The importance of methane derived carbon as a basal resource in chalk stream food webs: the effect of light availability and methane concentration.

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Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy

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Chapter 2 has been published and should be cited as:

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Chapter 3 has accepted for publication in Freshwater Biology and the co-authors are F Abdullahi, J Grey and M Trimmer

Chapter 5 has been submitted to ISME Journal and the co-authors are M Trimmer, K Purdy, P Chronopoulou, S Maanoja and J Grey

Abstract

Methane is oversaturated relative to the atmosphere in many rivers, yet its cycling and fate is poorly understood. While photosynthesis is the dominant source of autotrophic carbon to rivers, chemosynthesis and particularly methane oxidation could provide alternative sources of primary production where the riverbed is shaded or at depth beneath the sediment surface. I highlight geographically widespread methanotrophic carbon fixation within the gravel riverbeds of over 30 chalk rivers and in 15 of these, the potential for methane oxidation (methanotrophy) was also compared to photosynthesis and stable isotope analyses were used to trace methane into the wider food web. Detailed concurrent measurements of photosynthesis and methanotrophy in one large chalk river over a complete annual cycle, showed methanotrophy to be active to at least 15cm into the riverbed and to be strongly substrate limited. The seasonal trend in methanotrophic production reflected that of the riverine methane concentrations, and thus, the highest contribution to autotrophic production was in mid-summer. At the sediment surface, photosynthesis was limited by light for most of the year with heavy shading induced by dense beds of aquatic macrophytes and riparian vegetation. Across 15 rivers in mid-summer, methane derived carbon was estimated to contribute 18% of production (methanotrophic plus photosynthetic) in well illuminated riverbeds and 51% in the shaded areas (median values). With warming conditions and associated increasing methanogenesis in fine sediments, methanotrophy is predicted to prevent increased methane emissions from rivers due to the strong kinetic response of methane oxidation. The gross carbon fixation efficiency of methane oxidation was calculated as 50% and was conserved across eight rivers with varying methane oxidation capacities and ambient methane concentrations. Methanotrophic production is widespread, efficient and most important when ambient methane concentration is high and light availability is low.

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Chapter 1: Introduction

1.1 Integrating rivers into the global carbon cycle

It is now widely acknowledged that freshwaters perform an important role in the global carbon cycle through the mineralisation and storage of terrestrial carbon as well as its transportation to the ocean (Aufdenkampe *et al.*, 2011, Cole *et al.*, 2007, Richey *et al.*, 2002). It has been estimated that, globally 2.7 Pg C enters freshwaters from the surrounding catchment each year, and of that, only a third reaches the ocean and almost half is lost to the atmosphere (Figure 1.1) in the form of carbon dioxide or methane (Battin *et al.*, 2009).

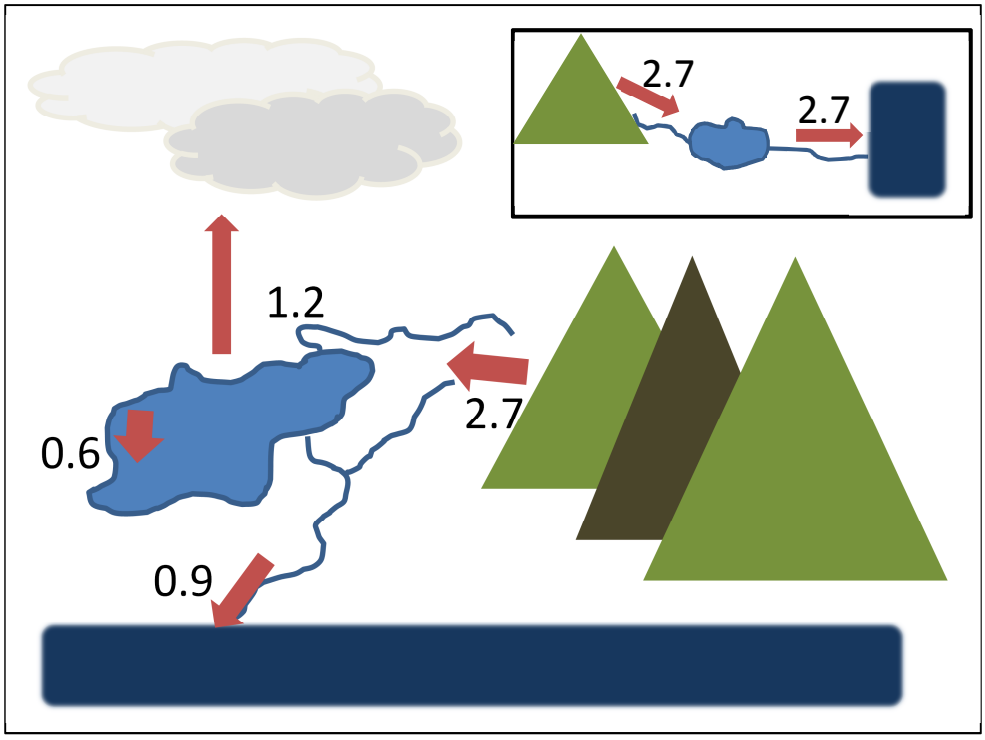

Figure 1.1: Estimates of net carbon fluxes through freshwaters in Pg C y^{-1} showing delivery from the land, evasion to the atmosphere, storage in freshwaters and transport to the ocean (redrawn from Battin *et al.* (2009)). Inset, top-right, the now redundant view of freshwaters as passive pipelines which transport terrestrial carbon to the ocean (redrawn from Cole *et al.* (2007)).

The need to quantify carbon gas emissions from freshwaters is particularly pressing because of the role carbon dioxide and methane play in global warming, and the difficulties faced in forming mitigation strategies when we do not properly understand the natural carbon cycle. Recent meta-analyses have estimated the freshwater methane and carbon dioxide emissions and found them to be substantial (2.05 Pg C y^{-1}) but there is a shortage of data for rivers (Bastviken *et al.*, 2011, Tranvik *et al.*, 2009). Although streams and rivers are small in their areal extent, accounting for just 3-5% of total freshwater surface area, they are hot-spots of biogeochemical cycling and account for ~17% of outgassing of carbon to the atmosphere (Aufdenkampe *et al.*, 2011). Momentum is now growing with regards to quantifying methane emissions from rivers (Crawford *et al.*, 2014, Garnier *et al.*, 2013, Sawakuchi *et al.*, 2014, Vihermaa & Waldron, 2013) but few have investigated the controls on these fluxes. It is crucial to study the processes that govern the magnitude of the methane sources and sinks in order to fully understand this section of the carbon cycle and how it might alter under future climate change scenarios. Here, I focus on quantifying the main methane sink in rivers, aerobic methane oxidation. Careful study of microbial methane oxidation is needed in order to understand the feedbacks and linkages with methanogenesis, temperature change and photosynthetic production which ultimately dictate the importance of methane as a carbon source for the benthic food web.

1.2 General overview of work to date

Most rivers are oversaturated in methane and so are net emitters of this potent greenhouse gas (Abril & Borges, 2005, De Angelis & Lilley, 1987, Devol *et al.*, 1990, Koné *et al.*, 2010, Rulik *et al.*, 2000, Sanders *et al.*, 2007) but little is known how much is oxidised before it escapes to the atmosphere. When methane undergoes microbial oxidation, carbon is fixed into organic matter which is then available to primary consumers in much the same way as for photosynthesis (Jones & Grey, 2011). River water methane measurements were combined with stable isotope analyses of common invertebrate consumers along with their putative

dietary resources and process measurements to conclude that methane could be contributing significant carbon to the benthic food web in a chalk stream (Trimmer *et al.*, 2009a, Trimmer *et al.*, 2010). This finding has the potential to stimulate re-evaluation of photosynthesis as the dominant basal resource in rivers. If methanotrophy is found to provide a significant portion of new carbon to riverbed sediments where photosynthesis is also active, this further broadens the range of aquatic ecosystem types that should be investigated with regards to the role of chemosynthetic production.

This thesis aims to expand upon these preliminary studies by answering the following questions:

Central research question: How do light availability and methane concentration affect the importance of methane-derived carbon to chalk river food webs?

- How widespread is methanotrophic production in chalk rivers and is it a significant source of carbon relative to the presumed dominant photosynthetic pathway?
- How do ambient methane concentration and water temperature affect the rate of methane oxidation?
- Does the importance of methanotrophic production relative to photosynthetic production change across rivers with different methane concentrations and between shaded and unshaded patches of the same river?
- How efficient is microbial methane oxidation at fixing carbon?

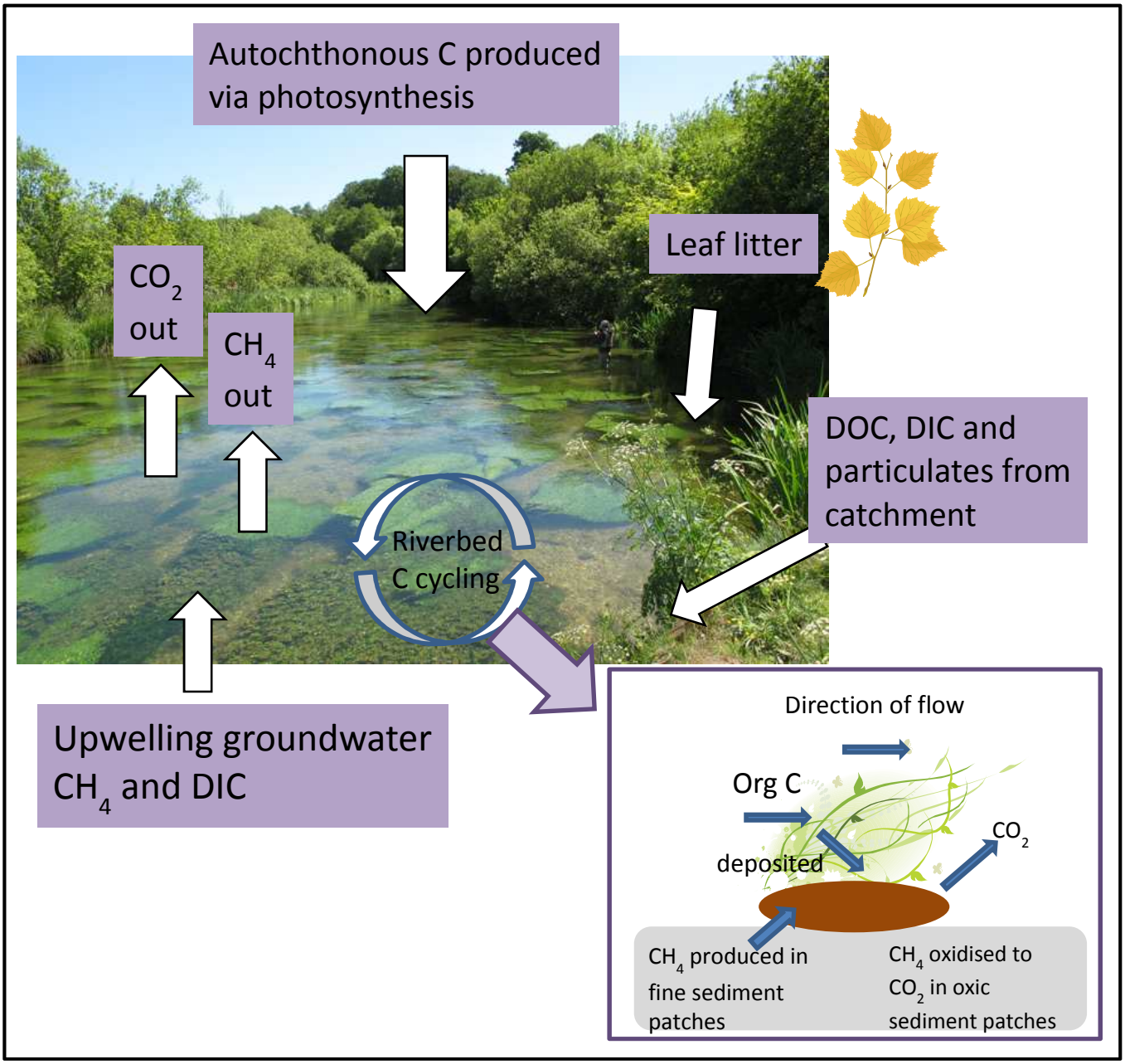

Figure 1.2: Schematic diagram showing major carbon sources and sinks in chalk rivers and (inset) diagram of potential sites for methane production and oxidation when fine sediment is deposited around aquatic macrophytes such as the *Callitriche* and *Ranunculus* stands shown in the photograph of the River Itchen, Hampshire, U.K.

1.3 Methane as a basal resource

Methane-derived carbon (MDC) can be traced through trophic levels using stable isotope analysis due to its distinct $\delta^{13}C$ value which arises from methanogenic archea discriminating against the heavier ^{13}C isotope during methanogenesis (Whiticar, 1999) and then further fractionation (0-16 ‰) when it is oxidised by methanotrophic bacteria (Summons *et al.*, 1994).

Using stable isotope analysis, primary consumers have been shown to assimilate MDC in a wide range of aquatic environments including lakes, marine methane seeps and wetlands (Jones & Grey, 2004, MacAvoy *et al.*, 2002, van Duinen *et al.*, 2013). More detailed studies have linked greater ^{13}C depletion in chironomid larvae with greater methanogenic and methanotrophic potential in the sediments, both within a single lake (Deines & Grey, 2006) and between lakes (Eller *et al.*, 2005). Evidence of MDC in riverine food webs is scarce, perhaps because the methane concentrations in most rivers, although over saturated relative to the atmosphere, are much lower than those found in standing water bodies (nM range in rivers, µM or mM range in stratifying lakes) and riverbed sediments are often well oxygenated (Jones & Mulholland, 1998). A rare example of a riverine study found MDC in aquatic insects feeding on detritus in a stream backwater (Kohzu *et al.*, 2004) but the stagnant conditions mean this is more comparable to a shallow lake than a free-flowing river channel. More recently, gas (methane concentration), process and ecological (SIA of basal resources and consumers) data have been linked together to highlight potential contribution of MDC to a riverine food web (Trimmer *et al.*, 2009a, Trimmer *et al.*, 2010). However, many questions remain unanswered and this thesis seeks to address some of the biggest questions arising from our existing understanding.

1.4 Methanotrophic bacteria

Methane is oxidised aerobically in the riverbed by a group of bacteria called methanotrophs which are obligate methylotrophs, meaning they use one-carbon compounds as their sole energy source (Hanson & Hanson, 1996). When oxygen is absent, sulphate, nitrate or nitrite can be used as alternative electron acceptors (Deutzmann & Schink, 2011, Ettwig *et al.*, 2010) but the majority of methane oxidation in freshwaters is aerobic (Conrad, 2009). There are thought to be two types of methanotrophic bacteria and they differ in the specific pathway through which they assimilate formaldehyde to formic acid; type I methanotrophs use the

ribulose monophosphate (RuMP) pathway and type II use the serine pathway. Both use the methane mono-oxygenase enzyme to catalyse the oxidation of methane to methanol and an increasing number of studies use the unique gene for this enzyme to identify the species present in a given sample (McDonald *et al.*, 2008). Strictly speaking, methanotrophs are not chemosynthetic because they do not assimilate carbon dioxide, instead they oxidise methane to methanol and then formaldehyde which is assimilated into their biomass (Hanson & Hanson, 1996). True chemoautotrophs such as ammonia or hydrogen-sulphide or iron oxidisers use carbon dioxide as their sole carbon source but in the absence of broad term for all non-photosynthetic forms of primary production, chemosynthesis is often used to cover methanotrophy. Preliminary investigations using the functional gene for particulate mono-oxygenase have shown that both types of methanotrophs are present in chalk streams (manuscript in prep., Chronopoulou, Shelley and Trimmer). They are thought to have different optimal conditions resulting in the dominance of one type over the other, but consistent patterns in mixed communities have not been found (Hanson & Hanson, 1996, Horz *et al.*, 2002).

