| **5% $CH_4$ amended** | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ |
| **Heat killed** | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ |
| **Unamended** | -* | - | - | - | - | - |

* (-) denotes no methane addition.

Triplicate microcosms, representing the initial bacterial communities and BHP compositions in the River Tyne surface sediments, were prepared with fresh sediment and sacrificially sampled; referred to as Time 0. TOC measurements (Chapter 3, Section 3.7.1) were performed in triplicate on fresh sediment and all BHP concentrations were normalised to the average of these values, 7.53% (range 7.23-7.99%).

### 6.3.2. Estuarine sediment microcosms to study the effect of salinity on methanotroph diversity and BHP composition

The microcosm set-up is described in full in Chapter 3, Section 3.3.2. Briefly, microcosms were prepared in triplicate according to the experimental conditions (Table 5-2: 5% $CH_4$ amended, unamended and heat killed controls + 5% $CH_4$ (abiotic controls)) and all incubations were carried out at 21°C and used River Tyne estuarine surface sediment as the inoculum.

**Table 6-2:** Salinity microcosm experimental conditions.

| | 1 g/L NaCl | 15 g/L NaCl | 35 g/L NaCl | 70 g/L NaCl | 120 g/L NaCl | 150 g/L NaCl |
|---|---|---|---|---|---|---|
| **5% $CH_4$ amended** | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ |
| **Heat killed** | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ | 5% $CH_4$ |
| **Unamended** | -* | - | - | - | - | - |

* (-) denotes no methane addition. The salinity values given are those of the medium prior to sediment addition.

Triplicate microcosms, representing the initial bacterial communities and BHP compositions in the River Tyne surface sediments, were prepared with fresh sediment and sacrificially sampled; referred to as Time 0. TOC measurements (Chapter 3, Section 3.7.1) were performed in triplicate on fresh sediment and all BHP concentrations were normalised to the average of these values, 7.36% (range 7.26-7.45%).

When all methane in the amended sets had been oxidised, the microcosms, along with their unamended and heat killed counterparts, were sacrificially sampled (Section 3.3.3). Microcosm sediments were freeze-dried (Chapter 3, Section 3.3.4) and BHPs were extracted from the sediment using a modified Bligh and Dyer extraction method (Chapter 3, Section 3.8.1). The polar fraction resulting from the SPE of total lipid extracts were prepared and then analysed using LC-MS (Chapter 3, Section 3.8.2 to 3.8.4). Microbial community analysis (Chapter 3, Section 3.6) was performed on microcosm sediment DNA extracts to assess methanotroph community composition, in response to pH and salinity, by targeting the *pmoA* gene.

## 6.4. Results

### 6.4.1. Estuarine sediment microcosms to study the effect of pH on methanotroph diversity and BHP composition

#### 6.4.1.1. Methane oxidation rates and community analysis of methanotrophs in River Tyne estuarine sediment microcosms

Methane oxidation rates in response to increasing pH (pH 4-9) were determined by monitoring the consumption of 5% methane in microcosm headspace (Chapter 3, Section 3.3.6; Figure 6-1).

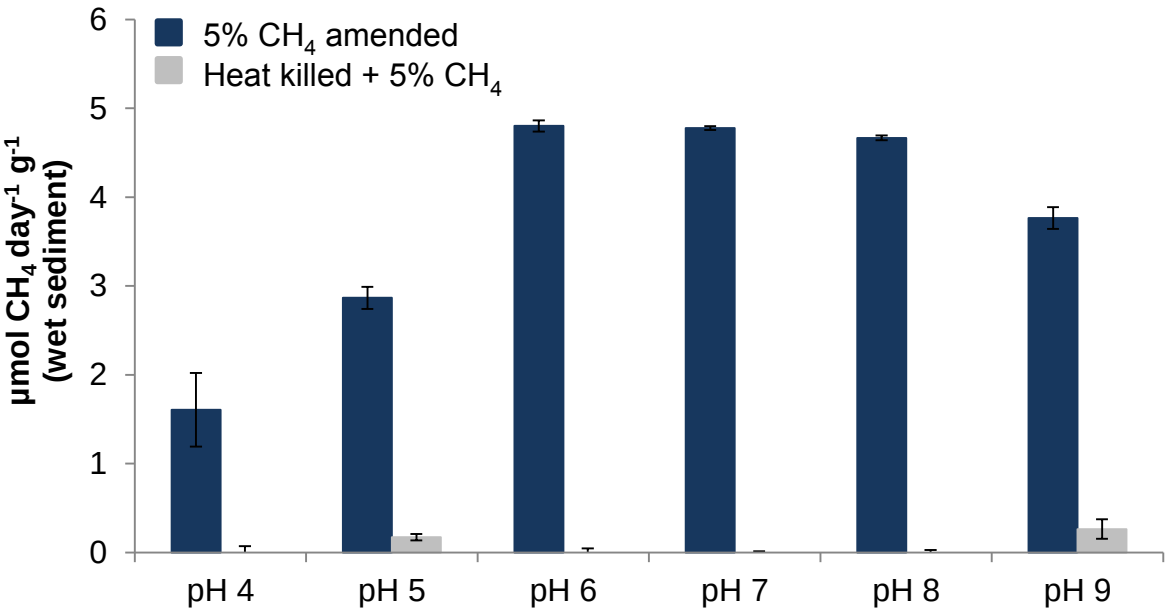



**Figure 6-1:** Methane oxidation rates in response to pH in River Tyne estuarine sediment microcosms. $CH_4$ amended microcosms (blue bars) and heat killed controls (grey bars). Error bars represent 1 x S.E. (N = 3). Data from Sherry et al., 2015 in review.

Rates of methane oxidation (µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment) in 5% methane amended microcosms were statistically compared to the corresponding heat killed controls using one-way analysis of variance (ANOVA) with Fisher's LSD.

Methane oxidation rates were highest at pH values close to those of the *in situ* pH of the sediment (pH 8), with the maximum rate observed at pH 6 (4.8 ± 0.1 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment, Figure 6-1). Rates of methane oxidation in 5% methane

amended microcosms were significantly higher than corresponding heat killed controls at all pH values ($p = 0.000$ for pH 4-9) and there were no significant differences between pairwise comparisons of 5% methane amended microcosms at pH 6, 7 and 8 ($p \geq 0.494$). One of the 5% methane-amended replicate microcosms at pH 4 oxidised the methane at a faster rate in 15 days compared to 26 days in the remaining two replicates. It was also observed that the pH drifted over the course of the experiment as follows in the microcosms, pH 4 to pH 5, pH 5 to pH 6, pH 6 to pH 7, pH 7 to pH 8, pH 8 remained stable (*in situ* pH of sediment), pH 9 to pH 8, however, all data are labelled according to the pH values at the start of the experiment.

PCR-DGGE profiles of the *pmoA* gene were very similar in microcosms with pH values close to the *in situ* pH conditions of the sediment (pH 6, 7 and 8) (Figure 6-2) which correlated with methane oxidation rates at pH 6, 7 and 8 which showed little variation (Figure 6-1). No gene was detected following PCR amplification in Time 0 microcosms. DGGE profiles at pH 6-8 strongly resembled those observed in the low temperature microcosms (4-30°C) and all methane concentrations (1-5%). At pH 6, band 13 (Figure 6.2) was consistent with the methanotroph identified at 1% methane concentration (Chapter 5, Figure 5.2, band 1) and at 21°C (Chapter 5, Figure 5-8, band 2) - an environmental clone C69, a putative *Methylobacter* sp., from a wetland soil (95 % sequence identity, HQ883363) (Yun et al., 2012). Band 14 present in pH 6, pH 7 and pH 8 PCR-DGGE profiles and band 11 at pH 5, shared 98% sequence identity to an uncultured bacterium clone RCL_mb661R_16 (EF212354) isolated from a landfill cover soil (Chen et al., 2007). The closest cultured representative was *Methylomonas methanica* with 92% *pmoA* sequence identity (EU722434). Band 12 in pH 5 microcosms was also a *Methylomonas* sp. with 98% sequence identity with an uncultured bacterium clone Der51 (EU071126) from a landfill cover soil. Although not excised and sequenced from the low temperature (4-30°C) and methane concentration experiments (1-5%), it appears likely that *Methylomonas sp.* represented by band 14 are also present in these other PCR-DGGE profiles (Chapter 5, Figure 5-2 and Figure 5-8).

The closest relatives to the *pmoA* sequences retrieved from bands 9 and 10 from pH 5 microcosms and band 15 from microcosms at pH 9 were *pmoA* from *Methylobacter* spp. Isolation sources of the *Methylobacter* spp. were soil environments including a landfill cover soil, a Canadian high arctic soil, and a flooded rice field soil (Wartiainen

et al., 2006; Ferrando and Tarlera, 2009; Martineau et al., 2010). Bands 7 and 8 from pH 4 microcosms were related to *Methylosoma* spp. and in particular band 8 was strongly selected for at this pH. Band 8 shared 92% sequence identity to an uncultured bacterium clone B15 (EU647285) isolated from a landfill soil cover (Lin et al., 2009). The closest cultured representative was *Methylosoma difficile* LC 2$^{T}$ (DQ119047) isolated from the littoral sediment of Lake Constance (Rahalkar et al., 2007). In contrast, microcosms incubated at pH 9 were dominated by *Methylomicrobium* spp., (bands 16-19). The closest cultured representatives included *Methylomicrobium alcaliphilum* strain 20Z (94%, FO082060) and *Methylomicrobium kenyense* strain AMO1 (89%, JN687579), and uncultured environmental representatives which were isolated from haloalkaliphilic environments, such as hypersaline and alkaline lakes, anoxic fjords and the marine environment.

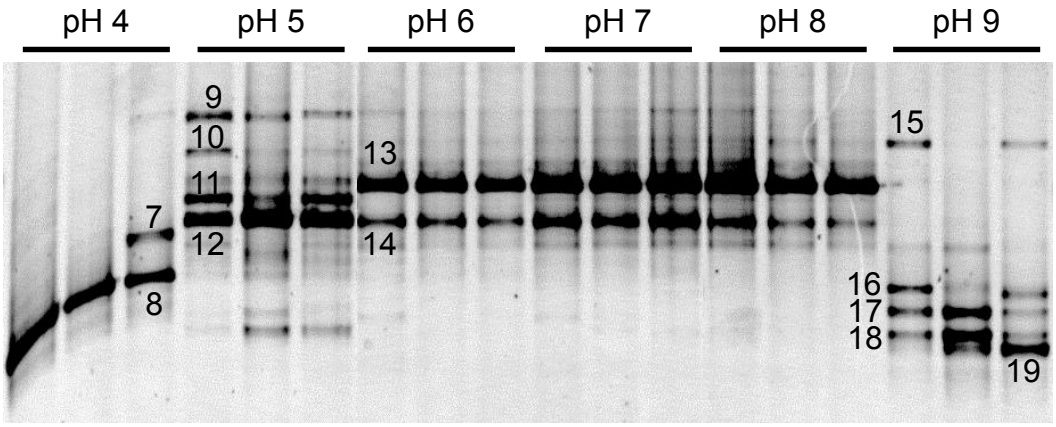



**Figure 6-2:** PCR-DGGE of the *pmoA* gene as an indicator of methanotroph community composition in aerobic River Tyne estuarine sediment microcosms in response to different pH.

### 6.4.2. 35-aminoBHP concentrations in River Tyne estuarine sediment microcosms subjected to variations in pH

Aminotriol (**V**) and related 'carbamylmethylestertriol' (**V$^{MeCarb}$**) and 'desmethylaminotriol' (**V$^{des}$**) compounds (Chapter 4, Section 4.4.1) were detected in microcosms at all pH values (Figure 6-3). Aminotetrol (**VI**) was detected in microcosm sediments at all pH values and 'carbamylmethylestertetrol' (**VI$^{MeCarb}$**) in the pH range 5-9, but not at pH 4 (Figure 6-5). Aminopentol (**VII**) was only detected at pH 4-7 and 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**) was detected at pH 6-8 (Figure 6-6).

### 6.4.2.1. Aminotriol and related compounds

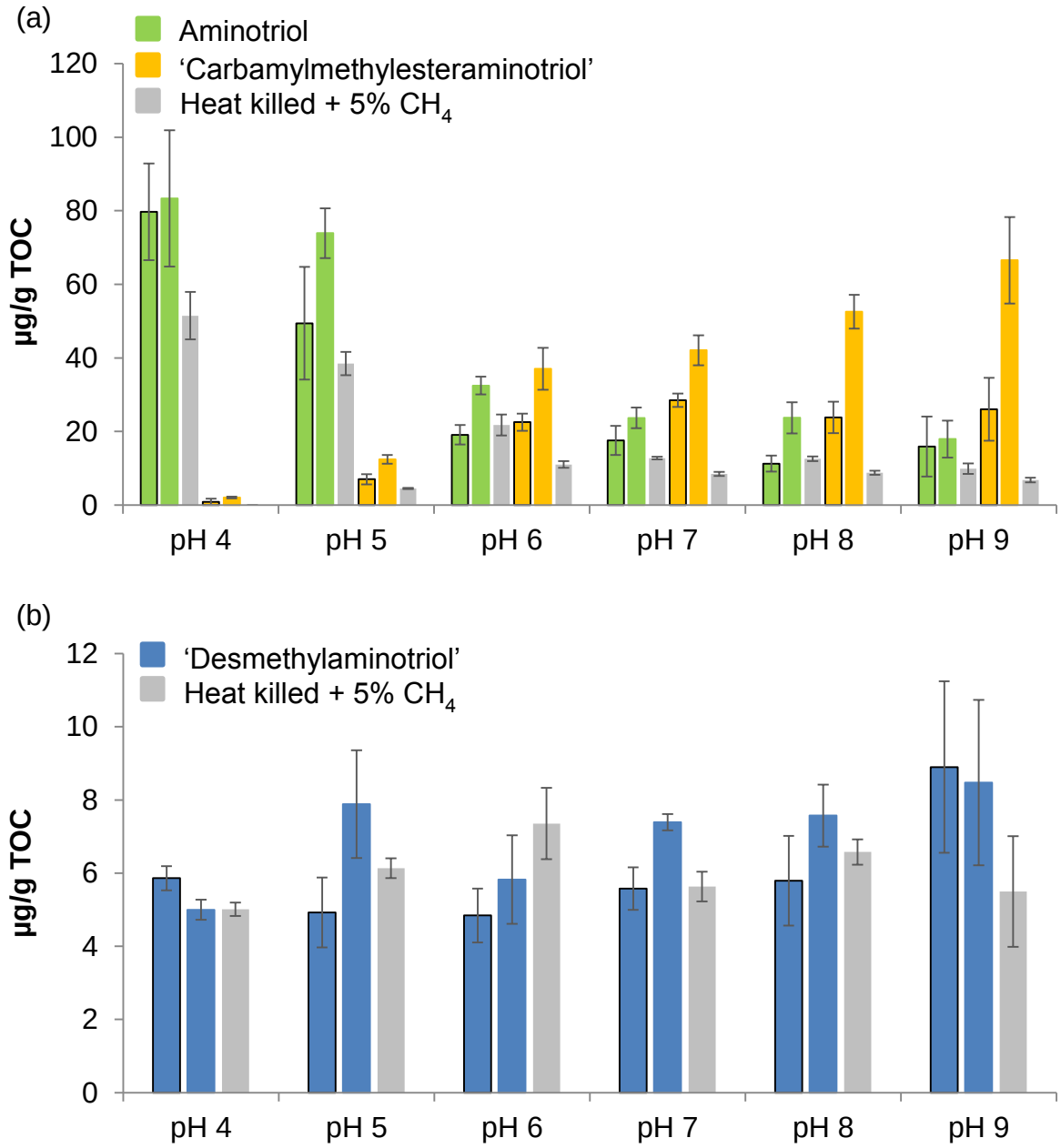



**Figure 6-3:** Concentration (µg/g TOC) of (a) aminotriol (**V**) and 'carbamylmethylestertriol' (**V**$^{\mathbf{MeCarb}}$); (b) 'desmethylaminotriol' (**V**$^{\mathbf{des}}$) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to pH (corresponding heat killed controls to the right, where compound present; corresponding Time 0 to left, black outline). Error bars represent 1 x S.E. (N = 3) except pH 9 heat killed (N = 2)* and Time 0 (N = 2, duplicate extraction).*

* As previously mentioned for the pH study (Chapter 3, Section 3.3.1), sediment was added to the nutrient medium to create a slurry which was then adjusted to the desired

pH using HCl or NaOH (1N). Therefore, an initial sediment microcosm for each pH value was created and was extracted twice. Herein, N = 2 for pH 9 heat killed due to a TLE replicate being lost.

Aminotriol (**V**) concentrations in 5% methane amended sediments decreased with increasing pH with maximum concentrations detected at pH 4 (Figure 6-4). The initial sediment concentrations of aminotriol (**V**) also followed a similar trend. Therefore, the level of enrichment of the compound compared with its corresponding initial sediment is not so pronounced. Conversely, 'carbamylmethylestertriol' ($\mathbf{V^{MeCarb}}$) concentrations increased with increasing pH with the maximum concentration of 67 ± 11.7 µg/g TOC measured at pH 9. The ratio of 'carbamylmethylestertriol' ($\mathbf{V^{MeCarb}}$) in 5% methane amended sediments compared to the corresponding initial sediments was also highest at pH 9. 'Desmethylaminotriol' ($\mathbf{V^{des}}$) concentrations in 5% methane amended sediments did not show significant variance over the pH range.

The concentration of aminotriol (**V**) in one pH 4 microcosm was approximately two times higher than the other two replicates (Figure 6-4). Replicate 2 behaved differently to the other two replicates and this corresponded with the replicate that oxidised 5% methane more rapidly (Section 6.4.1.1).

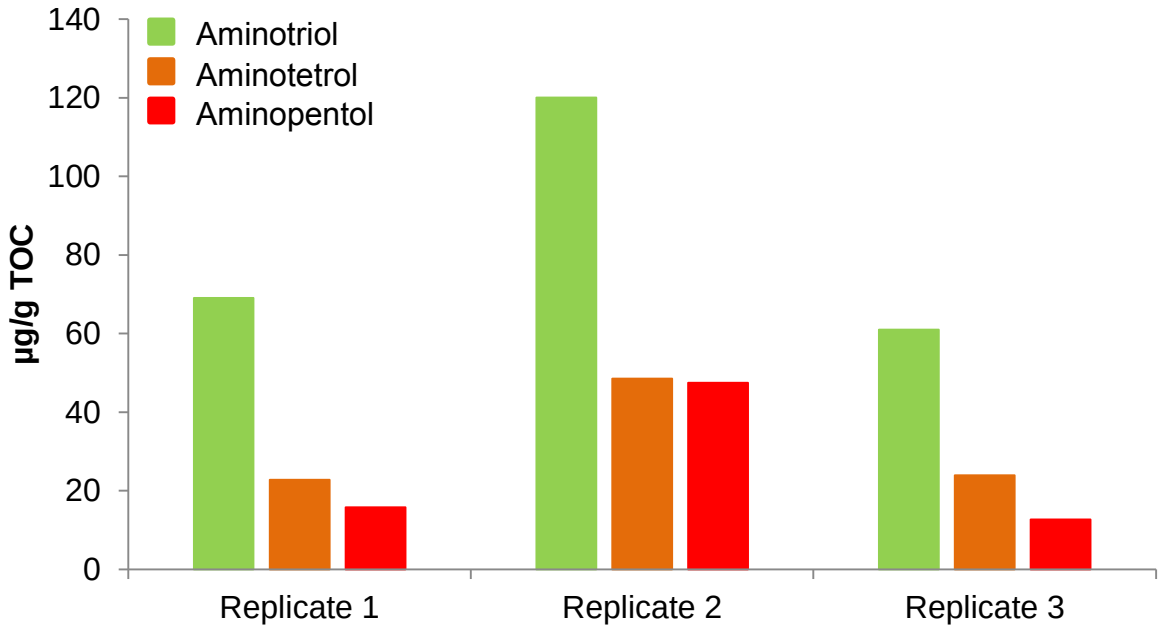



**Figure 6-4:** Concentration (µg/g TOC) of aminotriol (**V**), aminotetrol (**VI**) and aminopentol (**VII**) in 5% methane amended River Tyne estuarine sediment microcosms incubated at pH 4.

### 6.4.2.2. Aminotetrol and related compounds

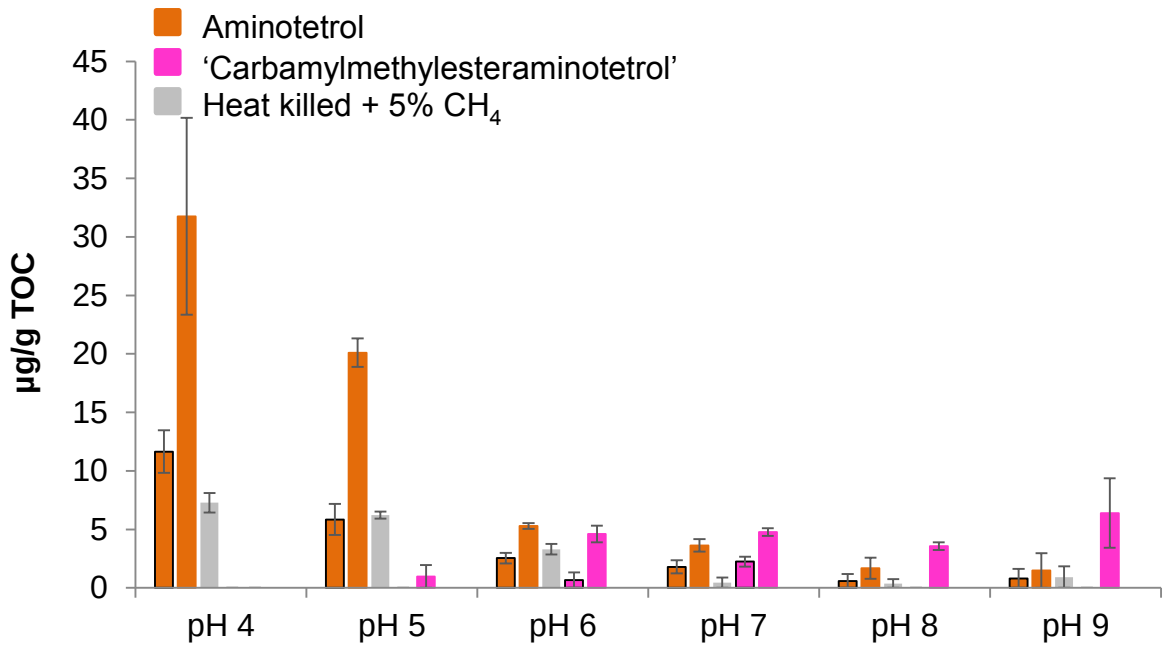



**Figure 6-5:** Concentration (µg/g TOC) of aminotetrol (**VI**) and 'carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to pH (corresponding heat killed controls to the right, where compound present; corresponding Time 0 to left, black outline, where compound present). Error bars represent 1 x S.E. (N = 3) except pH 9 heat killed (N = 2) and Time 0 (N = 2, duplicate extraction).

Aminotetrol (**VI**) concentrations in 5% methane amended sediments decreased with increasing pH with the maximum concentration detected at pH 4, 32 ± 8.4 µg/g TOC (Figure 6-5). The initial sediment concentrations of aminotetrol (**VI**) also followed a similar trend. The ratio of aminotetrol (**VI**) in 5% methane amended sediments compared to the corresponding initial sediment was also highest at pH 4 and 5. Conversely, 'carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$) concentrations generally increased with pH value, with no compound detected at pH 4, and the maximum concentration of 6.4 ± 3.0 µg/g TOC detected at pH 9 (Figure 6-5).

### 6.4.2.3. Aminopentol and related compounds

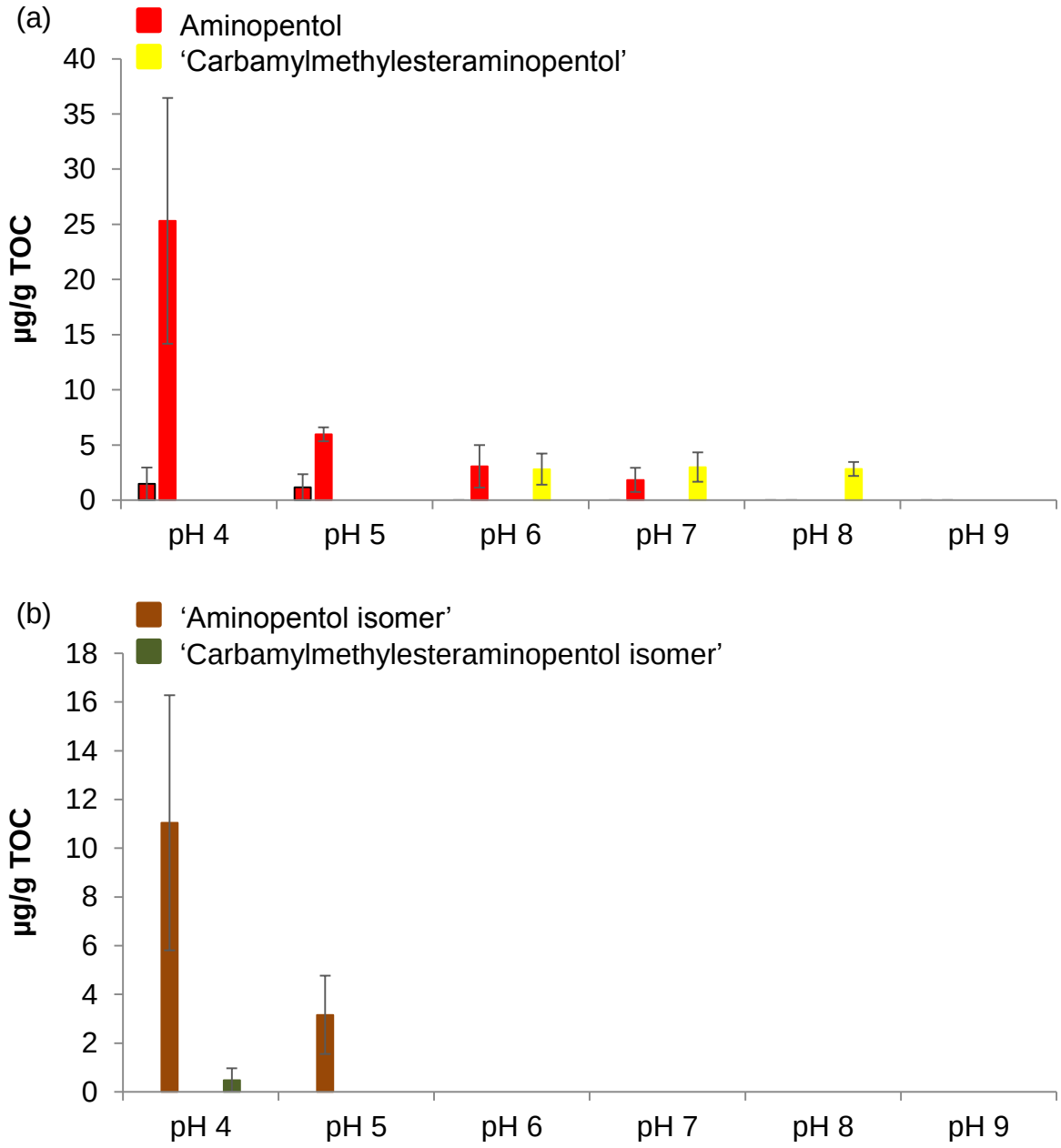



**Figure 6-6:** Concentration (µg/g TOC) of (a) aminopentol (**VII**) and 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**); (b) 'aminopentol isomer' (**VII$^{iso}$**) and 'carbamylmethylesterpentol isomer' (**VII$^{iso,MeCarb}$**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to pH (no compound present in heat killed controls; corresponding Time 0 to left, black outline, where compound present). Error bars represent 1 x S.E. (N = 3) except pH 9 heat killed (N = 2) and Time 0 (N = 2, duplicate extraction).

Aminopentol (**VII**) was detected at pH 4-7 but not at pH 8 and pH 9 (Figure 6-6a). The maximum concentration of 25 ± 11 µg/g TOC was detected in microcosms incubated at pH 4. The concentration of aminopentol (**VII**) in one pH 4 microcosm was more than three times higher than the other two replicates (Figure 6-4). 'Carbamylmethylesterpentol' (**VII$^{MeCarb}$**) was only detected in sediments incubated at pH 6-8. 'Aminopentol isomer' (**VII$^{iso}$**) was only detected at pH 4 and 5 and 'carbamylmethylesterpentol isomer' (**VII$^{iso,MeCarb}$**) only at pH 4 (Figure 6-6b).

### 6.4.3. Total BHP concentrations in River Tyne estuarine sediment microcosms subjected to variations in pH

Total BHP concentrations in microcosm sediments in response to pH were determined (Figure 6-7). The total BHP concentration of the initial sediment used for the experiment (Time 0) is also shown. For individual compound concentrations and relative abundances see appendix 3d.

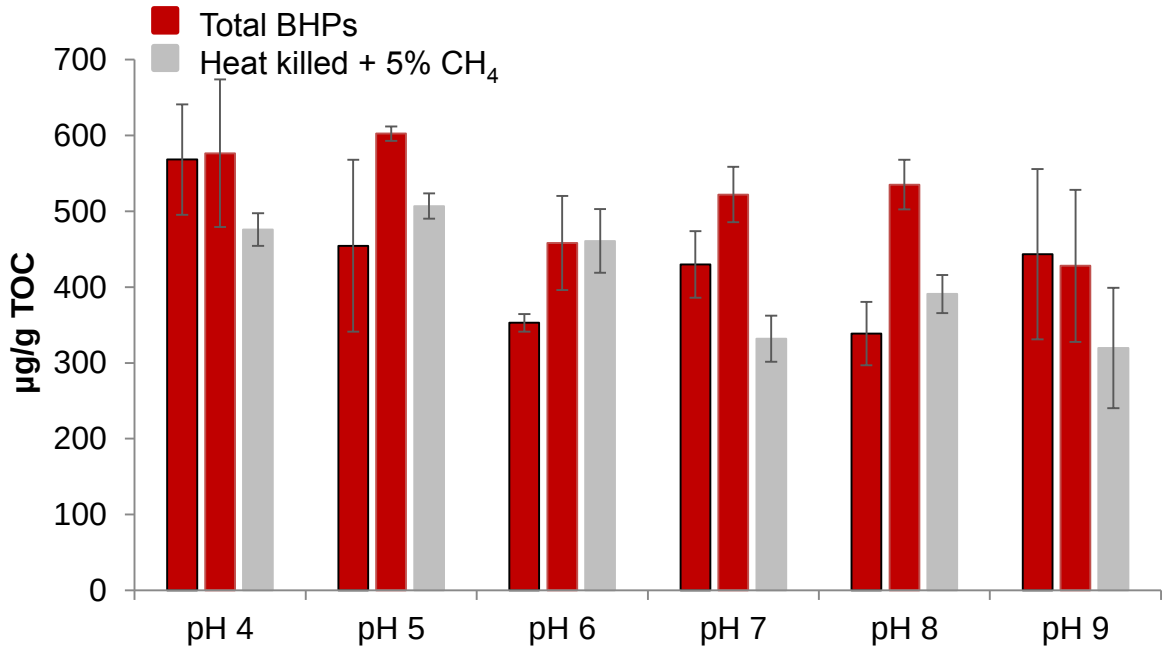



**Figure 6-7:** Total BHP concentrations (µg/g TOC) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to pH (heat killed controls to the right; Time 0 to left, black outline). Error bars represent 1 x S.E. (N = 3) except pH 9 heat killed (N = 2) and Time 0 (N = 2, duplicate extraction).

Total BHP concentrations in 5% methane amended microcosm sediments were statistically compared to the initial sediment (Time 0), unamended controls (data not

shown on Figure 6-7, see appendix 3d) and corresponding heat killed controls using one-way analysis of variance (ANOVA) with Fisher's LSD.

Total BHP concentrations were only significantly higher relative to the initial sediment in 5% methane amended sediments at pH 8 ($p = 0.019$) but were not significantly different at pH 4-7 and 9 ($p \geq 0.074$). There were no significant differences between total BHPs in 5% methane amended sediments compared with the equivalent heat killed controls at any pH value ($p \geq 0.168$). Total BHPs were significantly higher in 5% methane amended sediments at pH 5 ($p = 0.007$) and pH 7 ($p = 0.036$) compared with corresponding unamended controls but not at any other pH values ($p \geq 0.289$) (data not shown on Figure 6-7, see appendix 3d).

### 6.4.4. Estuarine sediment microcosms to study the effect of salinity on methanotroph diversity and BHP composition

#### 6.4.4.1. Methane oxidation rates and community analysis of methanotrophs in River Tyne estuarine sediment microcosms

Methane oxidation rates in response to increasing salinity (1-150 g/L NaCl) were determined by monitoring the consumption of 5% methane in microcosm headspace (Chapter 3, Section 3.3.6; Figure 6-8).

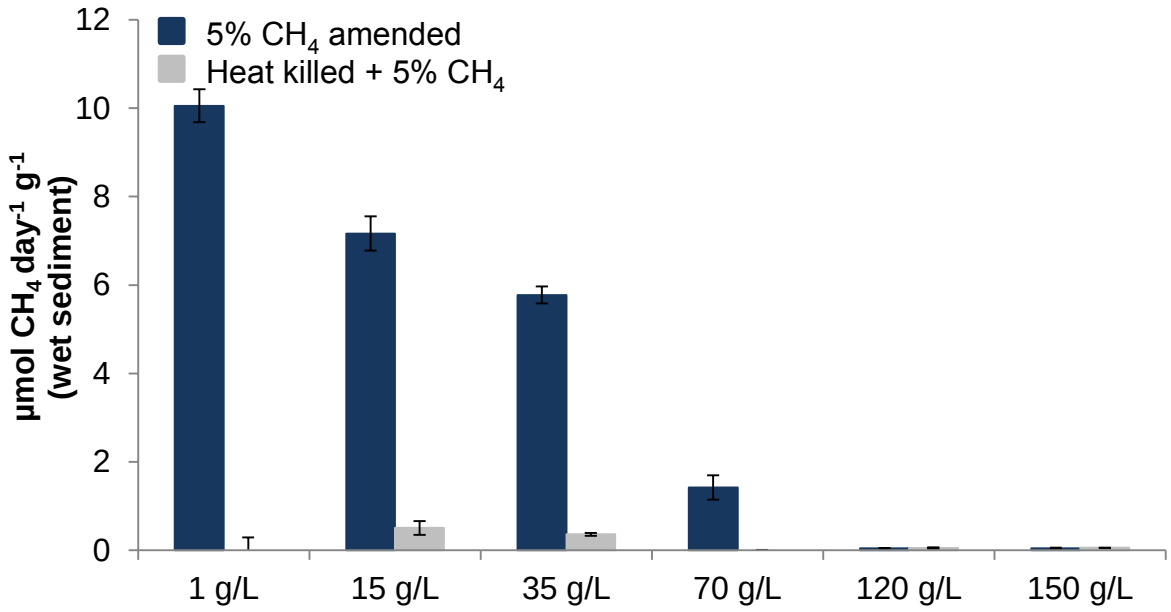



**Figure 6-8:** Methane oxidation rates in response to salinity in River Tyne estuarine sediment microcosms. 5% $CH_4$ amended microcosms (black bars) and heat killed controls (grey bars). Error bars represent 1 x S.E. (N = 3). Data from Sherry et al., 2015 in review.

Methane oxidation rates decreased with increasing salinity from 1-70 g/L NaCl with no methane oxidation being witnessed at 120 and 150 g/L NaCl (Figure 6-8). Rates of methane oxidation in methane amended microcosms were significantly higher than corresponding heat killed controls at 1-70 g/L NaCl ($p = 0.000$) but not at 120 and 150 g/L ($p \geq 0.974$).

The PCR-DGGE profiles of the *pmoA* gene across all salinities showed differences in methanotroph community composition (Figure 6-9), no gene was detected following PCR amplification in Time 0 microcosms. Microcosms containing 1 and 15 g/L NaCl were dominated by *Methylobacter* and *Methylomonas* (bands 20, 21, 22). At 1 g/L NaCl, band 20 was consistent with the methanotroph identified at 1% methane concentration (Chapter 5, Figure 5-2, band 1) and 21°C (Chapter 5, Figure 5-8, band 2) and at pH 6 (Section 6.4.1.1, band 13) - a putative *Methylobacter* sp. from a wetland soil (clone C69, 96 % sequence identity, HQ883363) (Yun et al., 2012). Band 21, which also appears to be present at low temperatures and present in all of the methane concentration microcosms, was most closely related to the *Methylomonas* sp. clone ProtK8 (DQ400901) from an igneous rock brackish aquifer (Chi Fru, 2008).

Microcosms containing 35 and 70 g/L NaCl were dominated by *Methylomicrobium* spp. The *Methylomicrobium* sp. in microcosms containing 35 g/L NaCl (band 23) was closely related to *Methylomicrobium japanense* NI (AB253367) (96-97% sequence identity) isolated from filtered seawater (Fuse et al., 1998; Kalyuzhnaya et al., 2008). The most closely related environmental *pmoA* sequence was an uncultured bacterium clone SI-*pmoA*_PHYLO4 (JN172109) (95% sequence identity) from an anoxic fjord. A *Methylomicrobium* sp. originally isolated from a soda lake in an unpublished study was dominant in microcosms containing 70 g/L NaCl.

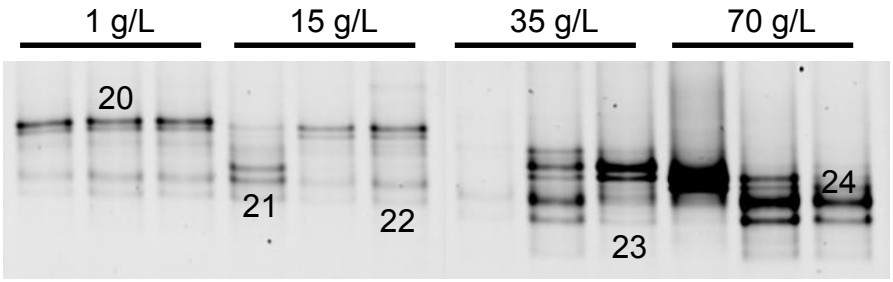



**Figure 6-9:** PCR-DGGE of the *pmoA* gene as an indicator of methanotroph community composition in aerobic River Tyne estuarine sediment microcosms in response to salinity.

### 6.4.5. 35-aminoBHP concentrations in River Tyne estuarine sediment microcosms subjected to variations in salinity

Aminotriol (**V**) and related 'carbamylmethylestertriol' ($\mathbf{V}^{\mathbf{MeCarb}}$) and 'desmethylaminotriol' ($\mathbf{V}^{\mathbf{des}}$) compounds (Chapter 4, Section 4.4.1) were detected at all salinities and in the initial sediment (Figure 6-10). Aminotetrol (**VI**) and aminopentol (**VII**) were detected across the salinity range tested (Figure 6-11 and Figure 6-12).

'Desmethylaminotriol' ($\mathbf{V}^{\mathbf{des}}$) and aminotetrol (**VI**) concentrations in 5% methane amended microcosm sediments were compared to the initial sediment (Time 0) and aminopentol (**VII**) concentrations in methane amended sediments were compared with each salinity using one-way analysis of variance (ANOVA) with Fisher's LSD.

### 6.4.5.1. Aminotriol and related compounds

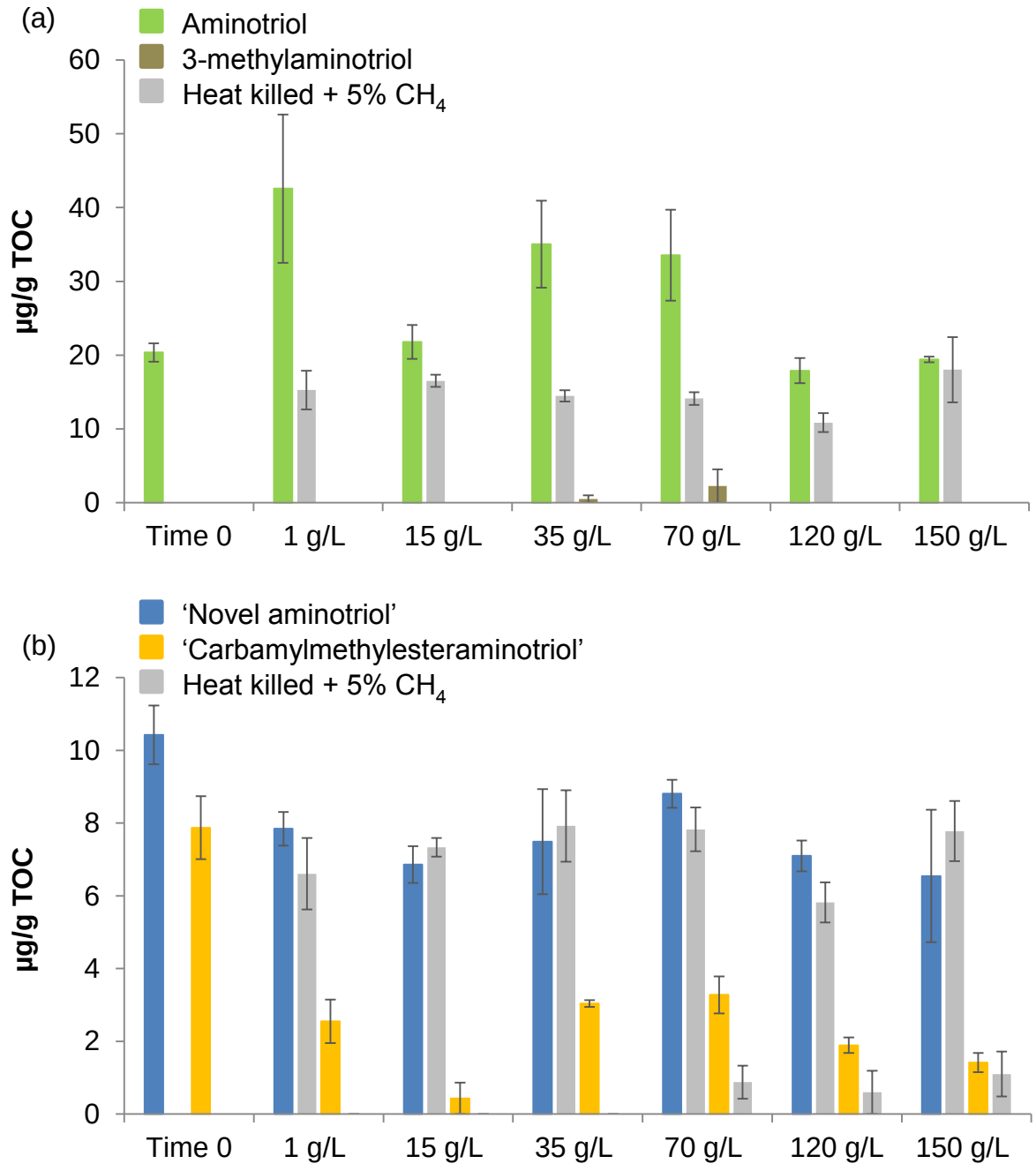



**Figure 6-10:** Concentration (µg/g TOC) of (a) aminotriol (**V**) and 3-methylaminotriol (**Va**); (b) 'desmethylaminotriol' ($\mathbf{V^{des}}$) and 'carbamylmethylestertriol' ($\mathbf{V^{MeCarb}}$) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to salinity (corresponding heat killed controls to the right, where compound present). Error bars represent 1 x S.E. (N = 3) except 150 g/L NaCl 5% $CH_4$ (N = 2)*.

* Herein, N = 2 for 150 g/L NaCl 5% $CH_4$ due to a TLE replicate being lost.

Aminotriol (**V**) concentrations were enriched in 5% methane amended sediments with 1 g/L NaCl relative to the initial sediments (Time 0), with a maximum concentration of 43 ± 10 µg/g TOC, but concentrations at 15 g/L NaCl were similar to those witnessed in the initial sediment (Figure 6-10a). Concentrations were higher in sediments at 35 and 70 g/L NaCl relative to initial sediments but concentrations witnessed at 120 and 150 g/L NaCl, where no methane oxidation was witnessed, were similar to those in the initial sediment. 3-Methylaminotriol (**Va**) was detected in 5% methane amended sediments containing 35 and 70 g/L NaCl with the highest concentration of 2.3 ± 2.3 µg/g TOC, detected at the higher salinity. The presence of 3-methylaminotriol (**Va**) was confirmed from its mass spectrum (data not shown), which was very similar to that of 2-methylaminotriol (Talbot et al., 2008), and its relative retention time compared to aminotriol (**V**). 'Desmethylaminotriol' concentrations ($\mathbf{V^{des}}$) were significantly lower in 5% methane amended sediments at all salinities ($p \leq 0.031$) apart from 70 g/L NaCl ($p = 0.165$) in pairwise comparisons with the initial sediments (Time 0) (Figure 6-10b). 'Carbamylmethylestertriol' ($\mathbf{V^{MeCarb}}$) concentrations were lower in 5% methane amended sediments at all salinities compared with the initial sediment.