1.5 The effect of light availability

Multiple studies have concluded that light affects methanotrophy but there is no solid consensus as to how; some say it inhibits the activity of methanotrophs (Dumestre *et al.*, 1999, Murase & Sugimoto, 2005) and others conclude that it increases their activity (King, 1990). In the surface layers of poorly mixed lakes and reservoirs, intense photosynthesis during daylight hours removes the carbon dioxide from the water and, as a result, the pH rises (Talling, 1976), and it is this change in pH (>pH 10.0) that inhibits methane oxidation. In shallow rivers, where the riverbed is bathed in light, methane, and oxygen, and the water column is well mixed, inhibition through extreme changes in pH is unlikely. Moreover, methanotrophic bacteria and photosynthetic organisms co-exist on the riverbed biofilm and

have been shown to perform their functions simultaneously in laboratory incubations with high light intensities (Trimmer *et al.*, 2010). There could even be a symbiotic relationship between methanotrophy and photosynthesis within the biofilm matrix whereby carbon dioxide, produced via methanotrophy, feeds directly into the photoautotrophs as has already been shown in mosses where active methanotrophs were found living within the internal structure of *Sphagnum* spp. (Raghoebarsing *et al.*, 2005). I hypothesise that methanotrophy will be unaffected by riverbed irradiance but its significance as a carbon source will increase with increased shading because of the relative decrease in photosynthesis.

Photosynthesis is the dominant form of autotrophic production in rivers (Odum, 1956) and as such, it is an important process to quantify in order to set methanotrophic production in context. We use short (<1 hour) light and dark laboratory incubations and quick response microelectrodes (Unisense) to quantify gross and net photosynthesis and dark respiration in discrete gravel samples (Figure 1.3). To accurately model riverbed photosynthetic production from laboratory measurements it is critical to understand the interplay between irradiances, chlorophyll pigments and carbon fixation. For example, a photosynthesis-irradiance (PI) curve is required to adjust laboratory measurements of photosynthesis to those expected on the riverbed where irradiances are site-specific (Hill *et al.*, 1995). Chlorophyll content is widely accepted as a proxy for photosynthetic biomass (Huot *et al.*, 2007) and the efficiency of the chlorophyll (i.e. units of oxygen produced per unit chlorophyll) can be used as an indicator of carbon quality (Huettel & Rusch, 2000). However, in terms of primary production and comparison with methanotrophy, net photosynthesis must be used because this is the measure of carbon fixation after consideration of that which is instantaneously respired.

Figure 1.3: Laboratory set-up for measuring photosynthesis in riverbed gravels. Light source positioned above four incubation chambers with 4-channel oxygen logger linked to microelectrodes (left) and a single incubation chamber (right). See chapter 2 for details of the method and equipment.

1.6 The effect of methane concentration

Where organisms are substrate limited, their metabolic rate will increase if they are exposed to increased substrate. The concentration of methane has been shown to correlate positively with the rate of methane oxidation in lakes (Deines *et al.*, 2007, Duc *et al.*, 2010), soils (Bender & Conrad, 1992, Bogner *et al.*, 1997) and wetlands (Sundh *et al.*, 1995). In such lotic aquatic environments there is usually a distinct oxycline where methane (often produced in anoxic sediments) meets oxygen (atmospheric equilibration in the mixed layer or oxic sediment layer) and this is where the peak in methanotrophic activity is found. In rivers with oxic gravel beds, the sites of methane production are continually shifting in size and location as a function of flow which dictates the location of depositional zones (Cotton *et al.*, 2006, Trimmer *et al.*,

2009b). Delineating the sites of methane oxidation is equally complex, particularly as we understand very little about the controls and drivers of methane oxidation in river beds. Nevertheless, the magnitude and spatial configuration of methane sources and sinks will play an important role in determining the net effect of methane cycling in the riverbed.

The seasonal variation in river water methane concentration (Sanders *et al.*, 2007), if combined with a kinetic response, as has been observed in other aquatic systems, will lead to increased methanotrophic production in the summer, when substrate is most abundant. Whether, seasonal or spatial (riverbed heterogeneity results in patches of methane production) changes in methane concentration in rivers will lead to changes in methanotrophic biomass, i.e. a change in the density of the community, is unknown. In soil cores, increased methanotrophic cell counts were observed in three month long incubations where methanotrophs were effectively grown on elevated methane concentrations (Kightley *et al.*, 1995) which shows methanotrophic communities can grow in response to changing methane concentration. Dose-response studies are much more common, where an immediate increase in the rate of oxidation with increased substrate is measured, indicating a substrate-limited population of methanotrophs (Deutzmann *et al.*, 2011). As far as we are aware, there are no studies on the response of methane oxidation in rivers to raised methane concentrations. The fate of methane in estuaries has been investigated, but the salinity gradient complicates the issue, apparently controlling methane oxidation rates more strongly than longitudinal changes in methane concentration (De Angelis & Scranton, 1993, Zhu, 2010). Whilst others have looked for indicators of methane oxidation in rivers (Buriánková *et al.*, 2012) there is only one study which has quantified the process by directly measuring its rate (Trimmer *et al.*, 2010). This thesis documents investigations into how methane concentration affects the rate of methane oxidation over time and space in riverbed sediments.

1.7 Chalk streams as the study ecosystems

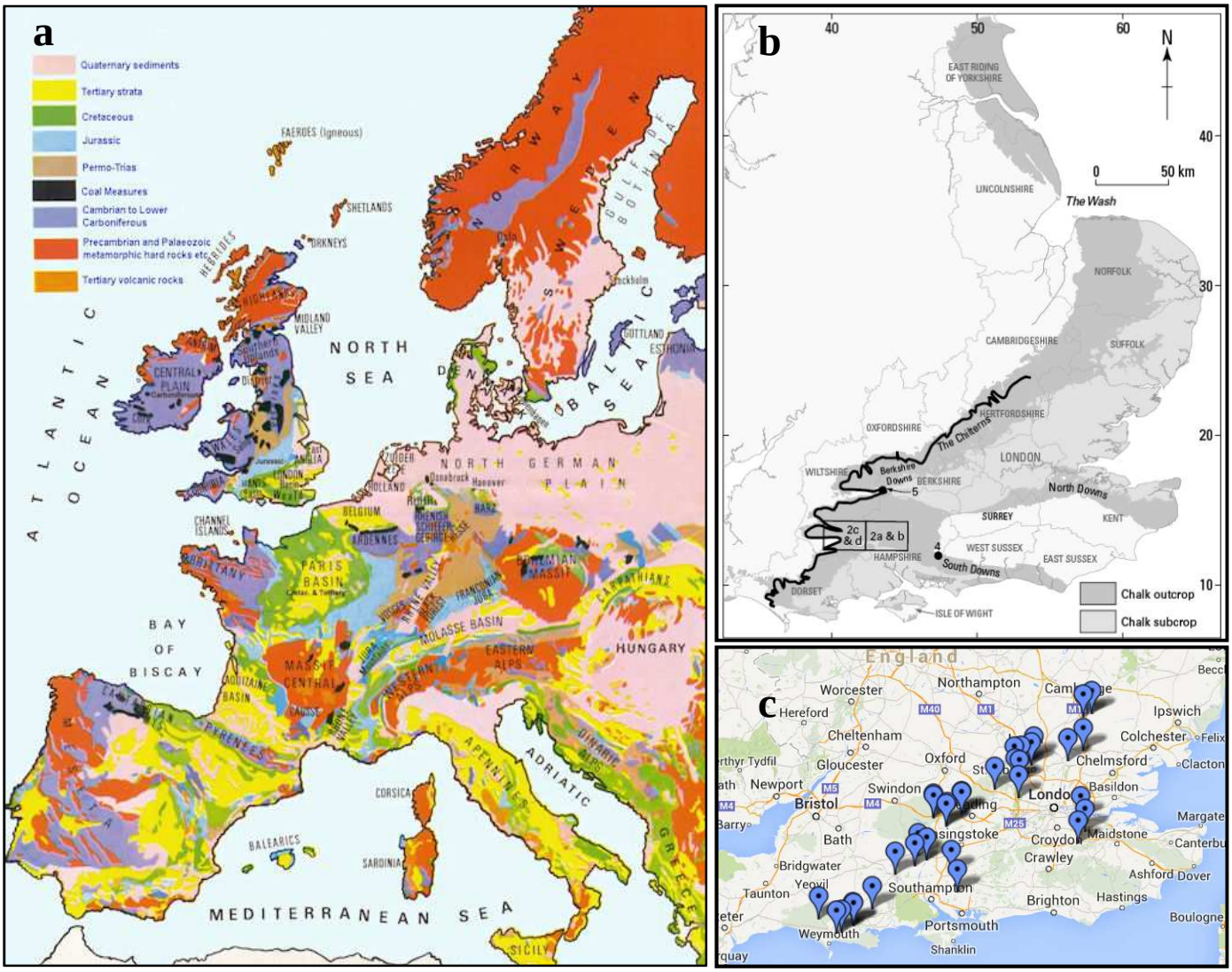

Figure 1.4 Distribution of chalk a) in Europe (marked as green, Cretaceous sediments (West, 2012)), b) in England (dark grey shows the chalk outcrops and light grey shows where the chalk is overlain by Quaternary sediments (Bromley & Gale, 1982)), and c) the study sites covered in this thesis marked on a Google Map image. For further details *see* chapters 4 and 5.

Chalk streams are iconic ecosystems of intrinsic value for biodiversity and have conservation status within the UK Biodiversity Action Plan due to their importance as a habitat for *Ranunculus* and *Callitriche* species (JNCC Report, No. 270). Chalk is a soft, porous sedimentary rock, primarily composed of calcium carbonate laid down in the Cretaceous period when shallow seas covered the lowlands of north-western Europe, which is where most

of the chalk outcrops are found today (*see* Figure 1.4a). Much of Southern England is underlain by chalk formations, but impermeable Quaternary sediments overlay the chalk along the Thames basin and eastern coastline (Figure 1.4b). All of the chalk stream sites visited for this thesis are marked on Figure 1.4c and cover all but the most northern (Lincolnshire and Norfolk) English counties dominated by chalk geology. They were chosen because of their clean gravel beds and the presence of the aquatic macrophyte *Ranunculus* and silt-intolerant cased caddis *Agapetus fuscipes* both of which are indicators of good ecological and hydrological status. Nutrient, pH and gas concentrations are presented in Chapter 4. Many of the sites were also chosen because they had previously been studied and so existing datasets and access arrangements made them ideal locations for further investigation.

In chalk catchments, rainwater percolates through the permeable rock, losing particulate material and gaining carbonate ions as it does so, before re-emerging as springs where the water table breaches the surface (Berrie, 1992). The location of the spring often moves upstream in winter when rainfall is typically higher than in summer, hence the term winterbourne for stretches of the channel which only experience flow in winter. All of the study sites were in the perennial section of the streams. The dominance of groundwater inputs into chalk rivers results in annually stable water temperature, chemistry and discharge relative to semi-permeable and impermeable catchments as overland flow is minimal (Sear *et al.*, 1999). Aquatic macrophytes are often abundant in chalk streams and in many rivers they fill much of the channel in the summer months (Figure 1.5), considerably altering flow and trapping sediments (Cotton *et al.*, 2006). The ultra-clear water means irradiances remain high through the water column (Trimmer *et al.*, 2010), which facilitates benthic photosynthetic production on the coarse gravel bed. Chalk riverbeds support the larval stages of many fly species some of which are intolerant to the high silt and toxin loadings found in surface run-off fed streams. Due to the abundance of prey (Tod & Schmid-Araya, 2009) and the quality of the

substrate for spawning, chalk streams are an important habitat for many fish species such as brown trout, Atlantic salmon and brook lamprey (Riley *et al.*, 2006). Given these appropriate conditions for classical photosynthetic production, it is therefore particularly surprising that methane-derived carbon appears to be a significant contributor to a proportion of the secondary production in this environment.

Figure 1.5: The River Lambourn, Berkshire, U.K. where *Ranunculus* spp. fill much of the channel in summer.

The source of the dissolved methane in chalk streams is thought to be a combination of over-saturated groundwater inputs (3-2,600 nmol CH_4 L^{-1} (median = 58 nmol L^{-1}) in chalk aquifers, Darling and Gooddy (2006)) and in-stream methanogenesis which is much more widespread in the summer months relative to the winter (Sanders *et al.*, 2007, Trimmer *et al.*, 2009b), both of which are microbial rather than thermogenic in origin. Chalk streams are likely to have lower methane concentrations than rivers which run over more permeable geologies because chalk riverbeds are characterised by their high oxygen concentrations and coarse gravel beds,

neither of which are optimal for methanogenesis. Most reported measurements of methane concentrations in streams and rivers are from very large catchments such as the Amazon (6,400 nmol L^{-1} (Devol *et al.*, 1988)), the Congo (1,720 nmol L^{-1} (Salter *et al.*, 2011)) and the Hudson (200-800 nmol L-1 (De Angelis & Scranton, 1993)) where suspended sediment loads are high and oxygen is depleted in the sediments. A small, but well-studied stream in the Czech Republic illustrates the importance of riverbed substrate type in influencing methane concentrations; in the upstream sections of the Sitka Stream where gravels form the riverbed, oxygen is high (65-90% saturation) and methane is comparable with chalk streams (40-80 nmol L^{-1}) but when clays and sands predominate the riverbed methane concentrations exceed 450,000 nmol L^{-1} (Buriánková *et al.*, 2012). It is therefore reasonable to predict that the range of methane concentrations found in English chalk streams is likely to be heavily influenced by the connectivity with the groundwater and the amount of fine sediment in the channel which will determine the extent of in-stream methanogenic activity.

1.8 Experimental approach

In order to fully answer the research questions measurements of potential rates of methane oxidation in riverbed sediments are not enough. It is likely that potential for methanotrophy will vary between and within rivers and over time (seasonally). Once the potential rates have been established, calculation of the carbon fixation efficiency will be required in order to convert the measure of methane oxidised into carbon fixed. As chalk streams have a hyporheic zone, methanotrophic production in the sub-surface gravels needs to be estimated for calculating the importance of methanotrophic production in a reach scale. Chalk riverbeds have a seasonally evolving mosaic of anoxic fine sediment accumulations within the wider coarse gravel beds (Malard *et al.*, 2002) and between these micro-sites the methanotrophic production is likely to vary. Temperature dependence is also poorly studied in riverbed carbon cycling and the few published studies focus solely on respiration (Acuna *et al.*, 2008, Perkins *et al.*, 2012),

leaving the interaction between temperature and methane sources and sinks largely unknown. This information is required to estimate seasonal trends in carbon fixation and longer term trends relating to climate change.

As MDC is a proposed alternative basal resource to allochthonous or autochthonous photosynthetic carbon, a thorough understanding of photosynthetic production in riverbed sediments is necessary in order to place the importance of methanotrophy in context. As with methanotrophy, photosynthesis is likely to vary spatially and temporally due to variation in light (Kirk, 1994) and temperature (Yvon-Durocher *et al.*, 2010).

1.9 Structural outline of the thesis

This research is divided into four main sections, outlined below:

Chapter 2: Widespread methanotrophic primary production in lowland chalk rivers

This chapter concerns the quantification of seasonal carbon fixation via methane oxidation and photosynthesis in the riverbed of the River Lambourn and potential methane oxidation data from a wider survey of 32 rivers. It has been published in Proceedings of the Royal Society: B.

Chapter 3: Temperature dependence of methane cycling in riverbed sediments

By incubating riverbed sediments at multiple temperatures and tracking methane concentration over time, I calculated the apparent activation energies for methane production (methanogenesis) and consumption (methane oxidation) in the riverbed of the River Itchen in Hampshire. Substrate availability (i.e. methane concentration) proved to be important in modulating the temperature dependence of methane oxidation. I explored the kinetics of methane oxidation in two different sediment patch types common across chalk rivers. The preliminary data for this paper were acquired by an undergraduate project student, Frah

Abudullahi under my supervision. This chapter is under review for publication in Freshwater Biology

Chapter 4: The effect of methane concentration and light availability on the contribution of methane derived carbon to the food web: A field survey.

In this chapter, a field survey approach was used to answer the central research question in a real stream setting. That is, how is the contribution of MDC to the food web affected by methane concentration and light availability? Here, production processes (i.e. methanotrophy and photosynthesis) were compared with stable isotope signatures of macroinvertebrates between adjacent well illuminated, open stretches, and darker, heavily shaded reaches of rivers. This comprehensive and detailed survey covered 15 rivers with varying methane concentrations, one of which was surveyed an additional six times over the year to explore the effect of seasonally changing methane concentrations and light levels incident on the riverbed. The fieldwork and some of the stable isotope preparation work were carried out along with Dr. Nicola Ings who is employed on a closely related NERC grant. We are currently preparing this chapter for publication.

Chapter 5: Constant carbon fixation efficiency by methanotrophic communities across eight rivers

In order to calculate the amount of carbon fixed via methanotrophy, the carbon fixation efficiency of the microbial process, i.e. how much organic carbon is produced for each unit of methane oxidised, is needed. I worked on this project along with a Masters by Research student (Susanna Maanoja), a Post Doctoral Research Assistant (Dr. Myrsini Chronopoulou) and my co-supervisor, Professor Mark Trimmer. I was responsible for all the experiments to determine the effectiveness of $^{13}CH_4$ as a tracer, the kinetics of methane oxidation and the GCFE of methane oxidation in the 8 rivers using the $^{13}CH_4$-$^{13}\Sigma DIC$ technique. This chapter has been

submitted as a paper to International Society of Microbial Ecology and is currently under review.

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Chapter 2: Widespread methanotrophic primary production in lowland chalk rivers

This chapter has been published in Proceedings of the Royal Society: B and as such, is formatted to their requirements. I am the lead author and Dr. J Grey and Prof. M. Trimmer were the other authors on this manuscript.

2.1 Summary

Methane is oversaturated relative to the atmosphere in many rivers, yet its cycling and fate is poorly understood. While photosynthesis is the dominant source of autotrophic carbon to rivers, chemosynthesis and particularly methane oxidation could provide alternative sources of primary production where the riverbed is heavily shaded or at depth beneath the sediment surface. Here we highlight geographically widespread methanotrophic carbon fixation within the gravel riverbeds of over 30 chalk rivers and in 15 of these, the potential for methane oxidation (methanotrophy) was also compared to photosynthesis. In addition, we performed detailed concurrent measurements of photosynthesis and methanotrophy in one large chalk river over a complete annual cycle, where we found methanotrophy to be active to at least 15cm into the riverbed and to be strongly substrate limited. The seasonal trend in methanotrophic activity reflected that of the riverine methane concentrations, and thus, the highest rates were measured in mid-summer. At the sediment surface, photosynthesis was limited by light for most of the year with heavy shading induced by dense beds of aquatic macrophytes. Across fifteen rivers, in late summer, we conservatively calculated that net methanotrophy was equivalent to between 1% and 46% of benthic net photosynthetic production within the gravel riverbed, with a median value of 4%. Hence, riverbed chemosynthesis, coupled to the oxidation of methane, is widespread and significant in English chalk rivers.

Key words Methane oxidation, carbon, photosynthesis, rivers, chemosynthesis.

2.2 Introduction

Inland waters have received relatively little attention in our attempts to quantify global carbon cycling, compared to the oceanic and terrestrial realms, yet they perform a significant role in carbon sequestration and mineralisation (Battin *et al.*, 2009, Cole *et al.*, 2007). Indeed, although modest in their areal extent, the close biogeochemical coupling with terrestrial systems means that globally, more carbon is buried in freshwaters than is sequestered on the ocean floor (Tranvik *et al.*, 2009). However, burial is often short-lived as a wide array of microbial communities metabolise the organic carbon and release it back to the atmosphere either as carbon dioxide or methane (Aufdenkampe *et al.*, 2011). Though data for rivers are comparatively scarce compared to lakes (Bastviken *et al.*, 2011), many that have been surveyed are often oversaturated in methane and carbon dioxide (Prairie, 2013), the partial pressures of which will be influenced by carbon biogeochemistry in both the mainstream, groundwater and broader catchment (Darling & Gooddy, 2006, Jones & Mulholland, 1998). Outgassing of these greenhouse carbon gases from rivers has been widely researched (Butman & Raymond, 2011, Miller *et al.*, 2007), but their cycling within rivers and bed sediments has not received as much attention (Cole *et al.*, 2007).

Traditionally, riverine production is recognised as being supported by a combination of allochthonous carbon from the surrounding catchment and autochthonous carbon produced within the river, both ultimately driven by photosynthesis (Odum, 1953). Recent work makes the case for a third driver of riverine metabolism whereby methanotrophy provides a significant portion of carbon to invertebrates in chalk rivers (Trimmer *et al.*, 2009a), as has been proposed for lakes (Bastviken *et al.*, 2003, Jones & Grey, 2011). Such a phenomenon may appear counterintuitive for chalk rivers, being well renowned for their high photosynthetic productivity. Chalk rivers are, however, also oversaturated in methane (Sanders *et al.*, 2007); the source of methane is thought to be a combination of local

methanogenesis in fine sediments (Sanders *et al.*, 2007) and upwelling groundwater which is enriched in methane relative to the atmosphere (Gooddy & Darling, 2005).

Riverbed sediments are known hotspots of biogeochemical cycling, having a concentration of organic matter and microorganisms several orders of magnitude greater than the overlying water column (Findlay, 1995). Unsurprisingly then, riverbed epilithic respiration may contribute significantly to whole stream metabolism (Naegeli & Uehlinger, 1997). Although a small number of studies have measured dissolved methane in riverbed porewaters (Pretty *et al.*, 2006, Rulik *et al.*, 2000), fewer have measured the potential for methane oxidation within the subsurface gravels. Our previous study at the River Lambourn revealed lower concentrations of methane in the gravel bed porewater than in the main channel which suggested that the gravel bed is a sink for methane (Trimmer *et al.*, 2010). Thus, in addition to altering the carbon gas balance of emissions from rivers, methanotrophy could account for a significant portion of the primary productivity i.e. chemosynthetic relative to photosynthetic production. We therefore chose this site to perform a detailed, seasonal study to assess the changing significance of methane-derived carbon as a proportion of photosynthetic production throughout the year. To explore the geographic extent of methane-derived carbon in chalk rivers, we made measurements of methane oxidation and photosynthetic potential in the gravel beds of chalk rivers spanning almost the entirety of the chalk aquifer in southern England.

2.3 Methods

2.3.1 Study sites and sampling

Thirty-two chalk rivers with permanent flow, submerged macrophytes, and clean gravel beds were selected from across southern England (Figure 2.1a). Of these, fifteen were chosen for more detailed measurements of benthic photosynthetic and methanotrophic carbon fixation. An additional site, on the River Lambourn, was further selected for a more detailed seasonal study which consisted of nine sampling trips between October 2010 and September 2011 and the wider survey was performed in August 2011. One of the sites for the one-off survey was also on the River Lambourn and will be referred to as Lambourn (at Westbrook).

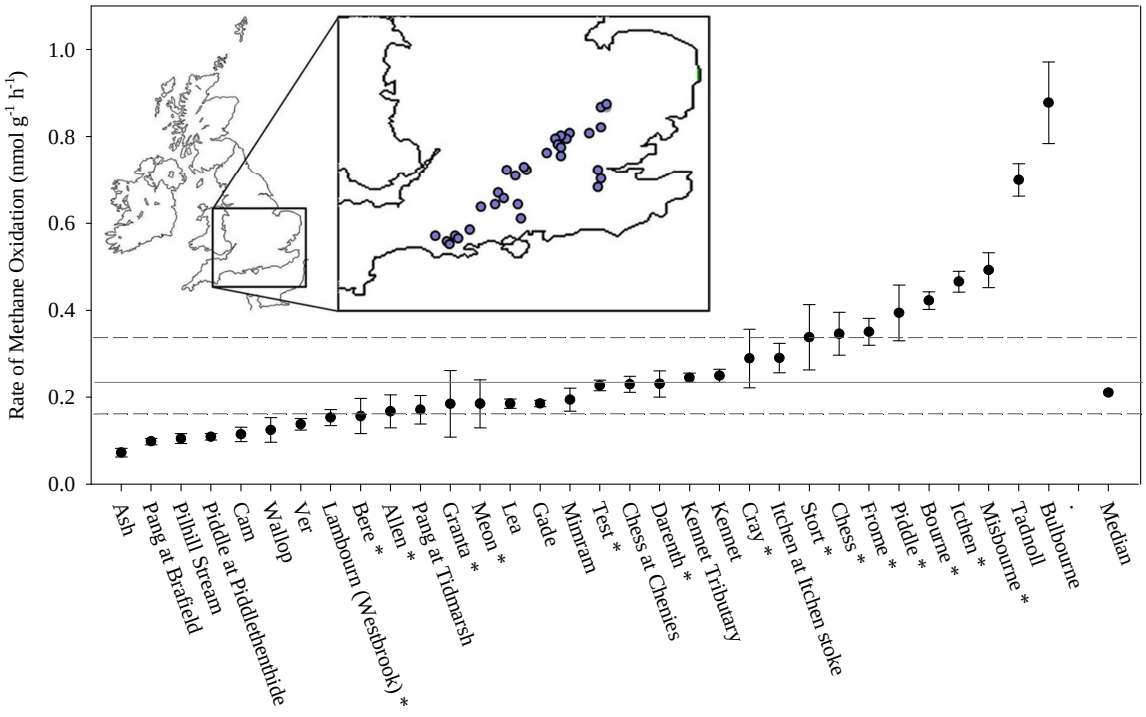

Figure 2.1: Mean rates of methane oxidation across 32 sites (± se, *n*=5) with the solid line showing the annual average rate from the detailed seasonal study in the River Lambourn and the dashed lines show the maximum and minimum seasonal rates. Rivers with * are those for which photosynthetic production was also measured. The map insert details location of these rivers across the chalk aquifer.

2.3.2 River water methane

Dissolved methane concentration in the river water was quantified by taking water samples (*n*=5) from the middle of the channel at mid-depth using polytetrafluoroethylene (PTFE) tubing attached to a 60 mL gas-tight syringe. The sample was then immediately discharged into a gas-tight vial (12 mL Exetainer, Labco) and allowed to overflow (3x) before being fixed (100 µL $ZnCl_2$ 50% w/v; bactericide) and sealed. A 2 mL headspace (analytical-grade helium) was introduced using a two-way valve and gas-tight syringe (Hamliton). After equilibration, gas samples (100 µL) were withdrawn from the headspace and injected into a gas chromatograph fitted with a flame-ionising detector (Agilent Technologies; (Sanders *et al.*, 2007)). Headspace concentrations of methane were calculated from peak areas calibrated against known standards (Scientific and Technical Gases), and the total amount in the vial (headspace plus water) and thus, the river water concentration was calculated using solubility coefficients (Yamamoto *et al.*, 1976).

2.3.3 Sediment sampling

To measure potential for methanotrophy, gravels from six discrete locations at each site (*n*=6) were gently kicked into a fine mesh net. Any large stones, detritus and invertebrates were removed, and the sediment was then stored in plastic zip-lock bags and placed into a portable fridge for transport back to the laboratory (<3 h). At the Lambourn, in order to measure methanotrophy with depth in the riverbed and the quality of allochthonous carbon, sediment cores were taken on each trip using a metal corer (internal dimensions: 18 cm x 5 cm) manually driven into the riverbed. The sediment core was then extruded and sectioned at 3 cm-intervals, the maximum practical spatial resolution due to some large stones (>2 cm). Seven replicate cores (resulting in 35 subsections) were taken on all trips except for October

(*n*=5) and February (*n*=6). Grain size was determined by sieving the dried samples through nine sieves (0.1 mm- 5 mm) and weighing the fractions.

2.3.4 Measuring rates of methane oxidation and estimating net methanotrophy

In the laboratory, sediment (~1 g) and river water (5 mL) were transferred into gas-tight vials (12.5 mL Exetainer, Labco) and sealed. The air headspace was enriched with methane (BOC) by adding 300 µL of 10,000ppm methane in helium to give a final concentration of 450 nmol L^{-1} in the water (Trimmer *et al.*, 2009a, Yamamoto *et al.*, 1976). The concentration of methane in the headspace of each vial was measured by gas chromatography with flame ionising detection (GC/FID; Agilent Technologies UK Ltd., South Queensferry, U.K.; (Sanders *et al.*, 2007)), immediately after spiking and then every 24 hours for 3-5 days (Trimmer *et al.*, 2009a). Between measurements the vials were incubated on rollers (Denley, Spiramix) in a dark and refrigerated room set to 9°C (± 1 °C) to mimic average river temperature. Following the final measurement, the samples were dried to a constant weight and all calculated rates of methanotrophy were normalised for dry mass. Control vials were set up to test for any potential for methane oxidation in the river water which was always found to be negligible (Trimmer *et al.*, 2009a).

The potential for methanotrophy was measured at a constant initial methane concentration in all incubations (across all rivers and throughout the year at the Lambourn). However, the seasonal study showed that the ambient methane concentration in the river displayed strong seasonal variation (Figure 2.2b). To investigate the effect of changing methane concentration on methanotrophy, incubations were set up as described above but with varying spikes of methane to give final concentrations in the water ranging from 4-80,000 nmol L^{-1}. We then used this linear relationship to normalise the measured rates of methane oxidation to the

ambient methane concentration for each month. Further, as part of a detailed parallel study using $^{13}CH_4$ (Trimmer *et al*., unpublished) the carbon fixation efficiency of methanotrophy in these chalk rivers is consistently around 50% (±2%) i.e. for each mole of methane oxidised 50% is fixed as new organic carbon. Accordingly, we multiplied our measured rates of methane oxidation by 0.5 to derive estimates of net methanotrophy to compare with our estimates of net photosynthetic production (detailed below). Although this is a potential method, performed in the laboratory, the gravels are well irrigated with both methane and oxygen (Pretty *et al.*, 2006), which was captured in our vials, and the strong kinetic effect enabled us to scale the potential activity accordingly**.** The average rate of methanotrophy for each core (seasonal study, Lambourn) or surface sediment sample (wider survey) was scaled over a depth of 15cm and surface area of a square metre. We have previously shown that methanotrophy in well oxygenated riverbeds is not thought to be light dependant (*see* Discussion) unlike stratified water bodies or wetlands where light has indirect effects through changing the position of the oxycline (King, 1990) and so hourly rates were multiplied by 24 to scale to daily rates. Diurnal temperature fluctuations were not included as methane oxidation is known to have no temperature dependence at these concentrations (*see* Chapter 3).

2.3.5 Measuring rates of net photosynthesis

To quantify the potential for photosynthesis in the sediments we measured oxygen evolution over timed light and dark incubations. Approximately 30 g of each sediment sample was placed inside incubation chambers fitted with a stirrer and a cable gland for holding an oxygen electrode (OX50, Unisense). The chambers were submerged in a temperature controlled bath (9°C) and the oxygen concentration was logged at 1 minute intervals for 45 minutes in the light (55 µmol quanta m^{-2} s^{-1} at the surface of the gravel) and then 45 minutes after the chambers were made dark (for further details *see* (Trimmer *et al.*, 2010)). Benthic

photosynthetic carbon fixation was calculated by taking one mole of net oxygen production to equate to one mole of carbon fixed. The rates per square metre were multiplied by the average daylight length for the month at the latitude of the study site to give µmol C m^{-2} d^{-1}. Given that we could isolate net methanotrophy we used net photosynthesis to calculate the respective contribution from each to net carbon fixation in the riverbed as that is what is of greatest significance in terms of export to higher trophic levels.