### 6.4.5.2. Aminotetrol and related compounds

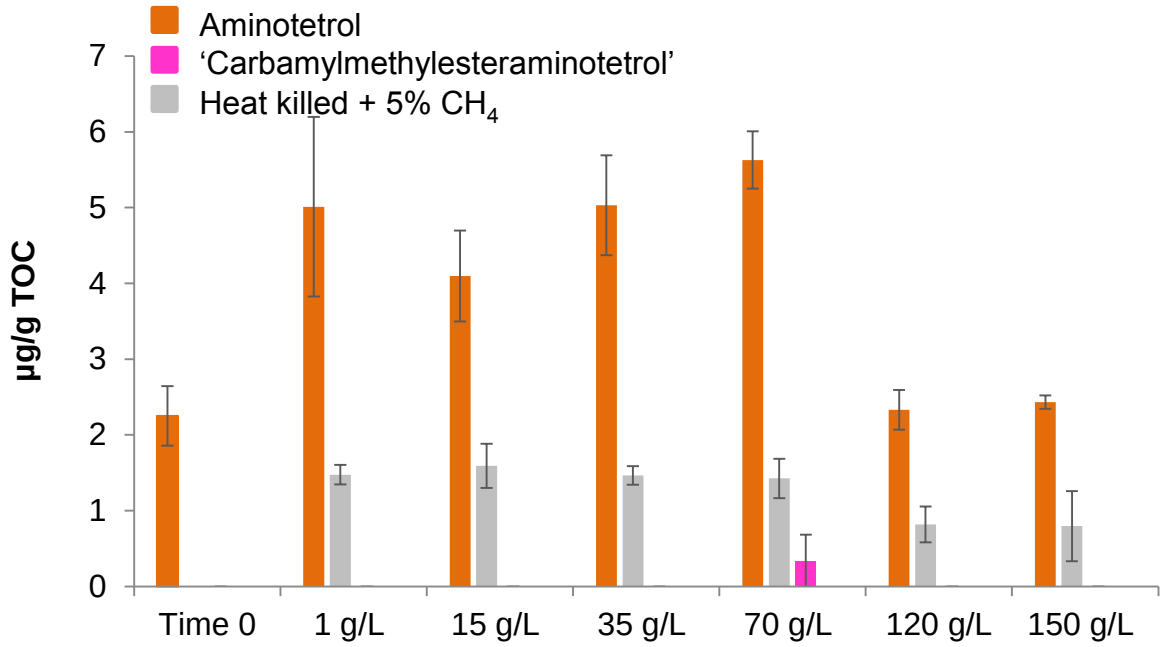



**Figure 6-11:** Concentration (µg/g TOC) of aminotetrol (**VI**) and 'carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to salinity (corresponding heat killed controls to the right, where compound present). Error bars represent 1 x S.E. (N = 3) except 150 g/L NaCl 5% $CH_4$ (N = 2).

Aminotetrol (**VI**) concentrations were significantly higher in 5% methane amended sediments with 1, 35 and 70 g/L NaCl relative to the initial sediments (Time 0) ($p \leq 0.008$), but there were no significant differences in microcosms with 15 g/L NaCl ($p = 0.058$) or 120 and 150 g/L NaCl ($p \geq 0.087$) (Figure 6-11). The maximum concentration of aminotetrol was detected at 70 g/L NaCl (Figure 6-11).

### 6.4.5.3. Aminopentol and related compounds

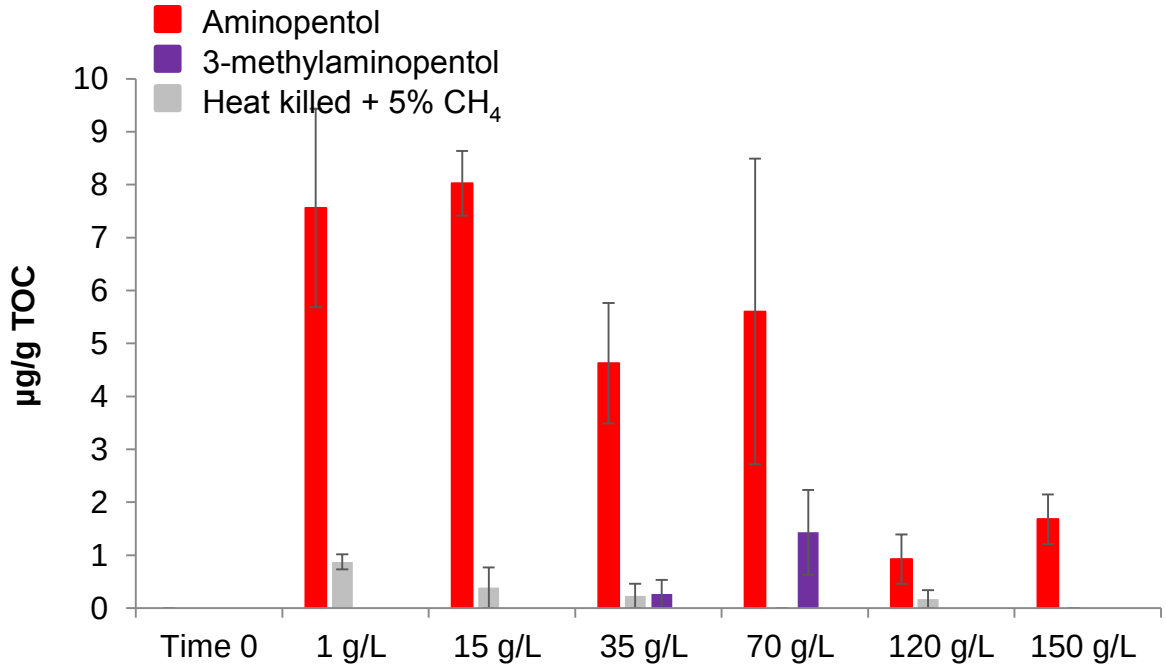



**Figure 6-12:** Concentration (µg/g TOC) of aminopentol (**VII**) and 3-methylaminopentol (**VIIa**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to salinity (corresponding heat killed controls to the right, where compound present). Error bars represent 1 x S.E. (N = 3) except 150 g/L NaCl 5% $CH_4$ (N = 2).

Aminopentol (**VII**) was detected at 1-150 g/L NaCl, but this compound was not detected in the initial sediments (Time 0) (Figure 6-12). The maximum concentration, 8.0 ± 0.6 µg/g TOC, was detected in microcosms with 15 g/L NaCl. Aminopentol (**VII**) concentrations were significantly higher in 5% methane amended sediments at 1 and 15g/L NaCl compared with 120 and 150 g/L NaCl ($p \leq 0.039$). 3-Methylaminopentol (**VIIa**) was detected only in 5% methane amended sediments with 35 and 70 g/L NaCl with the highest concentration, 1.4 ± 0.8 µg/g TOC, detected at the higher salinity. Trace amounts of 'desmethylaminopentol' (**VII**$^{des}$) and 'carbamylmethylesterpentol' (**VII**$^{MeCarb}$) were detected in one microcosm replicate containing 1 g/L NaCl (data not shown on Figure 6-12, see appendix 3e).

### 6.4.6. Total BHP concentrations in River Tyne estuarine sediment microcosms subjected to variations in salinity

Total BHP concentrations were determined in microcosm sediments in response to salinity (Figure 6-13). The total BHP concentration of the initial sediment used for the experiment (Time 0) is also shown. For individual compound concentrations and relative abundances see appendix 3e.

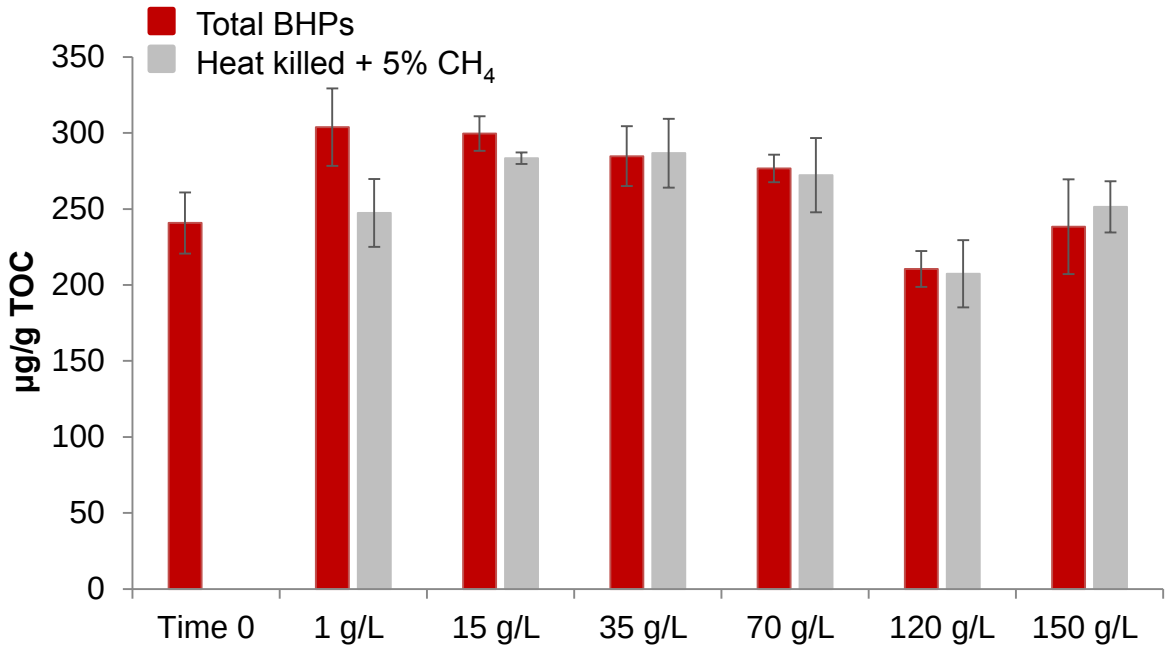



**Figure 6-13:** Total BHP concentrations (µg/g TOC) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms in response to salinity (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3) except 150 g/L NaCl 5% $CH_4$ (N = 2).

Total BHP concentrations in 5% methane amended microcosm sediments were statistically compared to the initial sediment (Time 0), unamended controls (data not shown on Figure 6-13, see appendix 3e) and corresponding heat killed controls using one-way analysis of variance (ANOVA) with Fisher's LSD.

Total BHP concentrations were significantly higher relative to the initial sediment in 5% methane amended microcosms at 1 g/L NaCl ($p = 0.025$) and 15 g/L NaCl ($p = 0.036$), with no significant differences across the range 35-150 g/L NaCl ($p \geq 0.112$). Total BHPs in 5% methane amended sediments were only significantly higher than in corresponding heat killed controls at 1 g/L NaCl ($p = 0.043$), with no significant differences at 15-150 g/L NaCl ($p \geq 0.551$). Total BHPs were not significantly higher in

5% methane amended sediments at all salinities tested compared to the corresponding unamended controls ($p \geq 0.126$ for all pairwise comparisons) (data not shown on Figure 6-13, see appendix 3e).

## 6.5. Discussion

### 6.5.1. Methane oxidation rates in River Tyne estuarine sediment microcosms in response to pH and salinity

Methane oxidation rates were highest at pH 6-8 close to *in situ* conditions (pH 8) in the River Tyne estuary (Figure 6-1) where indigenous methanotroph cell numbers were expected to be highest. Microcosm sediments at pH 4 were solely dominated by *Methylosoma* spp. that were able to withstand the acidic conditions which may be why lower rates were seen at this pH. At pH 9, methane oxidation rates were lower compared with pH 6-8 which may be because they were solely dominated by *Methylomicrobium* spp.

The rate of methane oxidation decreased with increasing salinity, 1-70 g/L NaCl, (Figure 6-8) and methane oxidation was not detected at 120 and 150 g/L NaCl. This suggests that methanotrophs in this study are unable to withstand salinities > 70 g/L NaCl. A decrease in the methane oxidation rate may be due to less methanotroph species in the River Tyne estuary being able to tolerate and grow at higher salinities representative of seawater, 35 g/L NaCl and hypersaline, 70 g/L NaCl, conditions. *Methylomicrobium* spp., enriched at 70 g/L NaCl, have been shown to grow at salinities of up to 88 g/L NaCl (Kalyuzhnaya et al., 2008) so it was predicted that this genus of methanotrophs would not grow at salinities of 120 and 150 g/L NaCl. However, *Methylohalobius crimeensis*, isolated from hypersaline lakes in the Crimean Peninsula, Ukraine, have shown the ability to oxidise methane at up to 150 g/L NaCl (Heyer et al., 2005).

### 6.5.2. Methanotroph community composition in River Tyne estuarine sediment microcosms in response to pH and salinity

Type I methanotrophs were identified at all pH values and across the salinity range 1-70 g/L NaCl, with no *pmoA* sequences related to Type II methanotrophs detected (Figure 6-2 and Figure 6-9). *Methylosoma* spp. were shown to be dominant in sediment

microcosms at pH 4 with the closest cultured representative being *Methylosoma difficile* LC 2$^{T}$ which has the optimal growth range of pH 5-9 (Rahalkar et al., 2007). The acidophilic *Methylovulum*-like strain M200, isolated from Dutch peatlands with optimal growth range of pH 4.1-7, had a close relationship with *Methylosoma difficile* LC 2$^{T}$ and probably represents a new strain belonging to this genus (Kip et al., 2011).

A *Methylobacter* sp. was present at pH 6 and was closely related to a *Methylobacter* sp. from a wetland soil (Figure 6-2). Microcosms incubated at pH 6, 7 and 8 were enriched with a methanotroph with the closest cultured representative of *Methylomonas methanica*; this was also present in sediments at pH 5 with an additional *Methylomonas* sp. identified at this pH. An acid-tolerant member of this genus, *Methylomonas paludis*, was recently isolated from an acidic wetland and had a pH growth range of 3.8-7.2 (Danilova et al., 2013; Danilova and Dedysh, 2014). *Methylobacter* spp. were also the closest relatives to the *pmoA* sequences retrieved from pH 5 microcosm sediments and also at pH 9.

*Methylobacter* and *Methylomonas* spp. also dominated at low salinities, 1 and 15 g/L NaCl (bands 20, 21 and 22, Figure 6-9). As previously mentioned (Chapter 5, Section 5.5.2), gene sequences identified in estuarine sediments from Newport Bay, USA, were *Methylomonas, Methylomicrobium* and *Methylobacter* and in the surface sediment from the Colne Estuary, UK, *Methylomonas* and *Methylobacter* were detected alongside *Methylomicrobium* and *Methylococcus* (Moussard et al., 2009). Therefore, the methanotrophs enriched in the microcosms in this study seem typical of those found in other estuarine environments (McDonald et al., 2005b; Moussard et al., 2009).

Microcosms incubated at pH 9 were dominated by *Methylomicrobium* spp. *Methylomicrobium* spp. in estuarine environments may have been passively introduced from marine settings. The closest cultured representatives included *Methylomicrobium alcaliphilum* strain 20Z and *Methylomicrobium kenyense* strain AMO1. These two alkaliphilic, halophilic species, isolated from highly alkaline soda lakes, have optimal growth in the pH range 7.2-9.5 and 9-10.5 respectively (Khmelenina et al., 1997; Sorokin et al., 2000; Kalyuzhnaya et al., 2008). Microcosms with 35 and 70 g/L NaCl were also dominated by *Methylomicrobium* spp. At 35 g/L NaCl the *Methylomicrobium* sp. enriched was closely related to *Methylomicrobium*

*japanense* NI, originally isolated from filtered seawater with optimum growth at 23-46 g/L NaCl but with growth seen at 87 g/L NaCl (Fuse et al., 1998; Kalyuzhnaya et al., 2008). At 70 g/L NaCl a *Methylomicrobium* sp. was also enriched.

### 6.5.3. The effect of pH on 35-aminoBHP signatures in River Tyne estuarine sediment microcosms

Aminotriol (**V**) is produced by a wide range of bacteria (e.g. Talbot and Farrimond, 2007; Talbot et al., 2008; Blumenberg et al., 2012). In methanotrophs it occurs in varying abundances in *Methylobacter* spp. (Chapter 4, Section 4.4.2) and *Methylomonas* spp. (Rohmer et al., 1984; Neunlist and Rohmer, 1985b; Talbot et al., 2001; van Winden et al., 2012c), genera enriched at pH 5-8, which may explain the differences in the aminotriol (**V**) concentrations detected.

Aminotriol (**V**) concentrations (Figure 6-3) in 5% methane amended sediments decreased with increasing pH whereas 'carbamylmethylestertriol' (**V$^{MeCarb}$**) increased, and was present in greater concentrations, than aminotriol (**V**) at values greater than pH 6. Concentrations detected at pH 6-8, close to the *in situ* sediment conditions, were similar to those detected in 5% methane amended sediments in the methane concentration and temperature studies (21°C) (Chapter 5, Section 5.4.2.1 and 5.4.5.1). High relative abundances of 'carbamylmethylester' compounds have so far only been detected in *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp. cultures to date (Chapter 4, Section 4.4.2), with 'carbamylmethylester' compounds being in higher abundance than the 'regular' 35-aminoBHPs in *Methylomarinovum* sp. IN45. The trends observed with increasing pH suggest that aminotriol (**V**) may preferentially be produced at lower pH values and the 'carbamylmethylester' form at higher pH values, or there may be an abiotic conversion between the two forms catalysed by acidic or alkaline conditions during the course of the experiment. However, the corresponding aminotriol (**V**) initial sediment (Time 0) concentrations at each pH also followed a similar trend to the methane amended sediments, with greater concentrations being detected at lower pH values which decreased with increasing pH. This suggests that microcosms at lower pH values (pH 4 and 5) may 'release' aminotriol (**V**) from the sediment matrix or lyse the bacterial cells thereby allowing greater recovery of the compound in the total lipid extract. This must be a rapid process as after preparation, Time 0 microcosms were frozen within a short time frame (no longer than 3 hours).

Other compounds such as BHT (**III**) do not show this trend and concentrations were not elevated at pH 4 and 5 in 5% methane amended or the initial sediments and showed no significant difference across the pH range (pH 4-9) (see appendix 3d for data). Adenosylhopane (**VIII**) concentrations were also not elevated in microcosms subjected to lower pH. This evidence suggests that other compounds do not seem to be affected by acidic conditions in the same way as aminotriol (**V**).

The dominant methanotroph at pH 4 (Figure 6-2) was related to a *Methylosoma* sp. and the BHP composition of this genus is yet to be determined. The 16S rRNA and *pmoA* gene sequences of the *Methylovulum*-like strain M200 had the closest identity to *Methylovulum miyakonense* but also had a close relationship to *Methylosoma difficile* LC $2^T$ and *Methylobacter* spp. and may represent a new strain belonging to the *Methylosoma* genus (Kip et al., 2011). The *Methylovulum*-like strain M200 was shown to have high abundances of aminotriol (**V**) (van Winden et al., 2012c). Therefore, the BHP composition detected at pH 4 may be a true reflection of this genus becoming enriched in microcosms due to its close relationship with the M200 strain. However, this would not explain why the corresponding initial sediment (Time 0) at pH 4 also had similar levels of aminotriol (**V**) as no enrichment of methanotrophs would be expected. Therefore, as previously mentioned, it could represent an abiotic process whereby reduction of the pH released aminotriol (**V**) from the sediment.

Aminotetrol (**VI**) is more diagnostic for methanotrophs than aminotriol (**V**), being found in almost all Type I methanotrophs (Neunlist and Rohmer, 1985a; Cvejic et al., 2000a; Talbot et al., 2001). A similar trend witnessed for aminotriol (**V**) (Figure 6-3) was also observed for aminotetrol (**VI**) with concentrations in 5% methane amended sediments decreasing with increasing pH (Figure 6-5). 'Carbamylmethylestertetrol' (**VI$^{MeCarb}$**) was detected at pH 5 and increased with pH with greater concentrations than aminotetrol (**VI**) at values of pH 7 and above. The corresponding initial sediment (Time 0) concentrations at pH 4 and 5 for aminotetrol (**VI**) were elevated compared to concentrations detected in the methane concentration and temperature studies (Chapter 5, Section 5.4.2.2 and 5.4.5.2), suggesting that microcosms at low pH values may also 'release' aminotetrol (**VI**) from the sediment matrix however this was less pronounced than for aminotriol (**V**). The ratio of aminotetrol (**VI**) in 5% methane amended sediments compared to the corresponding initial sediment was highest at pH

4 and 5 which seems to suggest that aminotetrol (**VI**) was enriched at pH 4 and 5 relative to microcosms subjected to higher pH values (pH 6-9).

Aminopentol (**VII**) concentrations (Figure 6-6) in 5% methane amended sediments decreased with increasing pH with no compound detected at pH 8 and 9. In microcosms incubated at pH 9, *Methylomicrobium* spp. were enriched and previous studies have showed that aminopentol (**VII**) was absent in all species of this genus (Talbot et al., 2001; Birgel et al., unpublished data). The concentration was greatest at pH 4 where *Methylosoma* spp. dominated. As previously mentioned, the BHP composition of this genus has not been investigated. The corresponding initial sediment (Time 0) concentrations at pH 4 and 5, did not show unusually high concentrations of aminopentol (**VII**) compared to what was observed for aminotriol (**V**) suggesting that any predicted 'release' of aminopentol (**VII**) from the sediment matrix is not evident at low pH values, presumably as the compound is not present rather than any preferential retention of the hexafunctionalised structure. 'Carbamylmethylesterpentol' (**VII$^{MeCarb}$**) was only present at pH 6-8 close to *in situ* sediment conditions. 'Aminopentol isomer' (**VII$^{iso}$**) was present at pH 4 and 5, where aminopentol (**VII**) concentrations were highest. It was not detected in the *Methylobacter* spp. pure cultures examined during this study (Chapter 4, Section 4.4.3), which was also the same methanotroph genus enriched at pH 5 (Figure 6-2). Interestingly, this isomer was also detected in the temperature study at 40°C (Chapter 5, Section 5.4.5.3, Figure 5-11) where the aminopentol (**VII**) concentration was greatest and a *Methylobacter* sp. enriched. It has also been found in other environmental settings where aminopentol (**VII**) is abundant relative to other BHPs (Talbot et al., 2014) and a *Methylovulum*-like sp. M200 (van Winden et al., 2012c). 'Carbamylmethylesterpentol isomer' (**VII$^{iso,MeCarb}$**) was also detected at pH 4.

No unsaturated compounds were detected at any pH which is perhaps unexpected as unsaturated $\Delta^{11}$ aminopentol (**VIIb**) was previously detected in *Methylobacter* spp., albeit in trace amounts (Chapter 4, Section 4.4.3); this genus was enriched in microcosms at pH 5-8. Unsaturated compounds may have been below the detection limit of the LC-MS or may not be produced by the *Methylobacter* species enriched at these pH values.

No C-3 methylated compounds were detected at any pH, but were previously detected in trace amounts in *Methylobacter* spp. cultures (Chapter 4, Section 4.4.2) enriched at pH 5-8 and *Methylomicrobium* spp. (Birgel et al., unpublished data) which were enriched at pH 9. The species enriched at pH 9 were closely related to a *Methylomicrobium alcaliphilum* strain 20Z and *Methylomicrobium kenyense* AMO1 strain both of which have been shown to produce C-3 methylated compounds in culture, especially 3-methylaminotriol (**Va**) (Birgel et al., unpublished data). In modern environmental settings, 3-methylhopanoids are frequently absent with 3-methylaminopentol (**VIIa**) only observed in one freshwater lake sediment (Talbot and Farrimond, 2007), three alkaline lakes (Farrimond et al., 2004) and a geothermal site (Gibson et al., 2008). This suggests that concentrations produced by methanotrophs may be too low to be detected in modern settings. However, the observations in modern environmental settings are at odds with widespread reports of 3-methyl degradation products, the C-3 methylated hopanes, in the sedimentary record (e.g. Collister et al., 1992; Farrimond et al., 2004; Eigenbrode et al., 2008).

### 6.5.4. The effect of salinity on 35-aminoBHP signatures in River Tyne estuarine sediment microcosms

The concentrations of 35-aminoBHPs vary with salinity (Figure 6-10 to Figure 6-12). Once again, the BHP compositions appear to be a reflection of the methanotroph communities that were enriched at specific salinities and do not directly correlate with methane oxidation rates and methanotrophic activity (Figure 6-8). Aminotriol (**V**) concentrations were enriched in 5% methane amended sediments at 1, 35 and 70 g/L NaCl compared with the initial sediment but displayed similar concentrations to initial sediments at 15 g/L NaCl (Figure 6-10); 'carbamylmethylestertriol' ($\mathbf{V}^{\mathbf{MeCarb}}$) concentrations were also lower at this salinity. The DGGE profiles of microcosm sediments at 1 and 15 g/L NaCl were similar (Figure 6-9) so it is perhaps surprising that the concentrations detected in sediments at these salinities were not also similar. This suggests that concentrations may be a reflection of the higher salinity and not the methanotroph community. The relative abundance of aminotriol (**V**) in pure cultures of *Methylomicrobium* is > 90% for all species recently examined (Birgel at al., unpublished data) whereas, abundances are much lower for *Methylobacter* spp. (Chapter 4, Section 4.4.3) and *Methylomonas* spp. (Rohmer et al., 1984; Neunlist and Rohmer, 1985b; Talbot et al., 2001; van Winden et al., 2012c). *Methylomicrobium* spp. dominated at 35

and 70 g/L NaCl therefore it is not unexpected that higher concentrations of aminotriol (**V**) were detected in these sediments compared to sediments with 15 g/L NaCl, where *Methylobacter* spp. and *Methylomonas* spp. dominated.

Aminotetrol (**VI**) concentrations were similar at 1-70 g/L NaCl. At 1 and 15 g/L NaCl similar DGGE profiles were generated (Figure 6-9) consequently, concentrations of aminotetrol (**VI**) were also expected to be similar as the same methanotroph genera were enriched at these salinities, *Methylobacter* and *Methylomonas* spp. At 35 and 70 g/L NaCl *Methylomicrobium* spp. were enriched and the relative abundance of aminotetrol (**VI**) in pure cultures of *Methylobacter* spp. (Chapter 4, Section 4.4.2) and *Methylomicrobium* spp. (Birgel et al., unpublished data) have been shown to be similar. Therefore, similar aminotetrol (**VI**) concentrations across the salinity range were perhaps expected.

Aminopentol (**VII**) concentrations decreased with increasing salinity (Figure 6-6) which corresponds with *Methylomicrobium* spp. dominating at 35 and 70 g/L NaCl (Figure 6-9). Aminopentol (**VII**) was absent in all *Methylomicrobium* spp. examined to date (Talbot et al., 2001; Birgel et al., unpublished data) so it was predicted that lower concentrations would be detected at higher salinities. However, the species enriched in microcosms in this study have not had their BHP compositions investigated. Aminopentol (**VII**) levels detected at higher salinities may be due to methanotrophs enriched at lower salinities also being present in the sediment. Conversely, the species of *Methylomicrobium* enriched in this study, may contradict current observations that aminopentol (**VII**) is universally absent in this genus.

No unsaturated compounds were detected at any of the salinities tested which is perhaps unexpected as unsaturated $\Delta^{11}$ aminopentol (**VIIb**) was detected in *Methylobacter* spp., albeit in trace amounts (Chapter 4, Section 4.4.2). 3-Methylaminotriol (**Va**) and 3-methylaminopentol (**VIIa**) were identified in sediments with 35 and 70 g/L NaCl where *Methylomicrobium* spp. were enriched. One of the species enriched at these salinities was closely related to *Methylomicrobium japanense* (Figure 6-9) with the 35-aminoBHP composition in pure culture yet to been determined. As previously mentioned (Section 6.5.3), *Methylomicrobium alcaliphilum* and *Methylomicrobium kenyense* cultures have been shown to produce C-3 methylated compounds, 3-methylaminotriol (**Va**) in significant amounts and 3-methylaminopentol

(**VIIa**) in trace amounts (Birgel et al., unpublished data). Therefore, production in other *Methylomicrobium* species may be likely. C-3 methylation in *Methylomicrobium* spp. may protect the cell from osmotic stress at high salinities, as it has been previously suggested that methylation plays a role in maintaining cell homeostasis (Welander et al., 2009).

### 6.5.5. Total BHP concentrations in River Tyne estuarine sediment microcosms in response to pH and salinity

Total BHP concentrations only showed a significant increase relative to initial sediment concentrations (Time 0) in microcosm sediments at pH 8 (Figure 6-7). As previously described (Section 6.4.1.1), the pH of microcosms drifted over the course of the experiment. Microcosms incubated at pH 8 showed no drift in pH which was expected as the *in situ* value of the sediment was pH 8. All other microcosm pH values drifted by one pH unit towards the *in situ* pH of the sediments. A significant increase in total BHPs relative to the initial sediment was also detected in the temperature study at 21°C (Chapter 6, Section 5.5.6) where the pH of the microcosms was predicted to be close to *in situ* sediment conditions.

Total BHP concentrations showed a significant increase relative to initial sediment concentrations (Time 0) in microcosm sediments with 1 and 15 g/L NaCl but significant differences were not detected at 35-150 g/L NaCl (Figure 6-13). Salinities of 1 g/L NaCl are representative of freshwater environments and those of 15 g/L NaCl are representative of brackish conditions. The salinity at the sampling location of the River Tyne estuary is brackish (7 g/L NaCl) so it is postulated that *in situ* bacteria present in the sediment would tolerate 15 g/L NaCl, still representative of brackish conditions. A significant increase in total BHPs was also witnessed in the temperature study at 21°C (7 g/L NaCl) relative to the initial sediment concentrations (Chapter 6, Section 5.5.6), similar to the increase in total BHPs that was observed here in the salinity experiments at 15g/L NaCl which were also incubated at 21°C.

The most abundant compound in all 5% methane amended, unamended and heat killed controls for both the pH and salinity studies was BHT (**III**) (see appendices 3d and 3e for data). As previously mentioned, this is one of the most commonly occurring BHPs and is produced by a wide range of bacteria (e.g. Sinninghe Damsté et al., 2004; Talbot and Farrimond, 2007; Talbot et al., 2008; Blumenberg et al., 2012; Eickhoff et

al., 2013). The second most abundant BHP was adenosylhopane (**VIII**); a compound that is produced by all organisms (Bradley et al., 2010) as the precursor for further side chain modification of the parent BHP structure; however, few microorganisms studied to date have been found to accumulate this compound.

Significant increases in total BHPs may have only been observed in microcosm sediments at pH 8 and 1 and 15 g/L NaCl as methanotrophs and other bacterial groups may be reduced in number at more extreme conditions markedly different from those of the *in situ* sediment conditions.

### 6.6. Conclusions

Only Type I methanotrophs were enriched in the River Tyne estuarine sediments with distinct shifts in community composition in response to changes in pH and salinity conditions, thus supporting the original hypothesis that community composition changes with pH and salinity. *Methylosoma* spp. dominated at pH 4 and *Methylobacter* spp. and *Methylomonas* spp. dominated across the pH range 5-8, with very similar *pmoA* PCR-DGGE profiles at pH values close to *in situ* conditions (pH 6-8). At 1-15 g/L NaCl, freshwater and brackish conditions, methanotroph community composition was similar with *Methylobacter* spp. and *Methylomonas* spp. dominating. *Methylomicrobium* spp. dominated at pH 9 and 35-70 g/L NaCl, representing seawater and hypersaline conditions. No Type II methanotrophs were detected in any of the microcosms in response to pH and salinity; this was also witnessed in methane concentration and temperature studies (Chapter 5, Section 5.4.1.1 and 5.4.4.1).

The shifts in methanotroph community composition were reflected in the BHP compositions and did not appear to correlate with methane oxidation rates (i.e. methanotrophic activity) which supports the original hypothesis. Aminotriol (**V**) and aminotetrol (**VI**) concentrations were greatest at pH 4 and decreased with increasing pH whereas the ‘carbamylmethylester’ compounds showed the converse relationship and increased with increasing pH. Comparable concentrations in corresponding initial sediments (Time 0) suggested that microcosms at low pH values may ‘release’ aminotriol (**V**) from the sediment matrix or lyse the bacterial cells allowing for greater recovery of the compound in the total lipid extract. This effect was less pronounced for aminotetrol (**VI**) and not apparent for aminopentol (**VII**) or other major BHP compounds.

Aminopentol (**VII**) concentrations were highest at pH 4 and decreased with increasing pH with no compound detected at pH 8 and 9; at pH 9 *Methylomicrobium* spp. dominated. Aminopentol (**VII**) concentrations decreased with increasing salinity (35 and 70 g/L NaCl) where *Methylomicrobium* spp. dominated. Methylated compounds, 3-methylaminotriol (**Va**) and 3-methylaminopentol (**VIIa**) were only detected in microcosms at 35 and 70 g/L NaCl where *Methylomicrobium* spp. dominated but not at pH 9.

# 7. The effect of bacterial growth phase on BHP composition

## 7.1. Introduction

Bacteria often alternate between growth and stationary phases which is the result of fluctuations in substrate availability in natural settings. When substrate(s) becomes limiting, bacteria can exhibit physiological and morphological changes in response to starvation (Roslev and King, 1995 and references therein). Gram-positive thermophilic sulphate-reducing bacteria produce spores in response to starvation whereas many Gram-negative bacteria develop resistant cells in dormancy (Navarro Llorens et al., 2010 and references therein). If nutrients become available again, cells will resume growth until nutrient depletion occurs, entering again into a stationary phase period.

Laboratory cultures and *in situ* studies have suggested that methanotrophs can survive long-term as differentiated resting states. However, other studies have suggested that long-term anaerobic survival can also occur in a non-differentiated state that rapidly responds to methane addition (Roslev and King, 1995 and references therein). Methanotrophic bacteria have been shown to survive nutrient-limiting conditions followed by fairly rapid aerobic methane oxidation, as witnessed in the Deepwater Horizon oil spill (Kessler et al., 2011).

Cultures of the cyanobacterium *Nostoc punctiforme* in which cells had differentiated into akinetes, survival structures that protect the cell from cold and desiccation, contained ~10-fold higher concentrations of 2-methylhopanoids compared with cultures that only contained vegetative cells (Doughty et al., 2009). This suggests hopanoids play a role in protecting the cell from environmental stress in this resting state (Doughty et al., 2009). A study of *Rhodopseudomonas palustris* TIE-1 suggested that stress conditions are necessary to regulate 2-methylBHP production with 2-methylBHT consistently higher in the presence of stressors in the wild type (Kulkarni et al., 2013). Studies have suggested that hopanoids play an important role in maintaining cell homeostasis particularly in the stationary phase. The deletion of the *sqhC* gene in *Rhodopseudomonas palustris* TIE-1, resulted in increased sensitivity to acidic and alkaline conditions relative to the wild type which was particularly noticeable in stationary phase (Welander et al., 2009). A study of *Methylococcus capsulatus* demonstrated that 3-methylhopanoids accumulate preferentially in late stationary

phase cells (Summons et al., 1994). In a different study involving the same methanotroph the deletion of the *hpnR* gene, required for C-3 methylation, indicated that 3-methylhopanoids were important for cell survival in late stationary phase (Welander and Summons, 2012).

In this study, 35-aminoBHP signatures were investigated at different time points to determine whether changes in composition occur after methane depletion in the microcosms, when methanotroph cells were presumably entering stationary phase. The findings from this study are pertinent as they give an approximation to conditions widely found in nature, where low nutrients and harsh conditions are commonplace and bacteria are thought to persist in a type of stationary state (Navarro Llorens et al., 2010).

## 7.2. Aims and hypotheses

### 7.2.1. Aims

This study investigates the 35-aminoBHP sedimentary signatures at different stages of growth from aerobic microcosm experiments inoculated with River Tyne estuarine sediment incubated at 30 and 50°C.

This chapter has two aims:

(1) to determine the effect of different stages of growth on the abundance of 35-aminoBHPs.

(2) to investigate the effect of incubation temperature on the BHP composition of mesophilic and thermophilic microorganisms during different stages of growth.

### 7.2.2. Hypotheses

- *35-aminoBHPs continue to be produced in stationary phase cells.*

In a pilot study, microcosms were incubated for a period of 20 days rather than being sacrificially sampled at the point where 5% methane had been oxidised, which was the regime used for all other aerobic microcosm studies (Chapters 5 and 6). At 30°C after 20 days, ~10 times the concentration of aminopentol (**VII**) was detected compared with the original temperature study (Chapter 5, Section 5.4.5.3).

- *3-methylaminopentol production increases in stationary phase cells.*

Studies of *Methylococcus capsulatus* have demonstrated that 3-methylhopanoids are important for cell survival in late stationary phase and accumulate in this growth phase (Summons et al., 1994; Welander and Summons, 2012).

### 7.3. Materials and methods

The experimental design was based on previous data from a pilot study which used River Tyne sediment sampled from anoxic depths (~30 cm), which was subsequently incubated under aerobic conditions, with a 5% methane amended headspace at 4, 30 and 60°C. This experiment was adapted, this time using aerobic River Tyne estuarine surface sediments as the inoculum. Additional time points were added to determine 35-aminoBHP composition in exponential and stationary phases. The experiment was performed at 30°C to replicate the previous study and also at 50°C to test the hypothesis that 3-methylaminopentol (**VIIa**) production increases during stationary phase; the original temperature study indicated production of 3-methylaminopentol (**VIIa**) at this temperature (Chapter 5, Section 5.4.5.3) but no significant methane oxidation at 60°C.

Microcosms were prepared (Chapter 3, Section 3.3.2) with some alterations (Chapter 3, Section 3.3.5.1). Briefly, six sets of 5% methane amended microcosms were prepared in triplicate to be sacrificially sampled during different stages of growth (Table 7-1). Unamended (to measure background levels of methane in the sediment) and heat killed controls + 5% $CH_4$ (abiotic controls) were prepared for both temperatures (30 and 50°C). Microcosms were monitored for methane for the duration of the experiment and sacrificed on completion of the experiment at 28 days.

**Table 7-1:** Growth stage sacrificial time points.

| Growth stage | 30°C | 50°C |
|---|---|---|
| **Exponential phase** | 5 days | 4 days |
| **5% $CH_4$ consumed** | 7 days | 8 days |
| **Stationary phase** | 11 days | 11 days |
| **Stationary phase** | 15 days | 15 days |
| **Stationary phase** | 20 days | 20 days |
| **Stationary phase** | 28 days<br>Unamended<br>Heat killed + 5% $CH_4$ | 28 days<br>Unamended<br>Heat killed + 5% $CH_4$ |

Triplicate microcosms, representing the initial BHP compositions in the River Tyne surface sediments, were prepared with fresh sediment and sacrificially sampled; referred to as Time 0. TOC measurements (Chapter 3, Section 3.7.1) were performed in triplicate on fresh sediment and all BHP concentrations were normalised to the average of these values, 8.51% (range 8.49-8.55%).

Microcosms were sacrificially sampled (Chapter 3, Section 3.3.4) at the point of most rapid methane oxidation (designated as exponential phase) and when 5% methane had been oxidised; these time points for both temperatures are shown in Table 7-1. The subsequent four sacrificial samples represented four time points during stationary phase. Microcosm sediments were freeze-dried (Chapter 3, Section 3.3.4) and BHPs were extracted from the sediment using a modified Bligh and Dyer extraction method (Chapter 3, Section 3.8.1). The polar fraction resulting from the SPE of total lipid extracts were prepared and then analysed using LC-MS (Chapter 3, Section 3.8.2 to 3.8.4).

## 7.4. Results

### 7.4.1. Methane oxidation in River Tyne estuarine sediment microcosms incubated at 30°C

Headspace methane concentrations were monitored over 28 days (Chapter 3, Section 3.3.6; Figure 7-1).

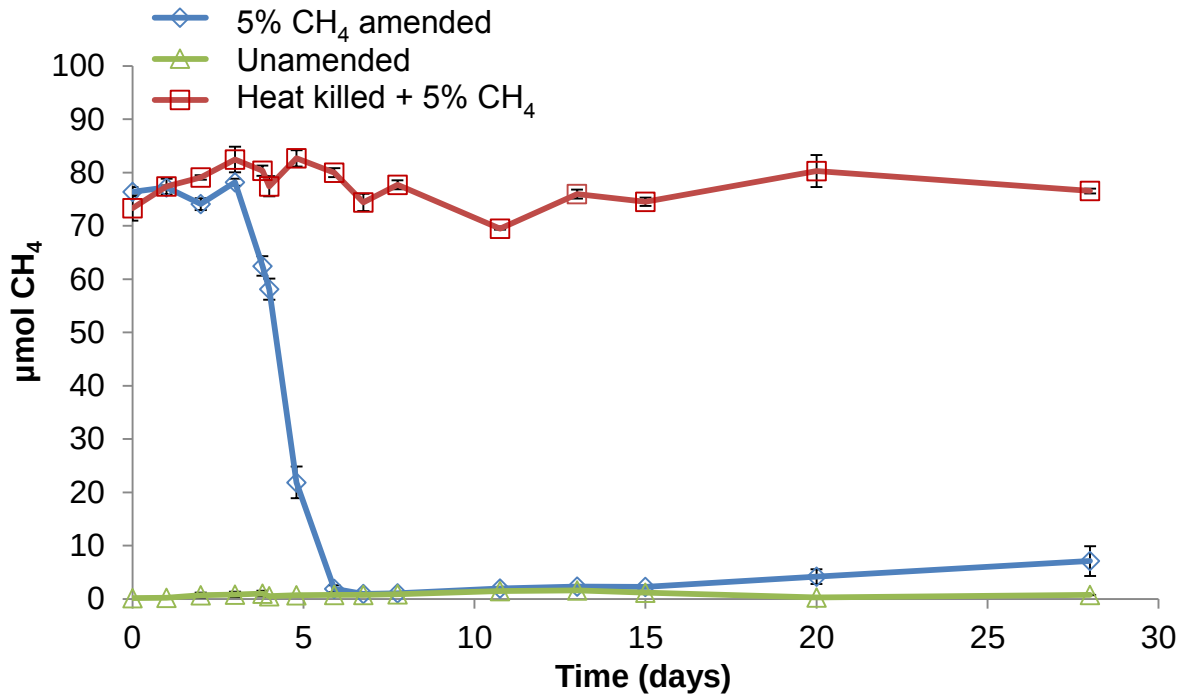



**Figure 7-1:** Methane concentration (µmol $CH_4$) in River Tyne estuarine sediment microcosms incubated at 30°C for a 28 day period. 5% $CH_4$ amended (blue line), unamended control (green line) and heat killed control (red line). Error bars represent 1 x S.E: Time 0 (N = 18); 5 days (N = 18); 7 days (N = 15); 11 days (N = 12); 15 days (N = 9); 20 days (N = 6); 28 days (N = 3), except unamended and heat killed control (N = 3).

The methane oxidation rate for the consumption of 5% methane was 6.21 ± 0.1 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment comparable to 5.27 ± 0.2 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment in the original temperature study at 30°C (Chapter 5, Section 5.4.4.1). A triplicate set of microcosms was sacrificed at day 5 where methane oxidation was most rapid (designated exponential phase) (Figure 7-1). At day 7, the 5% methane in the headspace had been consumed and a triplicate set was sacrificed at this time point. Additional triplicate sets of microcosms were sacrificed at days 11, 15, 20 and 28

representing increasing periods of starvation/nutrient limitation for the cells in the microcosms. At day 15, the concentration of methane in the headspace of the 5% methane amended microcosms began to rise slowly (Figure 7-1). At the end of the study, the unamended and heat killed controls were sacrificed.

### 7.4.2. 35-aminoBHP concentrations in River Tyne estuarine sediment microcosms incubated at 30°C at different stages of growth

Aminotriol (**V**), related 'carbamylmethylester' (**V$^{MeCarb}$**) and 'desmethyl' (**V$^{des}$**) compounds (Chapter 4, Section 4.4.1) and aminotetrol (**VI**) were detected at all time points and in the initial sediment (Time 0) (Figure 7-2 and Figure 7-3). Aminopentol (**VII**) was detected at all time points and in the initial sediment whereas 'aminopentol isomer' (**VII$^{iso}$**), unsaturated $\Delta^{11}$ aminopentol (**VIIb**) and 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**) were only detected at some time points (Figure 7-4). For 35-aminoBHP concentrations in unamended and heat killed control and all other BHPs in the study see appendix 3f.

Aminotriol (**V**), aminotetrol (**VI**) and aminopentol (**VII**) concentrations in 5% methane amended microcosm sediments were statistically compared to the initial sediment (Time 0) and across the time series using one-way analysis of variance (ANOVA) with Fisher's LSD.