2.3.6 Modelling riverbed irradiance and photosynthesis at the River Lambourn

Whilst the laboratory light source remained constant, the light regime at the detailed study site changed seasonally, so we needed to normalise our measured rates of photosynthetic production for *in situ* irradiance by modelling the riverbed light regime using a photosynthesis-irradiance curve and riverbed shading data from a previous study (Trimmer *et al.*, 2010) (see Supplementary Electronic Material). The ratios between modelled photosynthesis rates for each shading patch type over the annual cycle were used to convert the laboratory data to represent the whole riverbed surface-layer instead of just the open gravels. For the August 2011 survey of 15 rivers, we did not produce individual P-I curves for each site, so the estimates of photosynthesis are based solely on laboratory incubations and do not include the effect of shading; hence, we are probably over-estimating net benthic photosynthetic production and under-estimating the percentage accounted for by net methanotrophy.

With methanotrophic and photosynthetic carbon fixation now in µmol C m^{-2} d^{-1}, we divided the former by the latter and multiplied by 100 to give a percentage. When there was no NPP, i.e. respiration outstripped photosynthesis even in the light, methanotrophic C-fixation accounted for 100% of the new carbon produced in the gravels that would still be available to higher trophic levels.

2.3.7 Quantifying the quality of surface and subsurface chlorophyll-*a*

Although light would not penetrate beneath the top 1 cm and so neither would photosynthetic production, we measured chlorophyll-*a* and oxygen evolution at depth (>1cm) to provide a measure of the quality of allochthonous carbon carried into the dark gravel bed. Chlorophyll-*a* was extracted three times from the gravels with 30 mL of acetone (90% v/v with ultra-high purity water) over 24 hours in a dark refrigerator. Absorbance was measured at 750 nm to check for clarity, and at 650 nm for chlorophyll extinction (Dalsgaard, 2000). We divided the gross oxygen production rates by the chlorophyll-*a* content of the gravels to derive biomass specific photosynthetic production (nmol O_2 μg^{-1} Chl h^{-1}). Here, we used GPP because we wanted to quantify the overall capacity of the organisms associated with chlorophyll to produce oxygen.

2.4 Results

2.4.1 Study site characteristics

At the Lambourn, the temperature of the river water ranged from 6°C in December to 14°C in June, a much smaller range than that of the air temperature of -3°C and 28°C, reflecting the strong influence of groundwater typical for these chalk rivers. The macrophytes, predominantly *Ranunculus* spp., and riparian vegetation developed rapidly in late spring and shaded much of the riverbed by June (see Supplementary Electronic Material Figure S2.1) before dying back in the autumn as is typical for chalk rivers (Flynn *et al.*, 2002). There were no seasonal patterns in nutrient concentrations and the average (*n*=14) ammonia, nitrate and phosphate concentrations were 2.2 (± 0.02 s.e.) µmol L^{-1}, 489 (± 38 s.e.) µmol L^{-1}, and 1.2 (± 0.33 s.e.) µmol L^{-1} respectively (Environment Agency). Suspended solids remained low throughout the annual cycle (Oct 2010 to Sept 2011) at an average of 6 mg L^{-1} (Environment Agency).

The rivers surveyed in August 2011 covered a wide range of water temperatures (14-20°C), nitrate (0.2-2 mmol L^{-1}), ammonium (3-21 µmol L^{-1}) and phosphate (0.2-97 µmol L^{-1}) concentrations. The DIC (2.7-4.6 mmol L^{-1}) and pH (7.80-8.75) were high across all sites as would be expected for chalk rivers due to the dissolution of calcium carbonate as the water moves through the rock.

2.4.2 Dissolved methane concentration and methane oxidation

At all sites, the concentration of dissolved methane in the river water was oversaturated relative to atmospheric equilibration (3.2 nmol L^{-1} at 10°C), ranging from 23 nmol L^{-1} at the Misbourne to 150 nmol L^{-1} at the Piddle. The gravel biofilms oxidised methane at all 32 sites

but the activity varied across rivers, ranging from 0.07 nmol CH_4 g^{-1} h^{-1} at the Ash, to 0.88 nmol CH_4 g^{-1} h^{-1} at the Bulbourne, both in Hertfordshire (Figure 2.1). The detailed annual study showed that methane concentration was strongly seasonal in the Lambourn, peaking at 103 nmol L^{-1} in late June and falling to 27 nmol L^{-1} in December (Figure 2.2b), in agreement with our previous findings (Trimmer *et al.*, 2009a). At the Lambourn, the gravels oxidised methane throughout the year (Figure 2.2a) but the process was clearly substrate limited, with a linear increase in rate of methane oxidation both within (Figure 2.2c) and well beyond the riverine concentrations (up to 80 µmol CH_4 L^{-1}). This linear relationship was used to normalise the measured rates of methane oxidation at the Lambourn to the methane concentrations measured *in situ* (Figure 2.2d). The rates of methane oxidation from the one-off survey in August 2011 were not normalised for ambient methane concentration as the photosynthesis measurements were not be normalised to the ambient light regime. Finally, in the sediment cores from the Lambourn, the rate of methane oxidation decreased significantly with depth into the riverbed (see Supplementary Electronic Material for Table S2.2) with the rate tending towards zero at 35 cm beneath the surface.

Rate of methane oxidation = 0.107 – 0.00308(depth) Equation (1)

For our calculations on the wider survey we used the same approach as at the Lambourn seasonal site, integrating over the top 15cm of the riverbed, as there are few data on subsurface methane and oxygen concentrations in other chalk rivers, or indeed any other river on different geologies.

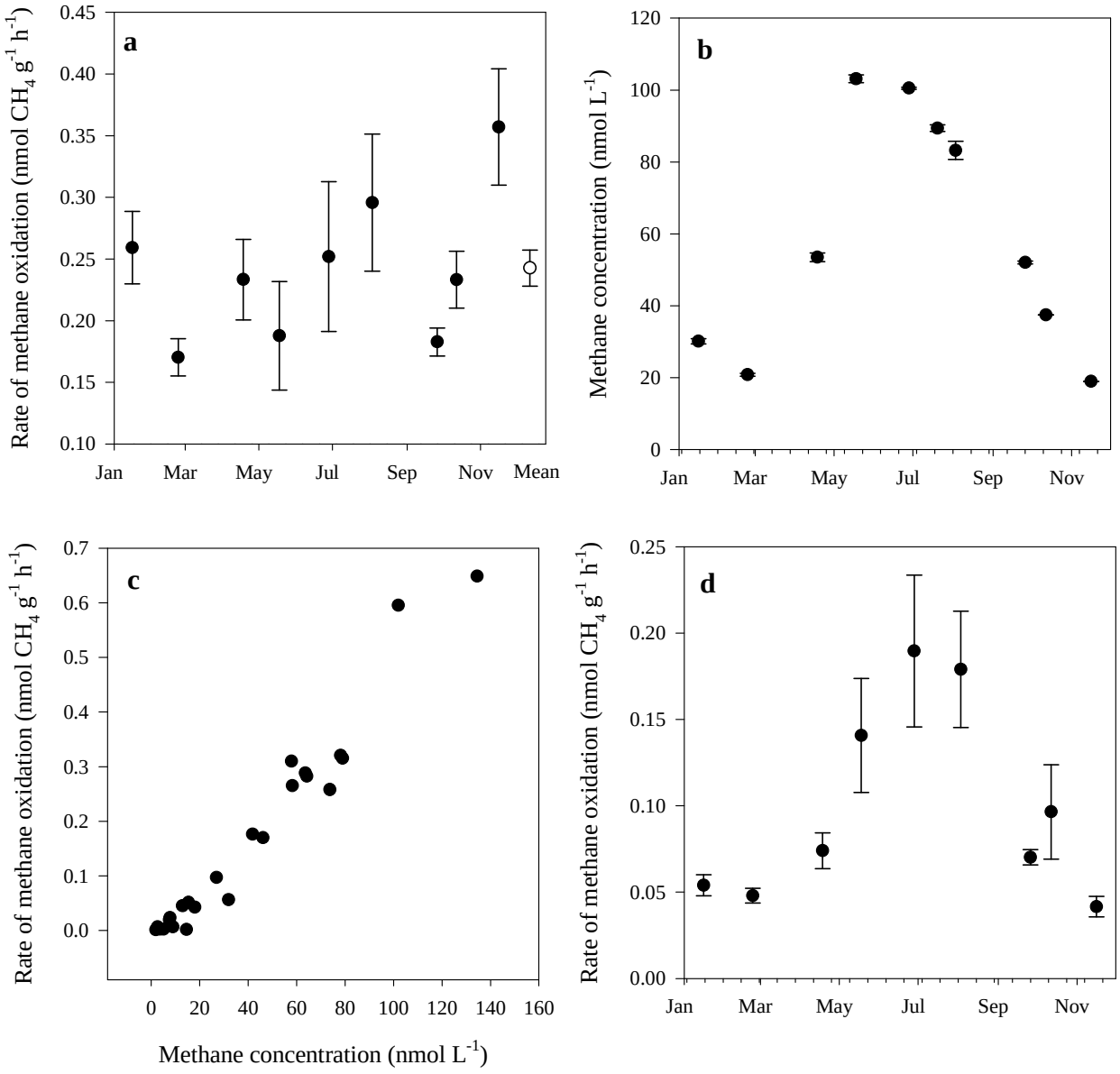

Figure 2.2: a) Filled circles show mean (± se, *n*=7) rate of methane oxidation across the year at the Lambourn under a constant methane concentration and the open circle is the mean of all data (± se, *n*=60). b) Mean (± se, *n*=5) ambient river water methane concentration. c) Rate of methane oxidation as a function of methane concentration at the start of the incubation. d) Mean (± se, *n*=7) methane oxidation normalised to changing methane concentrations in the river by using the relationship shown in 2c.

2.4.3 Photosynthesis

Net benthic photosynthetic production was measured in the surface gravels from all fifteen of the rivers surveyed in August 2011. Under laboratory conditions, which only simulate completely unshaded parts of the riverbed, the highest production was at the Lambourn (Westbrook) (319 nmol O_2 g^{-1} h^{-1}) and the lowest at the Granta (6 nmol O_2 g^{-1} h^{-1}) with the overall range in photosynthetic potential being explained by chlorophyll-*a* i.e. algal biomass. In the Lambourn, gross photosynthesis was measured in the surface sediments throughout the year with the highest rates in summer (Figure 2.3a). However, net photosynthesis was only observed in six out of the nine months (Figure 2.3a). In April, August and October, demand for oxygen via respiration outstripped the production via photosynthesis under illumination and so, the biofilm was net heterotrophic. The P-I curve clearly showed that the biofilm was light saturated at around 100µmol quanta m^{-2} s^{-1} (Figure 2.3b) which means for considerable periods of the summer, the open gravels are fully light saturated. The biomass specific photosynthetic production, i.e. moles of oxygen produced per unit chlorophyll, remained constant throughout the annual cycle so we know the photosynthetic kinetics of the biofilm did not vary significantly with season. The modelled benthic photosynthetic activity showed two peaks, one in spring and the other in autumn, with a trough in summer when dense stands of macrophytes heavily shade up to 80% of the riverbed (see Sup. Figure S2.2); a pattern which is widespread across the chalk rivers of southern England.

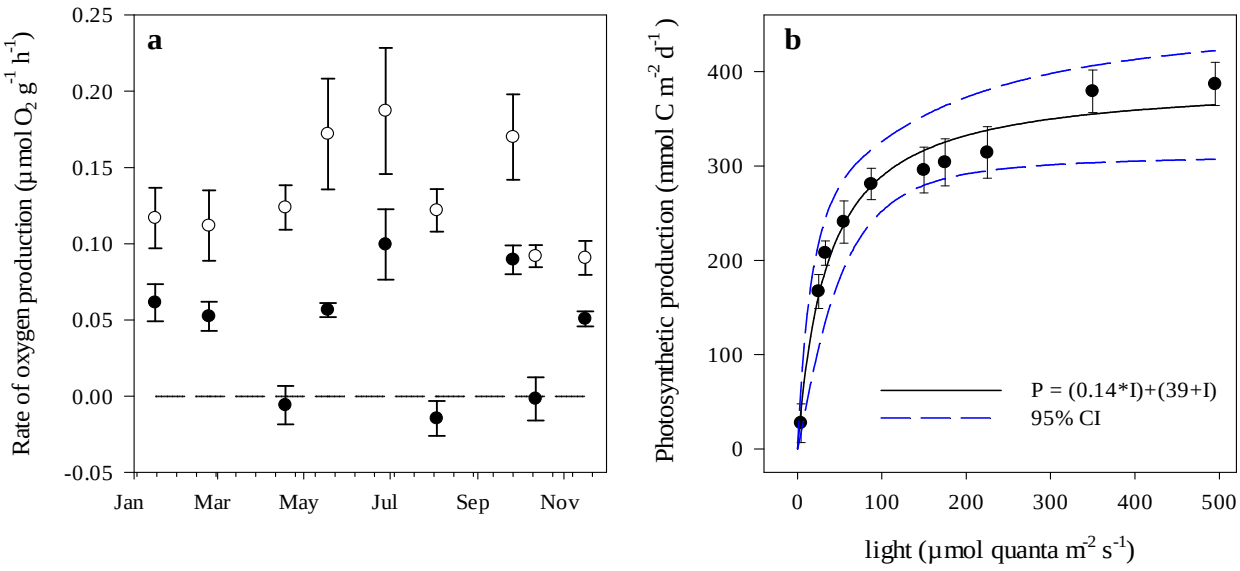

Figure 2.3: a) Mean (± se, *n*=7) rates of gross (open circles) and net (filled circles) photosynthesis in surface gravels. Dashed line indicates the compensation point; b) Photosynthesis-irradiance curve for the gravel biofilm community at the River Lambourn (r^2=0.92).

2.4.4 Viable subsurface chlorophyll

Chlorophyll-*a* was found at all depths within the Lambourn gravels throughout the year but decreased with depth from 7.4 µg Chl g^{-1} sediment at the surface, to 2.8 µg Chl g^{-1} sediment in the deepest section of the cores (Table 2.1). When exposed to light, all subsurface samples were able to produce oxygen which indicated the presence of viable photoautotrophic organisms at all depths. By normalising the rate of gross photosynthesis (i.e. taking into account the oxygen consumption via respiration) by chlorophyll content, to give biomass specific photosynthetic production, we found that the quality of the chlorophyll within the riverbed remained constant with depth (Table 2.1). This indicates rapid mixing between the subsurface pore water and overlying surface waters.

Table 2.1 Summary of mean grain size, methane oxidation, and chlorophyll quality in the sub-surface riverbed of the Lambourn. Here we have used the biomass specific photosynthetic potential (BSPP) to indicate the viability and quality of chlorophyll delivered to 15 cm into the riverbed. Note the decay in absolute amount of chlorophyll but consistency in BSPP with depth and the slight attenuation in methane oxidation (*see* Discussion).

Depth interval (cm)	Mean grain size (mm)	Chlorophyll -a ($\mu g\ g^{-1}$ sediment)	Methane oxidation at 450 nM (nmol $CH_4\ g^{-1}\ h^{-1}$)	Gross photosynthetic production (nmol $O_2\ g^{-1}\ h^{-1}$)	Biomass specific photosynthetic production (nmol $O_2\ \mu g^{-1}$ Chl)
0-3	9.7	7.4	0.723	133	22.5
3-6	6.7	5.6	0.72	79	17.7
6-9	5	3.7	0.576	50	19.9
9-12	4.8	3	0.528	37	20.3
12-15	5.1	2.8	0.507	26	21.5

2.4.5 Benthic primary production: net photosynthetic versus net methanotrophic carbon fixation

Across the 15 rivers, we estimated that between 260 and 960 µmol C m^{-2} d^{-1} was fixed via methane oxidation in August 2011. As a proportion of benthic NPP in the unshaded gravels, net methanotrophy accounted for between 1% and 46% of net carbon fixation (Figure 2.4). This is a conservative estimate as we did not take into account any shading from aquatic macrophytes or riparian vegetation.

Over the year in the Lambourn, net methanotrophy could potentially fix between 50 and 300 µmol C m^{-2} d^{-1} over the top 15 cm of the riverbed in winter and summer respectively (Figure 2.5a). Once normalised to the ambient methane concentration, the rate of methanotrophic carbon fixation followed the same seasonal pattern as the dissolved methane concentration in the river water, with a peak in summer and trough in winter. The NPP also peaked in mid-summer but with no NPP in April, August and October the relationship with season was weaker. As a proportion of carbon fixation via NPP, net methanotrophy fixed between 1% and 11% when there was NPP and 100% during periods of net heterotrophy (Figure 2.5b). This is not to say there was no photosynthesis, but there was no net carbon fixation because of rapid heterotrophic respiration within the biofilm. When integrated over the top 35 cm of riverbed (the inferred extent of methane and oxygen consumption in the riverbed;- here and *see* (Pretty *et al.*, 2006)), the contribution increased by 2.3 times and so, even when methane concentration in the water was lowest, and thus methanotrophy slowest (February 2011), net methanotrophy could produce the equivalent of > 3% of benthic NPP. Annually, carbon fixed via methanotrophy when integrated over the top 35 cm of the riverbed, was equivalent to 11% of benthic NPP.

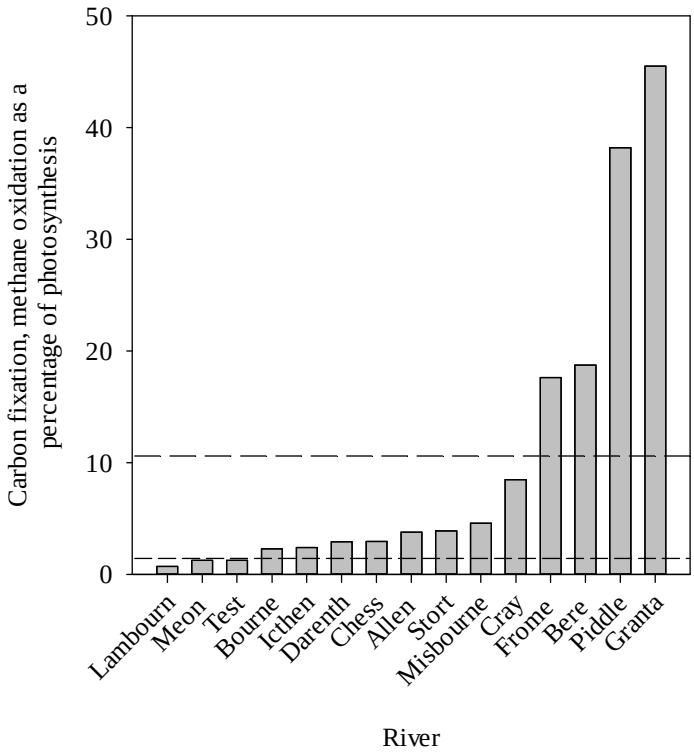

Figure 2.4: Estimated contribution of methane derived carbon in the wider survey (assuming 15 cm of methanotrophy).Dashed lines show the maximum and minimum seasonal range of methanotrophic carbon contribution from the detailed seasonal study in the River Lambourn.

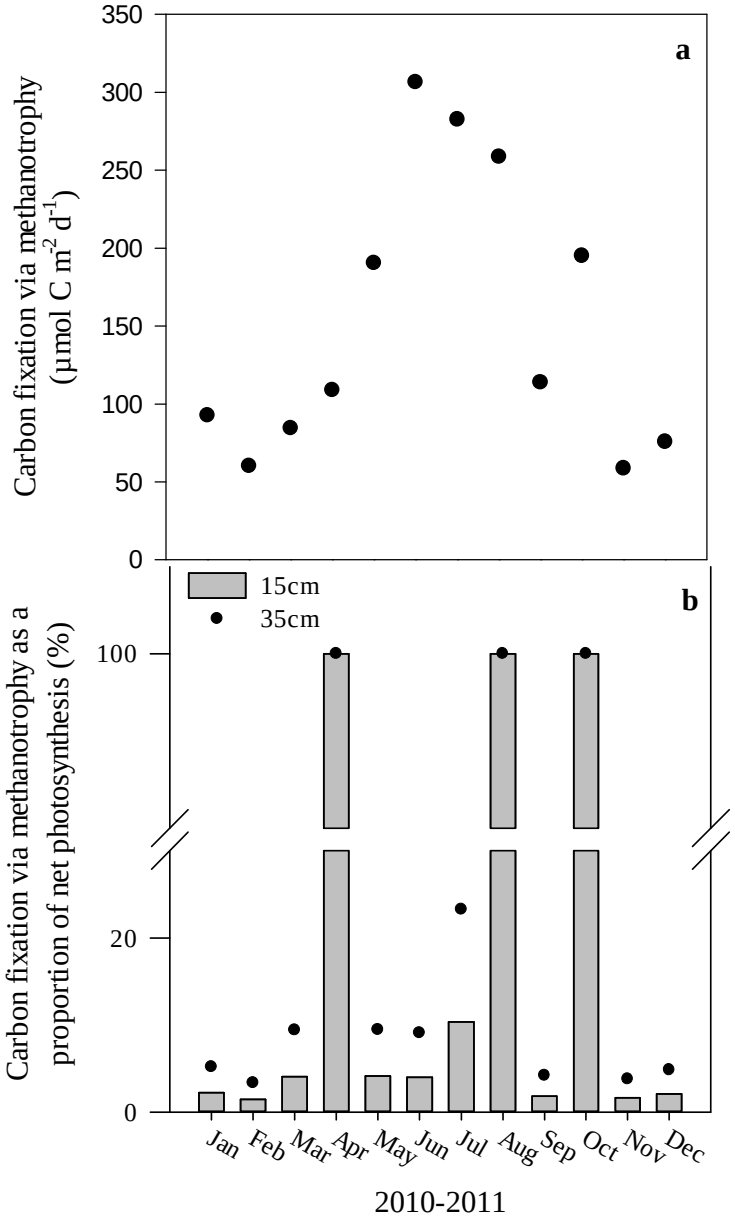

Figure 2.5: Carbon fixation via methanotrophy in the River Lambourn: a) integrated over the top 15 cm of the riverbed; b) as a proportion of that fixed via photosynthesis both over the first 15 cm (grey bars) and 35 cm (filled circles).

2.5 Discussion

Our study has highlighted geographically widespread methanotrophic carbon fixation within the riverbeds of over 30 chalk rivers. By measuring carbon fixation via photosynthesis, the well characterised, dominant source of benthic autotrophic carbon fixation in rivers at 15 of these sites, we were able to estimate the contribution of methanotrophy to the production of new biofilm carbon, the grazing community and ultimately the entire ecosystem. Although the input of allochthonous carbon (Thorp & Delong, 2002), as with most rivers, is an important source of energy to the system, here our focus was the production of new carbon. The decomposition of allochthonous carbon, trapped around the macrophyte stands ultimately produces methane (Sanders *et al.*, 2007) which is then available to methanotrophic bacteria as both an energy and carbon source (Hanson & Hanson, 1996). In this study, we have demonstrated that methanotrophy provides new carbon both at the riverbed surface, where photosynthesis is light-limited, especially in summer due to extensive shading, and deeper down in the riverbed where it is completely dark. Our results indicate a need to re-evaluate the long-held view that rivers receive their carbon through just two major mechanisms: photosynthetic detritus from the catchment (allochthonous carbon) and photosynthetic production within the river itself (autochthonous carbon) (Odum, 1953, Thorp & Delong, 1994).

While we have shown that the capacity for carbon fixation via methanotrophy in chalk rivers is widespread, it is strongly methane limited with a linear increase in activity observed well beyond the measured riverine methane concentrations. In contrast, the P-I curve shows that photosynthesis in the open gravels is light-saturated for much of the year. In short, in the summer, the photosynthetic organisms cannot exploit the higher light intensities but the methanotrophs appear to thrive on higher methane concentrations. Photo-inhibition studies on methanotrophy have often been in bottle incubations from stratifying water bodies (Dumestre

et al., 1999, Murase & Sugimoto, 2005) where strong gradients of methane and oxygen conflate the issue and high pH (caused by CO_2 removal due to high numbers of photosynthetic organisms) in illuminated bottles cannot be ruled out. The riverbed, however, has well-mixed oxygen and methane-rich water, we have previously measured simultaneous photosynthesis and methane oxidation in the laboratory (Trimmer *et al.*, 2010) and in our production calculations >80% of the length of the sediment core was from the dark subsurface. Our estimates for photosynthetic production over the fifteen riverbeds may be overestimates because we did not include the effect of shading as we were able to model with greater detail in the Lambourn.

The strong substrate limitation of methanotrophy at riverine methane concentrations implies that the methanotrophs could continue to mitigate the efflux of methane from rivers even where there are hot-spots of higher methane concentrations in fine sediment patches (Jones & Mulholland, 1998, Sanders *et al.*, 2007). Positive correlations between ambient methane concentrations and rates of methanotrophy have also been shown within (Deines *et al.*, 2007) and among lakes (Duc *et al.*, 2010), and in wetland sediments (Sundh *et al.*, 1995). The seasonal pattern in dissolved methane measured here agreed with our previous observations for similar chalk rivers in southern England. Although our seasonal study was restricted to the top 15 cm of the riverbed, data from earlier piezometer work indicated ideal conditions for methanotrophy (i.e. ample oxygen and methane) extend to at least 40 cm into the riverbed (Pretty *et al.*, 2006) and here, we estimate that methanotrophy extends to 35cm into the riverbed (Equation 1) which suggests the data presented in Figure 2.4 are underestimates of the potential contribution of methane-derived carbon to the food webs. The extensive river survey in August covered a greater range of both dissolved methane concentrations and methane oxidation rates, compared to the seasonal range in the Lambourn (*see* Figure 2.1). The methane oxidation rates were all measured with the same starting concentration of

methane and no normalisation for ambient methane concentration was carried out on the data, thus, the variation reflects real differences in capacity for methane oxidation across the 32 rivers and therefore capacity for methanotrophic carbon fixation.

The subsurface measurements of methanotrophy are strong evidence for new carbon fixation at depth and support our previous riverbed porewater gas data which had suggested a sink for methane at depth in the gravels (Trimmer *et al.*, 2009a). We know, however, the dark, subsurface gravels have good hydrological connectivity with the overlying water, as the viability of the chlorophyll pigments measured at depth (Table 2.1) (Huettel & Rusch, 2000) indicates rapid and continual delivery of fresh photoautotrophic organisms. The gravel beds of rivers are known to support a wide array of meiofauna and early ontogenetic stages of macroinvertebrates within the gravel interstices (Tod & Schmid-Araya, 2009) which are likely to graze on both new carbon, fixed via methanotrophy and high quality allochthonous import from above. Given the findings of our study, and by grazing the biofilm at depth, those fauna are likely to play an important role in delivering methane-derived carbon to higher trophic levels.

The seasonal distribution of macrophytes in rivers and their impact on hydrology, and nitrogen cycling has been studied extensively (Cotton *et al.*, 2006, Trimmer *et al.*, 2009b) but, as far as we are aware, this is the first study which considers their impact on riverbed primary productivity through shading. The modelled photosynthesis for the whole riverbed shows two peaks, one in spring and the other in early autumn (see Sup. Figure S2.2c), and is a temporal pattern previously observed for chalk stream secondary production (Wright, 1992). If the overhanging deciduous vegetation were to be included in the light model, thereby further reducing the summer riverbed irradiances, the summer trough in photosynthesis would be even deeper and given the constant yeild of oxygen per unit chlorophyll, the mid-summer biofilm could be less photosynthetically productive than those

in mid-winter. In short, throughout the annual cycle, both methane oxidation and photosynthesis are limited, by methane concentration and light intensity, respectively.

In combining estimates of both net photosynthetic and methanotrophic production, we placed our measurements of a relatively poorly understood process in the context of the traditionally accepted dominant source of autotrophic carbon fixation in clearwater rivers. At the surface, when the riverbed is illuminated, photosynthetic production completely dominates new carbon fixation. However, no river on Earth has a fully illuminated riverbed, irrespective of hour or season, and thus periods of darkness must be considered. Similarly, in permeable, well connected and oxygenated riverbeds, one cannot ignore the potential contribution of subsurface carbon fixation, namely, via methanotrophy, or even other chemosynthetic metabolism, to the total carbon budget. We have shown that just by considering the top 15 cm of the riverbed at the Lambourn, methanotrophy fixes carbon equivalent to 11 % of that fixed via benthic NPP in summer and conservative estimates from our wider survey suggest elsewhere this rises to at least 46% in August (the highest methane concentrations are usually observed in June). When considering periods of negative NPP, even in the unshaded gravels we begin to see how important other forms of production may be in these rivers which are famed for their photosynthetic autochthony.

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2.7 Supplementary Electronic Material

Supplementary Methods: Modelling photosynthesis across a seasonally shaded riverbed.

Firstly, we constructed a photosynthesis-irradiance curve to calculate the point at which photosynthesis in our samples reached light saturation(Guasch & Sabater, 1995, Jassby & Platt, 1976). We set up four replicate glass vials (60 mL) each with ~4 g sediment and filled with river water. The glass vials were immersed in a 45 L Perspex temperature controlled bath (9°C ± 0.5°C) and light sources (high-intensity tungsten 400W) were positioned either side of the tank. Using filters and variation in the distance between the light sources and the vials we created eleven different light intensities for each incubation. The experiment was repeated five times to give 20 replicates. After each experiment the chlorophyll-*a* was extracted as above. Irradiance was scattered against biomass specific photosynthetic production and the following relationship was fitted to the data:

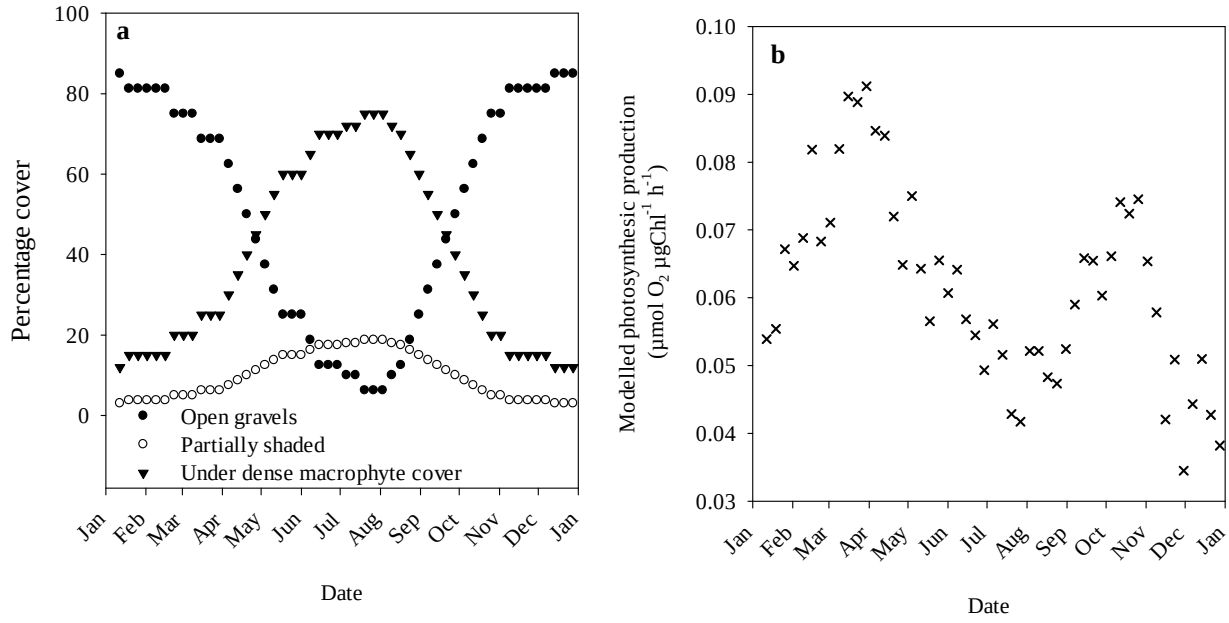
$$P=P_{max}\,[I]/\,(K_m + [I])$$

Where P is biomass specific photosynthetic rate, P_{max} is the maximum biomass specific photosynthetic rate, I is the light intensity and K_m is the half saturation constant.