### 7.4.2.1. Aminotriol and related compounds

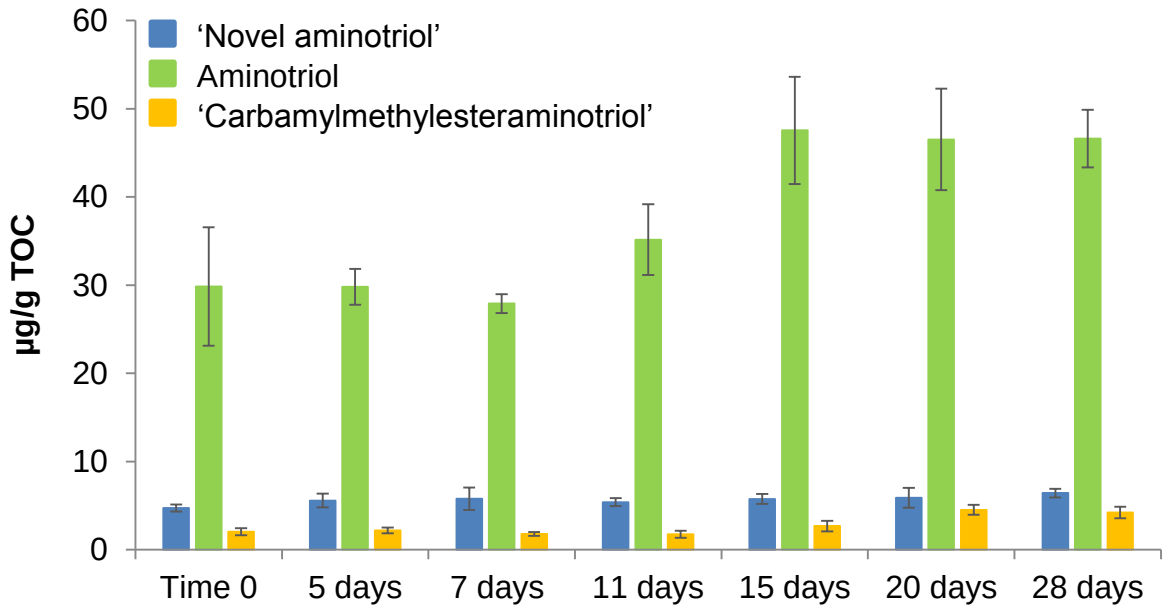



**Figure 7-2:** Concentration (µg/g TOC) of 'desmethylaminotriol' (**V$^{des}$**), aminotriol (**V)** and 'carbamylmethylestertriol' (**V$^{MeCarb}$**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms incubated at 30°C for a 28 day period. Error bars represent 1 x S.E. (N = 3).

Aminotriol (**V**) concentrations were significantly higher at days 15, 20 and 28 compared with the initial sediment (Time 0) ($p \leq 0.022$) and higher at day 15 compared with days 5 and 7 ($p \leq 0.016$); however, there was no significant difference in concentrations between 15, 20 and 28 days ($p \geq 0.877$). There was no significant difference in 'desmethylaminotriol' (**V$^{des}$**) concentrations in the initial sediment (Time 0) compared with any other time points ($p \geq 0.155$) (Figure 7-2). 'Carbamylmethylestertriol' (**V$^{MeCarb}$**) concentrations at days 5, 7 and 11 were similar to that of the initial sediment but were higher at days 15, 20 and 28.

### 7.4.2.2. Aminotetrol

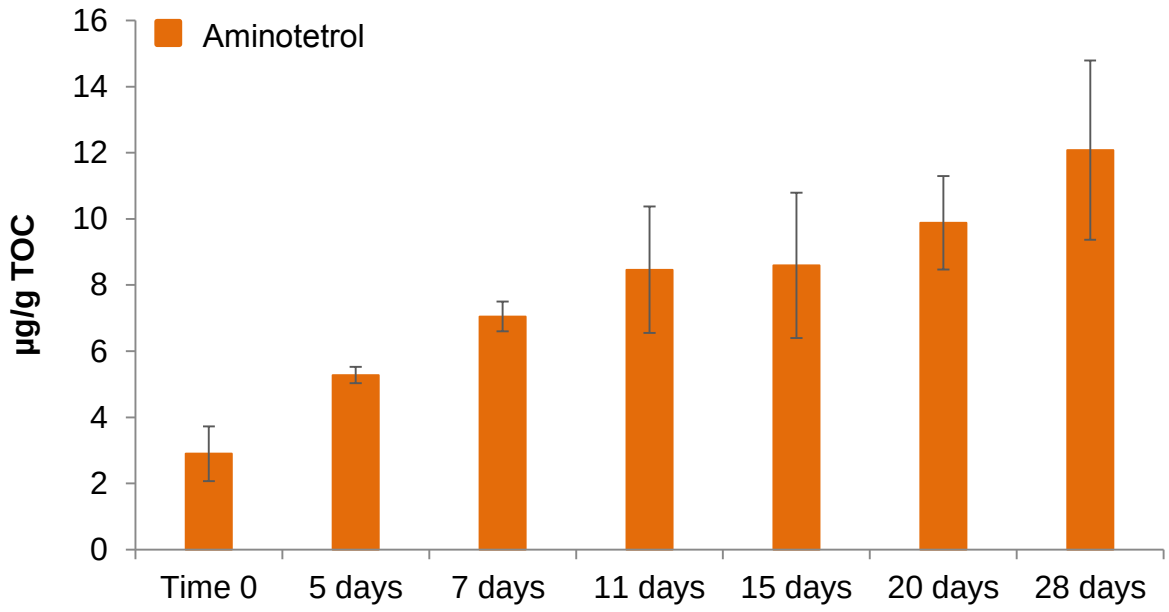



**Figure 7-3:** Concentration (µg/g TOC) of aminotetrol (**VI**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms incubated at 30°C for a 28 day period. Error bars represent 1 x S.E. (N = 3).

Aminotetrol (**VI**) concentrations were significantly higher at days 11, 15, 20 and 28 compared with the initial sediment (Time 0) ($p \leq 0.031$) (Figure 7-3). There was no significant difference in concentrations between days 11, 15, 20 and 28 ($p \geq 0.141$) but concentrations were significantly higher at day 28 compared with day 5 ($p = 0.011$) and 7 ($p = 0.047$). 'Carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$) was not detected.

### 7.4.2.3. Aminopentol and related compounds

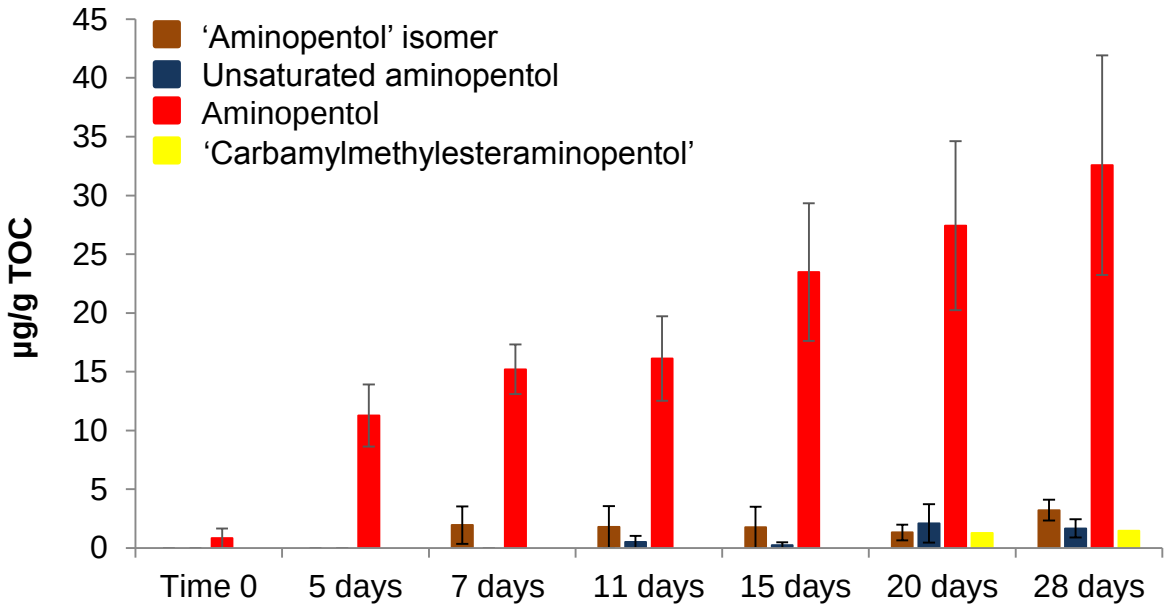



**Figure 7-4:** Concentration (µg/g TOC) of 'aminopentol isomer' (**VII$^{iso}$**), unsaturated $\Delta^{11}$ aminopentol (**VIIb**), aminopentol (**VII**) and 'carbamylmethylesterpentol' (**VII$^{MeCarb}$**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms incubated at 30°C for a 28 day period. Error bars represent 1 x S.E. (N = 3).

Aminopentol (**VII**) concentrations were greater at all time points relative to the initial sediment (Time 0) (Figure 7-4). There was no significant difference in concentrations between days 5, 7 and 11 ($p \geq 0.530$) and no difference between days 15, 20 and 28 ($p \geq 0.247$). Concentrations were significantly higher at day 28 compared with days 5, 7 and 11 ($p \leq 0.046$) and significantly higher at day 20 compared with day 5 ($p = 0.050$). The relative percentages of aminopentol (**VII**) increased during the study with the greatest percentage detected at day 28 as with absolute concentration (see appendix 3f). 'Aminopentol isomer' (**VII$^{iso}$**) was first detected at day 7 following the oxidation of 5% methane, and all time points thereafter, with the highest concentration of 3.2 ± 0.9 µg/g TOC detected at day 28. Unsaturated $\Delta^{11}$ aminopentol (**VIIb**) was only detected in low quantities in sediments after 5% methane had been oxidised at 7 days. 'Carbamylmethylesterpentol' (**VII$^{MeCarb}$**) was only detected at 20 and 28 days. 'Desmethylaminopentol' (**VII$^{des}$**) was detected in trace amounts in some replicates at days 7, 15, 20 and 28 and 3-methylaminopentol (**VIIa**) was detected in one replicate at day 20 (data not shown on Figure 7-4, see appendix 3f).

### 7.4.3. Methane oxidation in River Tyne estuarine sediment microcosms incubated at 50°C

Headspace methane concentrations were monitored over 28 days (Chapter 3, Section 3.3.6; Figure 7-5).

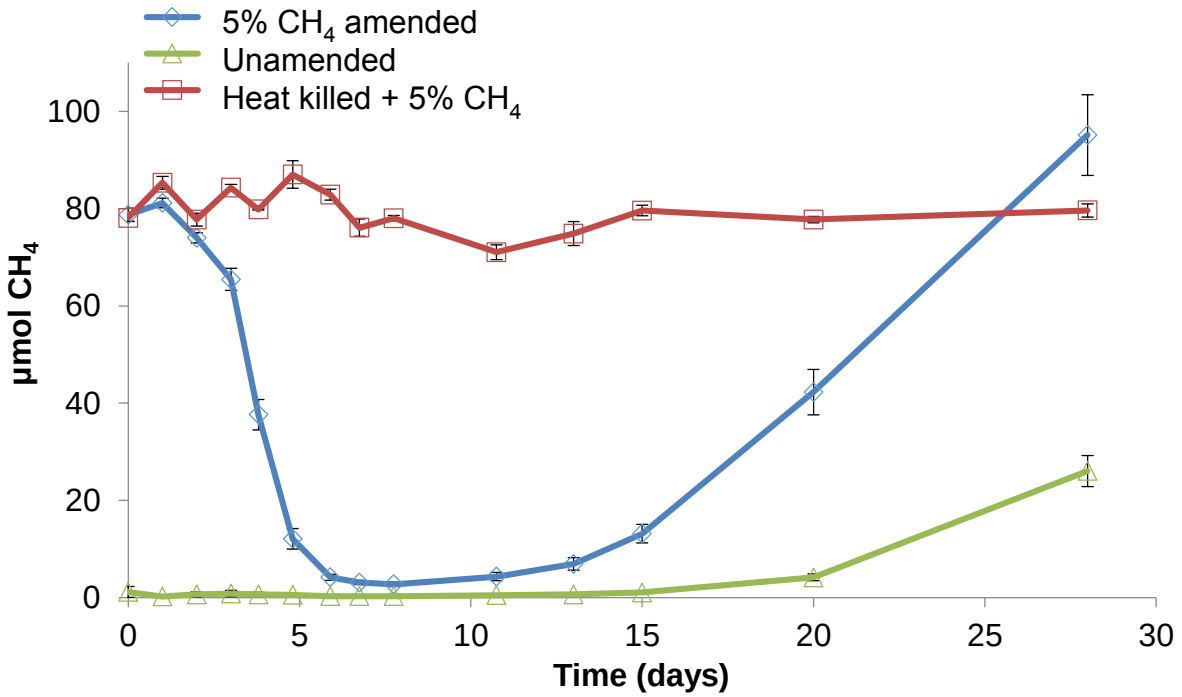



**Figure 7-5:** Methane concentration (µmol $CH_4$) in River Tyne estuarine sediment microcosms incubated at 50°C for a 28 day period. 5% $CH_4$ amended (blue line), unamended control (green line) and heat killed control (red line). Error bars represent 1 x S.E: Time 0 (N = 18); 4 days (N = 18); 8 days (N = 15); 11 days (N = 12); 15 days (N = 9); 20 days (N = 6); 8 days (N = 3), except unamended and heat killed control (N = 3).

The methane oxidation rate for the consumption of 5% methane, 3.75 ± 0.1 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment, was remarkably similar to the oxidation rate in microcosms incubated at 50°C in the original temperature study, 3.77 ± 0.1 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment (Chapter 5, Section 5.4.4.1). A triplicate set of microcosms were sacrificed at day 4 where methane oxidation was most rapid (designated exponential phase) (Figure 7-5) and at day 8, following 5% methane composition. After this time point, the concentration of methane in the headspace began to increase and continued to increase to a concentration higher than the headspace methane concentration at the start of the study (95.15 µmol $CH_4$). A triplicate set of microcosms was sacrificed at

days 11, 15, 20 and 28 which should be representative of stationary phase conditions. At the end of the study, the unamended and heat killed controls were sacrificed. The concentration of methane in the headspace of the unamended controls increased after 15 days.

### 7.4.4. 35-aminoBHP concentrations in River Tyne estuarine sediment microcosms incubated at 50°C at different stages of growth

Aminotriol (**V**) and related 'carbamylmethylester' ($\mathbf{V^{MeCarb}}$) and 'desmethyl' ($\mathbf{V^{des}}$) compounds (Chapter 4, Section 4.4.1) and aminotetrol (**VI**) were detected at all time points and in the initial sediment (Time 0) (Figure 7-6 and Figure 7-7). Aminopentol (**VII**), unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) and 3-methylaminopentol (**VIIa**) were detected at all time points but 'aminopentol isomer' ($\mathbf{VII^{iso}}$) was only detected at certain time points (Figure 7-8).

Aminotriol (**V**), desmethylaminotriol ($\mathbf{V^{des}}$) and aminotetrol (**VI**) concentrations in 5% methane amended microcosm sediments were statistically compared to the initial sediment (Time 0) and across the time series using one-way analysis of variance (ANOVA) with Fisher's LSD. Aminopentol (**VII**), 3-methylaminopentol (**VIIa**) and unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) concentrations were statistically compared across the time series.

### 7.4.4.1. Aminotriol and related compounds

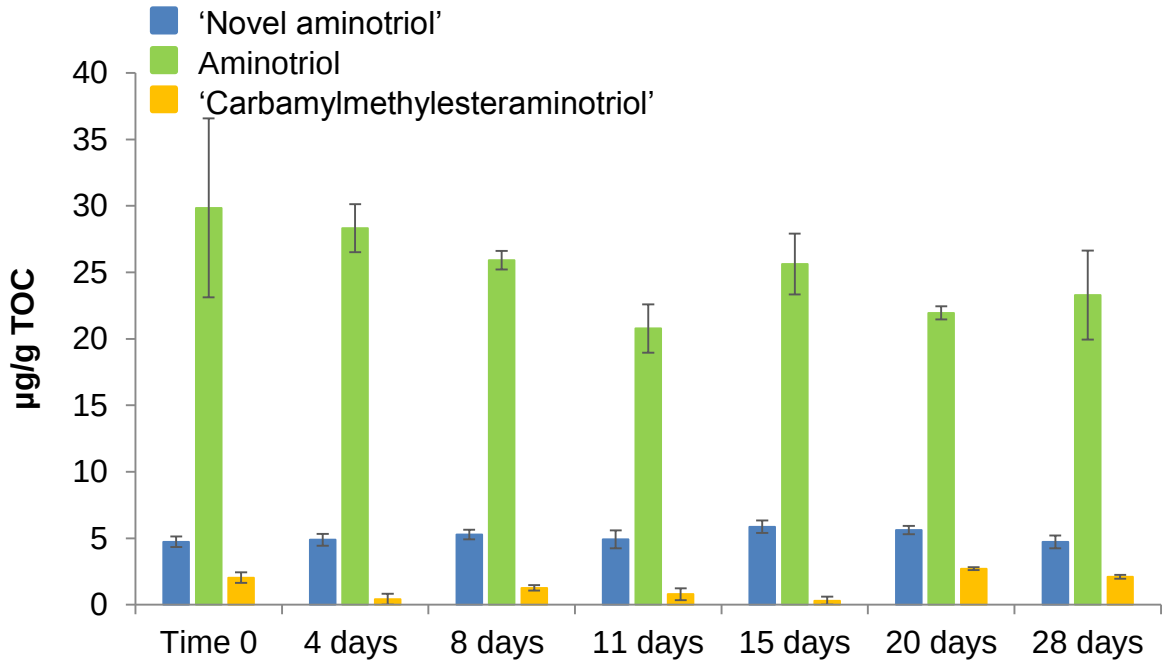



**Figure 7-6:** Concentration (µg/g TOC) of 'desmethylaminotriol' (**V$^{des}$**), aminotriol (**V)** and 'carbamylmethylestertriol' (**V$^{MeCarb}$**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms incubated at 50°C for a 28 day period. Error bars represent 1 x S.E. (N = 3).

Aminotriol (**V**) and 'desmethylaminotriol' (**V$^{des}$**) concentrations showed no significant differences across the time series (Figure 7-6). 'Carbamylmethylestertriol' (**V$^{MeCarb}$**) concentrations showed a small increase in concentration at days 15 and 20 but were similar to levels seen in the initial sediment.

### 7.4.4.2. Aminotetrol

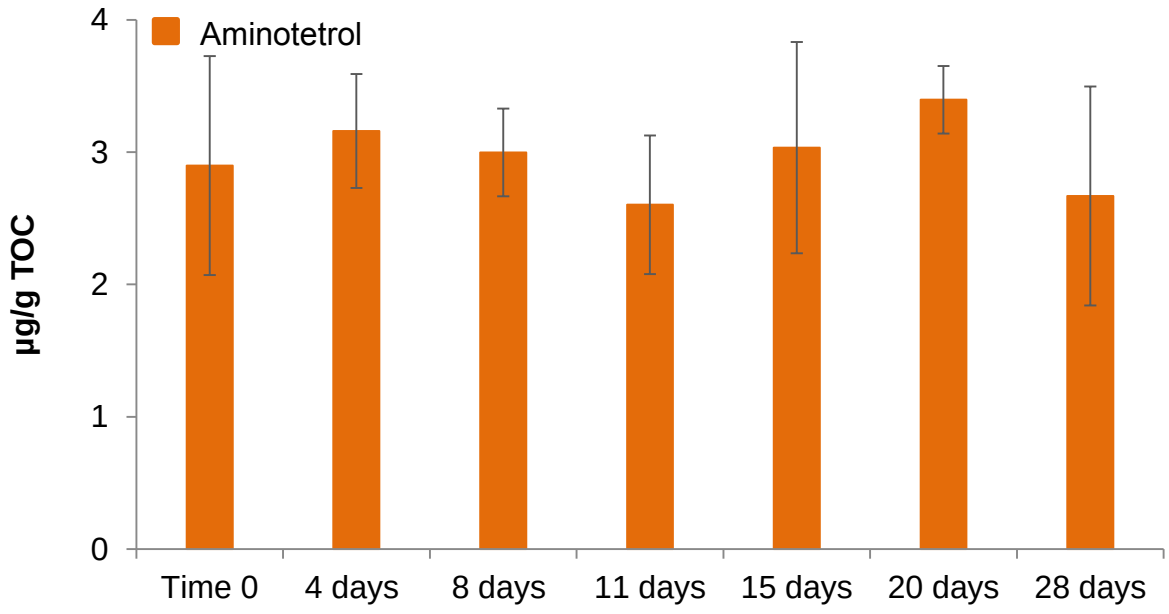



**Figure 7-7:** Concentration (µg/g TOC) of aminotetrol (**VI**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms incubated at 50°C for a 28 day period. Error bars represent 1 x S.E. (N = 3).

There were no significant differences in aminotetrol (**VII**) concentrations across the time series during the study (Figure 7-7). 'Carbamylmethylestertetrol' (**VI**$^{\mathbf{MeCarb}}$) was not detected.

### 7.4.4.3. Aminopentol and related compounds

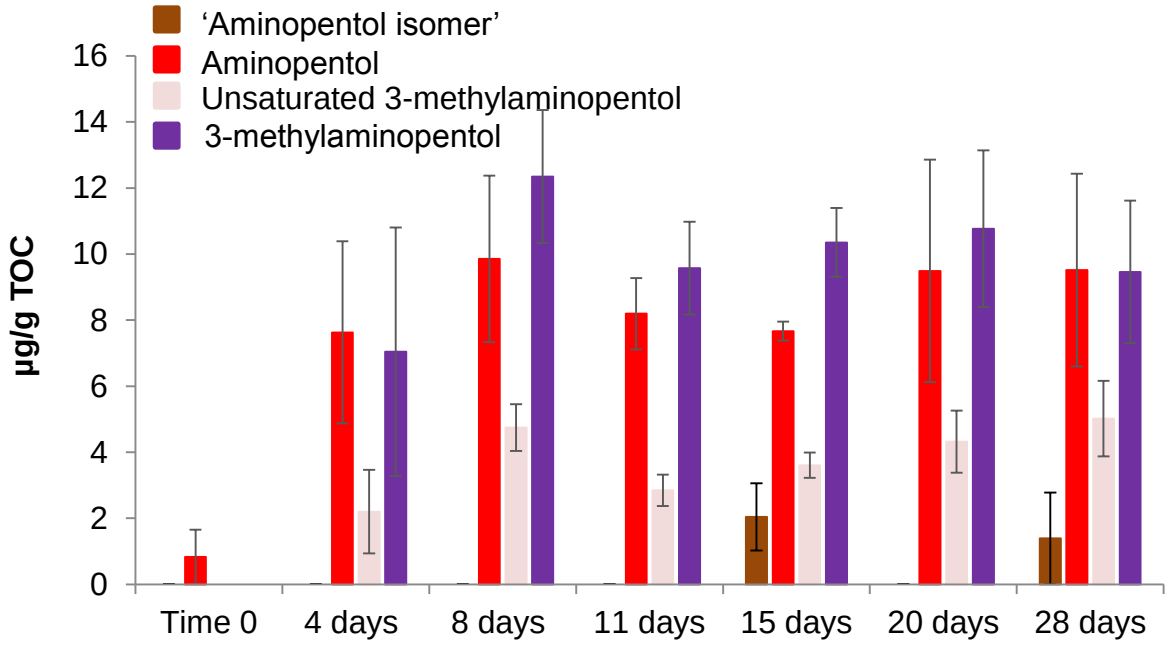



**Figure 7-8:** Concentration (µg/g TOC) of 'aminopentol isomer' (**VII$^{iso}$**), aminopentol (**VII**), unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) and 3-methylaminopentol (**VIIa**) in 5% $CH_4$ amended River Tyne estuarine sediment microcosms incubated at 50°C for a 28 day period. Error bars represent 1 x S.E. (N = 3).

Aminopentol (**VII**) concentrations were greater at all time points relative to the initial sediments (Time 0) with no significant differences detected between pairwise comparisons of all other time points (Figure 7-8). 3-Methylaminopentol (**VIIa**) and unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) were not detected in the initial sediment but were detected at all other time points. There were no significant differences between 3-methylaminopentol (**VIIa**) concentrations across the time series ($p \geq 0.127$). Unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) concentrations were significantly higher at day 28 compared with day 4 ($p = 0.043$) 'Aminopentol isomer' (**VII$^{iso}$**) was detected in sediments at day 15 and 28. 'Carbamylmethylesterpentol' (**VII$^{MeCarb}$**) was detected in one replicate at 20 days and 'desmethylaminopentol' (**VII$^{des}$**) was detected in trace amounts in two of three replicates at 20 days (data not shown on Figure 7-8, see appendix 3f).

## 7.5. Discussion

### 7.5.1. Methane oxidation in River Tyne estuarine sediment microcosms over a 28 day period

The methane oxidation rate for the consumption of 5% methane at 30°C was 6.21 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment (Section 7.4.1), slightly higher than in the original temperature study (5.27 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment, Chapter 5, Section 5.4.4.1). At day 7, 5% methane had been oxidised, however, methane levels began to rise from day 15 suggesting oxygen depletion in the system and ensuing anaerobic conditions (Figure 7-1). The increase in methane in the microcosm headspace may be a result of the breakdown of organic matter and subsequent methanogenesis in the sediments as previously witnessed in sediments from this location (Jones et al., 2008).

The methane oxidation rate for the consumption of 5% methane at 50°C was 3.75 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment (Section 7.4.1) compared to 3.77 µmol $CH_4$ $day^{-1}$ $g^{-1}$ wet sediment in the original temperature study (Chapter 5, Section 5.4.4.1). At day 8, 5% methane had been oxidised, however, after this time point, the concentration of methane in the headspace began to increase, more rapidly than at 30°C, and continued to increase to reach a concentration of 95.15 µmol methane which was higher than the headspace methane concentration at the start of the study (Figure 7-5). Furthermore, the concentration of methane in the headspace of the unamended control began to increase after 15 days. Anaerobic conditions ensued earlier and methane concentrations rapidly increased at 50°C compared with 30°C. In a study using anoxic sediment from the same location, rates of methane production were higher at 50°C compared with 30°C when $H_2/CO_2$ (hydrogenotrophic methanogenesis) was the substrate (Blake, 2010).

### 7.5.2. The effect of growth stage on 35-aminoBHP signatures in River Tyne estuarine sediment microcosms incubated at 30°C

Aminotriol (**V**) concentrations were significantly higher at days 15, 20 and 28 compared with days 5 and 7 (Figure 7-2), however, there was no significant difference in concentrations between 15, 20 and 28 days. This indicates that aminotriol (**V**) continued to be produced in ‘early stationary phase’ up to day 15, following 5% methane oxidation at day 7, but after day 15 ‘late stationary phase’, no further increase

was detected. However, because aminotriol (**V**) is produced by a wide range of bacteria, increases may also be due to other bacteria growing after methane has been consumed. Aminotriol (**V**) concentrations at day 7, when 5% methane had been oxidised, were similar to the original temperature experiment (Chapter 5, Section 5.4.5.1). 'Carbamylmethylestertriol' (**V**$^{\mathbf{MeCarb}}$) concentrations were also higher at days 15, 20 and 28 compared to earlier time points in the study once again indicating continued production in stationary phase, however, concentrations were lower than those detected in the original temperature experiment (Chapter 5, Section 5.4.5.1). This may be because the initial sediment concentrations were lower in this study or different species of methanotroph were present/enriched in the sediment.

Aminotetrol (**VI**) concentrations were significantly higher at day 28 compared with day 5 (exponential phase) and day 7, when 5% methane had been consumed (Figure 7-3). This indicated that aminotetrol (**VI**) continues to be produced in stationary phase but perhaps only significantly at a later stage compared with aminotriol (**V**). Aminotetrol (**VI**) concentrations at day 7, when 5% methane had been oxidised were higher than the original temperature experiment (Chapter 5, Section 5.4.5.2).

Aminopentol (**VII**) concentrations were greater at all time points relative to the initial sediment (Time 0) (Figure 7-4). Concentrations were significantly higher at day 28 compared with day 5 (exponential phase), day 7 when 5% methane had been consumed and day 11 (stationary phase). However, there was no significant difference in concentrations between 15, 20 and 28 days. This suggests that aminopentol (**VII**) production continued in 'early stationary phase' after 5% methane oxidation but little accumulation was observed after 15 days in 'late stationary phase', similar to aminotriol (**V**). The highest concentration of 'aminopentol isomer' (**VII**$^{\mathbf{iso}}$) was measured at day 28 where aminopentol (**VII**) concentrations were highest. 'Carbamylmethylesterpentol' (**VII**$^{\mathbf{MeCarb}}$) was only detected later in the study at 20 and 28 days but in the original temperature study was in greater concentration than aminopentol (**VII**) (Chapter 5, Section 5.4.5.3) at day 7 when 5% methane had been oxidised. Aminopentol (**VII**) concentrations were also lower in this study. As previously mentioned, this could be due to the different initial sediments used for the study or the species of methanotroph enriched.

Unsaturated $\Delta^{11}$ aminopentol (**VIIb**) was not detected at day 7, when 5% methane had been oxidised, also found in the original temperature experiment (Chapter 5, Section 5.4.5.3), but was detected in low quantities in sediments after this time, with the highest concentrations being found later in the study. Culture conditions can play a role in unsaturation with increased unsaturation in the pentacyclic ring witnessed in *Acetobacter aceti* ssp. *xylinum* in more oxygenated cultures (Rohmer and Ourisson, 1986). Furthermore, it has been suggested that unsaturation is the first step of hopanoid catabolism (Cvejic et al., 2000b) so perhaps these signatures could represent the first steps in degradation, though it is unlikely in the time scale of the study.

The continued production of 35-aminoBHPs following methane limitation and starvation, suggests that BHPs play an important role in maintaining cell homeostasis during the stationary phase. This was witnessed in *Rhodopseudomonas palustris* TIE-1, where the deletion of the *sqhC* gene, resulted in increased sensitivity to acidic and alkaline conditions which was particularly noticeable in stationary phase (Welander et al., 2009). In *Streptomyces coelicolor* A3(2) hopanoids were not produced in liquid culture but were produced on solid medium when sporulating (Poralla et al., 2000). This response is hypothesised to protect the cell by decreasing the water permeability of the cell membrane (Poralla et al., 2000).

3-Methylaminopentol (**VIIa**) was not detected in sediments incubated at 30°C, apart from one replicate at day 20, which was perhaps expected as this compound was not detected in sediments when 5% methane was oxidised in the original temperature study (Chapter 5, Section 5.4.5.3). It is assumed that the methanotroph enriched at this temperature would be a *Methylobacter* sp. as this genus was enriched at 30°C in the original temperature study (Chapter 5, Section 5.4.4.1; Sherry et al., 2015 in review) but this would need to be confirmed by targeting the *pmoA* gene in DNA extracted from microcosm sediments. 3-Methylaminopentol has been identified in trace amounts in *Methylobacter* spp. pure cultures (Chapter 4, Section 4.4.2) so this suggests that the postulated *Methylobacter* sp. enriched in this study at this temperature may not produce 3-methylaminopentol (**VIIa**) or it is present in too low abundance to be detected. This compound has been shown to accumulate in late stationary phase in *Methylococcus capsulatus* (Summons et al., 1994) with another study in the same methanotroph also suggesting their importance for late stationary

phase cell survival (Welander and Summons, 2012). Therefore, if this compound had been present in the methanotroph enriched at 30°C, continued production may have been detected after 5% methane was completely oxidised and starvation ensued.

### 7.5.3. The effect of growth stage on 35-aminoBHP signatures in River Tyne estuarine sediment microcosms incubated at 50°C

The concentrations of aminotriol (**V**) and aminotetrol (**VI**), at day 8 when 5% methane had been oxidised, were similar in this study compared to that of the original study at the same temperature (Chapter 5, Section 5.4.5.1 and 5.4.5.2). 3-Methylaminopentol (**VIIa**) concentrations were higher than aminopentol (**VII**) at the point of 5% methane oxidation for both studies although the absolute concentrations of both compounds were greater in this study compared with the original temperature experiment (Chapter 5, Section 5.4.5.3). The ratio of 3-methylaminopentol (**VIIa**) to unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) were similar for both studies too. Therefore, it was assumed that the methanotroph enriched at this temperature was a *Methylocaldum* sp. as this genus was enriched at 50°C in the original temperature study (Chapter 5, Section 5.4.4.1) but this would need to be confirmed by targeting the *pmoA* gene in DNA extracted from the microcosm sediments.

Aminotriol (**V**) and 'desmethylaminotriol' ($\mathbf{V^{des}}$) concentrations (Figure 7-6) showed no significant difference across all time points. Aminotetrol (**VI**) concentrations were not significantly higher than the initial sediment, which was also witnessed in the original temperature study (Chapter 5, Section 5.4.4.1), and there was no difference between all other time points during the study (Figure 7-7). This suggests that aminotriol (**V**) and aminotetrol (**VI**) were not abundant in the methanotroph(s) enriched at this temperature, thus agreeing with an assignment of a *Methylocaldum* sp. (cf. Cvejic et al., 2000a).

Aminopentol (**VII**), 3-methylaminopentol (**VIIa**) and unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) concentrations were greater than the initial sediment (Figure 7-8). However, there were no significant difference in aminopentol (**VII**) and 3-methylaminopentol (**VIIa**) concentrations between any of the subsequent time points. Unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) concentrations showed no significant difference after the point of 5% methane oxidation at day 8. The consistency of

aminopentol (**V**) and 3-methylaminopentol (**VIIa**) concentrations during the course of the study suggests that the methanotroph(s) enriched at this temperature had high constitutive levels of BHPs present, allowing the cell to survive and oxidise methane at thermophilic temperatures. Therefore, no increase in 35-aminoBHPs was detected after methane became limiting and cells entered a stationary state, as the cells presumably already have high levels of BHPs in order to survive adverse conditions. High constitutive level of hopanoids in *Zymomonas mobilis* were thought to allow ethanol tolerance, with no significant increase in hopanoid content seen with increasing ethanol concentration (Hermans et al., 1991; Moreau et al., 1997). 3-MethylBHPs appear to play a role in late stationary phase cell survival in the Type I methanotroph *Methylococcus capsulatus* Bath (Welander and Summons, 2012). However, the study does not indicate whether the abundance of 3-methylBHPs varied with growth phase; there may be high constitutive levels of methylated compounds present in the methanotroph with levels remaining consistent with growth.

### 7.5.4. Significance for interpretation of the sedimentary record

It is already known that BHPs in marine settings do not necessarily represent significant/intense *in situ* methane oxidation and, signatures found in these environments may often be the result of terrestrial inputs e.g. high aminopentol (**VII**) concentrations witnessed in the Congo and Amazon sedimentary fans (Talbot et al., 2014; Wagner et al., 2014). An additional complication to the interpretation of the BHPs signatures, is the evidence presented here suggesting that aminopentol (**VII**) production increases in stationary phase as a response to methane limiting conditions. This may suggest that aminopentol (**VII**) detected in marine environments does not represent periods of significant or prolonged *in situ*, or terrestrial methane oxidation but more a physiological response of the cells to survive persistent methane limiting/low oxygen conditions typically encountered by methanotrophs at seafloor methane seeps (Valentine, 2011).

At 30°C, the concentration of aminopentol (**VII**) at 28 days was double that detected at the point of 5% methane concentration at day 7 and, was 1.7 fold higher at 28 days for aminotriol (**V**) and aminotetrol (**VII**). If the witnessed increases in concentrations are even greater at lower temperatures, more representative of marine sediment-water interface temperatures, then the already low levels found in marine sediments may not

indicate much activity, with marine BHP concentrations typically much lower than soils, in the region of an order of magnitude so (Zhu et al., 2011).

### 7.6. Conclusions

Aminotriol (**V**) and aminopentol (**VII**) continued to be produced, after 5% methane was oxidised at day 7, in 'early stationary phase' up to day 15 but no increase was witnessed from day 15 to day 28. Aminotetrol (**VI**) continued to be produced later in the stationary phase. This supports the hypothesis that 35-aminoBHPs continue to be produced in stationary phase cells. The continued production of aminopentol (**VII**) in sediments incubated at 30°C after 5% methane is oxidised suggests that this compound may play an important role in maintaining cell homeostasis in methanotrophs in stationary phase when substrate becomes limiting. Aminotriol (**V**) and aminotetrol (**VI**) may also play a role in nutrient limiting conditions but isotope analysis would need to be performed to confirm the source of these compounds were methanotrophs.

Aminotriol (**V**) and aminotetrol (**VI**) concentrations were not greater in sediments incubated at 50°C relative to the initial sediment, suggesting that these compounds were not abundant in the methanotroph(s) enriched at this temperature. Aminopentol (**VII**), 3-methylaminopentol (**VIIa**) and unsaturated $\Delta^{11}$ 3-methylaminopentol (**VIIc**) concentrations increased relative to the initial sediment but differences in the concentrations were not observed at all other time points; this disproves the hypothesis that 3-methylaminopentol (**VIIa**) production increases in stationary phase cells. This suggests that the methanotroph(s) enriched at this temperature may have high constitutive levels of BHPs present allowing the cell to survive and oxidise methane at thermophilic temperatures.

This study suggests that methanotrophs that are mesophilic in nature continue to produce aminopentol (**VII**) in stationary phase after methane becomes limiting but, thermophilic methanotrophs may have high constitutive levels of compounds therefore, no increase is detected. Moreover, anoxic conditions could retard growth of methanotrophs preventing continued production of compounds.

# 8. BHP signatures of River Tyne estuarine sediment anaerobic microcosms - a long-term study

## 8.1. Introduction

Although BHP biosynthesis does not require oxygen, they are traditionally associated with aerobic bacteria (Ourisson et al., 1987) and a few facultative anaerobes, such as the facultatively anaerobic purple non-sulphur bacterium *Rhodomicrobium vannielii* which produces aminotriol (**V**) along with the $C_{30}$ hopanoids diploptene (**I**) and diplopterol (**II**) (Neunlist et al., 1985). Several years later the production of BHPs in microbial mats and deposits that formed in anoxic environments suggested production by anaerobic bacteria (Thiel et al., 2003). The detection of BHT and the $C_{30}$ hopanoids diploptene (**I**) and diplopterol (**II**) in enrichment cultures of planctomycetes, which anaerobically oxidize ammonium (Sinninghe Damsté et al., 2004), confirmed BHP production in anaerobic bacteria. The production of BHPs in two species of the anaerobic Fe(III)-reducing *Geobacter* bacteria was reported shortly after (Fischer et al., 2005; Härtner et al., 2005) with BHT (**III**), BHT cyclitol ether (**VII**) and BHT glucosamine (**VIII**) being the major components (Eickhoff et al., 2013). BHPs have also been detected in some species belonging to the *Desulfovibrio* genus, a group of sulphate-reducing bacteria widely distributed in marine sediments, waterlogged soils, and oil reservoirs (Blumenberg et al., 2006; Blumenberg et al., 2009a; Blumenberg et al., 2012). BHT (**III**) and aminotriol (**V**) are major components of this genus with traces of aminotetrol (**VI**) also detected. BHPs remain undetected in archaea to date.

There is limited evidence of the fate of BHP compounds in the environment. Adenosylhopane (**XIII**) has been shown to undergo significant diagenesis in the Congo Fan with increasing depth whereas the 35-aminoBHPs seem relatively resilient to degradation (Handley et al., 2010). Abiotic studies have shown the degradation of BHPs such as BHT (**III**) and aminotriol (**V**) in *Rhodopseudomonas palustris* TIE-1 when exposed to high temperatures and pressure (Eickhoff et al., 2014). In another study, the acid-catalysed transformation of BHT (**III**), BHT cyclitol ether (**VII**) and BHT glucosamine (**VIII**) to 'anyhydroBHTs' in *Zymomonas mobilis* was witnessed (Schaeffer et al., 2010). However, there have been no studies to date investigating anaerobic BHP producers in controlled experiments or on the rate of degradation of individual BHPs.

## 8.2. Aims and hypotheses

### 8.2.1. Aims

This study presents the BHP sedimentary signatures from anaerobic microcosm experiments inoculated with anoxic River Tyne estuarine sediment incubated under sulphate-reducing and methanogenic conditions.

This chapter has two aims:

(1) to investigate the types of BHP compounds produced in anaerobic systems.

(2) to determine if different compounds are produced or degraded at different rates over time.

### 8.2.2. Hypotheses

- *BHP compounds will show different susceptibility to degradation.*

In the sedimentary record, some BHP compounds appear more recalcitrant compared to others. For example, in the Congo Fan adenosylhopane (**XIII**) undergoes significant diagenesis with increasing depth down to 65 m below the sea floor whereas the 35-aminoBHPs seem relatively resilient to degradation (Handley et al., 2010).

- *Some BHPs will be enriched under sulphate-reducing conditions.*

BHT (**III**) and aminotriol (**V**) have been shown to be a major components in sulphate-reducing bacteria (Blumenberg et al., 2006; Blumenberg et al., 2009a; Blumenberg et al., 2012).

## 8.3. Materials and methods

### 8.3.1. Anaerobic estuarine sediment microcosms subjected to sulphate-reducing conditions to study BHP composition

Microcosm set-up is described in full in Chapter 3, Section 3.4.2.1. In brief, eight sets of identical microcosms were prepared using anoxic River Tyne estuarine sediments as the inoculum: sodium acetate amended, sodium molybdate inhibited + sodium acetate and heat killed controls + sodium acetate (abiotic controls) with sodium

sulphate added to all microcosms and sacrificed at the time points shown in Table 8-1. Microcosms were stored stopper down at ambient room temperature ~20°C.

**Table 8-1:** Sulphate-reducing microcosm experimental conditions.

| | **Time 0** | **28 days** | **56 days** | **94 days** | **433 days** | **465 days** | **619 days** | **706 days** |
|---|---|---|---|---|---|---|---|---|
| **Amended** | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ |
| **Sodium molybdate inhibited** | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ |
| **Heat killed** | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ | NaOAc $Na_2SO_4$ |

Sulphate levels were depleted at day 56 and two sets were allowed to turn methanogenic; subsequently these were sacrificed at 465 and 619 days (red box). Sodium sulphate, ($Na_2SO_4$, 84 mM), was added through the rubber stopper at day 58 to replenish sulphate in all remaining microcosms, apart from those left to turn methanogenic. Sodium acetate ($CH_3COONa$, 100 mM), was added through the rubber stopper at day 231, to all remaining microcosms, after sulphate-reduction plateaued.

TOC measurements (Chapter 3, Section 3.7.1) were performed on a triplicate of initial sediment, prior to carbon addition, and all BHP concentrations normalised to the average of these values, 7.26% (range 7.21-7.35%).

### 8.3.2. Anaerobic estuarine sediment microcosms subjected to methanogenic conditions to study BHP composition

Microcosm set-up is described in full in chapter 3, section 3.4.2.2. In brief, six sets of identical microcosms were prepared using anoxic River Tyne estuarine sediments as the inoculum: cellulose amended, BES inhibited + cellulose and heat killed controls + cellulose (abiotic controls), and sacrificed at the time points shown in Table 8-2. Microcosms were stored stopper down at ambient room temperature ~20°C.

**Table 8-2:** Methanogenic microcosm experimental conditions.

| | Time 0 | 30 days | 106 days | 225 days | 505 days | 665 days |
|---|---|---|---|---|---|---|
| **Amended** | Cellulose | Cellulose | Cellulose | Cellulose | Cellulose | Cellulose |
| **BES inhibited** | Cellulose | Cellulose | Cellulose | Cellulose | Cellulose | Cellulose |
| **Heat killed** | Cellulose | Cellulose | Cellulose | Cellulose | Cellulose | Cellulose |

Methane production was monitored via GC-FID (Chapter 3, Section 3.4.7). Methane accumulation slowed and appeared to be plateauing, therefore, on day 76, cellulose (0.32 g) was added after removing stopper under a constant stream of nitrogen.

TOC measurements (Chapter 3, Section 3.7.1) were performed on a triplicate of initial sediment, prior to carbon addition, and BHP concentrations normalised to the average of this, 6.53% (range 6.44-6.60%).

Sulphate-reducing and methanogenic microcosms, along with their unamended and heat killed counterparts, were sacrificially sampled (Section 3.4.3) according to Table 8-1 and Table 8-2 respectively. Microcosm sediments were freeze-dried (Chapter 3, Section 3.4.5) and BHPs were extracted from the sediment using a modified Bligh and Dyer extraction method (Chapter 3, Section 3.8.1). The polar fraction resulting from the SPE of total lipid extracts were prepared and then analysed using LC-MS (Chapter 3, Section 3.8.2 to 3.8.4).

## 8.4. Results

### 8.4.1. Anaerobic estuarine sediment microcosms subjected to sulphate-reducing conditions to study BHP composition

#### 8.4.1.1. Sulphate-reduction in River Tyne estuarine sediment microcosms

Sulphate concentrations were monitored periodically using Ion Chromatography (Chapter 3, Section 3.4.6) in sodium acetate amended, sodium molybdate inhibited and heat killed control microcosms over a 706 day period (Figure 8-1).

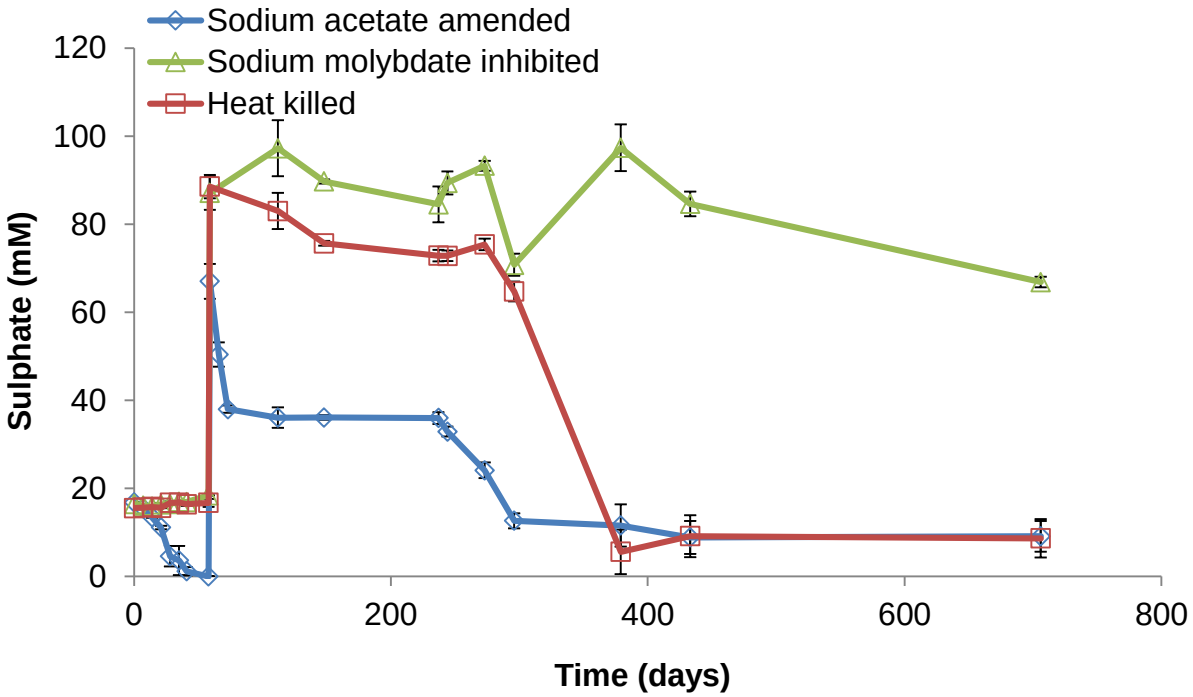



**Figure 8-1:** Sulphate concentrations (mM) in a set of River Tyne estuarine sediment microcosms incubated over a 706 day period. Sodium acetate amended (blue line), sodium molybdate inhibited (green line) and heat killed controls (red line). Error bars represent 1 x S.E (N = 3).

After 56 days, sulphate levels were depleted in sodium acetate amended microcosms (Figure 8-1). Sulphate levels were replenished but sulphate-reduction plateaued after the carbon source, sodium acetate, was depleted. This was replenished at 231 days after which sulphate-reduction resumed. A gradual decline in sulphate concentration over the course of the study from a maximum of 97.36 to 66.84 mM was detected in sodium molybdate inhibited microcosms. After 59 days, sulphate concentrations started to fall in heat killed controls and then rapidly decreased after 273 days to the lowest concentration of 5.58 mM at 379 days after which levels plateaued due to the carbon source, sodium acetate, being depleted. After this time, microcosms were left in this starved state.

The sulphate concentrations in sodium acetate amended, sodium molybdate inhibited and heat killed control microcosms for individual sets of microcosms at the time point of sacrifice is shown (Table 8-3).

**Table 8-3:** Sulphate concentrations (mM) at the point of sacrificial sampling for individual sets of sodium acetate amended microcosms plus their inhibited and heat killed controls.

| | **Sodium acetate amended** | **Sodium molybdate inhibited** | **Heat killed** |
|---|---|---|---|
| **Time 0** | 16.78 ± 0.16[a] | 16.47 ± 0.06 | 15.53 ± 0.06 |
| **28 days** | 2.30 ± 1.43 | 16.96 ± 0.04 | 18.84 ± 0.58 |
| **56 days** | 0.0 ± 0.0 | 15.18 ± 0.22 | 15.35 ± 0.39 |
| **58 days** | $Na_2SO_4$, 84 mM added | | |
| **94 days** | 48.44 ± 10.00 | 113.01 ± 20.11 | 86.75 ± 0.52 |
| **231 days** | $CH_3COONa$, 100 mM added | | |
| **433 days** | 12.53 ± 2.12 | 72.09 ± 2.72 | 0.33 ± 0.10 |
| **706 days** | 9.11 ± 3.51 | 66.84 ± 1.20 | 8.66 ± 4.37 |

[a] Error represents 1 x S.E. (N = 3) except 433 days heat killed (N = 2) due to a smashed microcosm.

Sulphate levels were depleted at day 56 and two sets were allowed to turn methanogenic; subsequently these were sacrificed at 465 and 619 days (Table 8-2).

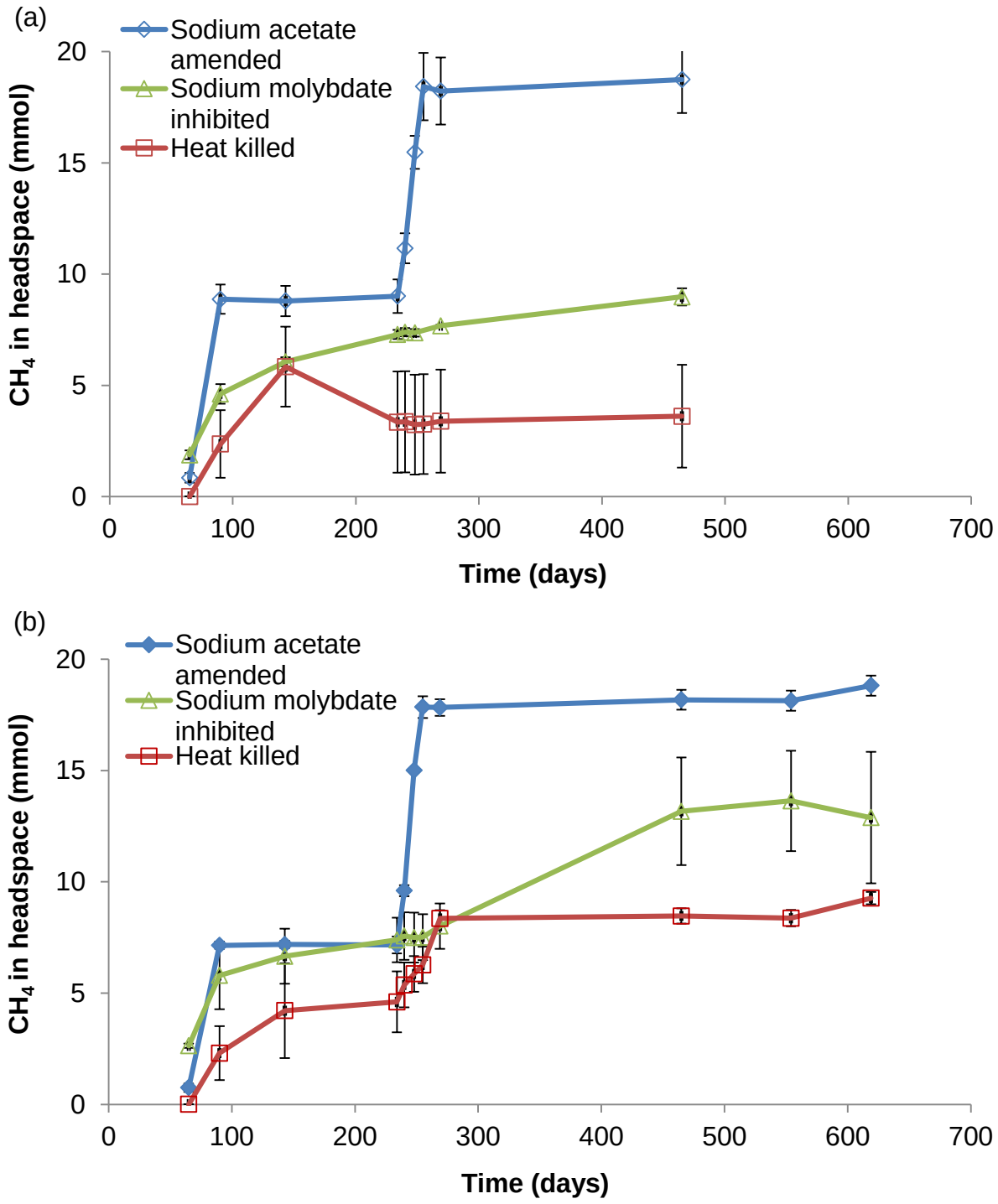



**Figure 8-2:** Cumulative methane production (mmol) in headspace of sulphate reducing River Tyne estuarine sediment microcosms allowed to turn methanogenic after 56 days sacrificed at (a) 465 days and (b) 619 days. Sodium acetate amended (blue line), sodium molybdate inhibited (green line) and heat killed controls (red line). Error bars represent 1 x S.E (N = 3).

For both sets of microcosms, methane production almost completely plateaued in sodium acetate amended microcosms between 90 and 234 days when sodium acetate had become depleted but rapidly increased once again when it was replenished at 231 days (Figure 8-1). It once again plateaued at 255 days when sodium acetate was once again depleted. The cumulative methane concentrations in the headspaces of both sets of sodium acetate amended microcosms were similar with a) 18.74 mmol for the set sacrificed at 465 days and (b) 18.81 mmol for the set sacrificed at 619 days. In sodium molybdate inhibited controls, cumulative methane concentrations were different when comparing both sets with (a) 9.0 mmol for the set sacrificed at 465 days and (b) 13.17 mmol at same time point for set that was later sacrificed at 619 days. In the heat killed controls, cumulative methane concentrations were once again different when comparing both sets with (a) 3.6 mmol for the set sacrificed at 465 days and (b) 9.27 mmol at same time point for set that was later sacrificed at 619 days.

### 8.4.2. Total BHP concentrations in River Tyne estuarine sediment microcosms subjected to sulphate-reducing conditions

Total BHP concentrations in sulphate-reducing microcosm sediments over a 706 day period were determined (Figure 8-3). For individual compound concentrations and relative abundances see appendix 3g, also includes the BHP data for inhibited controls that were analysed.

Total BHP concentrations, and individual compounds, in sodium acetate amended microcosm sediments were statistically compared to the initial sediment (Time 0) and corresponding heat killed controls using one-way analysis of variance (ANOVA) with Fisher's LSD.

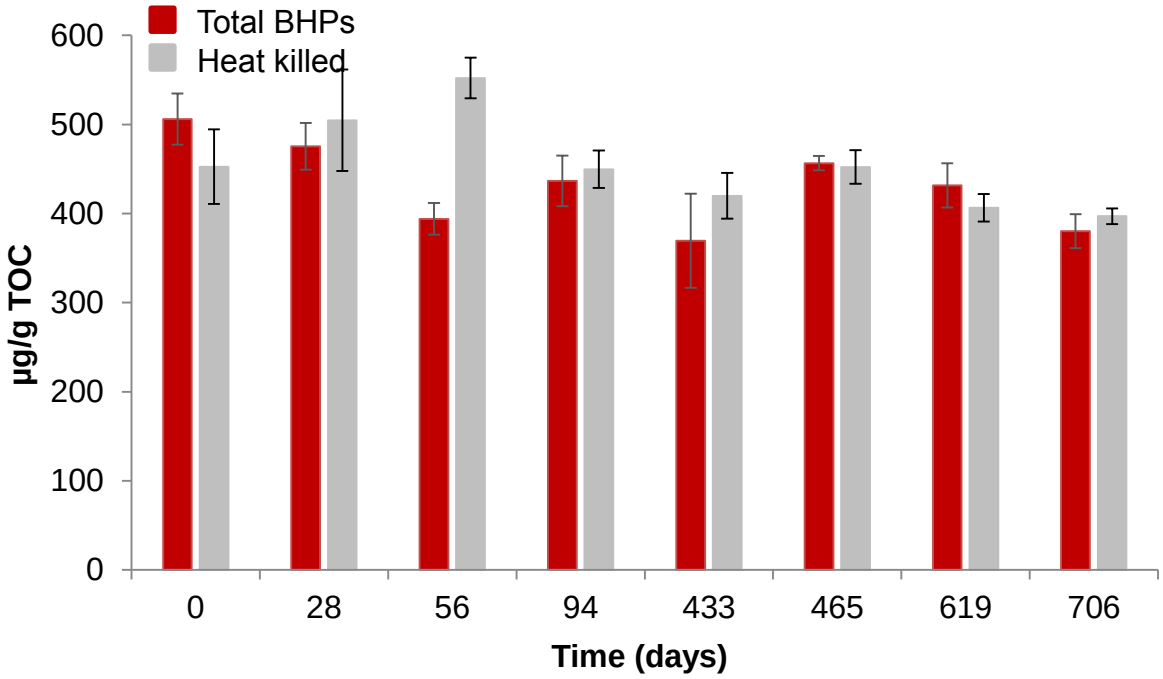



**Figure 8-3:** Total BHP concentrations (µg/g TOC) in sodium acetate amended River Tyne estuarine sediment microcosms over 706 days (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3) except 28 and 433 days heat killed (N = 2).*

* Herein, N = 2 for 28 and 433 days heat killed controls due to a TLE replicate being lost and a smashed microcosm respectively.

There was no significant difference in total BHP concentrations in sodium acetate amended microcosm sediments at Time 0 compared with 28, 94, 465 and 619 days ($p \geq 0.063$) but concentrations were significantly lower at 56, 433 and 706 days ($p \leq 0.007$). Total concentrations in sodium acetate amended sediments were not significantly different compared with their corresponding heat killed controls at all time points ($p \geq 0.176$) apart from 56 days ($p = 0.000$).

### 8.4.3. Individual BHP concentrations in River Tyne estuarine sediment microcosms subjected to sulphate-reducing conditions

BHT (**III**) concentrations were very similar in sodium acetate amended microcosms at all time points (appendix 3g). There was no significant difference in 2-methylBHT (**IIIa**) concentrations in sodium acetate amended microcosms at Time 0 compared with all other time points ($p \geq 0.272$). Aminotetrol (**VI**) concentrations in sodium acetate amended sediments were significantly higher in Time 0 sediments compared with all other time points ($p \leq 0.007$) apart from 28 days (appendix 3g). AnhydroBHT (**XIV**) was

not universally detected in all sediments and when present was in low concentrations (see appendix 3g for data).

### 8.4.3.1. Aminotriol

There was no significant difference in aminotriol (**V**) concentrations in sodium acetate amended sediments between Time 0 and 28 days ($p = 0.739$) but concentrations were significantly lower than Time 0 concentrations compared with all other time points ($p \leq 0.042$) (Figure 8-4). There was no significant difference between sodium acetate amended microcosms and their corresponding heat killed controls at all other time points ($p \geq 0.052$).

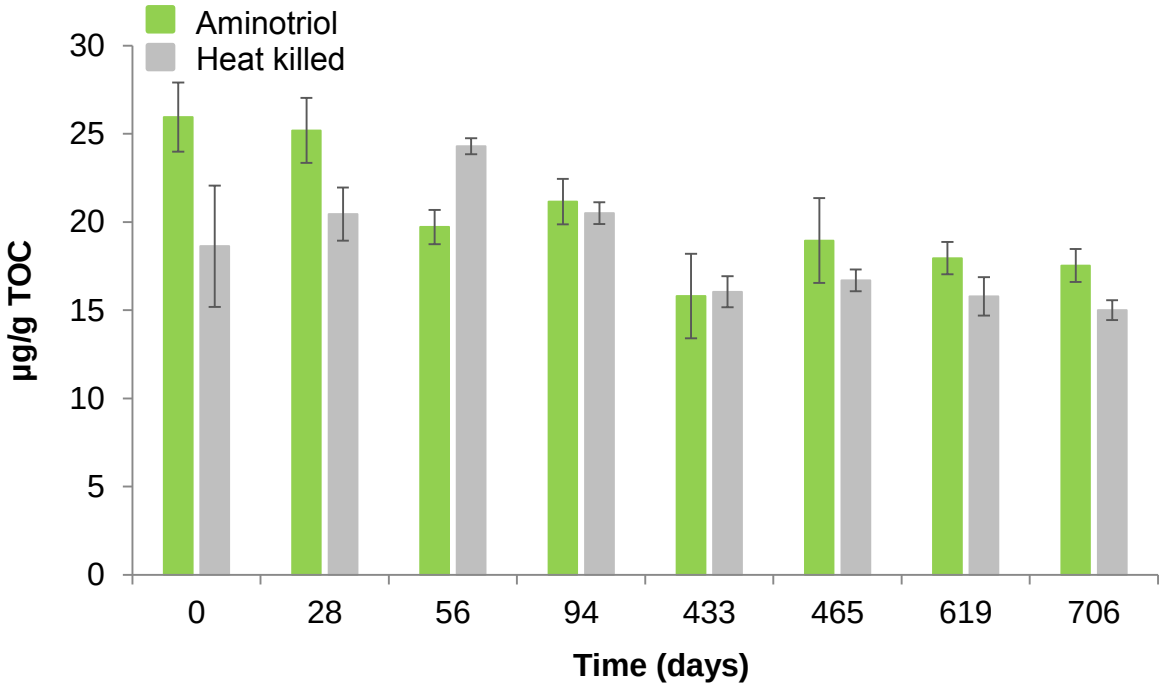



**Figure 8-4:** Concentration (µg/g TOC) of aminotriol (**V**) in sodium acetate amended River Tyne estuarine sediment microcosms over 706 days (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3) except 28 and 433 days heat killed (N = 2).

### 8.4.3.2. Adenosylhopane

There was no significant difference in adenosylhopane (**VIII**) concentrations in sodium acetate amended microcosms at Time 0 compared with all other time points ($p \geq 0.326$) (Figure 8-5). 'Adenosylhopane-type 2' (**IX**) concentrations (see appendix 3g for data) were also not significantly different in sodium acetate amended microcosms at Time 0 compared with all other time points ($p \geq 0.110$) apart from 706 days ($p = 0.047$).

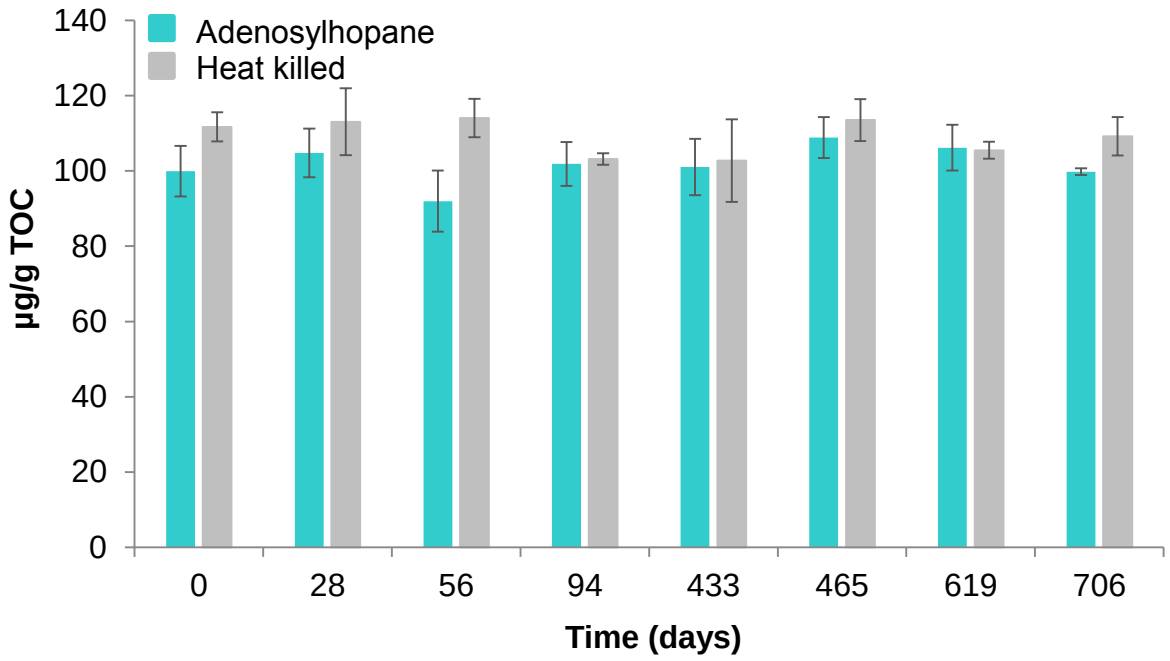



**Figure 8-5:** Concentration (µg/g TOC) of adenosylhopane (**VIII**) in sodium acetate amended River Tyne estuarine sediment microcosms over 706 days (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3) except 28 and 433 days heat killed (N = 2).

#### 8.4.3.3. BHT cyclitol ether and BHT glucosamine

BHT cyclitol ether (**XII**) concentrations in sodium acetate amended sediments were significantly higher in Time 0 sediments compared with all other time points (Figure 8-6a). Concentrations were also significantly higher in sediments at 28 days compared with 56, 433, 465, 619 and 706 days ($p \geq 0.031$) but not at 94 days. BHT glucosamine (**XIII**) concentrations in sodium acetate amended sediments were significantly higher in Time 0 sediments compared with those at 433 and 706 days (Figure 8-6b).

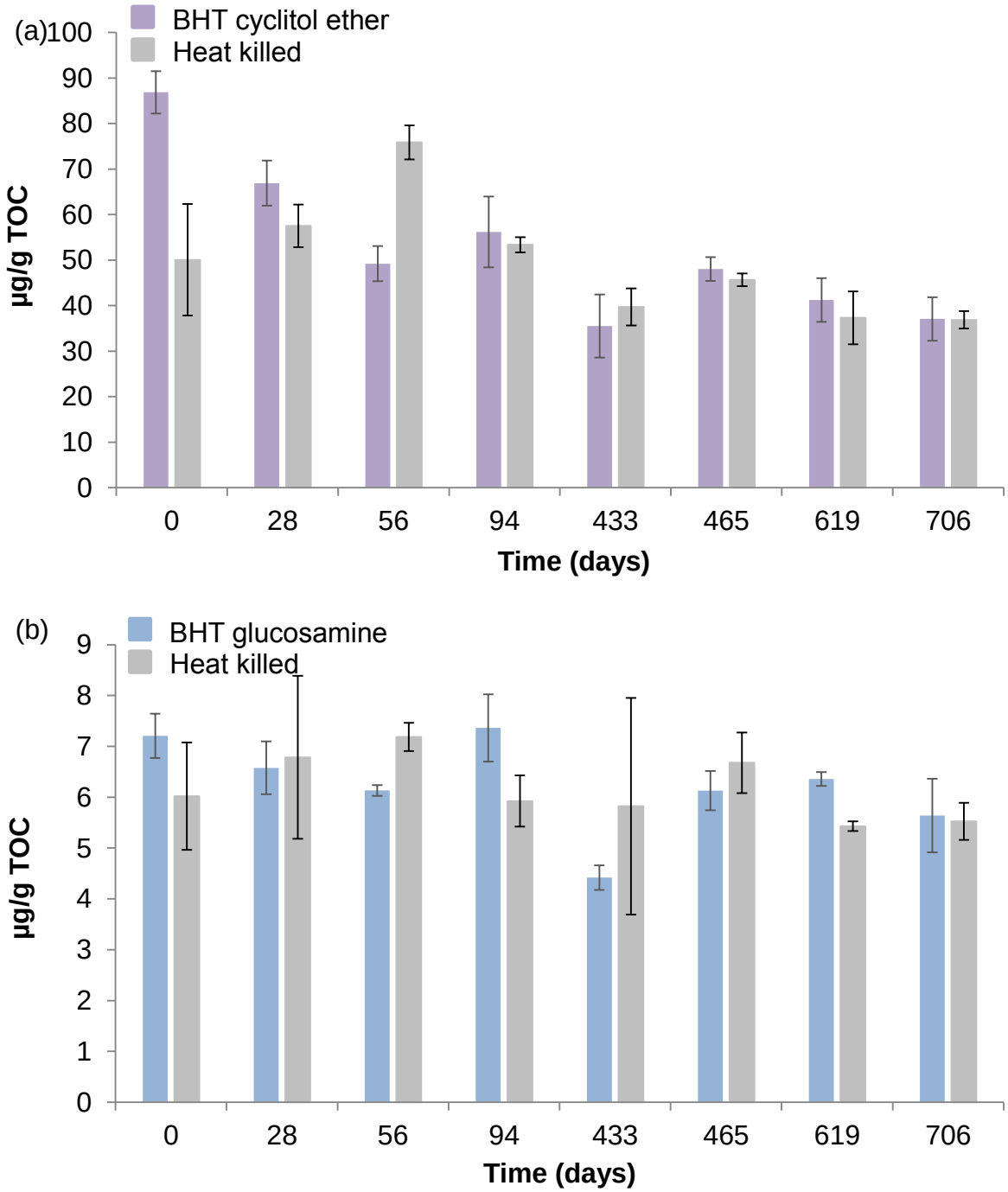



**Figure 8-6:** Concentration (µg/g TOC) of (a) BHT cyclitol ether (**XII**); (b) BHT glucosamine (**XIII**) in sodium acetate amended River Tyne estuarine sediment microcosms over 706 days (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3) except 28 and 433 days heat killed (N = 2).

BHpentol cyclitol ether (**XIIa**) concentrations (see appendix 3g for data) showed similar trends with maximum concentrations also being detected in Time 0 sediments followed by 28 days.

### 8.4.4. Anaerobic estuarine sediment microcosms subjected to methanogenic conditions to study BHP composition

#### 8.4.4.1. Cumulative methane production in River Tyne estuarine sediment microcosms

Cumulative methane production in the headspace was monitored periodically using GC-FID in cellulose amended, BES inhibited and heat killed control microcosms (Figure 8-7).

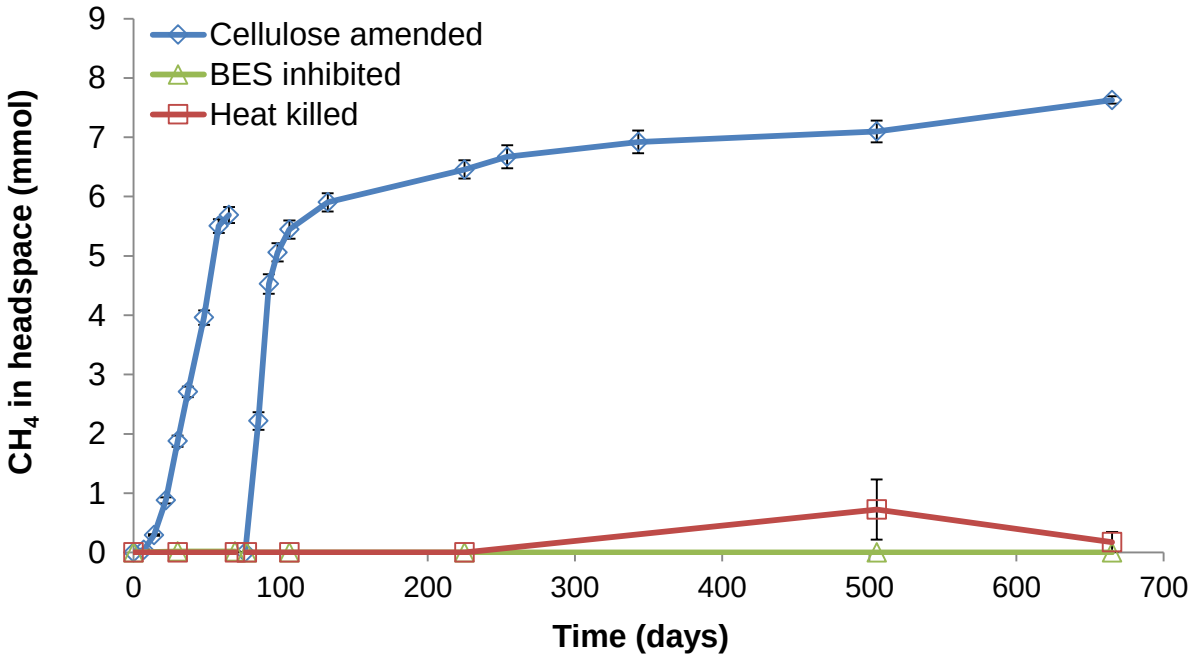



**Figure 8-7:** Cumulative methane production (mmol) in headspace of methanogenic River Tyne estuarine sediment microcosms over 665 days. Cellulose amended (blue line), BES inhibited (green line) and heat killed controls (red line). Error bars represent 1 x S.E: Time 0, N = 18; 30 days, N = 15; 106 days, N = 12; 225 days, N = 9; 505 days, N = 6; 665 days, N = 3.

After 65 days, the stoppers were removed from microcosms and additional cellulose was added as the carbon source, as rates of methane production appeared to be slowing (Figure 8-7). Methane production was still occurring at the end of the study with 7.63 ± 0.06 mmol $CH_4$ accumulated in the headspace of the last set of replicates after 665 days. In BES inhibited microcosms methane production was consistently very low with 0.0008 ± 0.0007 mmol $CH_4$ accumulated in the headspace of the last set of replicates after 665 days; methane production was also minimal in heat killed controls.

**Table 8-4:** Cumulative methane production (mmol) at the point of sacrificial sampling for individual sets of cellulose amended microcosms plus their inhibited and heat killed controls. Error bars represent 1 x S.E. (N = 3).

| | Cellulose amended | BES inhibited | Heat killed |
|---|---|---|---|
| **Time 0** | $4.0E^{-3} \pm 1.0E^{-4}$ | $3.0E^{-3} \pm 2.0E^{-4}$ | $1.79E^{-3} \pm 1.6E^{-4}$ |
| **30 days** | 1.51 ± 0.13 | $0.02 \pm 8.6E^{-4}$ | $1.1E^{-3} \pm 7.4E^{-5}$ |
| **77 days** | Cellulose added – $CH_4$ set to zero | | |
| **106 days** | 4.84 ± 0.27 | $2.0E^{-5} \pm 2.0E^{-6}$ | $3.0E^{-4} \pm 1.0E^{-4}$ |
| **225 days** | 6.06 ± 0.06 | $1.0E^{-4} \pm 1.0E^{-5}$ | $2.0E^{-4} \pm 1.0E^{-4}$ |
| **505 days** | 6.72 ± 0.16 | $1.0E^{-4} \pm 6.0E^{-6}$ | 0.73 ± 0.51 |
| **665 days** | 7.63 ± 0.06 | $8.0E^{-4} \pm 7.0E^{-4}$ | 0.17 ± 0.17 |

### 8.4.5. Total BHP concentrations in River Tyne estuarine sediment microcosms subjected to methanogenic conditions

Total BHP concentrations in methanogenic microcosm sediments over a 665 day period were determined (Figure 8-8). For individual compound concentrations and relative abundances see appendix 3h, also includes the BHP data for inhibited controls that were analysed.

Total BHP concentrations, and aminotriol (**V**), in cellulose amended microcosm sediments were statistically compared to the initial sediment (Time 0) and corresponding heat killed controls using one-way analysis of variance (ANOVA) with Fisher's LSD.

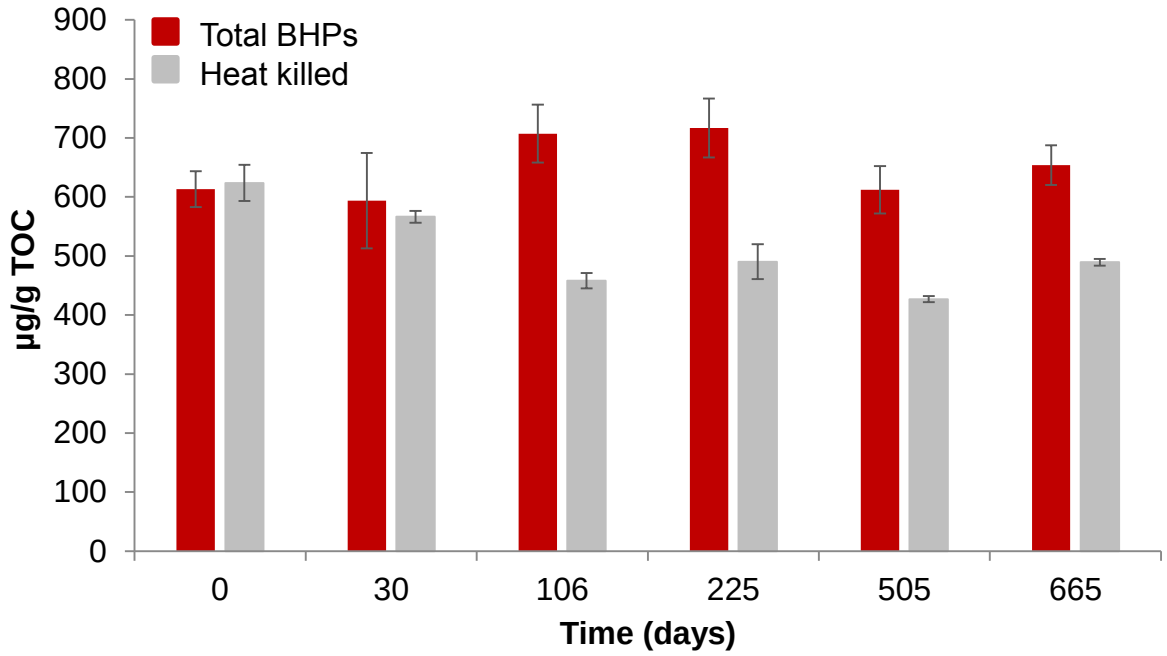



**Figure 8-8:** Total BHP concentrations (µg/g TOC) in cellulose amended River Tyne estuarine sediment microcosms over 665 days (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3).

There was no significant difference in total BHP concentrations in cellulose amended microcosm sediments at Time 0 compared with the other time points ($p \geq 0.065$). Total concentrations in cellulose amended sediments were significantly higher than their corresponding heat killed controls at 106, 225, 505 and 665 days ($p \leq 0.005$) but not at Time 0 or 30 days ($p \geq 0.614$).

### 8.4.6. Individual BHP concentrations in River Tyne estuarine sediment microcosms subjected to methanogenic conditions

There was no significant difference in BHT (**III**) concentrations in cellulose amended microcosm sediments at Time 0 compared with the other time points ($p \geq 0.301$) and 2-methylBHT (**IIIa**) ($p \geq 0.149$) during the study (see appendix 3h for data). Adenosylhopane (**VIII**) ($p \geq 0.113$) and 'adenosylhopane-type 2' (**IX**) ($p \geq 0.338$) concentrations (see appendix 3h for data) were also not significantly different in cellulose amended microcosms at Time 0 compared with all other time points.

### 8.4.6.1. Aminotriol

Aminotriol (**V**) concentrations were significantly higher in cellulose amended microcosms at 106, 225 and 665 days compared with Time 0 concentrations ($p \leq 0.029$) but not at 30 and 505 days ($p \geq 0.083$) (Figure 8-9).

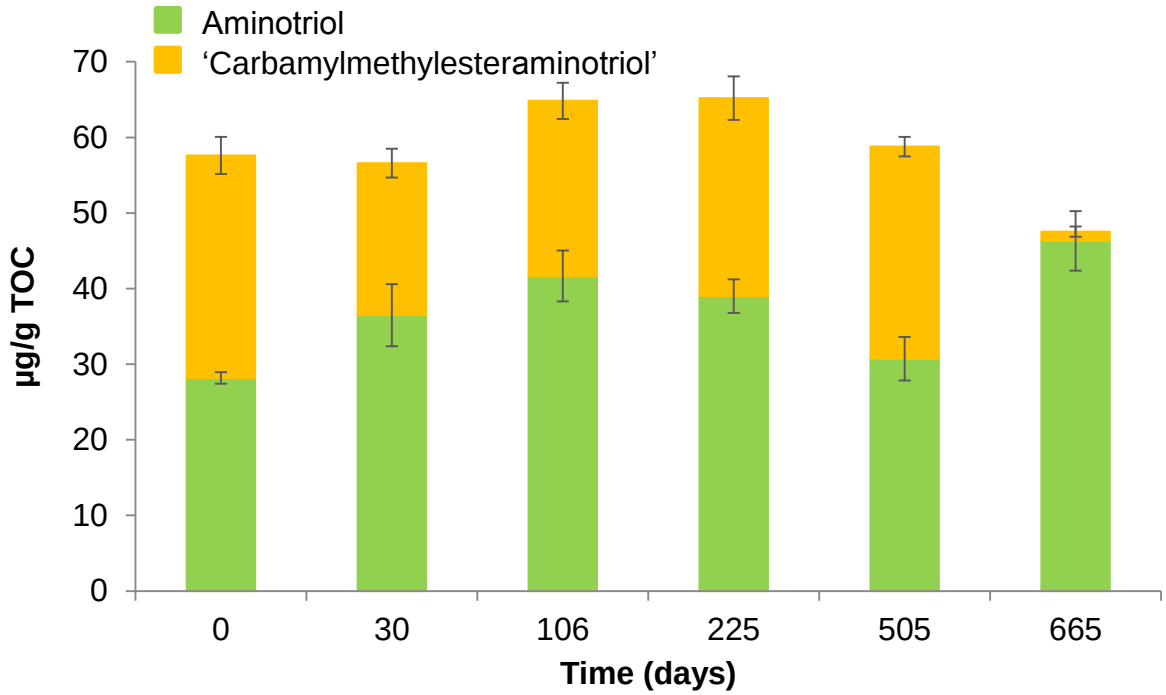



**Figure 8-9:** Concentration (µg/g TOC) of aminotriol (**V**) and 'carbamylmethylestertriol' ($\mathbf{V}^{\mathbf{MeCarb}}$) in cellulose amended River Tyne estuarine sediment microcosms over 665 days. Error bars represent 1 x S.E. (N = 3).

Aminotriol (**V**) and 'carbamylmethylestertriol' ($\mathbf{V}^{\mathbf{MeCarb}}$) concentrations are similar at Time 0 and 505 days. The 'carbamylmethylester' compound was ~51% of the total of both compounds at Time 0, ~35% at 30 and 106 days, ~41% at 225 days, ~33% at 505 days but < 3% at 665 days.

### 8.4.6.2. AnhydroBHT

AnhydroBHT (**XIV**) concentrations were quite low in sediments but appear higher in heat killed controls compared with corresponding cellulose amended sediments (Figure 8-10).

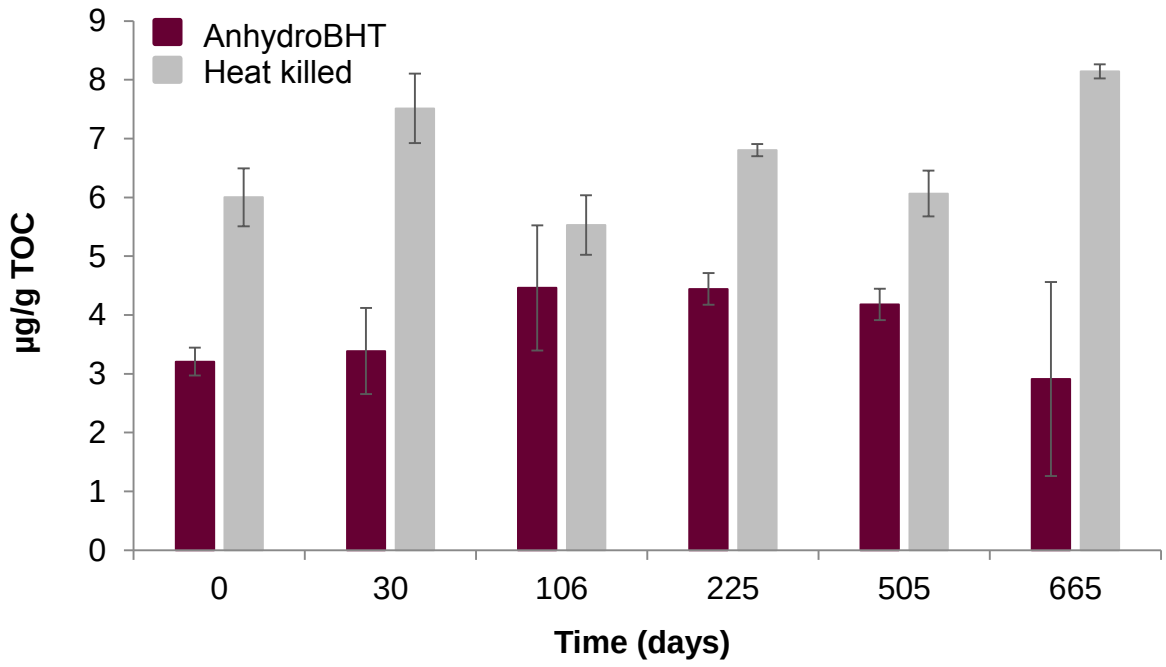



**Figure 8-10:** Concentration (µg/g TOC) of anhydroBHT (**XIV**) in cellulose amended River Tyne estuarine sediment microcosms over 665 days (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3).

#### 8.4.6.3. BHT cyclitol ether and BHT glucosamine

BHT cyclitol ether (**XII**) and BHT glucosamine (**XIII**) concentrations in methanogenic microcosm sediments over a 665 day period were determined (Figure 8-11).

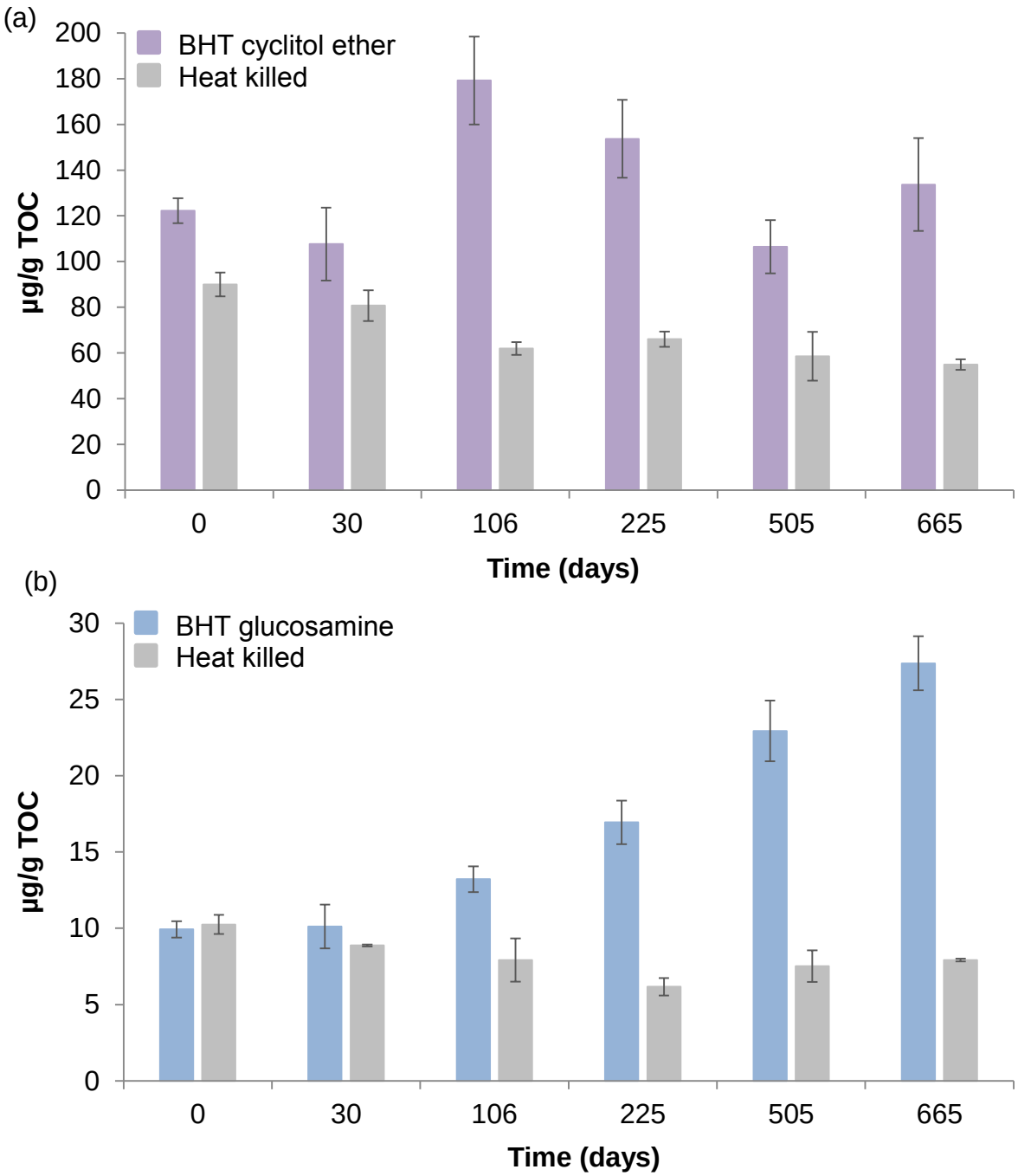



**Figure 8-11:** Concentration (µg/g TOC) of (a) BHT cyclitol ether (**XII**); (b) BHT glucosamine (**XIII**) in cellulose amended River Tyne estuarine sediment microcosms over 665 days (heat killed controls to the right). Error bars represent 1 x S.E. (N = 3).