Surface and riverbed irradiance data for the site, including the effect of shading in three different vegetation cover patch types was taken from a previous published study (Trimmer *et al.*, 2010). We estimated changing macrophyte cover over the year using studies of six English chalk streams (Flynn *et al.*, 2002, Wharton *et al.*, 2006, Wright *et al.*, 1982) and study site observations. By combining the surface irradiance data with our modelled riverbed shading, average weekly riverbed irradiances were estimated. Using the P-I curve, modelled weekly irradiances for each of the three shading patch types (open gravels, marginal shading and dense shading) were converted to estimates of *in situ* photosynthesis.

Supplementary Figures

Supplementary Figure S2.1: The study site on the River Lambourn in: a) January 2011; and b) July 2011.



Supplementary Figure S2.2: Modelling the seasonal changes in shading and photosynthesis over the River Lambourn bed: a) percentage cover of dense macrophyte stands and resulting seasonal shading patterns across the river bed; and b) weekly average photosynthesis across the whole river bed using the P-I curve and Figure S2.2a to account for changing incident light.

Supplementary Results

The biomass specific photosynthetic production (BSPP) (yield of oxygen per unit chlorophyll) did not vary over the year (see Supplementary Electronic Material Table S2.1) and so one photosynthesis-irradiance curve could be used to model changing photosynthesis with changing irradiances for all of the data and took the form:

$$P= 0.14[I]/ (39+ [I]) \qquad \text{Equation (2)}$$

The apparent half-saturation constant was 39 µmol photons m^{-2} s^{-1}. In order to estimate photosynthetic production across the whole riverbed, the modelled riverbed irradiances from Trimmer *et al.* (Trimmer *et al.*, 2010) were put into the P-I curve generated here to give predicted rates of photosynthesis throughout the year. The area of riverbed under dense shading varied considerably throughout the year (Electronic Supplementary Figure S2.2a). In July and August, over 70% of the riverbed was under dense shading with irradiance being only 6% of that in the unshaded parts. The overall modelled rate of riverbed photosynthesis showed an increase in early spring but then a summer minimum reflecting the widespread, dense shading (Supplementary Figure S2.2b). A second peak in photosynthetic productivity occurred in autumn when the macrophyte coverage declined, outpacing the concurrent decline in photosynthesis due to lowering irradiance. The lowest overall modelled rate of photosynthesis occurred in winter, despite over 80 % of the riverbed being completely unshaded but by then photosynthesis was light limited.

Supplementary Table S2.2

Summary of statistical analyses. Bold values are statistically significant. All organic carbon analysis was from August 2011 samples.

Test	Independent Variable	Dependant Variable	Random effect	Degrees of Freedom	F-value	p-value
Linear Regression	Methane concentration	Rate of Methanotrophy		10	120.2	**<0.001**
ANOVA	Month	Rate of Methanotrophy (normalised to riverine methane concentration)		291	27.6	**<0.001**
Linear Regression	Depth	Rate of Methanotrophy		298	11.0	**0.001**
ANOVA	Depth	Rate of Methanotrophy	Month	287	5.7	**<0.001**
Linear Regression	Depth	Organic carbon content		32	2.0	0.164
Linear Regression	$\delta^{13}C$ of the biofilm	Rate of Methanotrophy (Aug 2011)		32	5.0	**0.033**
Linear Regression	Depth	Chlorophyll-a content		297	88.9	**<0.001**
ANOVA	Month	Biomass specific photosynthetic production at the surface		51	2.0	0.061
Linear regression	Depth	Biomass specific photosynthetic production		297	0.01	0.935

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Chapter 3: Methane oxidation has the potential to mitigate any increase in methane production in rivers

This chapter moves on to focus on the effect of methane concentration and temperature on the rate of methane oxidation and how this varies between fine sediment and coarse gravels. This work was all done on the River Itchen which a large, well-studied river with a typical chalk stream riverbed characterised by clean gravels and patches of fine sediment deposited around dense macrophyte stands. This work has been submitted for publication in Freshwater Biology with myself as the first author, a undergraduate project student as the second, and my two supervisors, Dr. J. Grey and Prof. M. Trimmer as the final two authors.

3.1 Abstract

Many rivers are oversaturated in methane (CH_4) and carbon dioxide (CO_2) relative to the atmosphere but we know little about what controls the balance between these two important greenhouse gases and how they might respond to warming. Here, we characterise the potential response to temperature in the production of CO_2 and CH_4 and the subsequent oxidation of that CH_4 i.e. the sink and source components of the CH_4 cycle, in contrasting riverbed sediments; largely anoxic fine sediments, and oxic, coarse gravels. In the fine sediments, anaerobic production of both CH_4 and CO_2 increased with temperature, with apparent activation energies for each being 0.51 eV and 0.24 eV, respectively. The difference between the two resulted in a 4% increase in the ratio of CH_4:CO_2 production for a 1°C increase in temperature. In the coarse gravels, CH_4 oxidation showed no response to temperature at their characteristic CH_4 concentrations (30-200 nmol CH_4 L^{-1}), due to strong substrate limitation. In contrast, at higher CH_4 concentrations, more characteristic of the fine sediment patches (2-4 µmol CH_4 L^{-1}), CH_4 oxidation exhibited an increasingly strong response to temperature; eventually exceeding that

for CH_4 production. In the fine sediment, the surface layers had a CH_4 oxidation capacity over 100 times greater than the gravels and the kinetic response to differing porewater CH_4 concentrations meant CH_4 was oxidised some 2000 times faster in the fine sediment patches compared to the wider gravel riverbed. The calculated kinetic and temperature responses showed that with warming, methanogenesis is unlikely to outstrip methanotrophy and the ratio of CO_2 to CH_4 emitted could be conserved. Consequently, any change in the ratio of CH_4 to CO_2 emitted is more likely due to a bypassing of methanotrophy e.g. through ebullition or transport via plants, rather than an incapacity in the community of methanotrophs to match methanogenesis.

Keywords: carbon, methanotrophy, chemosynthesis, greenhouse gas, methanogenesis, gravel riverbed.

3.2 Introduction

Inland waters perform an important role in the global carbon cycle (Battin *et al.*, 2009) but their small areal extent coupled to a poor understanding of the magnitude of their carbon cycling processes has meant they are seldom integrated into global carbon models (Cole *et al.*, 2007). However, freshwaters are often oversaturated in two of the major greenhouse gases (GHG), methane (CH_4) and carbon dioxide (CO_2), and are net emitters to the atmospheric carbon pool, but there is a paucity of data for rivers particularly for methane (Bastviken *et al.*, 2011). Traditional global carbon cycling perspectives that disregard rivers as mere conduits have now been rejected (Aufdenkampe *et al.*, 2011) and rivers are now recognised as sites of rapid carbon cycling; whilst some carbon is ultimately exported to the ocean, much is metabolised within rivers and lost to the atmosphere (Battin *et al.*, 2009). Quantifying the rate of GHG production in rivers, and how this might change under future climate change scenarios such as warming and the impact of fine sediments mobilised via more frequent storms or indeed agricultural practice, is of paramount importance if we are to properly integrate rivers into the global carbon cycle.

Rivers receive much of their carbon from the surrounding catchment (Cole & Caraco, 2001, Jones *et al.*, 2001, Thorp & Delong, 2002) and a combination of changes in catchment land use and climate over the past century have increased the delivery of organic carbon to rivers, in both dissolved and particulate form (Bellamy *et al.*, 2005, Walling & Amos, 1999, Worrall *et al.*, 2004). Indeed, anthropogenic manipulation of catchments for urban or agricultural uses has been shown to have a greater effect on riverine metabolism than broad regional differences in catchment biogeography (Bernot *et al.*, 2010). Conversion from woodland and grassland to arable farmland, has been linked with increased siltation in rivers (Walling *et al.*, 2003) and this is problematic because fine sediment clogs gravel riverbeds (colmation) and becomes trapped around dense stands of macrophytes (Sand-jensen, 1998). As well as the destructive

biological consequences of habitat alteration on fish spawning and egg survival (Greig *et al.*, 2005, Soulsby *et al.*, 2001) and macroinvertebrate community structure (Kaller & Hartman, 2004), the anoxic sediments in some rivers produce as much CH_4 and CO_2 per unit area as peat bogs and heavily industrialised estuaries (Sanders *et al.*, 2007, Trimmer *et al.*, 2009b).

With such amplified allochthonous inputs the potential for rivers to emit CO_2 and CH_4 to the atmosphere is already high but how these supplements of carbon will interact with increased temperatures is largely unknown. Across both terrestrial and aquatic environments, respiration has a stronger temperature response than photosynthesis (Regaudie-de-Gioux & Duarte, 2012, Yvon-Durocher *et al.*, 2010a). In terrestrial environments, any potential temperature response in respiration is ultimately constrained by carbon fixed by photosynthesis whereas, due to their allochthonous carbon sources (Cole & Caraco, 2001), lakes and rivers have the potential to emit far more carbon as gases compared to the carbon they produce, thus becoming greater net sources of carbon to the atmosphere with increased temperature (Gudasz *et al.*, 2010, Trimmer *et al.*, 2012). Although less abundant than CO_2, atmospheric CH_4 is an especially potent GHG, with a warming potential, per mole, some 29 times higher over 100-years (Forster *et al.*, 2007). Moreover, as methanogenesis exhibits a stronger temperature response than respiration (Yvon-Durocher *et al.*, 2010b), moderate warming (approximately 2°C by the end of the 21st Century, (Stocker, 2013)) could result in a greater proportion of carbon being released from aquatic systems as CH_4 versus CO_2 (Yvon-Durocher *et al.*, 2014, Yvon-Durocher *et al.*, 2010b). Recent carbon gas budgets for lakes and reservoirs show that the global emissions of CO_2 and CH_4 (expressed in CO_2 equivalents) are currently on a par with one another (Tranvik *et al.*, 2009) and a further small increase in temperature could see CH_4 overtake CO_2 (Trimmer *et al.*, 2012). However, microbial CH_4 oxidation provides a sink for CH_4 and could attenuate some or all of the increase in CH_4 production due to warming.

The oxidation of CH_4 has been widely investigated in lakes (e.g. Bastviken *et al.*, 2008, Deutzmann *et al.*, 2011, Eller *et al.*, 2005) and more recently in rivers (Shelley *et al.*, 2014, Trimmer *et al.*, 2010) and its temperature dependence is often supressed under substrate limitation (Duc *et al.*, 2010, Lofton *et al.*, 2014). A recent meta-analysis of CH_4 emissions from aquatic ecosystems suggested that methanotrophy is unlikely to interact with temperature and CH_4 production in natural systems under *in situ* concentrations (Yvon-Durocher *et al.*, 2014) and our recently published kinetic response to the seasonal range of CH_4 concentrations in rivers confirms this substrate limitation in gravel riverbeds (Shelley *et al.*, 2014). Nevertheless, due to the contrasting environmental requirements of CH_4 oxidation and methanogenesis, the spatial configuration and magnitude of CH_4 sources and sinks will determine the final fate of CH_4 and therefore the overall balance of GHGs emitted from a river.

At our study site, a lowland river, the main riverbed is a mosaic of coarse gravel bed, with patches of predominantly anoxic fine sediment that accumulate around dense macrophyte stands (Figure 3.1, Sand-jensen, 1998) and the latter are known sources of both CH_4 and CO_2 (Jones & Mulholland, 1998, Sanders *et al.*, 2007, Trimmer *et al.*, 2009b). The coarse gravels also provide a sink for CH_4, oxidising CH_4 dissolved in the river water to CO_2 (Trimmer *et al.*, 2010). Benthic photosynthesis has long been recognised as a sink for CO_2 (Odum, 1956), but many riverbeds are net heterotrophic, with the sediments acting as a net source of CO_2 (Richey *et al.*, 2002). Here we sought to understand the interactions and feedbacks between CH_4 and CO_2 production, CH_4 oxidation and temperature in a naturally heterogeneous riverbed in order to better understand the role of rivers in global carbon cycling.

We broke our aims down into three research questions:

1. How will CH_4 and CO_2 production in riverbed sediments respond to warming?
2. How will riverbed CH_4 oxidation respond to increased temperature and CH_4 concentrations?
3. To what extent will CH_4 oxidation be capable of mitigating any increase in CH_4 production in riverbed sediments?

3.3 Methods

3.3.1 Study site and sample collection

Sediment was collected from the River Itchen at Ovington (51.083530 N, -1.1995214 W) in November, 2012. Here the river is 10-15 m wide, 0.5-1.2 m deep and there are dense stands of submerged macrophytes (Figure 3.1), predominantly *Ranunculus, Berula* and *Callitriche spp.* which trap fine sediment around their rhizomes and main stems.

We have already established that CH_4 oxidation is active throughout the oxic coarse gravels (Shelley *et al.,* 2014) and that methanogenesis occurs in the anoxic fine sediment patches (Sanders *et al.,* 2007) and so we decided to sample the sediment types separately to look at the cycling of CH_4 in the riverbed as a whole and how they interact with temperature. As the river water is well-oxygenated we also looked at the anoxic-oxic boundary layer on the surface of the fine sediment patches where we expected methanotrophic bacteria to be exploiting the high CH_4 concentrations (Jones & Mulholland, 1998, Sanders *et al.,* 2007, Trimmer *et al.,* 2010).

The coarse gravels were taken from the open gravel bed (Figure 3.1), using a kick-net and were then sieved (resulting in a sample with a grain size between 1.4 mm and 2.4 mm) and the fine sediment was collected from under the macrophytes stands using truncated syringes (60 mL) which were pushed into the sediment then sealed from beneath with a rubber bung. All sediment was refrigerated, and returned the laboratory within 3 hours.

Figure 3.1: Photograph of the site on the River Itchen showing the contrasting patches of fine sediment within the dense macrophyte stands (methane sources) and coarse gravel beds (methane sinks) which are typical for the chalk streams of southern England (Cotton *et al.*, 2006, Flynn *et al.*, 2002). Dr. J Grey provided the photograph.

3.3.2 Methane production: temperature dependence

We measured the rate of CH_4 and CO_2 production in anoxic slurries and while some of the CO_2 production will be from anaerobic respiration and fermentation, some will be released through acetoclastic methanogenesis (1:1 CH_4:CO_2 produced) and this must be considered when interpreting the temperature response of CO_2 production in these anoxic sediments. Moreover, a smaller, yet significant portion of methanogenesis could be hydrogenotrophic whereby CO_2 is reduced with H_2 to produce CH_4 and H_2O (Kotsyurbenko *et al.*, 2004). In the laboratory, the fine sediment cores were transferred to an anoxic glove-box (CV204; Belle Technologies, Portesham,

UK) and then, after careful extrusion to isolate the oxic surface layer from the rest of the anoxic core, ~2 g sediment and 2 mL of deoxygenated (10 minutes flushing with oxygen-free nitrogen (BOC)) river water were transferred into 12.5 mL gastight vials which were then sealed (*n*=32) with a nitrogen headspace. Within 30 minutes, gas samples (100 µL) were withdrawn from the headspace and injected into a gas chromatograph (GC) fitted with a flame-ionising detector (Agilent Technologies; for details *see* Sanders *et al.* (2007)). Headspace concentrations of CH_4 and CO_2 (after catalytic reduction over hot nickel (385°C) to CH_4) were calculated from peak areas calibrated against known standards (Scientific and Technical Gases), and the total amount in the vial (headspace plus water) was calculated using solubility coefficients (Weiss, 1974, Yamamoto *et al.*, 1976). The vials were placed on rollers (Denley, Spiramix 10) in temperature controlled rooms set at 3°C, 10°C, 15°C and 22°C and further gas measurements were made at 24, 48 and 72 h. After the final measurement, the vials were opened and the sediments dried to a constant weight.