The concentration of BHT cyclitol ether (**XII**) was greatest at 106 days, 179 ± 19 µg/g TOC (Figure 8-11a). BHpentol cyclitol ether (**XIIa**) and BHhexol cyclitol ether (**XIIb**) concentrations (see appendix 3h for data) showed similar trends with maximum concentrations also being detected at 106 days. However, BHT glucosamine (**XIII**) increased with time after 30 days, with the maximum concentration being detected at

665 days, 27 ± 1.8 µg/g TOC (Figure 8-11b). BHT cyclitol ether (**XII**) (Figure 8-11a) seemed to be effected by heat kill treatment to a greater extent compared with BHT glucosamine (**XIII**) (Figure 8-11b).

## 8.5. Discussion

### 8.5.1. Sulphate-reduction and methanogenesis in River Tyne estuarine sediment microcosms

In sodium acetate amended microcosms sulphate levels were depleted after 56 days (Figure 8-1). Bacteria in microcosm sediments were actively reducing sulphate after sulphate was replenished but plateaued again after sodium acetate was depleted, with sulphate-reduction resuming after the carbon source was replenished. A decline in sulphate concentration was detected in sodium molybdate inhibited microcosms with time but, very low compared to non-inhibited samples, suggesting that not all bacteria were inhibited and some still had the ability to reduce sulphate. Sulphate concentrations started to fall in heat killed controls at 59 days and then rapidly decreased after 273 days to the lowest concentration of 5.58 mM at 379 days (Figure 8-1). This suggests the presence of mesophilic endospore-forming sulphate-reducing bacteria in the estuarine sediments. They were apparently present in low numbers after heat kill treatment (20 min at 121°C, with elevated pressure; Chapter 3; Section 3.3.2) and then started to actively reduce sulphate after a period of time when left at room temperature ~20°C.

Two sets of sulphate-reducing microcosms were left to turn methanogenic after sulphate levels were depleted at 56 days with very similar levels of cumulative methane production seen in the headspace of both sets (Figure 8-2). Methane production was also seen in sodium molybdate controls. This was predicted as sodium molybdate inhibits sulphate-reduction which means that methanogenic bacteria take over as the dominant metabolic force (Madigan et al., 2009). Methane was also produced in heat killed controls suggesting the presence of heat-resistant mesophilic methanogens, that are resistant to heat kill treatment (20 min at 121°C, with elevated pressure; Chapter 3; Section 3.3.2). Methane production was immediately detected in these controls suggesting methanogenic activity (Figure 8-2) whereas sulphate-reduction in heat

killed controls displayed a dormant period before rapid reduction at 273 days (Figure 8-1).

In the methanogenic study, the heat killed controls showed little sign of methanogenic activity. These controls had been subjected to two rounds of autoclave treatment with overnight incubation at 40°C to allow the growth of vegetative cells, a process also repeated after cellulose was replenished. Conversely, the heat killed controls in the sulphate-reducing study, which had been left to turn methanogenic, had only been subjected to one round of autoclaving. The additional rounds of autoclaving may have killed a greater number of vegetative cells in the methanogenic study resulting in minimal methane production.

### 8.5.2. Total BHP compositions in River Tyne estuarine sediment microcosms under sulphate-reducing conditions

There was no significant difference in total BHP concentrations in sodium acetate amended microcosm sediments at Time 0 compared with 28, 94, 465 and 619 days but concentrations were significantly lower at 56, 433 and 706 days (Figure 8-3). Sulphate concentrations rapidly decreased after 273 days in heat killed controls (Figure 8-1) but no increase in total BHPs was detected. This suggests sulphate-reducers were only a small percentage of total bacteria in the sediment or the genus enriched did not produce BHPs. Another study using sediment from this location as the inoculum, showed only modest enrichment of sulphate reducers when degrading crude oil under anaerobic conditions (Sherry et al., 2013). Sequences from conventional sulphate-reducing bacteria such as *Desulfotomaculum, Desulfosporomusa, Desulfosporosinus*, *Desulfovibrio, Desulfobulbus, Desulfobacter* and *Desulfobacterium* were not dominant when actively reducing sulphate. Instead sequences closely related to *Gammaproteobacteria* and the *Firmicutes* were detected at highest frequency (Sherry et al., 2013).

There was no significant difference in BHT (**III**), 2-methylBHT (**IIIa**) (see appendix 3g for data) and adenosylhopane (**VIII**) (Figure 8-5) concentrations in sodium acetate amended microcosm sediments at Time 0 compared with the other time points. This suggests that these compounds are equally resistant to degradation over the period of the study (706 days). This included the two microcosm sets that had been left to turn methanogenic, 465 and 619 days, after sodium sulphate was depleted in the system

at 56 days. This indicates that a switch from sulphate-reducing to methanogenic conditions has no apparent effect on the degradation of the aforementioned compounds. It was previously shown that BHT (**III**), 2-methylBHT (**IIIa**) and adenosylhopane (**VIII**) were equally resistant to heat kill treatment too (Chapter 5, Section 5.5.7). This suggests that the duration of the microcosm study was too short to observe any significant degradation of these compounds. Moreover, BHT (**III**) appears recalcitrant in the environment and has been extracted from sediments ~50 Ma years old (van Dongen et al., 2006). Data from Congo deep-sea fan sediments has shown that adenosylhopane (**XIII**) undergoes significant diagenesis with increasing depth over a period of ~600 ka although BHT (**III**) and all other BHPs identified in that study seemed to be less susceptible to degradation (Handley et al., 2010).

No significant increase in aminotriol (**V**) was detected in sediments under sulphate-reducing conditions compared with the initial sediment even when sulphate-reduction was rapidly occurring (Figure 8-4). This suggests that the *Desulfovibrio* genus of this group of bacteria, which have been shown to produce aminotriol (**V**) and also BHT (**III**), were not enriched in the estuarine sediment (Blumenberg et al., 2006; Blumenberg et al., 2009a; Blumenberg et al., 2012).

### 8.5.3. Total BHP compositions in River Tyne estuarine sediment microcosms under methanogenic conditions

Although BHPs remain undetected in archaea, bacterial syntrophic partners may produce BHPs. However, no significant increase in total BHPs was detected in cellulose amended methanogenic microcosms during the period of study (Figure 8-8) suggesting that any bacteria present in the microcosm sediments did not produce BHPs or were not enriched to an extent to detect any changes in total BHP concentrations. Total BHP concentrations in cellulose amended sediments were highest, although not significantly, at 106 days before methane production began to slow and 225 days where methane production levels were plateauing, although methane continued to be produced during the course of the study (Figure 8-7).

There was no significant difference in BHT (**III**), 2-methylBHT (**IIIa**) and adenosylhopane (**VIII**) concentrations in cellulose amended microcosm sediments at Time 0 compared with the other time points (see appendix 3h for data), even when entering ‘stationary state’ when methane production slowed and began to plateau

(Figure 8-7). This suggests that these compounds are resistant to degradation over the period of the study under methanogenic conditions which mirrors what was observed in the sulphate-reducing study (Section 8.5.2).

AnhydroBHT (**XIV**) concentrations in the heat killed control sediments were higher than corresponding cellulose amended sediments (Figure 8-10). Previous studies have suggested that anhydroBHT (**XIV**) is an early degradation product and increased concentrations of this compound, with the concurrent decrease of BHT (**III**), have been observed in sediments in the environment with increasing depth and age (Watson, 2002; Bednarczyk et al., 2005; Cooke et al., 2008b). In an abiotic study, *Rhodopseudomonas palustris* strain TIE-1 was exposed to elevated pressure and temperature and BHT (**III**) was degraded to various isomers of anhydroBHT (**XIV**) (Eickhoff et al., 2014). This perhaps suggests that higher concentrations of anhydroBHT (**XIV**) in heat kill sediments is the result of the degradation of other compounds, namely BHT (**III**). However, anhydroBHT (**XIV**) concentrations at their greatest are only ~5 µg/g TOC higher in heat kill sediments compared with cellulose amended. These small changes detected in anhydroBHT (**XIV**) concentrations would not produce observable differences in BHT (**III**) concentrations, moreover, at no time point were BHT (**III**) concentrations significantly lower in heat kill sediments compared with cellulose amended sediments (see appendix 3h for data).

Aminotriol (**V**) concentrations were significantly higher in cellulose amended microcosm sediments at 106 and 225 days compared with Time 0 sediments which coincides with the highest total BHP concentrations detected (Figure 8-9). At 665 days aminotriol (**V**) concentrations were also significantly higher. However, at this time point 'carbamylmethylestertriol' ($\mathbf{V^{MeCarb}}$) was a notably smaller proportion of the combined total of aminotriol (**V**) and 'carbamylmethylestertriol' ($\mathbf{V^{MeCarb}}$) suggesting a conversion between these two forms (Figure 8-9). A conversion to the 'regular' aminotriol (**V**) form may suggest that this is the more stable form especially as this conversion occurred towards the end of the study. In the aerobic pH study (Chapter 6, Section 6.4.2.1), aminotriol (**V**) concentrations decreased with increasing pH values whereas 'carbamylmethylestertriol' ($\mathbf{V^{MeCarb}}$) concentrations increased with increasing pH. In the long-term studies, perhaps the physiological properties of the sediment change with time causing this conversion, for example a build-up of compounds resulting in more

acidic conditions. Furthermore, previous studies have shown transformations between compounds caused by acid catalysis (Schaeffer et al., 2008; Schaeffer et al., 2010).

### 8.5.4. BHT cyclitol ether and BHT glucosamine in River Tyne estuarine sediment microcosms

For the sulphate-reducing microcosm study, BHT cyclitol ether (**XII**) concentrations in sodium acetate amended sediments were significantly higher in initial sediments (Time 0) compared with all other time points (Figure 8-6a). Furthermore, concentrations in heat killed controls were similar to those of sodium acetate amended sediments later as the study progressed, > 56 days, which is at odds with what has been witnessed in the aerobic microcosm studies (e.g. Chapter 5, Section 5.4.8) where concentrations are consistently lower in heat killed controls. This suggests that the compound undergoes some degradation with time in sodium acetate amended sediments which may result in similar concentrations to those seen in heat killed control sediments. BHT glucosamine (**XIII**) concentrations in sodium acetate amended sediments were only significantly higher in Time 0 sediments compared with those at 433 and 706 days (Figure 8-6b) suggesting that this compound is more recalcitrant compared with its isomer BHT cyclitol ether (**XII**); this has been observed in the aerobic microcosm studies too (Chapter 5, Section 5.4.8).

In the methanogenic microcosm study, the concentration of BHT cyclitol ether (**XII**) was greatest at 106 days, 179 ± 19 µg/g TOC, with the second highest concentration at 225 days (Figure 8-11); the overall trend in concentration mirrors what was witnessed for total BHPs (Figure 8-8). BHT glucosamine (**XIII**) concentrations, however, increased with time after 30 days, with the maximum concentration being detected at 665 days, 27 ± 1.8 µg/g TOC. The results do not suggest a simple (abiotic) 1:1 conversion of BHT cyclitol ether (**XII**) to BHT glucosamine (**XIII**) and rather that this a bacterially mediated process. Furthermore, a postulated biosynthetic pathway suggests that BHT cyclitol ether (**XII**) is formed from BHT glucosamine (**XIII**) (Vincent et al., 2003). The concentration of BHT glucosamine (**VIII**) may increase with time as cellulose is broken down in the sediment to produce smaller subunits for syntrophic partners to metabolise. The production of both of these BHPs has been witnessed in two species of the anaerobic Fe(III)-reducing *Geobacter* bacteria (Fischer et al., 2005; Härtner et al., 2005) with BHT glucosamine (**VIII**) being one of the major components

of the BHP inventory of *Geobacter sulfurreducens* (Eickhoff et al., 2013), which could be the source of the compound in the microcosm study. BHT cyclitol ether (**XII**) (Figure 8-11a) seems to be affected by heat kill treatment to a greater extent compared with BHT glucosamine (**XIII**) (Figure 8-11b) which mirrors what was witnessed in aerobic studies (Chapter 5, Section 5.4.8).

## 8.6. Conclusions

Sulphate-reduction was monitored over a 706 day period with sulphate being replenished after depletion at day 56. Sulphate concentrations in heat killed controls rapidly decreased after 273 days to the lowest concentration. This suggests the presence of mesophilic endospore-forming sulphate-reducing bacteria in the sediments, which were initially dormant after heat kill treatment, but have been become active after a period of time.

Methane production in cellulose amended sediments occurred over a 665 day period with production slowing after 106 days; the heat killed controls showed little sign of methanogenic activity. Methane production was more pronounced in the heat killed controls of the sulphate-reducing microcosms that had been left to turn methanogenic perhaps due to less rounds of autoclaving.

In the sulphate-reducing and methanogenic studies major compounds such as BHT (**III**), 2-methylBHT (**IIIa**) and adenosylhopane (**VIII**) showed no significant degradation or increase over the course of the study. In the sulphate-reducing study, BHT cyclitol ether (**XII**) seemed to undergo some degradation with time whereas BHT glucosamine (**XIII**) seemed more recalcitrant supporting the hypothesis that BHPs will show different susceptibility to degradation. Furthermore, during the course of the sulphate-reducing study, no significant increase in aminotriol (**V**) was detected in sediments suggesting that the *Desulfovibrio* genus of this group of bacteria, which have been shown to produce aminotriol (**V**) and BHT (**III**) (Blumenberg et al., 2006; Blumenberg et al., 2009a; Blumenberg et al., 2012), were not enriched in the estuarine sediment. This rejects the hypothesis that some BHPs, namely the aforementioned compounds, will be enriched under sulphate-reducing conditions. In the methanogenic microcosm study, BHT glucosamine (**XIII**) concentrations increased with time after 30 days. This process seems to be bacterially mediated and is likely produced by a synthrophic

partner within in the sediment. A potential candidate for this is *Geobacter sulfurreducens* with BHT glucosamine (**VIII**) being one of the major component of its BHP inventory (Eickhoff et al., 2013). Studies have suggested that *Geobacter* grow under syntrophic association with methanogens (Kato et al., 2012).

# 9. Summary

## 9.1. Conclusions

In the final section of this thesis, the main outcomes of each results chapter are summarised along with general conclusions. Additionally, recommendations that might lead to future work are suggested.

### 9.1.1. Chapter 4. BHP signatures of marine methanotroph cultures

The main objective of this chapter was to determine the distribution and composition of BHPs in six strains of previously unscreened methanotrophic bacteria isolated from marine environments. The six strains investigated belonged to three Type I genera, *Methylobacter* (*Methylobacter marinus* A45, *Methylobacter* sp. BBA5.1 and *Methylobacter* sp. BB5.1), *Methylomarinum* (*Methylomarinum vadi* IT-4) and *Methylomarinovum* (*Methylomarinovum caldicuralii* IT-9 and *Methylomarinovum* sp. IN45). There were significant differences in BHP abundances between the two *Methylomarinovum* spp. indicating variation at species level within a genus, *Methylobacter* spp. also showed variance at species level too, thus disproving the original hypothesis that species of the same genus will have similar BHP compositions. This study has contributed to a greater knowledge of the BHP inventory of marine methanotrophs and has identified a suite of novel compounds that have since been identified in microcosm sediments and the environment.

Aminopentol (**VII**) was the most abundant BHP in *Methylobacter marinus* A45, *Methylobacter* sp. BBA5.1, *Methylobacter* sp. BB5.1 and *Methylomarinovum caldicuralii* IT-9 which is typically seen for Type I methanotrophs other than those from the *Methylomicrobium* genus (Talbot et al., 2001; Birgel et al., unpublished data) and the *Methylovulum*-like strain M200 (van Winden et al., 2012c). *Methylomarinum vadi* IT-4 and *Methylomarinovum* sp. IN45 disproves the hypothesis that aminopentol (**VII**) would be the most abundant BHP and are additional examples where this compound is not the most abundant BHP in a Type I methanotroph. This evidence indicates that aminopentol (**VII**) is not absent from all marine methanotrophs and absence of this compound may be restricted to *Methylomicrobium* spp. (Talbot et al., 2001; Birgel et al., unpublished data). This limits the use of aminopentol (**VII**) in marine environments as a proxy for aerobic methane oxidation as its absence does not imply this process

has not occurred. Marine-isolated methanotrophs from previously unexamined genera and/or species displayed marked differences in the relative abundances of BHPs.

All *Methylobacter* spp. contained traces of 3-methylaminopentol (**VIIa**). This compound has only been observed in *Methylococcus capsulatus* (Neunlist and Rohmer, 1985b), *Methylocaldum* spp. (Cvejic et al., 2000a) and most recently in trace amounts in *Methylomicrobium* spp. (Birgel et al., unpublished data). However, *Methylomarinum vadi* IT-4, *Methylomarinovum caldicuralii* IT-9 and *Methylomarinovum* sp. IN45 represent additional examples of Type I methanotrophs where aminopentol (**VII**) is present but 3-methylaminopentol (**VIIa**) is absent. *Methylomonas* spp. and *Methylovulum*-like strain M200 are other examples of Type I methanotrophs where 3-methylaminopentol (**VIIa**) is also absent (Neunlist and Rohmer, 1985b; Talbot et al., 2001; van Winden et al., 2012c); the absence of the *hpnR* gene in *Methylomonas methanica* S1 has also been confirmed in this study. Trace amounts of 3-methylaminotetrol (**VIa**) were observed in all *Methylobacter* spp. and *Methylomicrobium* spp. (Birgel et al., unpublished data) whereas significant amounts of 3-methylaminotriol (**Va**) were detected in *Methylomicrobium* spp.; this represents the first reports of 3-methylaminotriol (**Va**) in a methanotroph pure culture but has previously been detected in a Chinese soil (Zhu et al., 2011).

A suite of compounds related to aminotriol (**V**), aminotetrol (**VI**) and aminopentol (**VII**) but with a different C-35 terminal group tentatively identified as ‘carbamylmethylester’ on the basis of the APCI $MS^2$ and $MS^3$ spectra and with a peracetylated $[M+H]^+$ 16 Da higher than the regular compounds, were witnessed for the first time in methanotrophs and were present in all bacterial strains tested here. These ‘carbamylmethylester’ compounds were found in trace amounts in the *Methylobacter* spp. but higher abundances were seen in *Methylomarinum vadi* IT-4 and *Methylomarinovum* spp. The most abundant BHP in *Methylomarinum vadi* IT-4 was ‘carbamylmethylestertriol’ (**V**$^{\mathbf{MeCarb}}$) accompanied by almost equal amounts of aminotriol (**V**). The ‘carbamylmethylester’ compounds were in higher abundance than the ‘regular’ 35-aminoBHPs in *Methylomarinovum* sp. IN45 with ‘carbamylmethylestertetrol’ (**VI**$^{\mathbf{MeCarb}}$) being the most abundant compound. A suite of compounds related to aminotriol (**V**), aminotetrol (**VI**) and aminopentol (**VII**) but with peracetylated $[M+H]^+$ 14 Da lower, eluting just before their ‘regular’ equivalents, were also witnessed here for the first time in methanotrophs.

### 9.1.2. Chapter 5. Influence of methane concentration and temperature on BHP signatures of River Tyne estuarine sediment aerobic microcosms

The main objective of this chapter was to determine the effect methane concentration and temperature has on methanotroph community composition and the effect that variations in communities has on BHP composition, focusing on the methanotroph-derived 35-aminoBHPs.

Only Type I methanotrophs were enriched in River Tyne estuarine sediments at all methane concentrations and temperatures. This was originally hypothesised as Type I methanotrophs are characteristic of aquatic environments (e.g. McDonald et al., 1996; McDonald et al., 2005b; Moussard et al., 2009). *Methylobacter* spp. were a common feature in the estuarine microcosm sediments and were enriched at all methane concentrations, 0.1-5%, and temperatures of 4-40°C. A thermophilic *Methylocaldum* sp. was enriched at 50°C, probably introduced from terrestrial sources e.g. agricultural environments, and have also been previously detected in the temperate sediments of the Colne Estuary (Moussard et al., 2009). This supports the hypothesis that methanotroph community composition changes in response to temperature.

These changes in methanotroph community composition are reflected in the BHP compositions with greater concentrations of aminotriol (**V**) and aminotetrol (**VI**) being detected in sediments at 4-30°C compared with 40°C. This does not correlate with methane oxidation rates and methanotrophic activity and suggests variation in BHP compositions in sediments is controlled by changes in the species of *Methylobacter* enriched. Aminopentol (**VII**) concentrations increased with temperature with the maximum seen at 40°C. This once again suggests that BHP compositions vary at species level within the *Methylobacter* genus as a different species of *Methylobacter* was enriched at this temperature. At 50°C where a *Methylocaldum* sp. was enriched, 3-methylaminopentol (**VIIa**) was in greater abundance than its non-methylated equivalent which mirrors what has been witnessed in pure culture (Cvejic et al., 2000a). This supports the hypothesis that changes in methanotroph community composition is reflected in 35-aminoBHP composition.

Compounds such as BHT (**III**), 2-methylBHT (**IIIa**), adenosylhopane (**XIII**) and ‘adenosylhopane-type 2’ (**IX**) seem to be more resistant to heat kill treatment (20 min

at 121°C, with elevated pressure; Chapter 3; Section 3.3.2) compared with BHT cyclitol ether (**XII**).

### 9.1.3. Chapter 6. Influence of pH and salinity on BHP signatures of River Tyne estuarine sediment aerobic microcosms

The main objective of this chapter was to determine the effect pH and salinity has on methanotroph community composition and the effect that variations in communities has on BHP composition, focusing on the methanotroph-derived 35-aminoBHPs.

Once again, only Type I methanotrophs were enriched in River Tyne estuarine sediments with community composition changing with pH and salinity, thus supporting the original hypothesis. *Methylobacter* spp. were also a common feature in the estuarine microcosm sediments at pH 5-8 (closest to *in situ* conditions) accompanied by *Methylomonas* spp. at these values. *Methylosoma* spp. were enriched at pH 4 with the closest cultured representative of one of these species being *Methylosoma difficile* LC 2$^{T}$ (Rahalkar et al., 2007). *Methylosoma difficile* LC 2$^{T}$ is closely related to the acidophilic *Methylovulum*-like strain M200, isolated from Dutch peatlands with optimal growth range of pH 4.1-7.0 (Kip et al., 2011). *Methylomicrobium* spp. were enriched at pH 9 with the closest cultured representatives being *Methylomicrobium alcaliphilum* strain 20Z and *Methylomicrobium kenyense* strain AMO1. These two alkaliphilic, halophilic species, isolated from highly alkaline soda lakes, have optimal growth in the pH range 7.2-9.5 and 9-10.5 respectively (Khmelenina et al., 1997; Sorokin et al., 2000; Kalyuzhnaya et al., 2008).

Aminotriol (**V**), aminotetrol (**VI**) and aminopentol (**VII**) concentrations were greatest at pH 4 and decreased with increasing pH whereas the ‘carbamylmethylester’ compounds showed the inverse relationship. This suggests that these compounds play a significant role at higher pH conditions but this has yet to be demonstrated in samples from natural environments. However, initial sediment (Time 0) concentrations of ‘carbamylmethylester’ compounds were also higher at lower pH values. It was postulated, therefore, that acidic conditions may ‘release’ aminotriol (**V**), and to a lesser extent aminotetrol (**VI**), from the sediment matrix or burst the bacterial cell open allowing for greater recovery of the compound; the effect was minimal for aminopentol (**VII**).

At 1 and 15 g/L NaCl methanotroph community composition was similar with *Methylobacter* spp. and *Methylomonas* spp. dominating whereas *Methylomicrobium* spp. were enriched at 35 and 70 g/L NaCl. As stated previously, these genera are commonly identified in estuarine environments. Aminopentol (**VII**) concentrations decreased with increasing salinity at 35 and 70 g/L NaCl when a shift in methanotroph community composition to *Methylomicrobium* spp. was witnessed, which is consistent with recent observations in pure culture (Birgel et al., unpublished data). This supports the hypothesis that shifts in methanotroph community composition are reflected in BHP compositions.

At 35 and 70 g/L NaCl where *Methylomicrobium* spp. were enriched, 3-methylaminotriol (**Va**) and 3-methylaminopentol (**VIIa**) were detected in microcosms, but not at pH 9 where a different species of this genus was enriched. *Methylomicrobium* spp. have recently been shown to produce C-3 methylated compounds with significant amounts of 3-methylaminotriol (**Va**) detected and trace amounts of 3-methylaminopentol (**VIIa**) (Birgel et al., unpublished data).

### 9.1.4. Chapter 7. The effect of bacterial growth phase on BHP composition

This main objective of this chapter was to investigate the 35-aminoBHP sedimentary signatures at different time points over a 28 day period, incubated at mesophilic and thermophilic temperatures, 30 and 50°C.

Aminotriol (**V**) and aminopentol (**VII**) continued to be produced, after 5% methane was oxidised at day 7, in 'early stationary phase' up to day 15 but no increase was detected from day 15 to day 28. Aminotetrol (**VI**) continued to be produced later in the stationary phase. The continued production of 35-aminoBHPs in sediments incubated at 30°C, after 5% methane was oxidised, suggests that BHPs play an important role in maintaining cell homeostasis in stationary phase when substrate becomes limiting. However, aminotriol (**V**) and aminotetrol (**VI**) are produced by other bacteria and isotope analysis would need to be performed to confirm the source of these compounds were methanotrophs.

At 50°C, aminotriol (**V**) and aminotetrol (**VI**) concentrations were not higher than the initial sediment suggesting they were not abundant in the methanotroph(s) enriched. No difference in aminopentol (**VII**), 3-methylaminopentol (**VIIa**) and unsaturated $\Delta^{11}$ 3-

methylaminopentol (**VIIc**) concentrations were seen across all time points of the study but were greater than the initial sediment; this disproves the hypothesis that 3-methylaminopentol (**VIIa**) production increases in stationary phase cells. This suggests that the methanotroph(s) enriched at this temperature had high constitutive levels of BHPs present allowing the cell to survive and oxidise methane at thermophilic temperatures.

Methanotrophs that are mesophilic in nature appear to continue to produce aminopentol (**VII**) in stationary phase after methane becomes limiting but, thermophilic methanotrophs seem to have high constitutive levels of compounds therefore no increase is detected after methane becomes limiting.

### 9.1.5. Chapter 8. BHP signatures of River Tyne estuarine sediment anaerobic microcosms - a long-term study

The main objective of this chapter was to investigate the types of BHP compounds produced in anaerobic systems, namely sulphate-reducing and methanogenic conditions, and determine whether compounds are preserved or degraded at different rates over time, 706 and 665 days respectively.

In the sulphate-reducing and methanogenic studies major compounds such as BHT (**III**), 2-methylBHT (**IIIa**) and adenosylhopane (**VIII**) showed no significant degradation over the course of the study. This mirrors what was witnessed in the heat kill control sediments for the aerobic studies and suggest these compounds are recalcitrant over the course of the study.

In the sulphate-reducing study BHT cyclitol ether (**XII**) seemed to undergo some degradation with time whereas BHT glucosamine (**XIII**), although present at lower concentrations overall, seemed more recalcitrant supporting the hypothesis that BHPs will show different susceptibility to degradation. In the methanogenic microcosm study, BHT glucosamine (**XIII**) concentrations increased with time after 30 days. This process seemed to be bacterially mediated with one potential candidate for this being *Geobacter sulfurreducens*, where BHT glucosamine (**VIII**) has been found to be a major component of its BHP inventory (Eickhoff et al., 2013).

There was no significant increase in aminotriol (**V**) concentrations detected in sediments suggesting that the *Desulfovibrio* genus of sulphate-reducing bacteria, which have been shown to produce aminotriol (**V**) and BHT (**III**) (Blumenberg et al., 2006; Blumenberg et al., 2009a; Blumenberg et al., 2012), were not enriched in the estuarine sediment. This rejects the original hypothesis that some BHPs would be synthesised under sulphate-reducing conditions.

### 9.1.6. General conclusions

The overall aim of the research presented in this thesis has been to determine the effect environmentally-induced variations in methanotroph community composition in estuarine sediments has on BHP composition, focusing on the methanotroph-derived 35-aminoBHPs. The effect of growth phase on 35-aminoBHP signatures was also determined. Furthermore, the production of BHPs in anaerobic systems in long-term studies was investigated alongside the degradation of individual BHPs.

Aminopentol (**VII**) is present in all marine methanotrophs tested to date, and all Type I methanotrophs found in other environments, apart from *Methylomicrobium* spp. widely found in saline environments. The absence of aminopentol (**VII**) in certain methanotrophs (Talbot et al., 2001; Birgel et al., unpublished data) may explain its absence in several settings where Type I methanotrophs have been observed. For example, Type I methanotrophs were detected in all three units of Ace Lake sediments but aminopentol (**VII**) was only detected in sediments deposited under freshwater conditions (unit III) (Coolen et al., 2008). No aminopentol (**VII**) was detected in the methanotrophic symbionts in the gill tissue of a cold-seep mussel despite other lipid evidence suggesting a Type I methanotroph (Jahnke et al., 1995). Another study reported the absence of aminopentol (**VII**) in carbonate samples from the Alaminos Canyon, northern Gulf of Mexico (Birgel et al., 2011). Conversely, aminopentol (**VII**) was detected in the water column of the Baltic Sea with supporting evidence for the presence of Type I methanotrophs (Berndmeyer et al., 2013). Aminopentol (**VII**) was also detected in the water column of the Black Sea in the oxic-anoxic water transition (Blumenberg et al., 2007). Therefore, the absence of aminopentol (**VII**) is not evidence of the absence of methanotrophs or aerobic methane oxidation which limits its use in marine environments as a proxy for aerobic methane oxidation. However, its presence does not necessarily infer *in situ* methane oxidation either with high aminopentol (**VII)**

concentrations witnessed in the Congo and Amazon sedimentary fans thought to result from terrestrial inputs (Talbot et al., 2014; Wagner et al., 2014).

Type I methanotrophs were the dominant group enriched in River Tyne estuarine sediments as observed in other studies of this type of setting (e.g. McDonald et al., 2005b; Cunliffe et al., 2008; Moussard et al., 2009). *Methylobacter* spp. were a common feature with enrichment at 0.1-5% methane concentration and 4-40°C. They were also present at 1-15 g/L NaCl and pH 5-8 with *Methylomonas* spp. also enriched at these conditions. A *Methylocaldum* sp. was enriched at 50°C and *Methylomicrobium* spp. were enriched at pH 9 and 35 and 70 g/L NaCl whereas *Methylosoma* spp. were enriched at pH 4. The populations enriched in the sediments seem typical of methanotrophs found in estuarine environments with gene sequences identified in estuarine sediments from Newport Bay, USA, grouping with *Methylomonas, Methylomicrobium* and *Methylobacter* genera (Smith et al., 1997; McDonald et al., 2005b) and in the surface sediment from the Colne Estuary, UK, *Methylomonas* and *Methylobacter* were detected alongside *Methylomicrobium* and *Methylococcus* (Moussard et al., 2009).

There were significant differences in BHP compositions in a suite of marine methanotrophs examined at both genus and species level. A suite of compounds related to aminotriol (**V**), aminotetrol (**VI**) and aminopentol (**VII**) but with a different C-35 terminal group tentatively identified as ‘carbamylmethylester’, were witnessed for the first time in methanotrophs and were present in all marine bacterial strains tested. They have since been identified in microcosm sediments and environmental samples.

35-aminoBHPs do not appear to correlate with methane oxidation rates and methanotrophic activity which suggests that differences in BHP compositions in sediments is controlled by changes in the dominant genus and most likely at species level within the genus. Different *Methylobacter* spp. were enriched at 40°C compared with 4-30°C which was reflected in higher concentrations of aminotriol (**V**) and aminotetrol (**VI**) being detected in sediments at lower temperatures compared with 40°C. Aminopentol (**VII**) concentrations increased with temperature with the maximum seen at 40°C. This once again suggests that BHP compositions vary at species level within the *Methylobacter* genus. At 50°C where a *Methylocaldum* sp. was enriched, 3-methylaminopentol (**VIIa**) was in greater abundance than its non-methylated equivalent

which mirrors what has been witnessed in pure culture (Cvejic et al., 2000a). Aminopentol (**VII**) concentrations decreased with increasing salinity when a shift in methanotroph community composition to *Methylomicrobium* spp., from *Methylobacter* and *Methylomonas* spp. was witnessed, which is consistent with recent observations in *Methylomicrobium* pure cultures (Birgel et al., unpublished data). Aminopentol (**VII**) was also not detected at pH 9 where *Methylomicrobium* spp. were enriched.

35-aminoBHPs are produced by actively growing methanotrophs but evidence from the growth stage study indicates that BHP production continues in stationary phase as a response to methane limiting conditions. Alternatively, organisms other than methanotrophs produce BHPs following the depletion of methane. This study has implications for the interpretation of the sedimentary record where 35-aminoBHP signatures, namely the Type I methanotroph specific aminopentol (**VII**), witnessed in marine settings may not represent periods of significant or prolonged *in situ*, or terrestrial methane oxidation, but may represent a response to methane limiting conditions encountered during stationary phase.

In aerobic microcosm sediments, 3-methylaminopentol (**VIIa**) was only detected at higher temperatures, 50°C, where a *Methylocaldum* sp. was enriched (trace amounts at 40 and 60°C) and at higher salinities, 35 and 70 g/L NaCl, where *Methylomicrobium* spp. dominated. 3-methylaminotriol (**Va**) was also detected at these higher salinities too in agreement with recent data from several cultured species of *Methylomicrobium* (Birgel et al., unpublished). High concentrations of 3-methylBHPs seem to be restricted to quite specific conditions i.e., some alkaline lakes (Farrimond et al., 2004) and geothermal sites (Gibson et al., 2008) and observations in modern settings are at odds with widespread reports of 3-methyl degradation products, the C-3 methylated hopanes, in the sedimentary record (e.g. Collister et al., 1992; Farrimond et al., 2004; Eigenbrode et al., 2008). The reason for this disparity may be because of the suggestion that 3-methylBHPs are more strongly bound and less extractable than their non-methylated equivalents (Herrmann et al., 1996; Allen et al., 2010). Alternatively, environmental conditions at the time of deposition, such as hypersaline conditions, may have favoured 3-methylBHP producing methanotrophs i.e. *Methylomicrobium*.

Compounds such as BHT (**III**), 2-methylBHT (**IIIa**) and adenosylhopane (**XIII**) appear resistant to heat treatment in aerobic microcosms. BHT cyclitol ether (**XII**) seems to be

more susceptible to heat degradation whereas its isomer, BHT glucosamine (**XIII**) is more resistant. BHT (**III**), 2-methylBHT (**IIIa**), adenosylhopane (**XIII**) also seem resistant to degradation over the course of the long-term anaerobic studies for both sulphate-reducing and methanogenic conditions. BHT cyclitol ether (**XII**) seems to undergo some degradation with time under sulphate-reducing conditions.

## 9.2. Recommendations

The findings from this research has identified additional questions for future investigation. Some areas for additional research are highlighted below.

### 9.2.1. Short-term aerobic studies

#### 9.2.1.1. Extending methane concentration study

The maximum methane concentration tested could be extended beyond the current maximum of 5%. Beyond ~10%, oxygen may becoming limiting in the headspace which would mean the headspace would need to be flushed with air after methane oxidation and additional methane then added.

#### 9.2.1.2. Buffered microcosm medium for pH study

During the course of the pH study, the microcosms drifted by one pH unit towards the pH of the initial sediment (pH 8) due to the natural buffering capacity of the sediment. A way to prevent this drift would be to use 'Goods buffers' such as 4-morpholineethanesulfonic acid (MES) to buffer the medium at the desired pH value. The BHP signatures in the sediment would then be a true representation of the pH the microcosm was incubated at and may confirm the findings suggesting a sequential increase in the concentration of the 'carbamylmethylester' compounds at higher pH. A small pilot study was carried out using MES to buffer the nutrient medium at pH 5 and 6 which worked particularly well at pH 6. Buffered medium has been used to investigate the effect hopanoids play in membrane integrity and pH homeostasis in *Rhodopseudomonas palustris* TIE-1 because the original unbuffered medium used drifted during the course of the experiment (Welander et al., 2009).

Extending the pH range to encompass more acidic and alkaline conditions may also provide more evidence for extreme environments. Moreover, the effect acidic pH

values may have on the ‘release’ of some compounds from the sediment matrix also requires further investigation.

#### 9.2.1.3. Microbial community analysis of growth stage study

Microbiological analysis of microcosm sediments incubated at 30 and 50°C would determine the dominant methanotrophs enriched at these temperatures. It would also determine whether the methanotroph community composition changed with time especially when moving from aerobic to anaerobic conditions. Quantitative PCR (qPCR) would confirm whether the increase in concentration of 35-aminoBHPs later in the study (stationary phase) at 30°C, was the result of more methanotroph cells and consequently more BHPs, or more BHPs per methanotroph cell due to a stationary phase stress response.

#### 9.2.1.4. Extending time period of growth stage study

The growth stage study could be repeated but over a longer time period, perhaps up to a year, to see whether 35-aminoBHP concentrations continue to increase with time at 30°C. It would also be interesting to see whether the increases in compounds are detected at additional mesophilic temperatures and also pyschrotolerant temperatures, perhaps 5, 10 and 20°C, conditions more representative of typical marine sediment-water interface temperatures. It would be useful to determine the percentage increase of 35-aminoBHPs during stationary phase and if there are any trends with time.

#### 9.2.1.5. $^{13}$C labelled methane study

Microcosm studies could be repeated using $^{13}$C labelled methane as the carbon source. The labelled methane would be incorporated into methanotroph lipids and allow identification of BHPs that have been specifically produced by methanotrophs. For example, aminotriol (**V**) is synthesised by a wide range of bacterial sources (e.g. Talbot and Farrimond, 2007; Talbot et al., 2008; Blumenberg et al., 2012) and labelling would allow identification of the proportion that is produced solely by methanotrophs enriched in the sediment. This could also be done with natural abundance isotopes.

#### 9.2.1.6. Application to different environments

The microcosm studies used River Tyne estuarine sediment as the inoculum. To extend this work further, sediments could be sampled along the River Tyne, including truly marine sediments, to see whether methanotroph diversity and BHP signatures vary spatially. The microcosm experimental approach could be applied to terrestrial environments to determine whether Type II methanotrophs dominate in this environment and test how methanotroph community composition changes with environmental conditions.

### 9.2.2. Long-term anaerobic studies

#### 9.2.2.1. Microbial community analysis

Microbiological analysis of sulphate-reducing sediments would determine whether the *Desulfovibrio* genus of sulphate-reducing bacteria, which have been shown to produce aminotriol (**V**) and BHT (**III**) (Blumenberg et al., 2006; Blumenberg et al., 2009a; Blumenberg et al., 2012), were enriched in the estuarine sediment.

Analysis of methanogenic sediments would possibly confirm whether the increase in BHT glucosamine (**XIII**) witnessed was a bacterially mediated process. It may identify the type of bacteria responsible for the production and whether it was a member of the anaerobic Fe(III)-reducing *Geobacter* bacteria namely *Geobacter sulfurreducens* (Eickhoff et al., 2013). Isolating and sequencing the *sqhC* gene from DNA extracts would confirm whether this microorganism was enriched.

#### 9.2.2.2. GC-MS analysis of geohopanoids

For the long-term anaerobic microcosm studies no significant degradation was witnessed for the majority of BHP compounds. The next stage would be to look for geohopanoids (hopanols, hopanoic acids, hopanes and hopenes) using GC-MS to see whether any changes in abundance or composition is witnessed over the course of the study. A labelled carbon source could be used to trace diagenetic transformations in BHP structures also.

#### 9.2.2.3. Minimising heterogeneity of estuarine sediment

A volume of sediment contains areas of varying bacterial diversity which would result in differences in initial sediment communities in microcosm replicates at the beginning of the long-term studies. Larger scale mesocosms could be prepared allowing multiple sampling from one mesocosm over a time period, which would reduce heterogeneity between sediment replicates.