3.3.3 Methane oxidation: substrate limitation

To identify the extent of substrate limitation (i.e. CH_4 concentration) on the potential for CH_4 oxidation in the gravels, gastight vials (*n*=60) were set up with sediment (~2 g) and river water (5 mL) and spiked with pure (100%) CH_4 (BOC) to generate concentrations in the water of 3-10500 nmol L^{-1}. For the fine sediment, the oxic surface layer was homogenised and ~0.5 mL was transferred into a gastight vial (*n*=18) then river water (5mL) was added and the vials were then spiked generating CH_4 concentrations between 14 - 21,300 nmol L^{-1}. The vials were gently shaken for 30 seconds and the concentration of CH_4 in the headspace measured by GC/FID (as above), and then over time (as above). The vials were incubated on rollers at 10°C to mimic average river water temperature (Mackey & Berrie, 1991) and the change in total CH_4 (headspace and water phase) was used to calculate the rate of CH_4 oxidation.

3.3.4 Methane oxidation: temperature dependence in coarse gravels

Previous work in aquatic sediments had shown a supressed response to temperature by CH_4 oxidation, arguing that this was due to strong limitation by CH_4 (Duc *et al.*, 2010). Given that we typically measure 60 nmol CH_4 L^{-1} in gravel pore waters (Trimmer *et al.*, 2009a), we assumed a similar suppressed response to temperature. To test this we set up a series of temperature incubations with increasing CH_4 concentration (n=11). For each concentration, replicate vials (n=12) with gravel (2 g) and river water (5 mL) were set up and spiked, generating CH_4 concentrations in the water phase of 30-6250 nmol CH_4 L^{-1} and a total of 132 vials. After spiking, the vials were gently shaken and the headspace measured and vials incubated at four different temperatures as above.

3.3.5 Methane oxidation: temperature dependence in fine sediments

To measure the temperature dependence of CH_4 oxidation in the oxic surface layer of the fine sediments, vials were set up as above (~0.5 mL sediment, n=32) but the headspace was only enriched to one concentration of CH_4, that found in the immediately underlying anoxic pore water (~2.5 µmol CH_4 L^{-1}). The rate of CH_4 oxidation at four different temperatures was measured as above.

3.3.6 Sediment Organic Carbon Content

To quantify the organic carbon content of the sediment samples, we performed a persulphate oxidation reaction to convert all organic carbon to CO_2. Sediments were freeze dried for 24 hours, weighed (200 mg) into 20 mL crimp-top vials (Anatune) and H_2PO_4 (4 mL of 6% v/v) added to drive off any carbonate. After 48 hours, potassium orthophosphate (4 mL of 0.15 M) was added to each vial and the vials capped with butyl rubber stoppers and aluminium tear-away seals (Grace Discovery Sciences). The headspace was then purged with oxygen-free nitrogen (BOC) to degas any CO_2 from the liquid and then pure oxygen (BOC) (to ensure

complete oxidation) and the samples were then autoclaved at 120 °C for 3 hours. After cooling, the CO_2 concentration in the headspace was measured by GC/FID. The organic carbon content was calculated by using potassium biphthalate prepared in a series of concentrations (resulting in total carbon 0-5 mg C) and then treated in the same way as the samples.

3.3.7 Deriving the apparent Activation Energies for the measured processes

The rates of each measured process (e.g. nmol CH_4 oxidised g^{-1} gravel h^{-1}) were log transformed and the incubation temperatures were converted to the form $1/kT$, where T is absolute temperature in Kelvin and k is Boltzmann's Constant (8.62×10^{-5} eV K^{-1} (T)). The logged rates were then plotted on an Arrhenius plot against $1/kT$ where the negative slope of the linear regression line gives an estimate of the apparent activation energy in electron volts (1 eV = 96.49 kJ mol^{-1}) for each process. We acknowledge that this "apparent" or "realised" activation energy will always be lower than the theoretical sensitivity of the biochemical reaction to temperature because of environmental constraints, such as substrate limitation. Here, we are merely using it as an empirical index of temperature response following the similar approach used by Yvon-Durocher *et al.* (2010b).

3.4 Results

3.4.1 Methane production: temperature dependence

The rate of anaerobic CH_4 production in the fine sediment increased with temperature (Figure 3.2a, Table 3.1) from 22 to 80 nmol CH_4 g^{-1} h^{-1} (at 3°C and 22°C, respectively), as did the production of CO_2 (from 147-261 nmol CO_2 g^{-1} h^{-1}) but with a lower temperature dependency than that for CH_4 (Figure 3.2a, Table 3.2). The apparent activation energies for both CH_4 and CO_2 production in the fine sediments were 0.51 eV and 0.24 eV, respectively (Figure 3.2a and Table 3.2). As a result, the ratio of anaerobic CH_4:CO_2 production increased by 0.04 °C $^{-1}$, approximately doubling over the full range of temperatures investigated (Figure 3.2b). Acetoclastic methanogenesis is likely to be the dominant pathway for CH_4 production in these sediments because of the isotopic signature of the CH_4 produced (~-58‰, unpublished data, Trimmer *et al.*) and because they are from freshwaters rather than marine (Whiticar *et al.*, 1986). A conservative estimate that two thirds of CH_4 production was via this pathway could account for up to 20 % of the CO_2 and would lower the activation energy for non-methanogenic CO_2 production from 0.24 to 0.19eV.

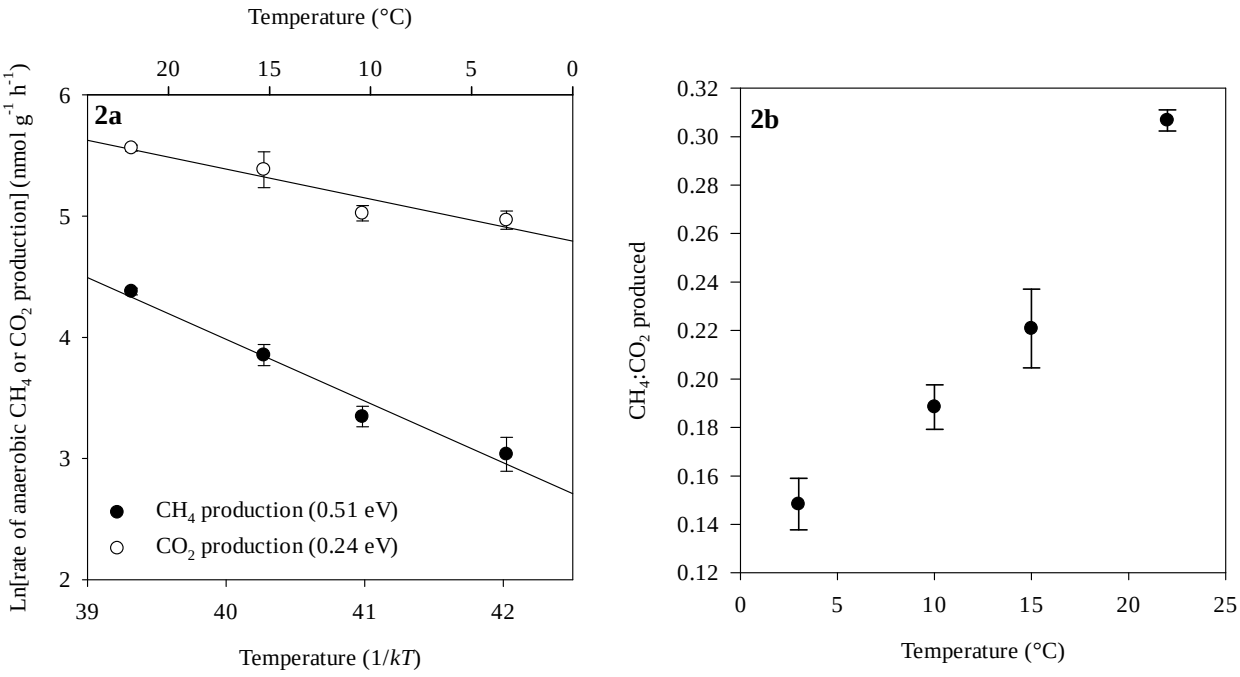

Figure 3.2: a) An Arrhenius plot showing the temperature dependency of methane and anaerobic CO_2 production. The slope of the regression line gives the activation energy (eV), b) The mean ratio of CH_4 to CO_2 gas production in the anoxic fine sediments with increasing temperature (error bars ± *SE*).

3.4.2 Kinetics of CH_4 oxidation in coarse gravels and fine sediments

In the gravels, we measured a linear increase in the initial rate of CH_4 oxidation with CH_4 concentration, from approximately atmospheric equilibration (2 nmol CH_4 L^{-1}) up to 10.5 CH_4 µmol L^{-1} (Figure 3.3a), suggesting strong substrate limitation at typical riverine concentrations (<200 nmol CH_4 L^{-1}). We also measured strong substrate limitation in the fine sediments but here the capacity for CH_4 oxidation was much greater and the activity became saturated at around 10 µmol CH_4 L^{-1} (Figure 3.3a). CH_4 oxidation in the fine sediments could be explained by simple Michaelis-Menten kinetics and took the form:

$$\text{Rate of } CH_4 \text{ oxidation} = (586C)/(3.68 + C)$$

Where C is the starting concentration of CH_4 and V_{max} equated to 586 nmol CH_4 g^{-1} h^{-1}, with an apparent K_m equivalent to 3.7 µmol CH_4 L^{-1}.

3.4.3 Temperature response of aerobic CH_4 oxidation

i) in the coarse gravels

At 10°C (~ average river temperature) and with CH_4 concentrations representative of the gravel bed (30-200 nmol L^{-1}), CH_4 oxidation was only 0.1 nmol CH_4 g^{-1} h^{-1} (*SE* ±0.03) and, there was no significant effect of temperature on the rate of CH_4 oxidation in the gravels (Table 3.1 and Figure 3.3b). Above this concentration, we measured an increasingly strong effect of temperature on the rate of oxidation which increased from a negligible sensitivity (0.05 eV, *SE*±0.031) at 260 nmol CH_4 L^{-1} to 0.6 eV (*SE* ±0.09) at 6250 nmol L^{-1} (Figure 3.3c, Table 3.2). At the highest concentration measured, the response to temperature was even greater than that for methanogenesis (0.6 eV to 0.51 eV).

ii) in the fine sediments

In the surface layer of fine sediments where the porewater CH_4 is high (2-4 µmol L^{-1}), the rates of CH_4 oxidation were some 1800-3100 times faster than those for the coarse gravels, ranging from 172 nmol CH_4 g^{-1} h^{-1} to 376 nmol CH_4 g^{-1} h^{-1} (over 3°C to 22°C), with an apparent activation energy of 0.3 eV (Figure 3.3b and Table 3.2). Even when the rates of CH_4 oxidation were normalised to the same starting concentration of 120 nmol CH_4 L^{-1}, the fine sediments still oxidised CH_4 112 times faster than the gravels, indicating a greater methanotrophic capacity in the fine sediment than in the gravel.

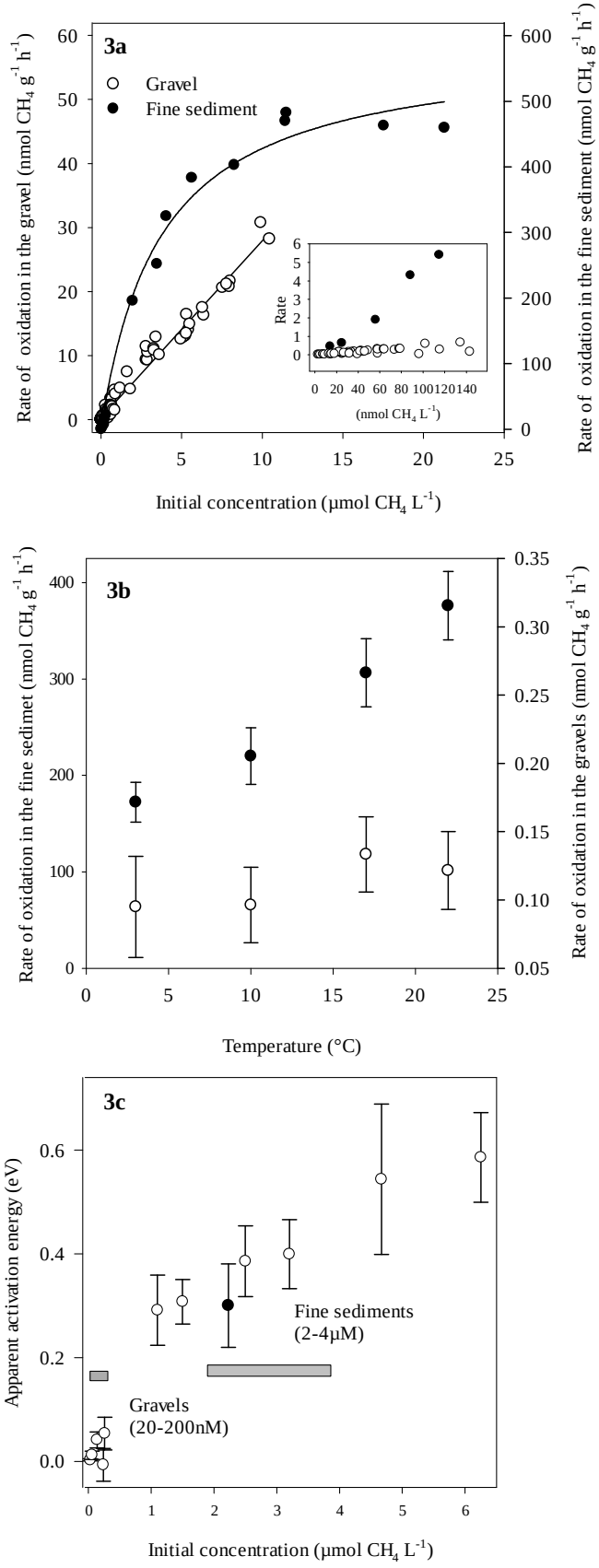

Figure 3.3: a) Initial rates of methane oxidation in the gravels (open circles) and fine sediments (filled circles) at a range of initial starting concentrations of methane. Inset shows CH_4 concentrations lower than 150 nmol L^{-1} i.e. those typical of the seasonal range found in many chalk rivers (Shelley *et al.*, 2014). Where *c* is the starting concentration of CH_4, the trend line for the gravels is a 1st order linear regression, where rate=0.3 + (2.8 *c*) and, for the fine sediments a Michaelis-Menten, where rate=$(586_{Vmax}\ c)/(3.68_{Km}+ c)$. b)Initial rates of methane oxidation in the gravels (open circles) and fine sediments, measured with starting CH_4 concentrations equivalent to those found in the pore waters of the two patch types (120 nmol CH_4 L^{-1} for the gravels and 2.5 µmol CH_4 L^{-1}). Rates in the fine sediment incubations are 1800-3100 times faster than those in the gravel incubations. c) Apparent activation energies of CH_4 oxidation in the gravels between 30-6250 nmol L^{-1} (open circles) and for the fine sediments at 2.2 µmol L^{-1} (filled circles) with the range of CH_4 concentrations expected in the gravels and the fine sediment patches marked with horizontal grey bars. Error bars ± *SE.*

3.4.4 Aerobic CO_2 production and organic carbon content

The fine sediments had a higher organic carbon content (18.2 mg C g^{-1}) and rates of aerobic CO_2 production (495-1278 nmol CO_2 g^{-1} h^{-1} at 3 and 22°C, respectively) compared to the coarse gravels (3.9 mg C g^{-1} and 28-145 nmol CO_2 g^{-1} h^{-1}). Normalising each rate of CO_2 production by the respective organic C content for each sediment type, showed that the fine sediments produced more CO_2 per unit of organic carbon (25-89 nmol CO_2 mg C^{-1} h^{-1} at 3 and 22°C, respectively) compared with the gravels (10-33 nmol CO_2 mg C^{-1} h^{-1}) indicating a denser population of micro-organisms in the fine sediments. However, aerobic CO_2 production in the coarse gravels exhibited a stronger temperature dependence (0.59 eV) than in the fine sediment patches (0.44 eV), both of which are at least twice that for anaerobic CO_2 production (Figure 3.4 versus Figure 3.2).