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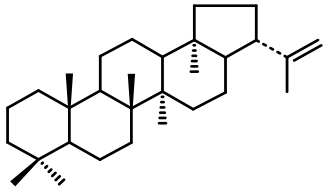
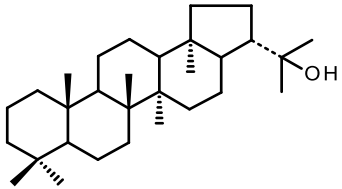
# Appendix

## Appendix 1: BHP structure names

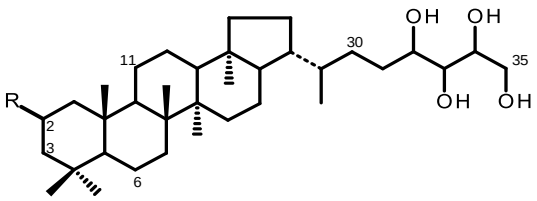


**I**: Diploptene

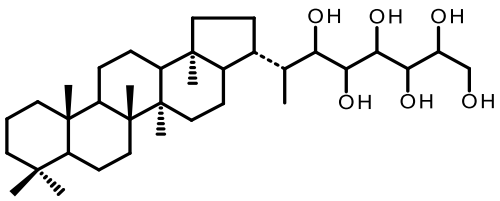



**II**: Diplopterol

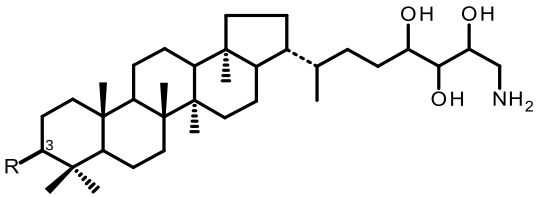




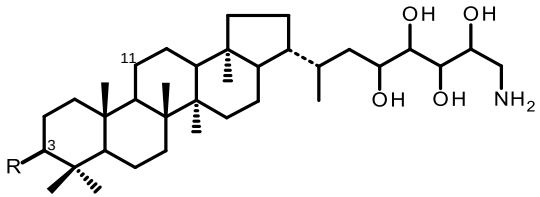

**III**: R=H, BHT
**IIIa**: R=$CH_3$, 2-methylBHT



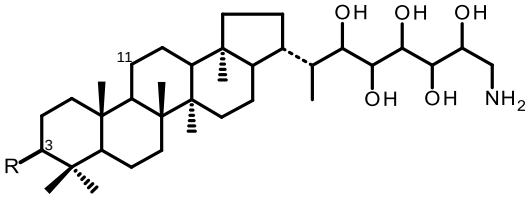

**IV**: BHhexol




**V**: R=H, aminotriol
**Va**: R=CH3, 3-methylaminotriol



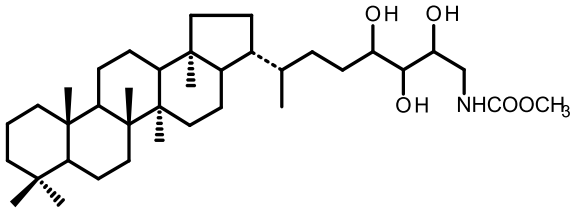

**VI**: R=H, aminotetrol
**VIa**: R=$CH_3$, 3-methylaminotetrol
**VIb**: R=H, aminotetrol with unsaturation at $\Delta^{11}$



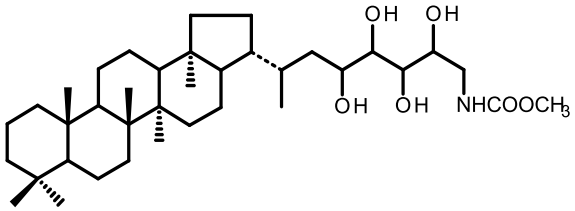

**VII**: R=H, aminopentol
**VIIa**: R=$CH_3$, 3-methylaminopentol
**VIIb**: R=H, aminopentol with unsaturation at $\Delta^{11}$
**VIIc**: R=$CH_3$, 3-methylaminopentol with unsaturation at $\Delta^{11}$



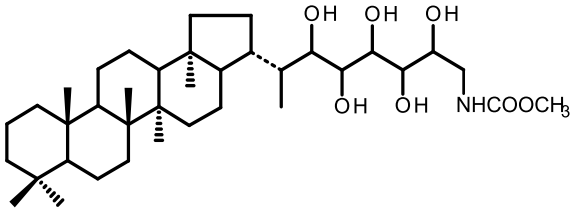
Tentative structures for novel compounds



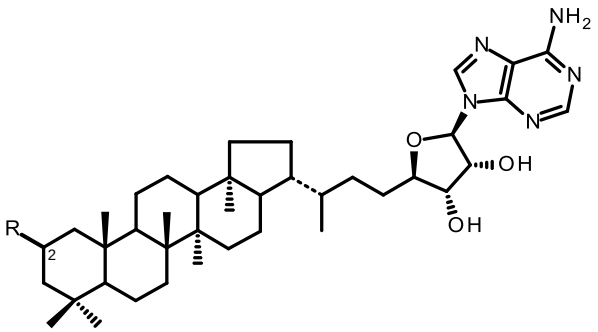

**V**$^{\textbf{MeCarb}}$:
'carbamylmethylesteraminotriol'



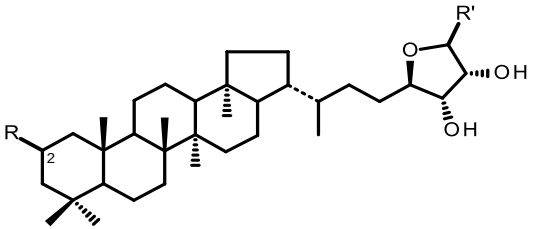

**VI**$^{\textbf{MeCarb}}$:
'carbamylmethylesteraminotetrol'



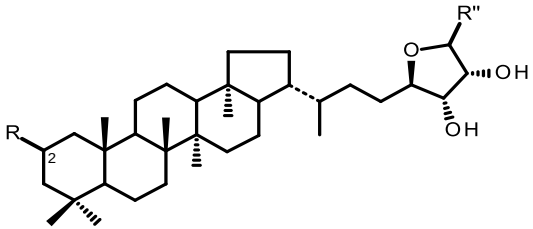

**VII**$^{\textbf{MeCarb}}$:
'carbamylmethylesteraminopentol'




**VIII**: R=H, adenosylhopane
**VIIIa**: R=$CH_3$, 2-methyladenosylhopane

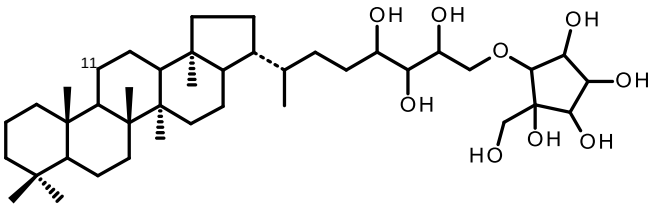



**IX**: R'=unknown structure
'adenosylhopane-type 2'
**IXa**: R'=unknown structure, R=$CH_3$
'2-methyladenosylhopane-type 2'

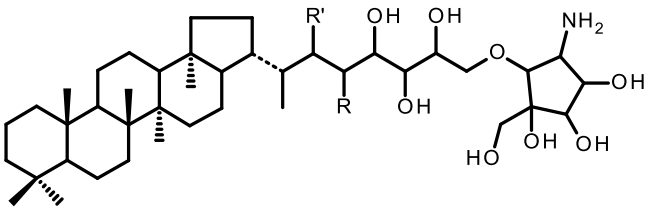



**X**: R''=unknown structure
'adenosylhopane-type 3'
**Xa**: R''=unknown structure, R=$CH_3$
'2-methyladenosylhopane-type 3'

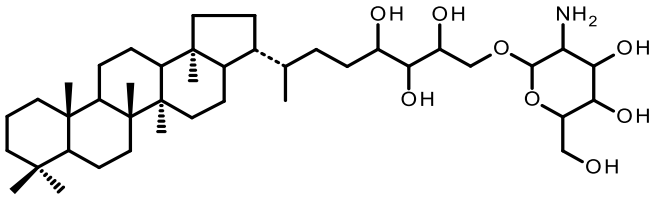





**XI**: BHT pentose
**XIa**: BHT pentose with at $\Delta^{11}$ unsaturation

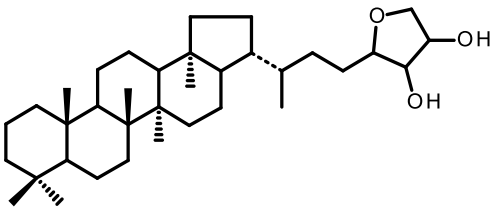



**XII**: R=H, R’=H, BHT cyclitol ether
**XIIa**: R=OH, R’=H, BHpentol cyclitol ether
**XIIb**: R=OH, R’=OH BHhexol cyclitol ether




**XIII**= BHT glucosamine






**XIV**: anhydroBHT

**Appendix 2: Nutrient medium**

| | |
|---|---|
| Basal medium | NaCl (7 g), $MgCl_2.6H_2O$ (1.2 g), $CaCl_2.2H_2O$ (0.1 g), $NH_4Cl$ (0.25 g), $KH_2PO_4$ (0.2 g), KCl (0.5 g) in 1L Milli-Q water. Autoclave 20 min, 121°C. |
| Non-chelated trace element mixture | $FeSO_4.7H_20$ (2100 mg), $H_3BO_3$ (30 mg), $MnCl_2.4H_2O$ (100 mg), $CoCl_2.6H_2O$ (190 mg), $NiCl_2.6H_2O$ (24 mg), $CuCl_2.2H_2O$ (2 mg), $ZnSO_4.7H_2O$ (144 mg), $Na_2MoO_4.7H_2O$ (36 mg) in 975 mL Milli-Q and 12.5 mL of 25% HCl. Autoclave 20 min, 121°C. |
| Selenite-tungstate solution | NaOH (400 mg), $NaSeO_3.5H_2O$ (6 mg) and $Na_2WO_4.2H_2O$ (8 mg) in 1L Milli-Q water. Autoclave 20 min, 121°C. |
| Bicarbonate solution | $NaHCO_3$ (2.52 g) in 30 mL Milli-Q water (degassed for 10 min prior addition). Autoclave 20 min, 121°C. |
| Vitamin solution | 4-aminobenzoic acid )4 mg), D(+)-biotin (1 mg), nicotinic acid (10 mg), calcium D(+)-pantothenate 5 mg, pyroxidine dihydrochloride (15 mg) and thiamine (10 mg) in 100 mL of NaPhosphate buffer (10 mM, pH 7.1). Filter sterilise (0.2 µm) and store in dark at 4°C. |
| Vitamin B12 | 5 mg cyanocobalamine in 100 mL Milli-Q water. Filter sterilise (0.2 µm) and store in dark at 4°C. |
| Oxygen scavenger | $Na_2S$ (2.4 g) in 50 mL Milli-Q water (degassed for 10 min prior addition). Autoclave 20 min, 121°C. |

The final medium was assembled by additions of non-chelated trace element mixture (1 mL) and selenite-tungstate solution (1 mL) to the basal medium with subsequent degassing with nitrogen for 1 h. Vitamin solution (1 mL), vitamin B12 solution (1 mL), bicarbonate solution (30 mL) and $Na_2S$ solution (2.5 mL) were added to the basal medium under a constant stream of nitrogen.

## Appendix 3a: Total BHPs (µg/g) extracted from bacterial biomass

| | *Methylomarinum vadi* IT-4 | *Methylomarinovum caldicuralii* IT-9 | *Methylomarinovum* sp. IN45 |
|---|---|---|---|
| **'Novel aminotriol'** | 257 | - | 13 |
| **Aminotriol** | 11880 | 50 | 280 |
| **'Methylcarbamoylaminotriol'** | 12093 | 64 | 1393 |
| **'Novel aminotetrol'** | 101 | 36 | 63 |
| **'Early eluting aminotetrol'** | 467 | 439 | 108 |
| **Aminotetrol** | 5925 | 3185 | 1460 |
| **'Methylcarbamoylaminotetrol'** | 6274 | 2933 | 5900 |
| **'Early eluting aminopentol'** | 819 | 481 | - |
| **'Earyl eluting methylcarbamoylaminopentol'** | 579 | 303 | 122 |
| **'Novel aminopentol'** | 188 | 69 | 17 |
| **Aminopentol** | 8192 | 8245 | 583 |
| **'Methylcarbamoylaminopentol'** | 7434 | 6807 | 1800 |
| **Total (ug/g)** | **54209** | **22612** | **11740** |

BHP concentrations (µg/g) represent a single extraction from bacterial biomass.

# Appendix 3b: Total BHPs (µg/g TOC) in response to methane concentration in River Tyne estuarine sediment microcosms

| | Time 0 | | | Unamended | | | 0.1% $CH_4$ | | | 0.1% $CH_4$ Heat killed | | | 0.5% $CH_4$ | | | 0.5% $CH_4$ Heat killed | | | 1% $CH_4$ | | | 1% $CH_4$ Heat killed | | | 5% $CH_4$ | | | 5% $CH_4$ Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 167 | 4.6 | 37 | 143 | 4.7 | 34 | 164 | 7.0 | 36 | 144 | 15 | 42 | 146 | 5.6 | 34 | 147 | 1.4 | 45 | 131 | 9.0 | 35 | 156 | 4.1 | 45 | 152 | 3.4 | 37 | 156 | 6.2 | 47 |
| **2-methylBHT** | 17 | 0.5 | 3.6 | 14 | 1.1 | 3.3 | 16 | 0.7 | 3.4 | 13 | 1.5 | 3.8 | 15 | 0.2 | 3.6 | 14 | 0.3 | 4.3 | 14 | 0.4 | 3.6 | 14 | 0.9 | 4.1 | 14 | 0.7 | 3.4 | 15 | 0.8 | 4.6 |
| **BHhexol** | 2.3 | 0.1 | 0.5 | 2.3 | 0.3 | 0.5 | 3.0 | 0.4 | 0.6 | 1.4 | 1.0 | 0.4 | 2.4 | 0.5 | 0.6 | 2.6 | 0.5 | 0.8 | 2.5 | 0.3 | 0.7 | 2.9 | 0.6 | 0.8 | 1.4 | 0.2 | 0.3 | 3.1 | 0.2 | 0.9 |
| **'Novel aminotriol'** | 6.6 | 0.4 | 1.4 | 6.5 | 0.6 | 1.5 | 6.7 | 0.8 | 1.5 | 5.1 | 0.4 | 1.5 | 5.7 | 0.3 | 1.3 | 5.3 | 0.4 | 1.6 | 4.7 | - | 1.2 | 5.2 | 0.3 | 1.5 | 5.6 | 0.4 | 1.4 | 5.0 | 0.2 | 1.5 |
| **Aminotriol** | 30 | 0.3 | 6.5 | 26 | 7.6 | 5.7 | 21 | 2.6 | 4.5 | 11 | 1.2 | 3.2 | 22 | 1.4 | 5.3 | 9.3 | 0.6 | 2.8 | 18 | 0.4 | 4.7 | 14 | 1.2 | 4.0 | 23 | 7.8 | 5.5 | 10 | 0.4 | 3.1 |
| **'Methylcarbamoylaminotriol'** | - | - | - | 24 | 1.0 | 5.6 | 22 | 1.4 | 4.8 | 3.9 | 0.6 | 1.2 | 23 | 0.7 | 5.5 | 1.5 | 1.3 | 0.4 | 21 | 0.4 | 5.7 | - | - | - | 26 | 3.0 | 6.3 | - | - | - |
| **Aminotetrol** | 2.2 | 0.1 | 0.5 | 1.5 | 0.9 | 0.3 | 2.3 | 0.3 | 0.5 | 0.7 | - | 0.2 | 2.0 | 0.3 | 0.5 | 0.6 | 0.3 | 0.2 | 1.6 | 0.1 | 0.4 | 1.7 | 0.4 | 0.5 | 2.5 | 0.9 | 0.6 | 0.6 | - | 0.2 |
| **Aminopentol** | 0.8 | 0.4 | 0.2 | - | - | - | 1.0 | 0.5 | 0.2 | - | - | - | 1.6 | 0.2 | 0.4 | - | - | - | 0.8 | 0.4 | 0.2 | 0.9 | 0.5 | 0.3 | 2.8 | 0.5 | 0.7 | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | 1.0 | 0.4 | 0.2 | 1.4 | 0.4 | 0.3 | - | - | - | 1.2 | 0.2 | 0.3 | - | - | - | 1.1 | 0.2 | 0.3 | - | - | - | 2.5 | 0.2 | 0.6 | - | - | - |
| **Adenosylhopane** | 84 | 3.7 | 18 | 86 | 14 | 20 | 81 | 2.2 | 18 | 81 | 3.8 | 24 | 71 | 8.1 | 17 | 67 | 2.6 | 20 | 68 | 0.5 | 18 | 65 | 1.7 | 19 | 72 | 0.9 | 18 | 65 | 0.5 | 20 |
| **2-methyladenosylhopane** | 4.2 | 0.5 | 0.9 | 3.7 | 0.8 | 0.8 | 3.0 | 0.4 | 0.6 | 4.1 | 0.4 | 1.2 | 1.7 | 0.8 | 0.4 | 2.8 | 0.2 | 0.9 | 3.2 | 0.3 | 0.8 | 3.7 | 0.6 | 1.0 | 2.4 | 1.2 | 0.6 | 2.8 | 0.3 | 0.8 |
| **Adenosylhopane-type 2** | 19 | 0.8 | 4.1 | 19 | 1.8 | 4.3 | 20 | 0.4 | 4.3 | 17 | 0.9 | 5.0 | 17 | 2.3 | 4.0 | 17 | 0.7 | 5.1 | 16 | 0.6 | 4.3 | 16 | 0.4 | 4.5 | 18 | 0.7 | 4.4 | 15 | 0.4 | 4.6 |
| **2-methyladenosylhopane-type 2** | 4.9 | 0.2 | 1.1 | 5.4 | 0.7 | 1.2 | 6.5 | 0.3 | 1.4 | 4.9 | 0.2 | 1.4 | 4.9 | 0.9 | 1.1 | 5.2 | 0.5 | 1.6 | 4.4 | 0.6 | 1.2 | 4.7 | 0.2 | 1.3 | 5.1 | 0.4 | 1.3 | 5.0 | 0.4 | 1.5 |
| **Adenosylhopane-type 3** | 0.7 | 0.4 | 0.1 | 1.4 | 0.4 | 0.3 | 0.9 | 0.1 | 0.2 | 1.0 | 0.5 | 0.3 | 1.1 | 0.2 | 0.3 | 1.0 | 0.1 | 0.3 | 0.6 | 0.4 | 0.2 | 1.0 | 0.2 | 0.3 | 0.7 | 0.3 | 0.2 | 0.9 | 0.1 | 0.3 |
| **BHT pentose with unsaturation** | 12 | 0.9 | 2.7 | 11 | 1.5 | 2.6 | 10 | 0.3 | 2.3 | 7.6 | 0.3 | 2.2 | 12 | 0.1 | 2.7 | 9.7 | 1.3 | 2.9 | 9.1 | 0.4 | 2.4 | 8.8 | 0.9 | 2.5 | 8.6 | 1.3 | 2.1 | 7.6 | 0.4 | 2.3 |
| **BHT pentose** | 18 | 1.1 | 3.9 | 15 | 1.6 | 3.4 | 16 | 0.6 | 3.4 | 11 | 1.6 | 3.3 | 15 | 1.3 | 3.5 | 12 | 0.8 | 3.8 | 13 | 1.4 | 3.6 | 12 | 0.9 | 3.6 | 13 | 1.1 | 3.1 | 11 | 1.0 | 3.3 |
| **BHT cyclitol ether** | 74 | 1.8 | 16 | 62 | 21 | 13 | 72 | 6.7 | 15 | 30 | 1.2 | 9.0 | 67 | 5.2 | 16 | 30 | 6.0 | 8.9 | 54 | 1.0 | 15 | 37 | 1.9 | 11 | 52 | 5.9 | 13 | 31 | 1.8 | 9.4 |
| **BHT glucoasamine** | 6.9 | 0.1 | 1.5 | 4.8 | 0.5 | 1.1 | 5.4 | 0.4 | 1.2 | 3.8 | 0.1 | 1.1 | 5.6 | 0.7 | 1.3 | 4.2 | 0.6 | 1.3 | 4.0 | 0.5 | 1.1 | 4.2 | 0.3 | 1.2 | 3.4 | 0.8 | 0.8 | 3.8 | 0.2 | 1.1 |
| **BHpentol cyclitol ether** | 4.3 | 0.7 | 0.9 | 4.7 | 2.4 | 1.0 | 5.2 | 0.5 | 1.1 | - | - | - | 4.8 | 0.6 | 1.1 | 0.6 | 0.6 | 0.2 | 3.0 | 0.4 | 0.8 | 0.4 | 0.4 | 0.1 | 2.9 | 0.8 | 0.7 | - | - | - |
| **BHhexol cyclitol ether** | 3.3 | 0.4 | 0.7 | 3.9 | 2.8 | 0.8 | 4.6 | 0.5 | 1.0 | 0.6 | 0.4 | 0.2 | 5.2 | 0.1 | 1.2 | 0.6 | 0.4 | 0.2 | 4.0 | 0.5 | 1.1 | 0.8 | 0.5 | 0.2 | 2.3 | 0.5 | 0.6 | 1.1 | 0.1 | 0.3 |
| **Total (µg/g TOC)** | **456** | 12 | | **433** | 49 | | **462** | 22 | | **340** | 17 | | **425** | 21 | | **329** | 10 | | **374** | 8.8 | | **349** | 5.1 | | **410** | 21 | | **334** | 7.8 | |

BHP concentrations (µg/g TOC) in column one represent the average value extracted from a triplicate set of microcosm sediments with total BHPs summed at the bottom of column. Standard error is in column two and the relative % of individual compounds in column three.

# Appendix 3c: Total BHPs (µg/g TOC) in response to temperature in River Tyne estuarine sediment microcosms

| | Time 0 | | | 4°C 5% $CH_4$ | | | 4°C Unamended | | | 4°C Heat killed | | | 21°C 5% $CH_4$ | | | 21°C Unamended | | | 21°C Heat killed | | | 30°C 5% $CH_4$ | | | 30°C Unamended | | | 30°C Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 103.9 | 1.6 | 34.7 | 111.1 | 5.7 | 32.1 | 114.0 | 4.7 | 31.3 | 112.4 | 8.7 | 38.8 | 116.7 | 5.3 | 29.9 | 104.5 | 3.4 | 33.0 | 113.1 | 2.2 | 39.3 | 113.4 | 8.7 | 29.9 | 104.3 | 3.9 | 32.4 | 122.5 | 4.5 | 42.6 |
| **2-methylBHT** | 10.6 | 0.3 | 3.6 | 12.2 | 0.9 | 3.5 | 12.2 | 0.4 | 3.4 | 11.3 | 0.7 | 3.9 | 12.2 | 0.7 | 3.1 | 10.5 | 0.1 | 3.3 | 10.4 | 0.4 | 3.6 | 11.3 | 0.5 | 3.0 | 10.6 | 0.9 | 3.3 | 12.4 | 0.3 | 4.3 |
| **BHhexol** | 0.7 | 0.4 | 0.3 | 1.3 | 0.7 | 0.4 | 1.9 | 1.1 | 0.5 | 3.5 | 0.7 | 1.2 | 1.3 | 0.7 | 0.3 | 1.6 | 1.0 | 0.5 | 4.8 | 0.4 | 1.7 | 0.6 | 0.6 | 0.2 | 4.0 | 0.4 | 1.2 | 3.8 | 0.5 | 1.3 |
| **'Novel aminotriol'** | 6.5 | 0.2 | 2.2 | 10.1 | 0.2 | 2.9 | 9.0 | 1.0 | 2.5 | 7.1 | 0.5 | 2.5 | 8.5 | 0.5 | 2.2 | 6.1 | 0.3 | 1.9 | 7.3 | 0.2 | 2.5 | 9.3 | 1.0 | 2.4 | 6.5 | 0.3 | 2.0 | 8.4 | 0.4 | 2.9 |
| **Aminotriol** | 18.6 | 1.7 | 6.2 | 26.0 | 5.0 | 7.4 | 29.3 | 0.5 | 8.1 | 13.0 | 0.9 | 4.5 | 33.4 | 5.5 | 8.5 | 21.0 | 1.4 | 6.6 | 12.7 | 0.2 | 4.4 | 28.6 | 2.3 | 7.5 | 16.1 | 0.9 | 5.0 | 13.4 | 0.8 | 4.7 |
| **'Methylcarbamoylaminotriol'** | 17.0 | 0.5 | 5.7 | 32.9 | 2.1 | 9.5 | 20.3 | 0.1 | 5.6 | 5.5 | 0.5 | 1.9 | 36.6 | 0.5 | 9.4 | 20.1 | 0.6 | 6.4 | 5.7 | 0.1 | 2.0 | 41.5 | 7.0 | 10.9 | 17.7 | 1.4 | 5.5 | 5.8 | 0.2 | 2.0 |
| **Aminotetrol** | 2.0 | 0.3 | 0.7 | 3.7 | 0.9 | 1.0 | 3.8 | 0.3 | 1.0 | 1.6 | 0.2 | 0.5 | 3.9 | 0.7 | 1.0 | 2.2 | 0.2 | 0.7 | 1.2 | 0.1 | 0.4 | 4.4 | 0.4 | 1.2 | 2.0 | 0.1 | 0.6 | 1.2 | - | 0.4 |
| **'Methylcarbamoylaminotetrol'** | 1.3 | 0.3 | 0.4 | 2.6 | 0.4 | 0.7 | 1.7 | 0.1 | 0.5 | - | - | - | 2.8 | 0.2 | 0.7 | 1.7 | - | 0.6 | 0.3 | 0.3 | 0.1 | 4.8 | 0.5 | 1.3 | 1.7 | 0.2 | 0.5 | - | - | - |
| **Unsaturated aminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aminopentol** | - | - | - | 0.6 | 0.3 | 0.2 | 1.0 | 0.2 | 0.3 | 0.2 | 0.2 | 0.1 | 2.1 | 0.7 | 0.5 | 1.1 | 0.6 | 0.3 | 0.6 | 0.3 | 0.2 | 3.4 | 0.7 | 0.9 | 1.9 | 0.5 | 0.6 | - | - | - |
| **Unsaturated 3-methylaminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **3-methylaminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | 0.1 | 0.1 | - | 0.3 | 0.3 | 0.1 | - | - | - | 1.4 | 0.3 | 0.4 | 0.4 | 0.4 | 0.1 | - | - | - | 3.7 | 0.6 | 1.0 | 1.8 | 0.3 | 0.5 | 0.1 | 0.1 | < 0.1 |
| **'Early eluting aminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Early eluting methylcarbamoylaminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Adenosylhopane** | 60.0 | 1.2 | 20.0 | 58.7 | 2.3 | 17.2 | 64.8 | 2.6 | 17.8 | 60.6 | 9.3 | 20.8 | 68.4 | 0.9 | 17.6 | 57.7 | 7.5 | 18.1 | 59.7 | 4.0 | 20.7 | 66.6 | 1.7 | 17.7 | 54.3 | 1.5 | 16.9 | 49.7 | 3.6 | 17.3 |
| **2-methyladenosylhopane** | 5.2 | 0.5 | 1.7 | 5.2 | 0.5 | 1.5 | 4.9 | 0.3 | 1.4 | 5.6 | 0.8 | 1.9 | 5.0 | 0.6 | 1.3 | 4.7 | 0.5 | 1.5 | 4.4 | 0.2 | 1.5 | 5.5 | 0.5 | 1.5 | 4.3 | 0.7 | 1.3 | 4.0 | 0.3 | 1.4 |
| **Adenosylhopane-type 2** | 13.6 | 0.7 | 4.6 | 14.0 | 0.7 | 4.0 | 15.1 | 0.6 | 4.2 | 12.3 | 0.7 | 4.3 | 13.8 | 0.7 | 3.6 | 12.6 | 0.9 | 4.0 | 13.0 | 0.4 | 4.5 | 14.5 | 0.2 | 3.9 | 12.9 | 0.5 | 4.0 | 14.7 | 0.9 | 5.1 |
| **2-methyladenosylhopane-type 2** | 4.9 | 0.2 | 1.6 | 4.0 | 0.1 | 1.2 | 4.9 | 0.3 | 1.3 | 3.5 | - | 1.2 | 4.3 | 0.3 | 1.1 | 4.2 | 0.4 | 1.3 | 3.8 | 0.5 | 1.3 | 4.8 | 0.1 | 1.3 | 14.8 | 11.4 | 4.4 | 4.3 | 0.3 | 1.5 |
| **Adenosylhopane-type 3** | 1.6 | 0.2 | 0.5 | 0.5 | 0.5 | 0.2 | 1.4 | 0.7 | 0.4 | 3.6 | 0.9 | 1.2 | 2.3 | 0.3 | 0.6 | 1.2 | 0.7 | 0.4 | 2.2 | 0.6 | 0.8 | 1.8 | 0.2 | 0.5 | 1.8 | 0.3 | 0.5 | 1.5 | 0.4 | 0.5 |
| **2-methyladenosylhopane-type 3** | 0.4 | 0.2 | 0.1 | - | - | - | 0.6 | 0.3 | 0.2 | 0.4 | 0.4 | 0.1 | 1.0 | - | 0.2 | 0.3 | 0.3 | 0.1 | - | - | - | 0.4 | 0.2 | 0.1 | 0.3 | 0.2 | 0.1 | - | - | - |
| **BHT pentose with unsaturation** | 6.4 | 0.5 | 2.1 | 5.5 | 0.4 | 1.6 | 7.1 | 0.8 | 1.9 | 8.2 | 1.3 | 2.8 | 6.2 | 0.7 | 1.6 | 7.9 | 0.4 | 2.5 | 8.7 | 0.5 | 3.0 | 6.1 | 0.6 | 1.6 | 7.7 | 0.7 | 2.4 | 8.0 | 1.2 | 2.8 |
| **BHT pentose** | 7.8 | 0.8 | 2.6 | 7.6 | 0.8 | 2.2 | 11.2 | 1.6 | 3.0 | 11.5 | 0.9 | 4.0 | 8.2 | 0.4 | 2.1 | 10.0 | 0.8 | 3.2 | 11.5 | 0.9 | 4.0 | 10.1 | 0.4 | 2.7 | 11.7 | 0.3 | 3.6 | 10.8 | 1.4 | 3.7 |
| **BHT cyclitol ether** | 32.5 | 4.8 | 10.8 | 43.5 | 8.4 | 12.3 | 51.4 | 5.2 | 14.1 | 25.2 | 2.3 | 8.7 | 53.0 | 6.3 | 13.5 | 40.5 | 4.0 | 12.9 | 23.4 | 0.2 | 8.1 | 40.8 | 5.4 | 10.7 | 39.5 | 2.7 | 12.3 | 22.0 | 1.5 | 7.6 |
| **BHT glucoasamine** | 4.1 | 0.3 | 1.4 | 4.4 | 0.4 | 1.3 | 4.2 | 0.1 | 1.2 | 3.6 | 0.3 | 1.3 | 4.5 | 0.3 | 1.2 | 4.0 | 0.2 | 1.3 | 4.3 | 0.3 | 1.5 | 4.5 | 0.5 | 1.2 | 4.2 | 0.3 | 1.3 | 4.6 | 0.5 | 1.6 |
| **BHpentol cyclitol ether** | 2.5 | 0.2 | 0.8 | 2.4 | 1.3 | 0.6 | 3.7 | 0.6 | 1.0 | - | - | - | 4.2 | 0.2 | 1.1 | 3.3 | 0.5 | 1.0 | - | - | - | 2.5 | 0.1 | 0.7 | 2.8 | 0.2 | 0.9 | - | - | - |
| **BHhexol cyclitol ether** | - | - | - | 0.7 | 0.3 | 0.2 | 1.3 | 0.7 | 0.3 | 0.8 | 0.4 | 0.3 | 1.0 | 0.2 | 0.3 | 1.1 | 0.7 | 0.3 | 0.8 | 0.1 | 0.3 | - | - | - | 2.0 | 0.1 | 0.6 | 0.6 | 0.3 | 0.2 |
| **Total (µg/gTOC)** | **300** | 7.0 | | **347** | 25 | | **364** | 19 | | **290** | 23 | | **391** | 19 | | **317** | 7.9 | | **288** | 1.6 | | **379** | 24 | | **323** | 8.9 | | **288** | 14 | |

| | 40°C 5% $CH_4$ | | | 40°C Unamended | | | 40°C Heat killed | | | 50°C 5% $CH_4$ | | | 50°C Unamended | | | 50°C Heat killed | | | 60°C 5% $CH_4$ | | | 60°C Unamended | | | 60°C Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 101 | 7.1 | 32 | 103 | 4.7 | 34 | 129 | 11 | 41 | 116 | 7.8 | 35 | 98 | 4.3 | 36 | 120 | 23.9 | 45 | 111 | 1.2 | 36 | 122 | 2.8 | 38 | 133 | 8.9 | 42 |
| **2-methylBHT** | 9.9 | 0.8 | 3.1 | 11 | 0.7 | 3.6 | 13.4 | 0.8 | 4.3 | 11 | 0.5 | 3.2 | 9.4 | 0.5 | 3.4 | 12 | 2.2 | 4.4 | 11 | 0.2 | 3.6 | 12 | 0.4 | 3.7 | 14 | 1.0 | 4.3 |
| **BHhexol** | 0.8 | 0.4 | 0.2 | 4.1 | 0.4 | 1.4 | 6.2 | 1.5 | 2.0 | 1.1 | 0.6 | 0.3 | 3.9 | 0.7 | 1.4 | 3.3 | 2.2 | 1.1 | 1.0 | 0.5 | 0.3 | 3.5 | 0.7 | 1.1 | 4.3 | 1.0 | 1.3 |
| **'Novel aminotriol'** | 6.2 | 0.3 | 2.0 | 6.2 | 0.5 | 2.1 | 8.3 | 0.7 | 2.7 | 6.7 | 0.7 | 2.0 | 6.2 | 0.3 | 2.3 | 7.5 | 1.2 | 2.8 | 6.4 | 0.4 | 2.1 | 7.0 | 0.3 | 2.2 | 7.4 | 0.5 | 2.3 |
| **Aminotriol** | 19 | 2.1 | 5.9 | 16 | 0.8 | 5.2 | 14 | 0.8 | 4.5 | 22 | 2.1 | 6.5 | 14 | 0.4 | 5.0 | 13 | 1.5 | 4.9 | 21 | 2.1 | 6.6 | 18 | 1.6 | 5.7 | 15 | 0.2 | 4.7 |
| **'Methylcarbamoylaminotriol'** | 17 | 1.2 | 5.4 | 16 | 2.5 | 5.3 | 5.2 | 0.5 | 1.7 | 11 | 0.8 | 3.4 | 13 | 1.7 | 4.7 | 3.5 | 0.5 | 1.3 | 9.6 | 0.9 | 3.1 | 11 | 0.5 | 3.5 | 3.8 | 0.5 | 1.2 |
| **Aminotetrol** | 3.2 | 0.6 | 1.0 | 1.7 | 0.1 | 0.6 | 1.4 | 0.2 | 0.4 | 2.5 | 0.3 | 0.8 | 1.3 | 0.2 | 0.5 | 0.9 | 0.2 | 0.4 | 2.3 | 0.2 | 0.8 | 2.0 | 0.3 | 0.6 | 1.5 | 0.1 | 0.5 |
| **'Methylcarbamoylaminotetrol'** | 1.7 | 0.2 | 0.5 | 1.5 | 0.1 | 0.5 | - | - | - | 0.9 | 0.5 | 0.3 | 1.1 | 0.2 | 0.4 | - | - | - | 0.5 | 0.3 | 0.2 | 0.7 | 0.4 | 0.2 | - | - | - |
| **'Early eluting aminopentol'** | 1.4 | 0.2 | 0.4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Early eluting methylcarbamoylaminopentol'** | 1.2 | 0.1 | 0.4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Unsaturated aminopentol** | 0.5 | 0.3 | 0.2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aminopentol** | 6.4 | 1.7 | 2.0 | 1.1 | 0.1 | 0.4 | - | - | - | 2.6 | 0.4 | 0.8 | 0.6 | 0.3 | 0.2 | 0.3 | 0.3 | 0.1 | - | - | - | 0.3 | 0.3 | 0.1 | - | - | - |
| **Unsaturated 3-methylaminopentol** | 1.2 | 0.5 | 0.4 | - | - | - | - | - | - | 1.5 | 0.3 | 0.5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **3-methylaminopentol** | 0.6 | 0.6 | 0.2 | - | - | - | - | - | - | 4.3 | 0.7 | 1.3 | - | - | - | - | - | - | 0.4 | 0.2 | 0.1 | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | 5.2 | 1.2 | 1.6 | 1.0 | 0.2 | 0.3 | - | - | - | 1.4 | 0.2 | 0.4 | 0.1 | 0.1 | < 0.1 | - | - | - | - | - | - | - | - | - | - | - | - |
| **Adenosylhopane** | 62 | 4.9 | 20 | 55 | 5.0 | 18.1 | 62 | 4.3 | 20 | 67 | 6.6 | 20 | 51 | 4.5 | 19 | 53 | 12 | 20 | 71 | 3.3 | 23 | 58 | 1.9 | 18 | 62 | 4.4 | 20 |
| **2-methyladenosylhopane** | 5.6 | 0.6 | 1.8 | 4.3 | 0.3 | 1.4 | 4.5 | 0.7 | 1.4 | 5.4 | 0.4 | 1.7 | 4.0 | 0.7 | 1.4 | 4.3 | 1.0 | 1.6 | 5.7 | 0.5 | 1.8 | 4.7 | 0.4 | 1.5 | 5.4 | 0.5 | 1.7 |
| **Adenosylhopane-type 2** | 11 | 0.2 | 3.4 | 12 | 0.5 | 4.0 | 14 | 1.0 | 4.3 | 14 | 0.3 | 4.1 | 12 | 0.4 | 4.5 | 13 | 0.6 | 5.1 | 13 | 0.6 | 4.2 | 14 | 0.5 | 4.3 | 14 | 0.9 | 4.4 |
| **2-methyladenosylhopane-type 2** | 3.9 | 0.1 | 1.2 | 3.6 | 0.4 | 1.2 | 4.1 | 0.1 | 1.3 | 3.7 | 0.1 | 1.1 | 3.2 | 0.2 | 1.2 | 3.5 | 0.1 | 1.4 | 4.0 | 0.2 | 1.3 | 4.2 | 0.2 | 1.3 | 4.8 | 0.2 | 1.5 |
| **Adenosylhopane-type 3** | 2.5 | 0.2 | 0.8 | 1.3 | 0.7 | 0.4 | 2.3 | 0.6 | 0.7 | 2.7 | 0.4 | 0.8 | 1.2 | 0.7 | 0.4 | - | - | - | 2.5 | 0.5 | 0.8 | 1.1 | 0.6 | 0.4 | 2.6 | - | 0.8 |
| **2-methyladenosylhopane-type 3** | 0.6 | 0.3 | 0.2 | - | - | - | - | - | - | 0.7 | 0.4 | 0.2 | 0.2 | 0.2 | 0.1 | - | - | - | 0.6 | 0.3 | 0.2 | - | - | - | 0.3 | 0.3 | 0.1 |
| **BHT pentose with unsaturation** | 5.9 | 0.3 | 1.9 | 8.7 | 0.2 | 2.9 | 8.0 | 0.9 | 2.6 | 7.9 | 0.6 | 2.4 | 7.8 | 0.8 | 2.8 | 6.0 | 2.2 | 2.2 | 7.6 | 0.4 | 2.4 | 8.5 | 0.4 | 2.7 | 9.0 | 1.1 | 2.8 |
| **BHT pentose** | 6.9 | 0.4 | 2.2 | 10 | 0.5 | 3.5 | 11 | 1.5 | 3.5 | 10 | 0.6 | 3.1 | 12 | 1.1 | 4.5 | 9.2 | 3.2 | 3.4 | 12 | 0.3 | 3.8 | 15 | 0.9 | 4.5 | 12 | 1.3 | 3.7 |
| **BHT cyclitol ether** | 37 | 8.9 | 11 | 36 | 0.8 | 12 | 24 | 1.3 | 7.8 | 33 | 1.5 | 9.9 | 27 | 3.0 | 10 | 14 | 4.5 | 5.1 | 28 | 2.4 | 9.0 | 31 | 3.0 | 9.6 | 22 | 1.9 | 7.1 |
| **BHT glucoasamine** | 3.8 | 0.3 | 1.2 | 4.3 | 0.8 | 1.4 | 4.2 | 0.6 | 1.3 | 4.4 | 0.3 | 1.3 | 4.1 | 0.1 | 1.5 | 3.5 | 0.6 | 1.3 | 4.2 | 0.4 | 1.4 | 4.2 | 0.2 | 1.3 | 4.5 | 0.3 | 1.4 |
| **BHpentol cyclitol ether** | 3.0 | 0.5 | 0.9 | 2.7 | 0.1 | 0.9 | - | - | - | - | - | - | 1.7 | 0.9 | 0.6 | - | - | - | - | - | - | 2.9 | 0.4 | 0.9 | - | - | - |
| **BHhexol cyclitol ether** | - | - | - | 2.3 | 0.1 | 0.8 | 1.1 | 0.2 | 0.3 | 0.2 | 0.1 | 0.1 | 1.1 | 0.6 | 0.4 | - | - | - | - | - | - | 1.2 | 0.2 | 0.4 | 0.7 | 0.4 | 0.2 |
| **Total (µg/gTOC)** | **318** | 24 | | **302** | 13 | | **313** | 23 | | **329** | 16 | | **273** | 11 | | **266** | 56 | | **312** | 5.3 | | **322** | 4.9 | | **315** | 18 | |

| | Time 0 | | | 8°C 5% $CH_4$ | | | 8°C Unamended | | | 8°C Heat killed | | | 15°C 5% $CH_4$ | | | 15°C Unamended | | | 15°C Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 106 | 11.7 | 26 | 126 | 12 | 27 | 145 | 36 | 23 | 162 | 18 | 35 | 145 | 3.6 | 25 | 126 | 5.0 | 24 | 161 | 13 | 31 |
| **2-methylBHT** | 13 | 1.7 | 3.2 | 14 | 0.8 | 3.1 | 17 | 4.8 | 2.6 | 17 | 1.9 | 3.7 | 17 | 1.0 | 2.9 | 16 | 0.8 | 3.0 | 19 | 1.2 | 3.6 |
| **BHhexol** | - | - | - | - | - | - | 1.1 | 1.1 | 0.1 | 6.4 | 1.0 | 1.4 | 1.0 | 0.5 | 0.2 | 1.3 | 0.8 | 0.3 | 9.2 | 4.4 | 1.7 |
| **'Novel aminotriol'** | 5.8 | 1.0 | 1.4 | 7.1 | 0.6 | 1.5 | 9.1 | 1.3 | 1.6 | 7.9 | 0.4 | 1.7 | 7.3 | 0.3 | 1.3 | 8.0 | 2.1 | 1.5 | 9.2 | 0.4 | 1.8 |
| **Aminotriol** | 33 | 4.6 | 8.3 | 35 | 5.2 | 7.4 | 48 | 10 | 7.8 | 21 | 1.7 | 4.5 | 48 | 2.8 | 8.4 | 35 | 4.5 | 6.7 | 19 | 1.2 | 3.6 |
| **'Methylcarbamoylaminotriol'** | 33 | 4.1 | 8.0 | 34 | 3.2 | 7.4 | 32 | 8.7 | 4.9 | 9.9 | 0.5 | 2.1 | 45 | 2.5 | 7.8 | 36 | 1.2 | 6.9 | 15 | 0.5 | 2.9 |
| **Aminotetrol** | 2.4 | 0.2 | 0.6 | 4.1 | 0.8 | 0.9 | 4.6 | 1.4 | 0.7 | 1.9 | 0.4 | 0.4 | 5.7 | 0.3 | 1.0 | 3.3 | 0.5 | 0.6 | 1.3 | 0.2 | 0.2 |
| **'Methylcarbamoylaminotetrol'** | 1.8 | 0.3 | 0.4 | 2.6 | 0.3 | 0.6 | 1.9 | 0.9 | 0.2 | 1.0 | 0.3 | 0.2 | 3.8 | 0.4 | 0.6 | 2.4 | 0.1 | 0.5 | 1.3 | 0.1 | 0.2 |
| **'Early eluting aminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Early eluting methylcarbamoylaminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Unsaturated aminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aminopentol** | - | - | - | 1.7 | 0.1 | 0.4 | 2.1 | 1.1 | 0.3 | 0.8 | 0.4 | 0.2 | 2.4 | 0.1 | 0.4 | 1.2 | 0.6 | 0.2 | 0.4 | 0.4 | 0.1 |
| **Unsaturated 3-methylaminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **3-methylaminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | 0.6 | 0.3 | 0.1 | 0.9 | 0.5 | 0.1 | 0.2 | 0.2 | 0.1 | 1.6 | 0.2 | 0.3 | 1.3 | 0.4 | 0.3 | - | - | - |
| **Adenosylhopane** | 77 | 7.5 | 19 | 81 | 8.3 | 18 | 117 | 29 | 19 | 87 | 10.6 | 18 | 94 | 7.4 | 16 | 96 | 12.3 | 18 | 114 | 3.8 | 22 |
| **2-methyladenosylhopane** | 7.1 | 0.8 | 1.7 | 7.8 | 0.7 | 1.7 | 10 | 2.0 | 1.8 | 7.9 | 1.3 | 1.7 | 8.5 | 0.4 | 1.5 | 8.1 | 1.0 | 1.5 | 10 | 0.1 | 2.0 |
| **Adenosylhopane-type 2** | 19 | 1.6 | 4.6 | 22 | 1.2 | 4.8 | 25 | 3.4 | 4.4 | 22 | 2.3 | 4.8 | 23 | 0.7 | 3.9 | 23 | 1.4 | 4.4 | 28 | 0.8 | 5.3 |
| **2-methyladenosylhopane-type 2** | 5.7 | 0.3 | 1.4 | 6.2 | 0.4 | 1.3 | 7.1 | 1.1 | 1.2 | 5.9 | 0.4 | 1.3 | 5.9 | 0.4 | 1.0 | 6.2 | 0.3 | 1.2 | 8.0 | 0.6 | 1.5 |
| **Adenosylhopane-type 3** | 2.3 | 0.7 | 0.6 | 1.2 | 0.6 | 0.3 | 3.3 | 1.6 | 0.5 | 2.0 | 0.4 | 0.4 | 2.6 | 0.6 | 0.4 | 3.6 | 0.6 | 0.7 | 4.0 | 0.2 | 0.8 |
| **2-methyladenosylhopane-type 3** | 0.5 | 0.3 | 0.1 | - | - | - | 0.7 | 0.7 | 0.1 | - | - | - | 0.4 | 0.4 | 0.1 | 0.9 | 0.1 | 0.2 | 0.7 | 0.4 | 0.1 |
| **BHT pentose with unsauration** | 8.3 | 1.0 | 2.0 | 12 | 1.0 | 2.6 | 12 | 3.9 | 1.9 | 18 | 1.9 | 3.7 | 11 | 0.5 | 2.0 | 13 | 0.4 | 2.5 | 18 | 0.5 | 3.4 |
| **BHT pentose** | 9.9 | 0.5 | 2.5 | 16 | 1.3 | 3.5 | 16 | 5.3 | 2.4 | 24 | 2.3 | 5.2 | 16 | 0.1 | 2.8 | 20 | 1.1 | 3.9 | 27 | 0.9 | 5.1 |
| **BHT cyclitol ether** | 70 | 4.4 | 17 | 78 | 8.4 | 17 | 152 | 44 | 24 | 60 | 9.9 | 13 | 125 | 11 | 21 | 103 | 7.4 | 20 | 68 | 7.3 | 13 |
| **BHT glucoasamine** | 6.3 | 0.3 | 1.6 | 5.8 | 0.4 | 1.3 | 6.1 | 1.9 | 1.0 | 6.0 | 0.5 | 1.3 | 7.0 | 0.3 | 1.2 | 7.1 | 0.2 | 1.4 | 5.8 | 0.1 | 1.1 |
| **BHpentol cyclitol ether** | 4.7 | 0.8 | 1.1 | 5.7 | 0.3 | 1.2 | 9.9 | 3.1 | 1.5 | 4.9 | 0.7 | 1.0 | 9.7 | 1.0 | 1.7 | 8.3 | 1.3 | 1.6 | 4.3 | 0.6 | 0.8 |
| **BHhexol cyclitol ether** | - | - | - | 1.9 | 0.1 | 0.4 | 5.3 | 2.9 | 0.7 | 3.4 | 0.2 | 0.7 | 2.2 | 1.0 | 0.4 | 3.7 | 1.1 | 0.7 | 2.7 | 0.4 | 0.5 |
| **Total (µg/gTOC)** | **405** | 31 | | **464** | 26 | | **627** | 158 | | **469** | 51 | | **582** | 14 | | **523** | 20 | | **526** | 24 | |

BHP concentrations (µg/g TOC) in column one represent the average value extracted from a triplicate set of microcosm sediments (50°C HK duplicate) with total BHPs summed at the bottom of column. Standard error is in column two and the relative % of individual compounds in column three.

# Appendix 3d: Total BHPs (µg/g TOC) in response to pH in River Tyne estuarine sediment microcosms

| | Time 0 pH 4 | | | pH 4 5% $CH_4$ | | | pH 4 Unamended | | | pH 4 Heat killed | | | Time 0 pH 5 | | | pH 5 5% $CH_4$ | | | pH 5 Unamended | | | pH 5 Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 155 | 21 | 27 | 149 | 14 | 26 | 158 | 4.1 | 32 | 147 | 9.6 | 31 | 137 | 32 | 30 | 174 | 5.1 | 29 | 145 | 36 | 36 | 172 | 6.0 | 34 |
| **2-methylBHT** | 16 | 3.0 | 2.7 | 13 | 1.6 | 2.3 | 15 | 0.8 | 3.1 | 12 | 0.3 | 2.6 | 14 | 4.3 | 3.0 | 16 | 0.8 | 2.7 | 14 | 3.7 | 3.3 | 15 | 0.5 | 3.0 |
| **BHhexol** | - | - | - | - | - | - | 2.6 | 1.3 | 0.5 | 1.6 | 1.6 | 0.3 | - | - | - | - | - | - | - | - | - | 0.7 | 0.7 | 0.2 |
| **'Novel aminotriol'** | 5.9 | 0.3 | 1.0 | 5.0 | 0.3 | 0.9 | 5.3 | 0.5 | 1.1 | 5.0 | 0.2 | 1.1 | 4.9 | 1.0 | 1.1 | 7.9 | 1.5 | 1.3 | 4.9 | 1.2 | 1.2 | 6.1 | 0.3 | 1.2 |
| **Aminotriol** | 80 | 13 | 14 | 83 | 18 | 14 | 46 | 4.0 | 9.5 | 51 | 6.5 | 11 | 49 | 15 | 11 | 74 | 6.8 | 12 | 33 | 4.3 | 8.5 | 38 | 3.2 | 7.6 |
| **'Methylcarbamoylaminotriol'** | 0.9 | 0.9 | 0.1 | 2.0 | 0.2 | 0.4 | 4.4 | 1.0 | 0.9 | - | - | - | 7.0 | 1.4 | 1.6 | 12 | 1.2 | 2.1 | 13 | 3.4 | 3.2 | 4.5 | 0.2 | 0.9 |
| **Aminotetrol** | 9.8 | 1.8 | 1.7 | 32 | 8.4 | 5.4 | 5.8 | 0.2 | 1.2 | 7.3 | 0.8 | 1.5 | 5.9 | 1.3 | 1.3 | 20 | 1.2 | 3.3 | 3.3 | 0.8 | 0.8 | 6.2 | 0.3 | 1.2 |
| **'Methylcarbamoylaminotetrol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1.0 | 1.0 | 0.2 | - | - | - | - | - | - |
| **'Early eluting aminopentol'** | - | - | - | 11 | 5.2 | 1.8 | - | - | - | - | - | - | - | - | - | 3.2 | 1.6 | 0.5 | - | - | - | - | - | - |
| **'Early eluting methylcarbamoylaminopentol'** | - | - | - | 0.5 | 0.5 | 0.1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aminopentol** | 1.5 | 1.5 | 0.2 | 25 | 11 | 4.0 | 1.3 | 1.3 | 0.3 | - | - | - | 1.2 | 1.2 | 0.2 | 6.0 | 0.6 | 1.0 | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Adenosylhopane** | 90 | 1.1 | 16 | 75 | 9.2 | 13 | 75 | 15 | 15 | 69 | 3.9 | 14 | 71 | 14 | 16 | 87 | 3.5 | 14 | 63 | 15 | 16 | 79 | 3.6 | 15 |
| **2-methyladenosylhopane** | - | - | - | 5.6 | 0.8 | 1.0 | 4.7 | 1.6 | 0.9 | 5.5 | 0.3 | 1.2 | 4.8 | 0.4 | 1.1 | 6.7 | 0.7 | 1.1 | 1.4 | 1.4 | 0.4 | 5.4 | 0.8 | 1.1 |
| **Adenosylhopane-type 2** | 18 | 1.8 | 3.1 | 13 | 1.2 | 2.4 | 18 | 0.9 | 3.7 | 12 | 1.3 | 2.5 | 14 | 3.1 | 3.1 | 18 | 0.8 | 3.0 | 17 | 2.4 | 4.4 | 15 | 0.9 | 2.9 |
| **2-methyladenosylhopane-type 2** | 5.0 | 0.9 | 0.9 | 4.6 | 0.2 | 0.8 | 5.0 | 0.1 | 1.0 | 3.5 | 0.2 | 0.7 | 4.0 | 0.1 | 0.9 | 5.6 | 0.3 | 0.9 | 5.5 | 0.7 | 1.4 | 4.4 | 0.4 | 0.9 |
| **Adenosylhopane-type 3** | - | - | - | 1.3 | 0.2 | 0.2 | 1.1 | 0.6 | 0.2 | 1.6 | 0.1 | 0.3 | 1.7 | 0.1 | 0.4 | 1.8 | 0.1 | 0.3 | 0.6 | 0.6 | 0.2 | 2.3 | 0.5 | 0.5 |
| **BHT pentose with unsaturation** | 14 | 2.5 | 2.5 | 13 | 1.0 | 2.4 | 16 | 3.2 | 3.2 | 13 | 1.1 | 2.8 | 13 | 2.4 | 2.9 | 15 | 2.1 | 2.5 | 9.8 | 1.0 | 2.5 | 15 | 0.4 | 3.0 |
| **BHT pentose** | 20 | 2.3 | 3.5 | 17 | 0.4 | 3.1 | 23 | 4.5 | 4.4 | 18 | 1.4 | 3.9 | 21 | 3.1 | 4.8 | 23 | 1.7 | 3.8 | 16 | 3.4 | 3.9 | 23 | 1.6 | 4.5 |
| **BHT cyclitol ether** | 134 | 20 | 23 | 109 | 23 | 19 | 100 | 14 | 20 | 110 | 8.9 | 23 | 91 | 28 | 20 | 115 | 1.8 | 19.1 | 66 | 9.2 | 17 | 104 | 5.5 | 20 |
| **BHT glucoasamine** | 7.7 | 0.3 | 1.4 | 6.0 | 1.1 | 1.0 | 7.8 | 0.6 | 1.6 | 7.3 | 0.1 | 1.5 | 6.6 | 2.1 | 1.4 | 6.5 | 0.1 | 1.1 | 6.9 | 0.8 | 1.8 | 6.7 | 0.6 | 1.3 |
| **BHpentol cyclitol ether** | 11 | 1.8 | 1.9 | 9.9 | 2.7 | 1.7 | 6.0 | 3.1 | 1.1 | 8.1 | 1.4 | 1.7 | 7.2 | 2.4 | 1.6 | 9.1 | 0.6 | 1.5 | - | - | - | 8.5 | 0.4 | 1.7 |
| **BHhexol cyclitol ether** | 1.2 | 1.2 | 0.2 | 0.4 | 0.4 | 0.1 | 4.8 | 2.5 | 0.9 | 3.1 | 3.1 | 0.6 | 0.7 | 0.7 | 0.1 | - | - | - | - | - | - | 1.0 | 1.0 | 0.2 |
| **Total (ug/gTOC)** | **568** | 73 | | **577** | 97 | | **501** | 47 | | **476** | 22 | | **455** | 113 | | **603** | 9.6 | | **399** | 71 | | **507** | 17 | |

| | Time 0 pH 6 | | | pH 6 5% $CH_4$ | | | pH 6 Unamended | | | pH 6 Heat killed | | | Time 0 pH 7 | | | pH 7 5% $CH_4$ | | | pH 7 Unamended | | | pH 7 Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 119 | 11 | 34 | 149 | 30 | 32 | 150 | 5.0 | 36 | 170 | 19 | 37 | 151 | 16 | 35 | 160 | 11 | 31 | 128 | 14 | 35 | 131 | 10 | 39 |
| **2-methylBHT** | 12 | 0.7 | 3.4 | 14 | 2.8 | 3.1 | 14.5 | 0.5 | 3.5 | 16 | 2.9 | 3.4 | 13 | 0.3 | 3.1 | 16 | 1.9 | 3.1 | 12 | 1.4 | 3.2 | 11 | 0.9 | 3.4 |
| **BHhexol** | - | - | - | 0.9 | 0.9 | 0.2 | - | - | - | - | - | - | - | - | - | 2.0 | 1.0 | 0.4 | - | - | - | 1.3 | 1.3 | 0.4 |
| **'Novel aminotriol'** | 4.8 | 0.7 | 1.4 | 5.8 | 1.2 | 1.3 | 6.2 | 0.5 | 1.5 | 7.4 | 1.0 | 1.6 | 5.6 | 0.6 | 1.3 | 7.4 | 0.2 | 1.4 | 5.3 | 0.4 | 1.5 | 5.6 | 0.4 | 1.7 |
| **Aminotriol** | 19 | 2.6 | 5.4 | 32 | 2.4 | 7.2 | 20 | 3.0 | 4.8 | 22 | 2.8 | 4.7 | 18 | 4.0 | 4.0 | 24 | 2.8 | 4.5 | 16 | 0.9 | 4.4 | 13 | 0.3 | 3.9 |
| **'Methylcarbamoylaminotriol'** | 23 | 2.3 | 6.4 | 37 | 5.7 | 8.1 | 25 | 0.8 | 6.0 | 11 | 0.9 | 2.4 | 29 | 1.8 | 6.7 | 42 | 4.1 | 8.0 | 24 | 1.9 | 6.5 | 8.5 | 0.6 | 2.6 |
| **Aminotetrol** | 2.6 | 0.5 | 0.7 | 5.3 | 0.3 | 1.2 | 2.2 | 0.7 | 0.5 | 3.3 | 0.4 | 0.7 | 1.8 | 0.6 | 0.4 | 3.6 | 0.5 | 0.7 | 1.9 | 0.3 | 0.5 | 0.4 | 0.4 | 0.2 |
| **'Methylcarbamoylaminotetrol'** | 0.7 | 0.7 | 0.2 | 4.6 | 0.7 | 1.0 | 0.7 | 0.7 | 0.2 | - | - | - | 2.3 | 0.4 | 0.5 | 4.8 | 0.3 | 0.9 | 0.9 | 0.9 | 0.2 | - | - | - |
| **'Early eluting aminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Early eluting methylcarbamoylaminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aminopentol** | - | - | - | 3.1 | 1.9 | 0.8 | - | - | - | - | - | - | - | - | - | 1.8 | 1.1 | 0.3 | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | 2.8 | 1.4 | 0.6 | - | - | - | - | - | - | - | - | - | 3.0 | 1.3 | 0.6 | - | - | - | - | - | - |
| **Adenosylhopane** | 56 | 6.9 | 16 | 56 | 20 | 12 | 76 | 6.4 | 18 | 79 | 4.5 | 17 | 73 | 2.5 | 17 | 79 | 3.1 | 15 | 66 | 9.5 | 18 | 60 | 8.7 | 18 |
| **2-methyladenosylhopane** | 1.2 | 1.2 | 0.4 | 3.7 | 1.9 | 0.8 | 6.2 | 0.7 | 1.5 | 6.1 | 0.6 | 1.3 | 6.0 | 0.5 | 1.4 | 5.8 | 0.7 | 1.1 | 4.9 | 0.7 | 1.3 | 4.4 | 0.7 | 1.3 |
| **Adenosylhopane-type 2** | 12 | 1.4 | 3.5 | 15 | 2.5 | 3.2 | 16 | 0.3 | 3.8 | 17 | 1.6 | 3.6 | 15 | 1.5 | 3.6 | 17 | 1.2 | 3.3 | 14 | 1.6 | 3.8 | 12 | 0.8 | 3.6 |
| **2-methyladenosylhopane-type 2** | 3.4 | 0.2 | 1.0 | 4.0 | 0.6 | 0.9 | 4.6 | 0.1 | 1.1 | 4.5 | 0.2 | 1.0 | 4.7 | 0.1 | 1.1 | 5.0 | 0.7 | 1.0 | 4.1 | 0.4 | 1.1 | 3.4 | 0.3 | 1.0 |
| **Adenosylhopane-type 3** | - | - | - | 1.4 | 0.7 | 0.3 | 2.3 | 0.2 | 0.6 | 2.4 | 0.2 | 0.5 | 1.5 | 1.5 | 0.3 | 0.7 | 0.7 | 0.1 | 1.6 | 0.1 | 0.4 | 1.5 | 0.3 | 0.5 |
| **BHT pentose with unsaturation** | 12 | 1.1 | 3.4 | 12 | 2.4 | 2.6 | 10 | 0.9 | 2.5 | 16 | 2.6 | 3.5 | 12 | 0.2 | 2.8 | 16 | 3.2 | 3.1 | 11 | 0.9 | 2.9 | 12 | 0.9 | 3.7 |
| **BHT pentose** | 18 | 0.1 | 5.0 | 19 | 3.9 | 4.2 | 17 | 2.2 | 4.1 | 24 | 3.0 | 5.3 | 18 | 0.7 | 4.2 | 26 | 4.5 | 4.9 | 16 | 1.6 | 4.4 | 16 | 1.0 | 4.9 |
| **BHT cyclitol ether** | 61 | 5.2 | 17 | 84 | 7.6 | 18 | 59 | 5.5 | 14 | 77 | 3.3 | 17 | 69 | 12 | 16 | 92 | 1.8 | 18 | 57 | 4.6 | 16 | 48 | 6.4 | 14 |
| **BHT glucoasamine** | 5.0 | 0.5 | 1.4 | 6.2 | 1.1 | 1.3 | 6.2 | 0.5 | 1.5 | 5.5 | 0.8 | 1.2 | 5.5 | 0.2 | 1.3 | 5.9 | 0.9 | 1.1 | 3.3 | 1.7 | 0.9 | 4.5 | 0.4 | 1.4 |
| **BHpentol cyclitol ether** | 3.0 | 3.0 | 0.9 | - | - | - | - | - | - | - | - | - | 5.4 | 1.2 | 1.2 | 8.3 | 0.5 | 1.6 | 1.8 | 1.8 | 0.6 | - | - | - |
| **BHhexol cyclitol ether** | 0.5 | 0.5 | 0.2 | 1.8 | 1.8 | 0.5 | - | - | - | - | - | - | - | - | - | 1.1 | 1.1 | 0.2 | - | - | - | - | - | - |
| **Total (ug/gTOC)** | **353** | 11 | | **458** | 62 | | **417** | 21 | | **461** | 42 | | **430** | 44 | | **522** | 36 | | **366** | 30 | | **332** | 31 | |

| | Time 0 pH 8 | | | pH 8 5% $CH_4$ | | | pH 8 Unamended | | | pH 8 Heat killed | | | Time 0 pH 9 | | | pH 9 5% $CH_4$ | | | pH 9 Unamended | | | pH 9 Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 119 | 14 | 35 | 176 | 13 | 33 | 158 | 11 | 35 | 156 | 6.7 | 40 | 149 | 43 | 33 | 139 | 37 | 32 | 159 | 13 | 36 | 136 | 35 | 43 |
| **2-methylBHT** | 10 | 0.7 | 3.1 | 16 | 0.4 | 3.0 | 15 | 1.4 | 3.2 | 14 | 1.1 | 3.7 | 13 | 3.8 | 2.8 | 12 | 3.1 | 2.7 | 15 | 1.0 | 3.5 | 12 | 3.1 | 3.9 |
| **BHhexol** | - | - | - | 0.8 | 0.8 | 0.2 | - | - | - | 0.8 | 0.8 | 0.2 | - | - | - | - | - | - | - | - | - | 1.6 | 1.6 | 0.4 |
| **'Novel aminotriol'** | 5.8 | 1.2 | 1.7 | 7.6 | 0.8 | 1.4 | 6.2 | 0.5 | 1.3 | 6.6 | 0.3 | 1.7 | 8.9 | 2.3 | 2.0 | 8.5 | 2.3 | 2.0 | 6.0 | 0.2 | 1.4 | 5.5 | 1.5 | 1.7 |
| **Aminotriol** | 11 | 2.2 | 3.3 | 24 | 4.2 | 4.4 | 18 | 0.4 | 4.0 | 13 | 0.6 | 3.3 | 16 | 8.1 | 3.3 | 18 | 5.1 | 4.1 | 19 | 0.8 | 4.4 | 9.9 | 1.4 | 3.2 |
| **'Methylcarbamoylaminotriol'** | 24 | 4.3 | 7.0 | 53 | 4.6 | 9.8 | 29 | 1.4 | 6.4 | 8.8 | 0.6 | 2.3 | 26 | 8.5 | 5.8 | 67 | 11.7 | 16 | 28 | 1.9 | 6.4 | 6.8 | 0.6 | 2.2 |
| **Aminotetrol** | 0.6 | 0.6 | 0.2 | 1.7 | 0.9 | 0.3 | 1.8 | 0.2 | 0.4 | 0.4 | 0.4 | 0.1 | 0.8 | 0.8 | 0.1 | 1.5 | 1.5 | 0.2 | 1.8 | 0.2 | 0.4 | 0.9 | 0.9 | 0.2 |
| **'Methylcarbamoylaminotetrol'** | - | - | - | 3.6 | 0.3 | 0.7 | - | - | - | - | - | - | - | - | - | 6.4 | 3.0 | 1.4 | 1.7 | 0.9 | 0.4 | - | - | - |
| **'Early eluting aminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Early eluting methylcarbamoylaminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | 2.8 | 0.6 | 0.5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Adenosylhopane** | 63 | 7.8 | 19 | 82 | 4.9 | 15 | 79 | 1.7 | 17 | 77 | 4.3 | 20 | 88 | 7.8 | 21 | 71 | 13 | 17 | 67 | 5.0 | 15 | 60 | 15 | 19 |
| **2-methyladenosylhopane** | 4.5 | 0.9 | 1.3 | 3.6 | 1.8 | 0.6 | 5.4 | 0.8 | 1.2 | 5.2 | 0.4 | 1.3 | 6.0 | 0.1 | 1.4 | 4.3 | 0.5 | 1.1 | 3.8 | - | 0.9 | 4.2 | 1.2 | 1.3 |
| **Adenosylhopane-type 2** | 15 | 0.5 | 4.4 | 18 | 1.4 | 3.3 | 18 | 0.7 | 3.9 | 15 | 0.1 | 3.9 | 18 | 2.7 | 4.2 | 16 | 3.1 | 3.9 | 17 | 1.4 | 3.8 | 14 | 2.9 | 4.4 |
| **2-methyladenosylhopane-type 2** | 4.5 | 0.5 | 1.3 | 5.3 | 0.3 | 1.0 | 4.9 | 0.3 | 1.1 | 4.6 | 0.3 | 1.2 | 5.6 | 0.5 | 1.3 | 4.6 | 0.8 | 1.1 | 4.7 | 0.8 | 1.1 | 3.9 | 0.4 | 1.3 |
| **Adenosylhopane-type 3** | 1.6 | 0.2 | 0.5 | 0.8 | 0.8 | 0.1 | 2.0 | 0.1 | 0.4 | 2.3 | 0.3 | 0.6 | 3.0 | 0.6 | 0.7 | 0.5 | 0.5 | 0.2 | 0.4 | 0.4 | 0.1 | 1.5 | 0.4 | 0.5 |
| **BHT pentose with unsaturation** | 11 | 0.5 | 3.3 | 16 | 2.2 | 3.0 | 12 | 1.5 | 2.7 | 14 | 2.1 | 3.5 | 15 | 4.0 | 3.3 | 9.0 | 1.4 | 2.2 | 12 | 1.7 | 2.8 | 9.9 | 2.8 | 3.1 |
| **BHT pentose** | 17 | 1.7 | 5.1 | 22 | 2.9 | 4.1 | 17 | 2.8 | 3.7 | 18 | 3.1 | 4.6 | 21 | 6.1 | 4.6 | 14 | 4.0 | 3.2 | 19 | 0.4 | 4.4 | 15 | 7.1 | 4.5 |
| **BHT cyclitol ether** | 48 | 7.2 | 14 | 90 | 5.3 | 17 | 79 | 13 | 17 | 51 | 7.1 | 13 | 66 | 22.3 | 14 | 52 | 16 | 12 | 76 | 6.9 | 17 | 34 | 4.8 | 11 |
| **BHT glucoasamine** | 4.2 | 0.6 | 1.3 | 7.8 | 0.7 | 1.5 | 5.7 | 0.1 | 1.3 | 4.4 | 0.1 | 1.1 | 7.9 | 2.1 | 1.8 | 4.7 | 0.9 | 1.1 | 6.1 | 0.7 | 1.4 | 3.9 | 1.2 | 1.2 |
| **BHpentol cyclitol ether** | - | - | - | 6.5 | 3.3 | 1.2 | 2.7 | 2.7 | 0.6 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **BHhexol cyclitol ether** | - | - | - | - | - | - | 3.4 | 2.5 | 0.7 | 0.9 | 0.9 | 0.2 | - | - | - | - | - | - | 0.7 | 0.7 | 0.2 | - | - | - |
| **Total (ug/gTOC)** | **339** | 42 | | **535** | 33 | | **458** | 28 | | **391** | 25 | | **443** | 112 | | **428** | 100 | | **437** | 17 | | **320** | 79 | |

BHP concentrations (µg/g TOC) in column one represent the average value extracted from a triplicate set of microcosm sediments (Time 0 all duplicate and pH 9 HK duplicate) with total BHPs summed at the bottom of column. Standard error is in column two and the relative % of individual compounds in column three.

# Appendix 3e: Total BHPs (µg/g TOC) in response to salinity in River Tyne estuarine sediment microcosms

| | Time 0 | | | 1 g/L NaCl 5% $CH_4$ | | | 1 g/L NaCl Unamended | | | 1 g/L NaCl Heat killed | | | 15 g/L NaCl 5% $CH_4$ | | | 15 g/L NaCl Unamended | | | 15 g/L NaCl Heat killed | | | 35 g/L NaCl 5% $CH_4$ | | | 35 g/L NaCl Unamended | | | 35 g/L NaCl Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 99 | 5.1 | 41 | 111 | 4.1 | 37 | 119 | 5.9 | 40 | 116 | 8.4 | 47 | 123 | 4.9 | 41 | 115 | 9.7 | 42 | 137 | 5.7 | 48 | 111 | 6.1 | 39 | 118 | 6.0 | 42 | 139 | 8.5 | 49 |
| **2-methylBHT** | 11 | 0.5 | 4.4 | 12 | 0.7 | 3.9 | 13 | 0.6 | 4.5 | 11 | 1.2 | 4.5 | 14 | 0.3 | 4.6 | 13 | 0.1 | 4.7 | 15 | 0.5 | 5.1 | 13 | 0.3 | 4.5 | 13 | 1.5 | 4.5 | 15 | 0.5 | 5.2 |
| **BHhexol** | 0.9 | 0.9 | 0.3 | 3.7 | 0.1 | 1.2 | 3.2 | 0.3 | 1.1 | 3.3 | 0.2 | 1.3 | 4.2 | 0.2 | 1.4 | 4.2 | 0.6 | 1.5 | 3.7 | 0.3 | 1.3 | 2.7 | 0.7 | 0.9 | 4.0 | 0.2 | 1.4 | 3.9 | 0.5 | 1.4 |
| **'Novel aminotriol'** | 10 | 0.8 | 4.3 | 7.8 | 0.5 | 2.6 | 7.3 | 0.4 | 2.4 | 6.6 | 1.0 | 2.7 | 6.9 | 0.5 | 2.3 | 5.8 | 0.5 | 2.2 | 7.3 | 0.3 | 2.6 | 7.5 | 1.4 | 2.6 | 7.2 | 0.5 | 2.6 | 7.9 | 1.0 | 2.7 |
| **Aminotriol** | 20 | 1.2 | 8.5 | 43 | 10 | 14 | 28 | 3.9 | 9.4 | 15 | 2.6 | 6.1 | 22 | 2.3 | 7.3 | 22 | 1.8 | 8.2 | 17 | 0.8 | 5.8 | 35 | 5.9 | 12 | 21 | 1.5 | 7.4 | 14 | 0.8 | 5.1 |
| **3-methylaminotriol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.5 | 0.5 | 0.2 | - | - | - | - | - | - |
| **'Methylcarbamoylaminotriol'** | 7.9 | 0.9 | 3.3 | 2.5 | 0.6 | 0.9 | 1.9 | 0.3 | 0.6 | - | - | - | 0.4 | 0.4 | 0.1 | 1.7 | 0.3 | 0.6 | - | - | - | 3.0 | 0.1 | 1.1 | 1.5 | 0.2 | 0.6 | - | - | - |
| **Aminotetrol** | 2.3 | 0.4 | 0.9 | 5.0 | 1.2 | 1.6 | 3.4 | 0.7 | 1.1 | 1.5 | 0.1 | 0.6 | 4.1 | 0.6 | 1.4 | 2.6 | 0.5 | 1.0 | 1.6 | 0.3 | 0.6 | 5.0 | 0.7 | 1.8 | 2.0 | 0.5 | 0.7 | 1.5 | 0.1 | 0.5 |
| **'Methylcarbamoylaminotetrol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Novel aminopentol'** | - | - | - | 0.2 | 0.2 | 0.1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aminopentol** | - | - | - | 7.6 | 1.9 | 2.4 | 2.6 | 0.1 | 0.9 | 0.9 | 0.1 | 0.3 | 8.0 | 0.6 | 2.7 | 1.6 | 0.0 | 0.6 | 0.4 | 0.4 | 0.1 | 4.6 | 1.1 | 1.6 | 1.2 | 0.3 | 0.4 | 0.2 | 0.2 | 0.1 |
| **3-methylaminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.3 | 0.3 | 0.1 | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | 0.2 | 0.2 | 0.1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Adenosylhopane** | 30 | 3.9 | 12 | 38 | 6.5 | 13 | 40 | 2.9 | 13 | 34 | 1.8 | 14 | 42 | 2.8 | 14 | 35 | 5.6 | 13 | 40 | 3.0 | 14 | 36 | 2.8 | 13 | 38 | 4.8 | 14 | 41 | 4.4 | 14 |
| **2-methyladenosylhopane** | 1.8 | 1.0 | 0.7 | 1.3 | 1.3 | 0.4 | 4.3 | 0.4 | 1.4 | 2.1 | 1.2 | 0.8 | 0.0 | 0.0 | 0.0 | 2.1 | 2.1 | 0.7 | 1.3 | 1.3 | 0.4 | 1.5 | 1.0 | 0.6 | 0.9 | 0.8 | 0.3 | 1.4 | 1.4 | 0.4 |
| **Adenosylhopane-type 2** | 11 | 1.1 | 4.8 | 12 | 0.5 | 3.9 | 12.5 | 0.3 | 4.2 | 11 | 0.2 | 4.5 | 13 | 0.5 | 4.5 | 12 | 0.6 | 4.5 | 14 | 1.1 | 5.0 | 12 | 0.5 | 4.2 | 13 | 1.0 | 4.6 | 13 | 0.7 | 4.6 |
| **2-methyladenosylhopane-type 2** | 2.9 | 0.1 | 1.2 | 3.6 | 0.5 | 1.2 | 4.0 | 0.3 | 1.3 | 3.4 | 0.2 | 1.4 | 4.1 | 0.5 | 1.4 | 3.4 | 0.1 | 1.2 | 4.0 | 0.4 | 1.4 | 4.4 | 0.5 | 1.6 | 4.1 | 0.1 | 1.5 | 4.3 | 0.7 | 1.5 |
| **Adenosylhopane-type 3** | 1.2 | 0.8 | 0.5 | 1.1 | 0.6 | 0.4 | 0.3 | 0.3 | 0.1 | 0.6 | 0.3 | 0.3 | 0.5 | 0.3 | 0.2 | 1.5 | 0.2 | 0.5 | 0.7 | 0.3 | 0.2 | 0.6 | 0.3 | 0.2 | 1.1 | 0.6 | 0.4 | 1.2 | 0.6 | 0.4 |
| **2-methyladenosylhopane-type 3** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **BHT pentose with unsaturation** | 4.2 | 0.9 | 1.7 | 7.4 | 1.0 | 2.5 | 7.6 | 0.9 | 2.5 | 5.3 | 0.9 | 2.1 | 8.9 | 1.1 | 3.0 | 8.1 | 2.9 | 2.9 | 5.9 | 1.5 | 2.1 | 5.8 | 1.4 | 2.0 | 7.9 | 1.2 | 2.7 | 8.0 | 1.6 | 2.7 |
| **BHT pentose** | 7.0 | 0.8 | 2.9 | 8.7 | 0.7 | 2.9 | 9.5 | 1.4 | 3.2 | 8.1 | 0.7 | 3.3 | 12 | 1.4 | 3.8 | 9.3 | 3.3 | 3.3 | 7.8 | 1.5 | 2.8 | 7.8 | 1.5 | 2.7 | 9.9 | 1.5 | 3.5 | 10 | 1.2 | 3.6 |
| **BHT cyclitol ether** | 27 | 3.4 | 11 | 34 | 5.5 | 11 | 36 | 4.9 | 12 | 24 | 4.4 | 9.5 | 30 | 4.5 | 9.9 | 31 | 3.7 | 11 | 25 | 2.9 | 9.0 | 31 | 6.2 | 11 | 31 | 4.6 | 11 | 21 | 1.5 | 7.2 |
| **BHT glucoasamine** | 3.7 | 0.8 | 1.5 | 4.2 | 0.6 | 1.4 | 4.8 | 0.4 | 1.6 | 3.5 | 1.0 | 1.4 | 6.0 | 0.9 | 2.0 | 4.8 | 0.8 | 1.7 | 3.1 | 1.6 | 1.1 | 3.6 | 0.6 | 1.2 | 5.3 | 0.9 | 1.9 | 4.5 | 0.9 | 1.6 |
| **BHpentol cyclitol ether** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1.5 | 1.5 | 0.5 | - | - | - |
| **BHhexol cyclitol ether** | - | - | - | 1.0 | 1.0 | 0.3 | 2.4 | 0.4 | 0.8 | 0.7 | 0.7 | 0.3 | 1.1 | 1.1 | 0.4 | 1.6 | 1.6 | 0.5 | - | - | - | - | - | - | 2.1 | 1.1 | 0.7 | - | - | - |
| **Total (ug/gTOC)** | **241** | 20 | | **304** | 25 | | **300** | 19 | | **247** | 22 | | **300** | 11 | | **275** | 28 | | **283** | 3.8 | | **285** | 20 | | **283** | 17 | | **287** | 23 | |

| | 70 g/L NaCl 5% $CH_4$ | | | 70 g/L NaCl Unamended | | | 70 g/L NaCl Heat killed | | | 120 g/L NaCl 5% $CH_4$ | | | 120 g/L NaCl Unamended | | | 120 g/L NaCl Heat killed | | | 150 g/L NaCl 5% $CH_4$ | | | 150 g/L NaCl Unamended | | | 150 g/L NaCl Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 112 | 7.4 | 40 | 96 | 4.1 | 41 | 129 | 10 | 47 | 94 | 5.1 | 45 | 109 | 11.8 | 43 | 101 | 9.4 | 49 | 104 | 14 | 44 | 114 | 3.0 | 43 | 121 | 8.8 | 48 |
| **2-methylBHT** | 12 | 1.1 | 4.3 | 10 | 0.6 | 4.3 | 13 | 1.5 | 4.8 | 9.6 | 1.5 | 4.6 | 11 | 1.9 | 4.3 | 10 | 0.8 | 5.1 | 11 | 1.1 | 4.5 | 11 | 0.7 | 4.1 | 12 | 1.1 | 4.6 |
| **BHhexol** | 1.9 | 0.9 | 0.7 | 3.2 | 0.3 | 1.4 | 4.2 | 2.1 | 1.4 | 1.2 | 0.6 | 0.6 | 3.9 | 0.7 | 1.5 | 3.1 | 1.8 | 1.4 | 1.8 | 1.8 | 0.7 | 3.5 | 0.5 | 1.3 | 4.0 | 1.1 | 1.5 |
| **'Novel aminotriol'** | 8.8 | 0.4 | 3.2 | 6.9 | 0.5 | 2.9 | 7.8 | 0.6 | 2.9 | 7.1 | 0.4 | 3.4 | 6.5 | 1.2 | 2.6 | 5.8 | 0.5 | 2.8 | 6.5 | 1.8 | 2.7 | 6.1 | 0.7 | 2.3 | 7.8 | 0.8 | 3.1 |
| **Aminotriol** | 34 | 6.2 | 12 | 18 | 2.8 | 7.4 | 14 | 0.9 | 5.2 | 18 | 1.7 | 8.5 | 16 | 3.1 | 6.3 | 11 | 1.3 | 5.2 | 19 | 0.4 | 8.3 | 24 | 4.1 | 8.8 | 18 | 4.4 | 7.4 |
| **3-methylaminotriol** | 2.3 | 2.3 | 0.8 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Methylcarbamoylaminotriol'** | 3.3 | 0.5 | 1.2 | 1.8 | 0.1 | 0.8 | 0.9 | 0.5 | 0.3 | 1.9 | 0.2 | 0.9 | 2.0 | 0.2 | 0.8 | 0.6 | 0.6 | 0.2 | 1.4 | 0.3 | 0.6 | 2.6 | 0.1 | 1.0 | 1.1 | 0.6 | 0.4 |
| **Aminotetrol** | 5.6 | 0.4 | 2.0 | 1.7 | 0.3 | 0.7 | 1.4 | 0.3 | 0.5 | 2.3 | 0.3 | 1.1 | 1.9 | 0.4 | 0.7 | 0.8 | 0.2 | 0.4 | 2.4 | 0.1 | 1.0 | 2.7 | 0.7 | 1.0 | 0.8 | 0.5 | 0.3 |
| **'Methylcarbamoylaminotetrol'** | 0.3 | 0.3 | 0.1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Novel aminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aminopentol** | 5.6 | 2.9 | 2.1 | 1.3 | 0.2 | 0.6 | - | - | - | 0.9 | 0.5 | 0.5 | 1.1 | 0.1 | 0.4 | 0.2 | 0.2 | 0.1 | 1.7 | 0.5 | 0.7 | 0.6 | 0.6 | 0.2 | - | - | - |
| **3-methylaminopentol** | 1.4 | 0.8 | 0.5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Adenosylhopane** | 33 | 1.3 | 12 | 32 | 4.2 | 13 | 36 | 0.6 | 14 | 30 | 1.1 | 15 | 39 | 2.6 | 16 | 32 | 2.2 | 15 | 38 | 4.1 | 16 | 33 | 2.8 | 12 | 39 | 3.7 | 16 |
| **2-methyladenosylhopane** | 0.7 | 0.7 | 0.3 | - | - | - | 2.4 | 0.4 | 0.9 | - | - | - | 1.9 | 1.0 | 0.8 | 2.2 | 1.2 | 1.2 | - | - | - | 1.2 | 1.2 | 0.5 | 1.1 | 0.6 | 0.5 |
| **Adenosylhopane-type 2** | 12 | 0.8 | 4.5 | 11 | 0.5 | 4.8 | 12 | 0.4 | 4.6 | 9.6 | 0.4 | 4.6 | 11 | 0.4 | 4.5 | 11 | 0.6 | 5.3 | 12 | 0.8 | 5.0 | 12 | 0.2 | 4.4 | 12 | 1.1 | 4.7 |
| **2-methyladenosylhopane-type 2** | 3.8 | 0.1 | 1.4 | 3.2 | 0.3 | 1.4 | 4.5 | 0.3 | 1.7 | 3.0 | 0.1 | 1.5 | 4.0 | 0.6 | 1.6 | 3.2 | 0.5 | 1.6 | 3.3 | 0.2 | 1.4 | 3.4 | 0.3 | 1.3 | 3.6 | 0.3 | 1.4 |
| **Adenosylhopane-type 3** | 1.2 | 0.7 | 0.4 | 0.5 | 0.5 | 0.2 | 1.6 | 0.9 | 0.6 | 0.9 | 0.5 | 0.5 | 1.7 | 0.6 | 0.7 | 1.1 | 0.6 | 0.5 | - | - | - | 1.8 | 0.3 | 0.7 | 1.4 | 0.2 | 0.6 |
| **2-methyladenosylhopane-type 3** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.1 | 0.1 | - | - | - | - |
| **BHT pentose with unsaturation** | 6.0 | 1.7 | 2.1 | 5.9 | 1.4 | 2.4 | 7.8 | 2.5 | 2.7 | 3.5 | 0.7 | 1.6 | 6.8 | 1.5 | 2.7 | 4.7 | 1.6 | 2.2 | 4.3 | 1.7 | 1.7 | 6.7 | 0.8 | 2.5 | 4.4 | 2.3 | 1.6 |
| **BHT pentose** | 6.8 | 1.3 | 2.4 | 8.2 | 1.9 | 3.4 | 9.9 | 2.9 | 3.5 | 5.3 | 1.1 | 2.5 | 9.8 | 2.6 | 3.8 | 5.3 | 1.7 | 2.4 | 6.9 | 2.3 | 2.8 | 8.0 | 1.0 | 3.0 | 6.3 | 3.2 | 2.4 |
| **BHT cyclitol ether** | 22 | 2.0 | 8.1 | 32 | 8.0 | 13 | 22 | 3.9 | 8.1 | 20 | 4.3 | 9.3 | 22 | 3.6 | 8.7 | 12 | 3.0 | 5.8 | 21 | 1.8 | 8.9 | 33 | 4.7 | 12 | 15 | 3.7 | 5.9 |
| **BHT glucoasamine** | 4.0 | 0.8 | 1.4 | 3.8 | 0.4 | 1.6 | 4.2 | 0.3 | 1.5 | 3.1 | 0.7 | 1.5 | 4.3 | 0.7 | 1.7 | 3.3 | 0.8 | 1.6 | 4.2 | - | 1.8 | 4.4 | 0.1 | 1.7 | 4.5 | 1.0 | 1.7 |
| **BHpentol cyclitol ether** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **BHhexol cyclitol ether** | - | - | - | 1.0 | 1.0 | 0.4 | - | - | - | - | - | - | 1.0 | 1.0 | 0.4 | - | - | - | - | - | - | - | - | - | - | - | - |
| **Total (ug/gTOC)** | **277** | 9.1 | | **237** | 23 | | **272** | 25 | | **211** | 12 | | **253** | 23 | | **207** | 22 | | **238** | 31 | | **267** | 6.5 | | **251** | 17 | |

BHP concentrations (µg/g TOC) in column one represent the average value extracted from a triplicate set of microcosm sediments (15 g/L Unamended NaCl and 150 g/L NaCl 5% $CH_4$ duplicate) with total BHPs summed at the bottom of column. Standard error is in column two and the relative % of individual compounds in column three.

# Appendix 3f: Total BHPs (µg/g TOC) in response to bacterial growth phase River Tyne estuarine sediment microcosms

**30°C**

| | Time 0 | | | 5 days 5% $CH_4$ | | | 7 days 5% $CH_4$ | | | 11 days 5% $CH_4$ | | | 15 days 5% $CH_4$ | | | 20 days 5% $CH_4$ | | | 28 days 5% $CH_4$ | | | Unamended | | | Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 127 | 7.2 | 30 | 153 | 4.7 | 29 | 131 | 5.5 | 31 | 138 | 8.4 | 30 | 169 | 18 | 30 | 163 | 26 | 29 | 151 | 17 | 27 | 151 | 2.5 | 32 | 152 | 6.1 | 40 |
| **2-methylBHT** | 12.9 | 0.8 | 3.0 | 15.8 | 1.4 | 3.0 | 13 | 0.6 | 3.1 | 14 | 0.4 | 3.1 | 17 | 2.0 | 2.9 | 17 | 3.5 | 3.0 | 15 | 1.6 | 2.7 | 14 | 0.3 | 3.0 | 15 | 0.2 | 3.9 |
| **BHhexol** | 1.2 | 0.6 | 0.3 | - | - | - | - | - | - | - | - | - | 2.6 | 1.5 | 0.5 | 3.9 | 0.5 | 0.7 | 1.2 | 1.2 | 0.2 | 1.7 | 0.9 | 0.4 | 2.1 | 1.0 | 0.5 |
| **'Novel aminotriol'** | 4.7 | 0.4 | 1.1 | 5.6 | 0.8 | 1.1 | 5.8 | 1.3 | 1.4 | 5.4 | 0.4 | 1.2 | 5.8 | 0.6 | 1.0 | 5.9 | 1.1 | 1.0 | 6.4 | 0.5 | 1.2 | 5.6 | 0.4 | 1.2 | 6.0 | 0.5 | 1.6 |
| **Aminotriol** | 30 | 6.7 | 6.8 | 30 | 2.0 | 5.7 | 28 | 1.1 | 6.5 | 35 | 4.0 | 7.6 | 48 | 6.1 | 8.5 | 47 | 5.7 | 8.7 | 47 | 3.3 | 8.4 | 43 | 3.3 | 9.0 | 13 | 0.8 | 3.4 |
| **'Methylcarbamoylaminotriol'** | 2.0 | 0.4 | 0.5 | 2.2 | 0.3 | 0.4 | 1.8 | 0.2 | 0.4 | 1.8 | 0.4 | 0.4 | 2.7 | 0.6 | 0.5 | 4.5 | 0.6 | 0.8 | 4.2 | 0.7 | 0.8 | 2.6 | 0.6 | 0.5 | 0.7 | 0.4 | 0.2 |
| **Aminotetrol** | 2.9 | 0.8 | 0.7 | 5.3 | 0.2 | 1.0 | 7.0 | 0.5 | 1.6 | 8.5 | 1.9 | 1.8 | 8.6 | 2.2 | 1.5 | 9.9 | 1.4 | 1.8 | 12 | 2.7 | 2.2 | 4.3 | 0.4 | 0.9 | 0.4 | 0.4 | 0.1 |
| **'Early eluting aminopentol'** | - | - | - | - | - | - | 1.9 | 1.6 | 0.3 | 1.8 | 1.8 | 0.4 | 1.8 | 1.8 | 0.3 | 1.3 | 0.7 | 0.2 | 3.2 | 0.9 | 0.6 | - | - | - | - | - | - |
| **'Novel aminopentol'** | - | - | - | - | - | - | 0.6 | 0.6 | 0.2 | - | - | - | 0.6 | 0.6 | 0.1 | 0.3 | 0.3 | 0.1 | 0.9 | 0.4 | 0.2 | - | - | - | - | - | - |
| **Unsaturated aminopentol** | - | - | - | - | - | - | - | - | - | 0.5 | 0.5 | 0.1 | 0.2 | 0.2 | < 0.1 | 2.1 | 1.6 | 0.4 | 1.7 | 0.8 | 0.3 | - | - | - | - | - | - |
| **Aminopentol** | 0.8 | 0.8 | 0.2 | 11 | 2.6 | 2.1 | 15 | 2.1 | 3.6 | 16 | 3.6 | 3.5 | 23 | 5.9 | 4.1 | 27 | 7.2 | 4.8 | 33 | 9.3 | 6.0 | 3.0 | 0.4 | 0.6 | - | - | - |
| **Unsaturated 3-methylaminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **3-methylaminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.4 | 0.4 | 0.1 | - | - | - | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1.3 | 0.7 | 0.2 | 1.5 | 0.8 | 0.3 | - | - | - | - | - | - |
| **Adenosylhopane** | 81 | 3.4 | 19 | 107 | 12 | 20 | 73 | 6.4 | 17 | 78 | 5.1 | 17 | 92 | 7.6 | 16 | 82 | 14 | 15 | 90 | 9.9 | 16.1 | 85 | 2.2 | 18 | 84 | 2.9 | 22 |
| **2-methyladenosylhopane** | 6.5 | 0.8 | 1.5 | 8.1 | 0.9 | 1.5 | 5.8 | 0.6 | 1.3 | 7.0 | 1.0 | 1.5 | 7.8 | 1.0 | 1.4 | 6.0 | 0.1 | 1.1 | 6.2 | 1.1 | 1.1 | 7.0 | 0.5 | 1.5 | 6.3 | 0.6 | 1.7 |
| **Adenosylhopane-type 2** | 18 | 1.1 | 4.3 | 22 | 2.3 | 4.1 | 17 | 1.2 | 3.9 | 17 | 1.3 | 3.8 | 24 | 3.4 | 4.1 | 20 | 3.7 | 3.5 | 21 | 3.5 | 3.8 | 19 | 1.3 | 4.0 | 19 | 1.4 | 4.9 |
| **2-methyladenosylhopane-type 2** | 3.9 | 0.2 | 0.9 | 5.8 | 0.9 | 1.1 | 3.7 | 0.4 | 0.9 | 3.9 | 0.1 | 0.9 | 5.0 | 0.6 | 0.9 | 4.4 | 0.5 | 0.8 | 4.5 | 0.4 | 0.8 | 3.9 | 0.2 | 0.8 | 4.5 | 0.7 | 1.2 |
| **Adenosylhopane-type 3** | 2.3 | 0.4 | 0.5 | 2.8 | 0.4 | 0.5 | 1.5 | 0.1 | 0.3 | 2.6 | 0.5 | 0.6 | 2.7 | 0.4 | 0.5 | 1.1 | 0.6 | 0.2 | 2.2 | 0.3 | 0.4 | 1.9 | 0.3 | 0.4 | 2.8 | 0.2 | 0.7 |
| **BHT pentose with unsaturation** | 14 | 1.1 | 3.3 | 17 | 1.9 | 3.3 | 13 | 0.8 | 3.0 | 13 | 0.4 | 2.9 | 16 | 2.0 | 2.9 | 18 | 2.8 | 3.2 | 16 | 0.9 | 2.9 | 14 | 0.3 | 2.9 | 14 | 0.8 | 3.8 |
| **BHT pentose** | 19.5 | 0.8 | 4.6 | 24 | 2.5 | 4.6 | 21 | 1.9 | 5.0 | 19 | 1.1 | 4.1 | 23 | 0.8 | 4.0 | 25 | 4.5 | 4.4 | 25 | 0.7 | 4.5 | 21 | 0.8 | 4.3 | 20 | 1.6 | 5.3 |
| **BHT cyclitol ether** | 85 | 18 | 20 | 99 | 4.4 | 19 | 74 | 5.7 | 17 | 83 | 5.4 | 18 | 107 | 1.2 | 19 | 103 | 18 | 18 | 96 | 3.0 | 17 | 86 | 10.7 | 18 | 33 | 3.4 | 8.8 |
| **BHT glucoasamine** | 6.0 | 0.5 | 1.4 | 6.6 | 0.2 | 1.3 | 5.9 | 0.5 | 1.4 | 5.5 | 0.3 | 1.2 | 6.7 | 1.2 | 1.2 | 5.9 | 0.9 | 1.1 | 6.3 | 0.5 | 1.1 | 5.8 | 0.4 | 1.2 | 4.2 | 0.3 | 1.1 |
| **BHpentol cyclitol ether** | 5.9 | 1.2 | 1.4 | 9.0 | 0.4 | 1.7 | 5.2 | 0.3 | 1.2 | 7.3 | 0.5 | 1.6 | 5.1 | 2.6 | 0.9 | 6.0 | 1.0 | 1.1 | 7.4 | 0.8 | 1.3 | 6.4 | 1.5 | 1.3 | - | - | - |
| **BHhexol cyclitol ether** | 2.9 | 0.9 | 0.7 | 4.4 | 0.5 | 0.8 | 2.4 | 0.1 | 0.6 | 2.6 | 0.1 | 0.6 | - | - | - | 4.6 | 0.7 | 0.8 | 4.1 | 2.1 | 0.8 | 1.3 | 1.3 | 0.3 | - | - | - |
| **Total (ug/gTOC)** | **426** | 38 | | **529** | 30 | | **427** | 20 | | **460** | 27 | | **569** | 25 | | **559** | 83 | | **555** | 21 | | **477** | 9 | | **376** | 13 | |

**50°C**

| | 4 days 5% $CH_4$ | | | 8 days 5% $CH_4$ | | | 11 days 5% $CH_4$ | | | 15 days 5% $CH_4$ | | | 20 days 5% $CH_4$ | | | 28 days 5% $CH_4$ | | | Unamended | | | Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **BHT** | 151 | 21 | 32 | 149 | 19 | 31 | 169 | 19 | 34 | 190 | 27 | 34 | 156 | 4.5 | 32 | 163 | 4.3 | 33 | 177 | 19 | 36 | 149 | 2.0 | 41 |
| **2-methylBHT** | 15 | 2.6 | 3.1 | 13 | 1.0 | 2.8 | 16 | 1.7 | 3.3 | 17 | 2.7 | 3.0 | 14 | 0.5 | 2.9 | 15 | 0.7 | 3.0 | 17 | 2.0 | 3.4 | 15 | 0.3 | 4.2 |
| **BHhexol** | 0.6 | 0.6 | 0.1 | 2.0 | 1.0 | 0.4 | 3.6 | 0.7 | 0.7 | 1.5 | 1.5 | 0.3 | 1.6 | 1.6 | 0.3 | 2.1 | 1.1 | 0.4 | 4.2 | 2.1 | 0.8 | 3.2 | 0.9 | 0.9 |
| **'Novel aminotriol'** | 4.9 | 0.4 | 1.1 | 5.3 | 0.4 | 1.1 | 4.9 | 0.7 | 1.0 | 5.9 | 0.5 | 1.1 | 5.6 | 0.3 | 1.2 | 4.7 | 0.5 | 1.0 | 5.5 | 0.4 | 1.1 | 5.7 | 0.2 | 1.6 |
| **Aminotriol** | 28 | 1.8 | 6.1 | 26 | 0.7 | 5.5 | 21 | 1.8 | 4.3 | 26 | 2.3 | 4.7 | 22 | 0.5 | 4.5 | 23 | 3.3 | 4.7 | 24 | 0.3 | 4.9 | 12 | 0.3 | 3.3 |
| **'Methylcarbamoylaminotriol'** | 0.4 | 0.4 | 0.1 | 1.3 | 0.2 | 0.3 | 0.8 | 0.4 | 0.2 | 0.3 | 0.3 | 0.1 | 2.7 | 0.1 | 0.6 | 2.1 | 0.1 | 0.4 | 1.9 | 0.4 | 0.4 | 0.6 | 0.3 | 0.1 |
| **Aminotetrol** | 3.2 | 0.4 | 0.7 | 3.0 | 0.3 | 0.6 | 2.6 | 0.5 | 0.5 | 3.0 | 0.8 | 0.6 | 3.4 | 0.3 | 0.7 | 2.7 | 0.8 | 0.5 | 2.3 | 0.2 | 0.5 | 0.3 | 0.3 | 0.1 |
| **'Early eluting aminopentol'** | - | - | - | - | - | - | - | - | - | 2.0 | 1.0 | 0.4 | - | - | - | 1.4 | 1.4 | 0.3 | - | - | - | - | - | - |
| **'Novel aminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | 0.6 | 0.4 | 0.1 | - | - | - | - | - | - | - | - | - |
| **Unsaturated aminopentol** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aminopentol** | 7.6 | 2.8 | 1.6 | 9.9 | 2.5 | 2.1 | 8.2 | 1.1 | 1.6 | 7.7 | 0.3 | 1.4 | 9.5 | 3.4 | 2.0 | 9.5 | 2.9 | 1.9 | 1.3 | 0.7 | 0.3 | - | - | - |
| **Unsaturated 3-methylaminopentol** | 2.2 | 1.3 | 0.4 | 4.7 | 0.7 | 1.0 | 2.8 | 0.5 | 0.6 | 3.6 | 0.4 | 0.7 | 4.3 | 0.9 | 0.9 | 5.0 | 1.1 | 1.0 | - | - | - | - | - | - |
| **3-methylaminopentol** | 7.0 | 3.8 | 1.4 | 12 | 2.0 | 2.6 | 9.6 | 1.4 | 1.9 | 10 | 1.0 | 1.9 | 11 | 2.4 | 2.2 | 9.5 | 2.2 | 1.9 | - | - | - | - | - | - |
| **'Methylcarbamoylaminopentol'** | - | - | - | - | - | - | - | - | - | - | - | - | 0.4 | 0.4 | 0.1 | - | - | - | - | - | - | - | - | - |
| **Adenosylhopane** | 87 | 7.6 | 19 | 88 | 6.6 | 19 | 98 | 11 | 20 | 99 | 7.7 | 18 | 92 | 11 | 19 | 93 | 6.0 | 19 | 99 | 4.8 | 20 | 79 | 2.3 | 22 |
| **2-methyladenosylhopane** | 4.2 | 2.2 | 1.0 | 7.4 | 0.5 | 1.6 | 9.1 | 0.9 | 1.8 | 9.2 | 1.2 | 1.7 | 7.5 | 0.7 | 1.5 | 7.7 | 1.0 | 1.6 | 7.4 | 0.8 | 1.5 | 7.6 | 0.9 | 2.1 |
| **Adenosylhopane-type 2** | 21 | 2.6 | 4.5 | 19 | 2.0 | 4.1 | 23 | 3.2 | 4.6 | 24 | 3.4 | 4.3 | 19 | 1.9 | 3.9 | 22 | 1.9 | 4.5 | 22 | 2.4 | 4.4 | 18 | 0.7 | 5.0 |
| **2-methyladenosylhopane-type 2** | 4.5 | 0.7 | 0.9 | 4.7 | 0.8 | 1.0 | 5.3 | 0.9 | 1.0 | 5.3 | 1.0 | 1.0 | 3.9 | 0.3 | 0.8 | 4.4 | 0.6 | 0.9 | 5.5 | 0.4 | 1.1 | 3.9 | 0.2 | 1.1 |
| **Adenosylhopane-type 3** | 1.9 | 0.2 | 0.4 | 2.3 | 0.3 | 0.5 | 3.1 | 0.5 | 0.6 | 2.8 | 0.3 | 0.5 | 3.2 | 0.2 | 0.7 | 3.5 | 0.7 | 0.7 | 2.9 | 0.5 | 0.6 | 2.5 | 0.3 | 0.7 |
| **BHT pentose with unsaturation** | 15 | 0.9 | 3.3 | 18 | 1.0 | 3.8 | 19 | 0.9 | 3.8 | 17 | 0.8 | 3.1 | 18 | 1.5 | 3.7 | 17 | 0.2 | 3.4 | 18 | 2.3 | 3.7 | 13 | 1.2 | 3.6 |
| **BHT pentose** | 23 | 1.4 | 5.0 | 24 | 0.3 | 5.2 | 27 | 2.7 | 5.4 | 27 | 1.8 | 4.9 | 26 | 2.4 | 5.3 | 24 | 0.3 | 5.0 | 25 | 2.1 | 5.2 | 19 | 2.2 | 5.4 |
| **BHT cyclitol ether** | 79 | 9.0 | 17 | 71 | 1.9 | 15 | 70 | 16 | 14 | 86 | 10 | 16 | 77 | 3.7 | 16 | 72 | 4.5 | 15 | 68 | 8.4 | 14 | 28 | 2.8 | 7.7 |
| **BHT glucoasamine** | 6.1 | 0.1 | 1.3 | 5.7 | 0.2 | 1.2 | 5.8 | 0.4 | 1.2 | 8.3 | 1.5 | 1.5 | 5.0 | 0.3 | 1.0 | 6.6 | 0.6 | 1.3 | 6.1 | 0.5 | 1.3 | 3.5 | 0.2 | 1.0 |
| **BHpentol cyclitol ether** | 5.9 | 0.5 | 1.3 | 3.5 | 1.8 | 0.8 | - | - | - | 3.5 | 1.8 | 0.7 | 4.2 | 2.1 | 0.9 | 2.2 | 2.2 | 0.4 | 1.9 | 1.9 | 0.4 | - | - | - |
| **BHhexol cyclitol ether** | 2.2 | 0.3 | 0.5 | 2.2 | 1.2 | 0.5 | - | - | - | 0.6 | 0.6 | 0.1 | 2.1 | 1.1 | 0.4 | - | - | - | 1.0 | 1.0 | 0.2 | - | - | - |
| **Total (ug/gTOC)** | **470** | 52 | | **474** | 25 | | **497** | 58 | | **549** | 56 | | **489** | 8.1 | | **491** | 3.7 | | **490** | 41 | | **360** | 14 | |

BHP concentrations (µg/g TOC) in column one represent the average value extracted from a triplicate set of microcosm sediments with total BHPs summed at the bottom of column. Standard error is in column two and the relative % of individual compounds in column three.

# Appendix 3g: Total BHPs (µg/g TOC) in sulphate-reducing River Tyne estuarine sediment microcosms

| | Time 0 Amended | | | Time 0 Inhibited | | | Time 0 Heat killed | | | 28 days Amended | | | 28 days Heat killed | | | 56 days Amended | | | 56 days Inhibited | | | 56 days Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **AnhydroBHT** | 0.5 | 0.5 | 0.1 | - | - | - | 0.8 | 0.8 | 0.2 | 0.5 | 0.5 | 0.1 | 1.6 | 1.6 | 0.3 | - | - | - | 0.6 | 0.6 | 0.1 | 1.0 | 1.0 | 0.2 |
| **BHT** | 146 | 12 | 29 | 147 | 0.5 | 32 | 158 | 16 | 35 | 140 | 8.2 | 29 | 164 | 18 | 32 | 124 | 5.2 | 32 | 137 | 8.2 | 33 | 183 | 11 | 33 |
| **2-methylBHT** | 13 | 1.2 | 2.6 | 15 | 0.8 | 3.2 | 13 | 1.7 | 3.0 | 13 | 0.6 | 2.8 | 15 | 2.1 | 2.9 | 11 | 1.0 | 2.9 | 13 | 1.0 | 3.0 | 17 | 0.6 | 3.0 |
| **BHhexol** | 2.8 | 0.5 | 0.5 | 5 | 0.4 | 1.0 | 2.1 | 1.1 | 0.4 | 2.6 | 1.4 | 0.5 | 5.3 | 0.9 | 1.0 | 1.7 | 1.0 | 0.5 | 4.7 | 0.3 | 1.1 | 5.9 | 0.6 | 1.1 |
| **'Novel aminotriol'** | 15 | 3.0 | 2.9 | 11 | 2.2 | 2.3 | 11 | 1.0 | 2.5 | 13 | 1.5 | 2.7 | 12 | 1.9 | 2.3 | 8.8 | 0.7 | 2.2 | 8.0 | 0.1 | 1.9 | 13 | 0.7 | 2.3 |
| **Aminotriol** | 26 | 2.0 | 5.1 | 24 | 1.1 | 5.2 | 19 | 3.4 | 4.1 | 25 | 1.8 | 5.3 | 20 | 1.5 | 4.1 | 20 | 1.0 | 5.0 | 22 | 2.0 | 5.1 | 24 | 0.5 | 4.4 |
| **'Methylcarbamoylaminotriol'** | 13 | 0.6 | 2.6 | 5.1 | 0.8 | 1.1 | 2.4 | 0.6 | 0.5 | 10 | 1.0 | 2.2 | 3.2 | 0.2 | 0.6 | 8.9 | 0.6 | 2.3 | 4.8 | 0.7 | 1.1 | 3.0 | 0.1 | 0.5 |
| **Aminotetrol** | 3.5 | 0.1 | 0.7 | 3.0 | 0.2 | 0.6 | 2.1 | 0.4 | 0.5 | 2.8 | 0.1 | 0.6 | 1.8 | 0.2 | 0.4 | 1.8 | 0.3 | 0.5 | 2.7 | 0.4 | 0.6 | 2.7 | 0.1 | 0.5 |
| **Adenosylhopane** | 100 | 6.7 | 20 | 88 | 8.3 | 19 | 112 | 3.9 | 25 | 105 | 6.5 | 22 | 113 | 8.9 | 22 | 92 | 8.1 | 23 | 88 | 7.7 | 21 | 114 | 5.1 | 21 |
| **2-methyladenosylhopane** | 7.6 | 0.6 | 1.5 | 7.0 | 0.5 | 1.5 | 8.4 | 0.5 | 1.9 | 8.5 | 0.1 | 1.8 | 9.2 | 0.7 | 1.8 | 7.6 | 1.3 | 1.9 | 6.4 | 0.5 | 1.5 | 8.8 | 0.6 | 1.6 |
| **Adenosylhopane-type 2** | 16 | 0.3 | 3.2 | 17 | 1.0 | 3.6 | 16 | 0.4 | 3.6 | 16 | 0.9 | 3.4 | 18 | 1.4 | 3.5 | 15 | 1.3 | 3.9 | 14 | 0.8 | 3.3 | 19 | 1.0 | 3.4 |
| **2-methyladenosylhopane-type 2** | 4.1 | 0.1 | 0.8 | 4.7 | 0.1 | 1.0 | 4.1 | 0.2 | 0.9 | 4.4 | 0.2 | 0.9 | 4.4 | 0.1 | 0.9 | 3.9 | 0.3 | 1.0 | 4.3 | 0.2 | 1.0 | 5.2 | 0.3 | 0.9 |
| **Adenosylhopane-type 3** | 3.1 | 0.5 | 0.6 | 2.9 | 0.4 | 0.6 | 3.3 | 0.6 | 0.8 | 3.3 | 0.2 | 0.7 | 3.1 | 0.8 | 0.6 | 2.7 | 0.4 | 0.7 | 2.6 | 0.4 | 0.6 | 3.8 | 0.5 | 0.7 |
| **2-methyladenosylhopane-type 3** | - | - | - | 0.3 | 0.3 | 0.1 | 0.5 | 0.5 | 0.1 | - | - | - | - | - | - | 0.4 | 0.4 | 0.1 | 0.9 | 0.5 | 0.2 | 0.6 | 0.6 | 0.1 |
| **BHT pentose with unsat.** | 19 | 1.0 | 3.8 | 19 | 0.4 | 4.2 | 16 | 3.8 | 3.5 | 19 | 2.8 | 4.0 | 26 | 4.5 | 5.0 | 13 | 1.4 | 3.4 | 17 | 1.0 | 4.1 | 24 | 1.8 | 4.3 |
| **BHT pentose** | 30 | 2.3 | 5.9 | 29 | 0.4 | 6.2 | 24 | 4.7 | 5.2 | 28 | 2.8 | 5.9 | 36 | 6.3 | 7.1 | 21 | 1.6 | 5.3 | 27 | 2.3 | 6.3 | 34 | 0.8 | 6.1 |
| **BHT cyclitol ether** | 87 | 4.7 | 17 | 68 | 3.7 | 15 | 50 | 12 | 11 | 67 | 5.0 | 14 | 58 | 4.7 | 11 | 49 | 3.8 | 12 | 56 | 10 | 13 | 76 | 3.8 | 14 |
| **BHT glucoasamine** | 7.2 | 0.4 | 1.4 | 7.1 | 0.2 | 1.5 | 6.0 | 1.1 | 1.3 | 6.6 | 0.5 | 1.4 | 6.8 | 1.6 | 1.3 | 6.1 | 0.1 | 1.6 | 7.0 | 0.2 | 1.7 | 7.2 | 0.3 | 1.3 |
| **BHpentol cyclitol ether** | 8.3 | 0.5 | 1.6 | 6.0 | 0.3 | 1.3 | 3.2 | 1.8 | 0.7 | 7.0 | 0.6 | 1.5 | 5.6 | 1.0 | 1.1 | 4.4 | 1.0 | 1.1 | 5.2 | 1.0 | 1.2 | 7.9 | 0.5 | 1.4 |
| **BHhexol cyclitol ether** | 3.4 | 1.9 | 0.6 | 4.4 | 0.3 | 1.0 | 0.9 | 0.9 | 0.2 | 3.2 | 2.0 | 0.7 | 3.8 | 0.7 | 0.8 | 1.4 | 1.4 | 0.4 | 3.8 | 0.9 | 0.9 | 3.5 | 1.8 | 0.7 |
| **Total (ug/gTOC)** | **506** | 29 | | **462** | 14 | | **453** | 42 | | **475** | 26 | | **505** | 57 | | **394** | 18 | | **423** | 28 | | **552** | 23 | |

| | 94 days Amended | | | 94 days Inhibited | | | 94 days Heat killed | | | 433 days Amended | | | 433 days Inhibited | | | 433 days Heat killed | | | 465 days Amended | | | 465 days Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **AnhydroBHT** | 1.3 | 0.7 | 0.3 | 0.7 | 0.7 | 0.1 | 1.6 | 0.8 | 0.3 | 1.5 | 0.8 | 0.4 | 1.8 | 0.2 | 0.4 | 2.1 | 0.4 | 0.5 | 0.7 | 0.7 | 0.2 | 2.9 | 0.1 | 0.6 |
| **BHT** | 137 | 8.2 | 31 | 145 | 8.3 | 31 | 159 | 12 | 35 | 120 | 19 | 32 | 138 | 23 | 29 | 161 | 3.7 | 39 | 145 | 1.0 | 32 | 151 | 7.1 | 33 |
| **2-methylBHT** | 13 | 0.7 | 3.0 | 13 | 0.8 | 2.8 | 15 | 1.1 | 3.2 | 12 | 2.4 | 3.2 | 13 | 2.4 | 2.7 | 13 | 0.6 | 3.1 | 14 | 0.3 | 3.2 | 14 | 0.5 | 3.0 |
| **BHhexol** | 0.8 | 0.8 | 0.2 | 3.7 | 0.2 | 0.8 | 1.4 | 1.4 | 0.3 | 1.2 | 1.2 | 0.3 | 3.7 | 1.0 | 0.8 | - | - | - | 4.6 | 0.8 | 1.0 | 5.4 | 0.7 | 1.2 |
| **'Novel aminotriol'** | 11 | 0.7 | 2.6 | 8.3 | 0.4 | 1.8 | 8.1 | 0.6 | 1.8 | 9.9 | 2.2 | 2.6 | 10 | 1.1 | 2.2 | 7.6 | 0.9 | 1.8 | 12 | 0.9 | 2.7 | 6.9 | 0.5 | 1.5 |
| **Aminotriol** | 21 | 1.3 | 4.9 | 24 | 0.7 | 5.2 | 21 | 0.6 | 4.6 | 16 | 2.4 | 4.3 | 30 | 1.4 | 6.5 | 16 | 0.9 | 3.8 | 19 | 2.4 | 4.1 | 17 | 0.6 | 3.7 |
| **'Methylcarbamoylaminotriol'** | 10 | 0.2 | 2.4 | 5.3 | 0.3 | 1.1 | 2.4 | 0.3 | 0.5 | 5.8 | 1.2 | 1.5 | 4.9 | 0.3 | 1.1 | 0.9 | 0.9 | 0.2 | 10 | 0.4 | 2.2 | 0.5 | 0.3 | 0.1 |
| **Aminotetrol** | 1.8 | 0.1 | 0.4 | 2.9 | 0.1 | 0.6 | 2.1 | 0.2 | 0.5 | 1.9 | 0.4 | 0.5 | 3.9 | 0.3 | 0.8 | 0.8 | 0.8 | 0.2 | 2.1 | 0.2 | 0.5 | 0.7 | 0.4 | 0.2 |
| **Adenosylhopane** | 102 | 5.8 | 23 | 100 | 11 | 21 | 103 | 1.5 | 23 | 101 | 7.5 | 28 | 98 | 5.4 | 21 | 103 | 11 | 24 | 109 | 5.4 | 24 | 113 | 5.6 | 25 |
| **2-methyladenosylhopane** | 8.3 | 1.0 | 1.9 | 7.8 | 0.4 | 1.7 | 7.9 | 0.0 | 1.8 | 8.0 | 0.4 | 2.2 | 8.0 | 0.6 | 1.7 | 8.3 | 2.1 | 2.0 | 8.6 | 0.7 | 1.9 | 9.0 | 0.3 | 2.0 |
| **Adenosylhopane-type 2** | 15 | 0.6 | 3.4 | 16 | 1.4 | 3.3 | 15 | 0.4 | 3.4 | 14 | 0.9 | 4.0 | 15 | 0.9 | 3.2 | 15 | 1.7 | 3.5 | 16 | 0.4 | 3.5 | 17 | 1.8 | 3.6 |
| **2-methyladenosylhopane-type 2** | 3.8 | 0.3 | 0.9 | 4.5 | 0.5 | 0.9 | 3.7 | 0.1 | 0.8 | 3.1 | 0.1 | 0.9 | 4.4 | 0.3 | 1.0 | 4.0 | 0.3 | 1.0 | 4.7 | 0.4 | 1.0 | 4.2 | 0.7 | 0.9 |
| **Adenosylhopane-type 3** | 3.1 | 0.3 | 0.7 | 2.7 | 0.5 | 0.6 | 2.9 | 0.5 | 0.7 | 3.8 | 0.5 | 1.0 | 2.8 | 0.4 | 0.6 | 2.6 | 0.3 | 0.6 | 4.2 | 0.9 | 0.9 | 3.5 | 0.4 | 0.8 |
| **2-methyladenosylhopane-type 3** | - | - | - | 0.2 | 0.2 | 0.1 | 0.3 | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 | 0.6 | 0.3 | 0.1 | - | - | - | 0.9 | 0.5 | 0.2 | 0.5 | 0.5 | 0.1 |
| **BHT pentose with unsat.** | 16 | 1.4 | 3.7 | 22 | 2.5 | 4.6 | 17 | 1.7 | 3.7 | 12 | 3.0 | 3.2 | 20 | 2.4 | 4.3 | 15 | 2.2 | 3.5 | 17 | 1.6 | 3.8 | 20 | 1.5 | 4.4 |
| **BHT pentose** | 22 | 1.6 | 5.1 | 32 | 3.5 | 6.7 | 27 | 2.7 | 5.9 | 17 | 4.3 | 4.4 | 29 | 2.9 | 6.3 | 22 | 2.9 | 5.2 | 27 | 2.4 | 6.0 | 30 | 2.2 | 6.6 |
| **BHT cyclitol ether** | 56 | 7.8 | 13 | 69 | 4.9 | 15 | 53 | 1.7 | 12 | 36 | 6.9 | 9.5 | 65 | 3.9 | 14 | 40 | 4.0 | 9.4 | 48 | 2.6 | 11 | 46 | 1.4 | 10 |
| **BHT glucoasamine** | 7.4 | 0.7 | 1.7 | 6.8 | 0.3 | 1.4 | 5.9 | 0.5 | 1.3 | 4.4 | 0.2 | 1.2 | 7.9 | 0.3 | 1.7 | 5.8 | 2.1 | 1.4 | 6.1 | 0.4 | 1.3 | 6.7 | 0.6 | 1.5 |
| **BHpentol cyclitol ether** | 5.5 | 0.7 | 1.3 | 6.4 | 0.5 | 1.4 | 3.5 | 1.7 | 0.8 | 2.1 | 1.1 | 0.5 | 5.9 | 0.1 | 1.3 | 3.9 | 0.4 | 0.9 | 4.6 | 0.3 | 1.0 | 3.3 | 1.7 | 0.8 |
| **BHhexol cyclitol ether** | 0.7 | 0.7 | 0.2 | 3.7 | 0.2 | 0.8 | - | - | - | - | - | - | 2.2 | 0.5 | 0.5 | - | - | - | 1.4 | 1.4 | 0.3 | 2.6 | 0.3 | 0.6 |
| **Total (ug/gTOC)** | **437** | 28 | | **474** | 33 | | **450** | 21 | | **370** | 53 | | **464** | 19 | | **420** | 26 | | **457** | 8.2 | | **452** | 19 | |

| | 619 days Amended | | | 619 days Inhibited | | | 619 days Heat killed | | | 706 days Amended | | | 706 days Inhibited | | | 706 days Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **AnhydroBHT** | 1.2 | 0.6 | 0.3 | 0.4 | 0.4 | 0.1 | 1.6 | 0.8 | 0.4 | 0.6 | 0.6 | 0.1 | 0.6 | 0.6 | 0.1 | 2.4 | 0.4 | 0.6 |
| **BHT** | 142 | 7.5 | 33 | 151 | 8.8 | 32 | 149 | 7.8 | 37 | 124 | 5.8 | 33 | 151 | 10 | 32 | 141 | 0.9 | 35 |
| **2-methylBHT** | 14 | 1.6 | 3.1 | 14 | 0.2 | 2.9 | 13 | 0.4 | 3.1 | 11 | 1.3 | 3.0 | 13 | 1.4 | 2.8 | 11 | 0.3 | 2.9 |
| **BHhexol** | 2.3 | 1.3 | 0.5 | 3.3 | 1.7 | 0.7 | 1.9 | 1.5 | 0.4 | 1.9 | 1.0 | 0.5 | 3.8 | 0.8 | 0.8 | 1.7 | 0.9 | 0.4 |
| **'Novel aminotriol'** | 11 | 1.6 | 2.5 | 8.0 | 0.6 | 1.7 | 6.0 | 0.4 | 1.5 | 8.0 | 0.8 | 2.1 | 10 | 0.8 | 2.2 | 6.3 | 0.7 | 1.6 |
| **Aminotriol** | 18 | 0.9 | 4.2 | 26 | 0.7 | 5.6 | 16 | 1.1 | 3.9 | 18 | 0.9 | 4.6 | 29 | 2.0 | 6.1 | 15 | 0.6 | 3.8 |
| **'Methylcarbamoylaminotriol'** | 9.9 | 0.4 | 2.3 | 4.0 | 0.1 | 0.9 | 0.2 | 0.2 | < 0.1 | 5.8 | 0.2 | 1.5 | 4.8 | 0.2 | 1.0 | 1.7 | 0.1 | 0.4 |
| **Aminotetrol** | 2.3 | 0.2 | 0.5 | 3.2 | 0.3 | 0.7 | 1.4 | 0.1 | 0.3 | 2.2 | 0.5 | 0.6 | 3.4 | 0.2 | 0.7 | 1.1 | 0.6 | 0.3 |
| **Adenosylhopane** | 106 | 6.1 | 25 | 100 | 5.9 | 21 | 105 | 2.3 | 26 | 100 | 0.9 | 26 | 101 | 7.1 | 21 | 109 | 5.1 | 27 |
| **2-methyladenosylhopane** | 8.8 | 0.7 | 2.0 | 8.3 | 0.6 | 1.8 | 7.5 | 0.4 | 1.8 | 8.4 | 0.2 | 2.2 | 7.6 | 0.9 | 1.6 | 8.4 | 0.5 | 2.1 |
| **Adenosylhopane-type 2** | 15 | 1.0 | 3.5 | 15 | 1.1 | 3.2 | 17 | 0.6 | 4.1 | 14 | 0.6 | 3.7 | 15 | 1.0 | 3.3 | 16 | 0.4 | 3.9 |
| **2-methyladenosylhopane-type 2** | 4.3 | 0.2 | 1.0 | 4.4 | 0.5 | 0.9 | 4.2 | 0.2 | 1.0 | 3.5 | 0.4 | 0.9 | 4.0 | 0.1 | 0.8 | 4.1 | 0.2 | 1.0 |
| **Adenosylhopane-type 3** | 4.1 | 0.1 | 0.9 | 3.7 | 0.1 | 0.8 | 3.5 | 0.2 | 0.8 | 3.2 | 0.3 | 0.8 | 3.8 | 0.3 | 0.8 | 3.4 | 0.3 | 0.9 |
| **2-methyladenosylhopane-type 3** | 0.8 | 0.4 | 0.2 | 0.5 | 0.3 | 0.1 | 0.3 | 0.3 | 0.1 | 1.1 | 0.2 | 0.3 | 0.4 | 0.4 | 0.1 | 0.2 | 0.2 | 0.1 |
| **BHT pentose with unsat.** | 16 | 2.8 | 3.7 | 21 | 0.3 | 4.5 | 14 | 1.0 | 3.4 | 13 | 1.4 | 3.5 | 18 | 1.1 | 3.9 | 13 | 0.2 | 3.3 |
| **BHT pentose** | 23 | 4.0 | 5.3 | 29 | 0.8 | 6.3 | 22 | 1.6 | 5.4 | 19 | 2.3 | 5.1 | 27 | 2.9 | 5.7 | 20 | 1.4 | 5.1 |
| **BHT cyclitol ether** | 41 | 4.8 | 9.5 | 59 | 2.3 | 13 | 37 | 5.8 | 9.1 | 37 | 4.8 | 9.7 | 62 | 4.4 | 13 | 37 | 1.9 | 9.3 |
| **BHT glucoasamine** | 6.4 | 0.1 | 1.5 | 7.1 | 0.1 | 1.5 | 5.4 | 0.1 | 1.3 | 5.6 | 0.7 | 1.5 | 7.6 | 0.6 | 1.6 | 5.5 | 0.4 | 1.4 |
| **BHpentol cyclitol ether** | 4.1 | 0.6 | 0.9 | 5.1 | 0.3 | 1.1 | 2.2 | 1.4 | 0.5 | 2.5 | 1.3 | 0.6 | 5.8 | 0.2 | 1.2 | - | - | - |
| **BHhexol cyclitol ether** | 0.9 | 0.9 | 0.2 | 3.0 | 0.7 | 0.6 | - | - | - | 0.6 | 0.6 | 0.2 | 2.3 | 0.6 | 0.5 | - | - | - |
| **Total (ug/gTOC)** | **432** | 25 | | **467** | 15 | | **406** | 15 | | **380** | 19 | | **470** | 31 | | **397** | 8.9 | |

BHP concentrations (µg/g TOC) in column one represent the average value extracted from a triplicate set of microcosm sediments (28 and 433 days HK duplicate) with total BHPs summed at the bottom of column. Standard error is in column two and the relative % of individual compounds in column three.

# Appendix 3h: Total BHPs (µg/g TOC) in methanogenic River Tyne estuarine sediment microcosms

| | Time 0 Amended | | | Time 0 Inhibited | | | Time 0 Heat killed | | | 30 days Amended | | | 30 days Heat killed | | | 106 days Amended | | | 106 days Heat killed | | | 225 days Amended | | | 225 days Inhibited | | | 225 days Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **AnhydroBHT** | 3.2 | 0.2 | 0.5 | 3.2 | 0.1 | 0.5 | 6.0 | 0.5 | 1.0 | 3.4 | 0.7 | 0.6 | 7.5 | 0.6 | 1.3 | 4.5 | 1.1 | 0.6 | 5.5 | 0.5 | 1.2 | 4.4 | 0.3 | 0.6 | 2.5 | 1.3 | 0.5 | 6.8 | 0.1 | 1.4 |
| **BHT** | 199 | 9.6 | 32 | 201 | 11 | 33 | 228 | 7.3 | 37 | 178 | 27 | 30 | 213 | 5.5 | 38 | 197 | 14 | 28 | 184 | 5.8 | 40 | 211 | 8.1 | 30 | 132 | 4.9 | 28 | 196 | 17 | 40 |
| **2-methylBHT** | 21 | 1.8 | 3.5 | 21 | 0.7 | 3.4 | 22 | 1.0 | 3.6 | 20 | 2.8 | 3.3 | 21 | 0.7 | 3.7 | 20 | 1.6 | 2.8 | 18 | 0.7 | 4 | 22 | 1.0 | 3.1 | 13 | 0.7 | 2.8 | 19 | 1.4 | 3.8 |
| **BHhexol** | 1.5 | 1.5 | 0.2 | - | - | - | 6.1 | 0.8 | 1.0 | 1.3 | 1.3 | 0.2 | 3.1 | 2.0 | 0.6 | 1.0 | 1.0 | 0.2 | 1.7 | 1.7 | 0.4 | 1.3 | 1.3 | 0.2 | - | - | - | 1.3 | 1.3 | 0.2 |
| **'Novel aminotriol'** | 9.7 | 0.6 | 1.6 | 7.2 | 1.1 | 1.2 | 7.8 | 0.9 | 1.2 | 8.9 | 1.7 | 0.9 | 6.7 | 0.5 | 1.2 | 7.8 | 0.4 | 1.1 | 4.4 | 0.2 | 1.0 | 9.9 | 0.8 | 1.4 | 5.2 | 0.4 | 1.1 | 4.7 | 0.3 | 1.0 |
| **Aminotriol** | 28 | 0.8 | 4.6 | 49 | 5.9 | 7.9 | 25 | 1.9 | 4.0 | 36 | 4.1 | 6.2 | 26 | 2.6 | 4.6 | 42 | 3.4 | 5.9 | 20 | 0.2 | 4.4 | 39 | 2.2 | 5.5 | 52 | 1.3 | 11 | 22 | 0.4 | 4.6 |
| **'Methylcarbamoylaminotriol'** | 29 | 2.5 | 4.8 | - | - | - | 0.3 | 0.3 | < 0.1 | 20 | 1.9 | 3.4 | - | - | - | 23 | 2.4 | 3.3 | - | - | - | 26 | 2.9 | 3.6 | - | - | - | - | - | - |
| **Aminotetrol** | 3.1 | 0.6 | 0.5 | 5.5 | 0.4 | 0.9 | 1.8 | 0.9 | 0.3 | 4.6 | 0.7 | 0.8 | 1.6 | 0.8 | 0.3 | 5.0 | 0.3 | 0.7 | 1.3 | 0.6 | 0.3 | 4.9 | 0.7 | 0.7 | 7.8 | 0.9 | 1.7 | - | - | - |
| **Adenosylhopane** | 95 | 3.4 | 16 | 98 | 11 | 16 | 117 | 13 | 19 | 101 | 14 | 17 | 106 | 5.4 | 19 | 101 | 11 | 14 | 90 | 2.4 | 20 | 108 | 11 | 15 | 67 | 9.6 | 14 | 93 | 7.4 | 19 |
| **2-methyladenosylhopane** | - | - | - | 9.3 | 0.3 | 1.5 | 11 | 0.7 | 1.8 | 9.9 | 1.5 | 1.7 | 6.8 | 3.5 | 1.2 | 4.6 | 2.9 | 0.7 | 4.6 | 2.3 | 1.0 | 11 | 1.0 | 1.5 | 2.5 | 2.5 | 0.5 | 7.7 | 1.0 | 1.6 |
| **Adenosylhopane-type 2** | 20 | 1.2 | 3.3 | 19 | 1.0 | 3.1 | 21 | 1.4 | 3.4 | 22 | 3.2 | 3.6 | 19 | 0.9 | 3.4 | 19 | 2.2 | 2.7 | 16 | 0.5 | 3.5 | 22 | 2.1 | 3.1 | 11 | 2.2 | 2.3 | 16 | 0.5 | 3.3 |
| **2-methyladenosylhopane-type 2** | 6.9 | 0.8 | 1.1 | 5.6 | 0.4 | 0.9 | 5.5 | 0.5 | 0.9 | 5.7 | 0.7 | 1.0 | 4.9 | 0.2 | 0.9 | 6.4 | 1.2 | 0.9 | 4.9 | 0.4 | 1.1 | 6.2 | 1.0 | 0.9 | 1.7 | 0.9 | 0.4 | 4.6 | 0.2 | 1.0 |
| **Adenosylhopane-type 3** | 1.3 | 0.7 | 0.2 | 2.5 | 1.3 | 0.4 | 4.5 | 0.6 | 0.7 | 2.5 | 1.3 | 0.6 | 3.6 | 0.9 | 0.6 | 1.5 | 1.5 | 0.2 | 3.9 | - | 0.8 | 4.5 | 0.2 | 0.6 | 1.7 | 0.9 | 0.4 | 3.6 | 0.1 | 0.7 |
| **2-methyladenosylhopane-type 3** | - | - | - | - | - | - | - | - | - | - | - | 0.3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **BHT pentose with unsat.** | 19 | 1.2 | 3.1 | 17 | 1.0 | 2.8 | 21 | 0.7 | 3.4 | 17 | 3.3 | 2.8 | 22 | 1.7 | 3.8 | 24 | 1.8 | 3.4 | 15 | 1.4 | 3.2 | 23 | 3.1 | 3.1 | 19 | 2.2 | 4.1 | 18 | 1.6 | 3.7 |
| **BHT pentose** | 28 | 0.8 | 4.5 | 26 | 1.5 | 4.4 | 31 | 2.0 | 5.0 | 31 | 4.9 | 5.1 | 29 | 1.0 | 5.1 | 32 | 1.4 | 4.5 | 20 | 2.5 | 4.3 | 33 | 0.9 | 4.7 | 22 | 1.5 | 4.6 | 24 | 1.7 | 4.9 |
| **BHT cyclitol ether** | 122 | 5.5 | 20 | 124 | 14 | 20 | 90 | 5.2 | 14 | 108 | 16 | 18 | 81 | 6.7 | 14 | 179 | 19 | 25 | 62 | 2.8 | 14 | 154 | 17 | 21 | 113 | 8.1 | 24 | 66 | 3.3 | 14 |
| **BHT glucoasamine** | 9.9 | 0.5 | 1.6 | 11 | 2.0 | 1.8 | 10 | 0.6 | 1.6 | 10 | 1.4 | 1.7 | 8.9 | 0.1 | 1.6 | 13 | 0.8 | 1.9 | 7.9 | 1.4 | 1.7 | 17 | 1.4 | 2.4 | 9.9 | 0.2 | 2.1 | 6.2 | 0.6 | 1.3 |
| **BHpentol cyclitol ether** | 13 | 1.8 | 2.1 | 12 | 2.0 | 1.9 | 9.4 | 0.4 | 1.5 | 11 | 1.5 | 1.9 | 5.3 | 2.8 | 0.9 | 18 | 1.8 | 2.5 | - | - | - | 14 | 2.3 | 1.9 | 7.9 | 1.0 | 1.7 | - | - | - |
| **BHhexol cyclitol ether** | 3.1 | 1.6 | 0.5 | 1.1 | 1.1 | 0.2 | 4.9 | 0.5 | 0.8 | 4.4 | 1.0 | 0.7 | 1.6 | 1.6 | 0.3 | 8.8 | 1.8 | 1.3 | - | - | - | 6.1 | 1.2 | 0.8 | - | - | - | 1.2 | 1.2 | 0.2 |
| **Total (ug/gTOC)** | **613** | 30 | | **612** | 43 | | **624** | 31 | | **594** | 81 | | **566** | 10 | | **707** | 49 | | **458** | 13 | | **717** | 50 | | **468** | 17 | | **490** | 30 | |

| | 505 days Amended | | | 505 days Heat killed | | | 665 days Amended | | | 665 days Inhibited | | | 665 days Heat killed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ± | % | | ± | % | | ± | % | | ± | % | | ± | % |
| **AnhydroBHT** | 4.2 | 0.3 | 0.7 | 6.1 | 0.4 | 1.4 | 2.9 | 1.7 | 0.4 | 11 | 5.5 | 2.0 | 8.1 | 0.1 | 1.7 |
| **BHT** | 194 | 5.2 | 32 | 164 | 7.2 | 38 | 186 | 4.4 | 29 | 145 | 9.5 | 30 | 198 | 1.4 | 41 |
| **2-methylBHT** | 22 | 0.4 | 3.6 | 16 | 0.1 | 3.7 | 18 | 1.0 | 2.7 | 14 | 1.2 | 2.9 | 18 | 0.5 | 3.7 |
| **BHhexol** | - | - | - | 1.3 | 1.3 | 0.3 | 0.8 | 0.8 | 0.1 | - | - | - | 1.7 | 1.7 | 0.3 |
| **'Novel aminotriol'** | 14 | 0.8 | 2.4 | 3.5 | 0.2 | 0.8 | 7.2 | 0.7 | 1.1 | 4.8 | 1.7 | 1.0 | 4.8 | 0.1 | 1.0 |
| **Aminotriol** | 31 | 2.9 | 5.0 | 17 | 1.2 | 3.9 | 46 | 4.0 | 7.1 | 56 | 9.1 | 11 | 19 | 1.3 | 3.9 |
| **'Methylcarbamoylaminotriol'** | 28 | 1.3 | 4.6 | - | - | - | 1.2 | 0.7 | 0.2 | 2.0 | 1.0 | 0.4 | - | - | - |
| **Aminotetrol** | 3.9 | 0.1 | 0.6 | - | - | - | 5.9 | 0.7 | 0.9 | 6.4 | 0.6 | 1.3 | - | - | - |
| **Adenosylhopane** | 92 | 13 | 15 | 94 | 6.5 | 22 | 120 | 1.0 | 18 | 77 | 8.8 | 16 | 102 | 4.0 | 21 |
| **2-methyladenosylhopane** | 3.7 | 1.9 | 0.6 | 4.7 | 2.4 | 1.1 | 8.1 | 1.6 | 1.2 | 5.2 | 2.6 | 1.0 | 5.7 | 0.5 | 1.2 |
| **Adenosylhopane-type 2** | 22 | 1.6 | 3.6 | 15 | 1.4 | 3.5 | 23 | 0.3 | 3.5 | 13 | 2.2 | 2.5 | 17 | 0.8 | 3.5 |
| **2-methyladenosylhopane-type 2** | 5.8 | 0.4 | 0.9 | 4.1 | 0.3 | 1.0 | 6.6 | 0.9 | 1.0 | 3.1 | 0.8 | 0.6 | 4.9 | 0.2 | 1.0 |
| **Adenosylhopane-type 3** | 3.4 | 0.9 | 0.5 | 2.8 | 0.3 | 0.6 | 3.0 | 1.5 | 0.5 | 1.5 | 1.5 | 0.3 | 3.6 | 0.9 | 0.7 |
| **2-methyladenosylhopane-type 3** | - | - | - | - | - | - | 0.3 | 0.3 | 0.1 | - | - | - | - | - | - |
| **BHT pentose with unsat.** | 20 | 0.5 | 3.3 | 14 | 2.1 | 3.4 | 20 | 1.7 | 3.1 | 14 | 1.8 | 3.0 | 18 | 1.9 | 3.6 |
| **BHT pentose** | 27 | 1.5 | 4.4 | 18 | 2.2 | 4.3 | 30 | 1.7 | 4.6 | 20 | 2.6 | 4.2 | 26 | 4.0 | 5.2 |
| **BHT cyclitol ether** | 106 | 12 | 17 | 59 | 11 | 14 | 134 | 20 | 20 | 99 | 7.9 | 20 | 55 | 2.3 | 11 |
| **BHT glucoasamine** | 23 | 2.0 | 3.7 | 7.5 | 1.0 | 1.8 | 27 | 1.8 | 4.2 | 8.1 | 0.9 | 1.6 | 7.9 | 0.1 | 1.6 |
| **BHpentol cyclitol ether** | 12 | 1.4 | 1.9 | - | - | - | 11 | 1.6 | 1.7 | 8.6 | 1.7 | 1.7 | - | - | - |
| **BHhexol cyclitol ether** | - | - | - | - | - | - | 2.5 | 1.3 | 0.4 | - | - | - | - | - | - |
| **Total (ug/gTOC)** | **612** | 40 | | **427** | 5.2 | | **654** | 33 | | **489** | 53 | | **490** | 5.7 | |

BHP concentrations (µg/g TOC) in column one represent the average value extracted from a triplicate set of microcosm sediments with total BHPs summed at the bottom of column. Standard error is in column two and the relative % of individual compounds in column three.