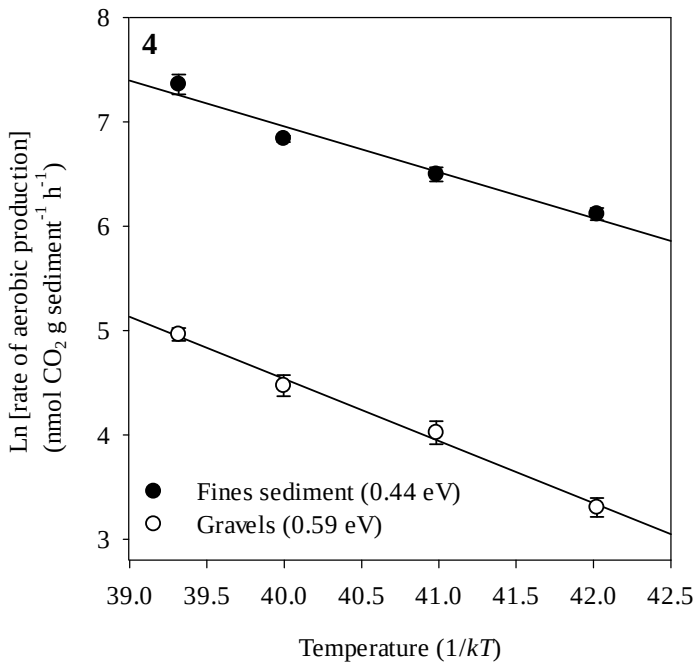

Figure 3.4: An Arrhenius plot showing the temperature dependency of aerobic CO_2 production in the two different sediment types. Mean values and error bars ± *SE*. The fine sediment (filled circles) CO_2 production is faster but has a weaker response to temperature than the coarse gravels.

Explanatory variable	Response variable	*df*	*F*	*p*	Intercept (a_1)	Slope (b_1)
Temperature (°C)	CH_4 production	1,29	154.18	0.00	6.08	3.11
Temperature (°C)	CH_4 oxidation (30-240nM data pooled)	1,46	0.45	0.50	0.102	0.00188
Temperature (°C)	CH_4 oxidation (260-6250nM data pooled)	1,106	35.01	0.00	0.390	0.259
Initial concentration (CH_4)	CH_4 oxidation (10°C)	1,41	838.9	0.00	0.701	0.00266
Temperature (°C)	Anaerobic CO_2 production (fine sediments)	1,29	22.04	0.00	116	6.64
Temperature (°C)	Aerobic CO_2 production (gravels)	1,30	147.1	0.00	3.2	6.18
Temperature (°C)	Aerobic CO_2 production (fine sediments)	1,30	11	0.002	506	41.4

Table 3.1: Response in rate of activity to temperature or initial concentration of CH_4. The dimensions of the slopes (b_1) are nmol g^{-1} h^{-1} $°C^{-1}$ except for the relationship between initial CH_4 concentration and CH_4 oxidation where the dimensions are nmol CH_4 g^{-1} h^{-1}.

Process	Apparent activation energy derived between 3°C to 22°C(eV)	Standard error	r^2
Anaerobic CO_2 production (fine sediment)	0.24	0.04	0.49
Anaerobic CH_4 production (fine sediment)	0.51	0.05	0.79
Aerobic CO_2 production (fine sediment)	0.44	0.03	0.51
Aerobic CO_2 production (gravels)	0.59	0.06	0.86
CH_4 oxidation (gravels <200nmol CH_4 L^{-1})	0.01*	0.02	0.01
CH_4 oxidation (gravels 260-6250 nmol CH_4 L^{-1})	0.05-0.59		
CH_4 oxidation (fine sediment at 2.5 µmol CH_4 L^{-1})	0.30	0.02	0.99

Table 3.2: Apparent activation energies for each measured process (nmol g^{-1} h^{-1} $(1/kT)^{-1}$) and the standard error and the r^2 of the regression line. * indicates that the slope, was not significantly different to zero. The full range of activation energies for CH_4 oxidation at the 11 CH_4 concentrations are shown on Figure 3.3c.

3.5 Discussion

Here, we have quantified the sources and sinks of CH_4, their temperature dependencies, and the interplay between the three, in contrasting types of sediments in a large riverbed. CH_4 production responds twice as strongly as CO_2 production to temperature and although strong substrate limitation supresses the temperature dependency of CH_4 oxidation in the gravel riverbed, in the fine sediment patches, where CH_4 concentrations are much higher, this is not the case. There is a marked interaction between methanotrophy and temperature which is dependent on substrate, which differs with sediment type. While we only calculated these detailed process measurements in one riverbed, local sources and sinks for dissolved CH_4 have previously been identified in rivers right across southern England (Shelley *et al.*, 2014) and so we are confident that this phenomenon is more widely spread.

3.5.1 CH_4 and CO_2 production

The difference in the temperature dependencies of CH_4 and CO_2 production (Fig. 2a) means that if emissions were driven by temperature alone, the predicted temperature rise of 2°C by the end of this century (scenarios RCP4.5, 6.0 and 8.5, Stocker, 2013), could result in an 8% increase in the proportion of carbon emitted as CH_4 (rather than CO_2) from the patches of fine sediment. Further, the delivery of fine sediment is likely to increase under some climate change scenarios and ongoing intensification of agriculture (Goudie, 2006, Sanders *et al.*, 2007). Despite this potential for methanogenesis, its apparent activation energy presented here (0.51 eV) is much lower than those calculated for other aquatic sediments: 1.7-2.0 eV in lake sediments (Lofton *et al.*, 2014); 1.3-2.8 eV in peat slurries (Dunfield *et al.*, 1993)); 0.85 eV in freshwater mesocosms (Yvon-Durocher *et al.*, 2010b); and 0.96 eV for a meta-analysis of 127 aquatic field sites (Yvon-Durocher *et al.*, 2014). This could be explained by poor substrate quality as has previously been demonstrated in wetland sediments (Bergman *et al.*, 1998,

Valentine *et al.*, 1994), lakes (Duc *et al.*, 2010) and rice paddy soils (Fey & Conrad, 2003). The more labile DOC is often leached from the allochthonous sediments into rivers long before they are deposited on the riverbed (Stanley *et al.*, 2012), resulting in increasingly refractory particulate carbon over time. Further evidence for the relatively poor carbon quality in the fine sediment can be seen in the aerobic CO_2 measurements where the apparent activation energy was higher on the gravels (0.59 eV, close to that of general heterotrophic metabolism 0.65 eV (Yvon-Durocher *et al.*, 2010a)) than that in the fine sediment (0.44 eV) which we already know to be mainly of terrestrial origin (Collins & Walling, 2007).

3.5.2 Methane oxidation

The capacity for CH_4 oxidation in gravel riverbeds has only recently begun to be investigated (Shelley *et al.*, 2014, Trimmer *et al.*, 2010) and here, we followed the kinetic response in two contrasting sediment types, which differ markedly in their porewater CH_4 concentrations. Our calculated V_{max} for CH_4 oxidation in the fine sediments (586 nmol CH_4 g^{-1} h^{-1}) is comparable with those reported around CH_4 seeps in Lake Constance (511 nmol CH_4 g^{-1} h^{-1},Deutzmann *et al.*, 2011) and landfill soils (743 nmol CH_4 g^{-1} h^{-1}, Bogner *et al.*, 1997) and although we did not find a plateau in the kinetic response in the gravels, it was clearly much slower than the fine sediment (Figure 3.3a), probably due to lower densities of methanotrophic bacteria on the gravel particles (Deutzmann *et al.*, 2011). Methanotrophs exploit the thin oxic layer enveloping the anoxic sites of methanogenesis and as CH_4 production increases, the increased substrate will stimulate faster oxidation of CH_4 (Megonigal & Schlesinger, 2002). At 10°C CH_4 oxidation (at 2.5 µmol CH_4 L^{-1}) was ~8 times faster than CH_4 production, illustrating the capability of the methanotrophic community to counteract any local increase in CH_4. The effect of substrate limitation on the temperature dependence of CH_4 oxidation gradually

weakened as CH_4 concentrations rose, eventually, at the top end of our concentration gradient (0.58 eV at 6350 nmol CH_4 L^{-1}), superseding that calculated for methanogenesis (0.51 eV).

Substrate limitation often supresses the temperature dependence of CH_4 oxidation, as has previously been documented in lake sediments (Duc *et al.*, 2010) and hypolimnetic waters (Lofton *et al.*, 2014) but our experiment at 11 different CH_4 concentrations (spanning the ambient range) gives a much more detailed picture of the potential interaction between temperature and substrate availability which, in rivers, is ultimately governed by sediment type. At the lowest CH_4 concentrations observed in rivers, typical of clean gravel beds (Jones & Mulholland, 1998, Trimmer *et al.*, 2009a), methanotrophy does not respond to temperature but there is a marked kinetic effect (*see* Figure 3.3a) and at only marginally higher concentrations (where substrate is still limiting) there is a temperature effect too.

These findings counter the recently published statement by Yvon-Durocher *et al.* (2014) that methanotrophy does not interact with CH_4 production and temperature under the substrate-limiting conditions encountered in aquatic ecosystems. Moreover, in other aquatic ecosystems, where anoxic, fine sediments constitute a much higher proportion of the bed (e.g. lakes and wetlands), the kinetics of methanotrophy enable it to offset temperature-induced increases in CH_4 (Duc *et al.*, 2010, Lofton *et al.*, 2014, Megonigal & Schlesinger, 2002), resulting in no change in CH_4:CO_2 emissions with increasing temperature. We propose that surface flux measurements which show an increase in CH_4 emissions with warming (Yvon-Durocher *et al.*, 2010b) must be as a result of CH_4 oxidation being physically bypassed, either through plants (Chanton *et al.*, 1993, Nouchi *et al.*, 1990, Sanders *et al.*, 2007), which play a globally important role in the CH_4 cycle (Carmichael *et al.*, 2014), or ebullition (Crawford *et al.*, 2014, Prairie, 2013) rather than the failure of methanotrophy to oxidise any increase in CH_4. Indeed, the seasonal pattern in river water CH_4 concentration often with a peak in summer (Devol *et al.*, 1990, Koné *et al.*, 2010, Trimmer *et al.*, 2009a) shows that some CH_4 is avoiding

oxidation, and in clear-water rivers, where water column methanotrophy is negligible, once the CH_4 is in the main channel water, the oxic gravel beds are the final barrier to CH_4 degassing to the atmosphere. Although the rates of oxidation in the gravels are comparatively slow, relative to the surface fine sediment layer, the large three-dimensional volume (to some 35 cm in depth (Shelley *et al.*, 2014)) of oxic gravels means they do oxidise a substantial amount of CH_4 to CO_2 (Shelley *et al.*, 2014), the less potent of the two GHGs and therefore provide an important role in carbon cycling that has, until recently, largely been undocumented.

The data presented here are novel as we are the first to consider the effect of warming on the individual CH_4 cycling processes and the feedbacks between them as substrate concentrations vary across a heterogeneous riverbed. We have demonstrated the potential for CH_4 oxidation to respond rapidly to increasing CH_4 production, mitigating efflux of CH_4 diffusing through the anoxic-oxic sediment layer. These data show the importance of detailed process measurements in understanding carbon cycling in aquatic systems; surface flux measurements may be useful to track trends in emissions but a more thorough approach is necessary to fully comprehend the direction and magnitude of the interactions in the methane cycle within these dynamic natural environments.

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Chapter 4: The effect of light availability and methane concentration on the importance of methane derived carbon in chalk rivers: a spatial and seasonal survey.

In this chapter, I exploited the natural variation in methane concentrations across English chalk streams in order to explore its effect on the importance of methane-derived carbon relative to photosynthetic carbon. To quantify the influence of light availability on the importance of methane-derived carbon to the food web, adjacent shaded and unshaded stretches of each river were sampled. The fieldwork and some of the laboratory analyses were performed in collaboration with Dr. Nicola Ings as part of a wider NERC grant. This chapter has not yet been submitted for publication.

4.1 Introduction

Methane gas is oversaturated relative to the atmosphere in many aquatic environments and it is increasingly apparent that methanotrophy provides a mechanism for channelling the energy from this single-carbon compound into aquatic food webs (Jones & Grey, 2011). Methanotrophic bacteria are unique in being able to use methane as their sole carbon and energy source (Hanson & Hanson, 1996); they oxidise it to carbon dioxide and in doing so fix methane into organic carbon. The fastest rates of aerobic methane oxidation occur at the interface between anoxic sites of methane production and the oxic layer above, and this has been widely studied in stratifying lakes (Bastviken *et al.*, 2003), wetlands (Segers, 1998), marine methane seeps (Sassen *et al.*, 1998) and soils (Bender & Conrad, 1995). There have been relatively few studies on methane oxidation in rivers but earlier parts of this thesis focus on the activity of methanotrophs and have found widespread capacity for methanotrophic production in the riverbed gravels of chalk rivers. In this chapter we go one step further by employing stable isotopes to assess the contribution of methane derived carbon (MDC) to the food web and investigating whether light availability and methane concentration affect the size of this contribution.

Using stable isotope analysis, the distinctive depleted $\delta^{13}C$ value associated with methane (Whiticar, 1999), can be used to estimate its contribution to the biomass of specific food web components such as pelagic and benthic invertebrates (Jones *et al.*, 2008, Kankaala *et al.*, 2006), and fish (McLeod & Wing, 2007, Ravinet *et al.*, 2010). Although stable isotope analysis is widely used to indicate MDC, the most robust food web studies use it in conjunction with process measurements or molecular techniques (Bastviken *et al.*, 2003, Eller *et al.*, 2005, van Duinen *et al.*, 2013). The need for supporting evidence is particularly acute when stable isotope data indicate the contribution of MDC to organism biomass may be small or that the isotopic signal in the methane may not be particularly distinct from other basal resources (e.g. the $\delta^{13}C$ value of groundwater methane can be >-40‰ (Zhang *et al.*, 1998)) leading to ambiguity.

Rivers are often over-saturated with methane (Jones & Mulholland, 1998, Koné *et al.*, 2010, Shelley *et al.*, 2014) but to a much lesser extent than still water bodies such as wetlands (Van der Nat & Middelburg, 2000), reservoirs (Abril *et al.*, 2005) and lakes (Huttunen *et al.*, 2003). However, there is now evidence that secondary production in some rivers is, in part, fuelled by MDC (Trimmer *et al.*, 2009). Studies indicating high incorporation of MDC from the profundal zones of lakes are perhaps not surprising because of high (mM concentrations) methane concentrations and capacity for methane oxidation, and because they are below the euphotic zone meaning any photosynthetic carbon must be transported there i.e. there is no fresh photosynthetic carbon being produced *in situ*. The findings of Trimmer *et al.* (2009) showed that MDC may be contributing ~30% of the organic carbon of the abundant grazing cased-caddis, *Agapetus fuscipes* in a free-flowing chalk river, where methane concentrations are relatively low (30-150 nM) and benthic photosynthetic production is high (Trimmer *et al.*, 2010). Subsequent detailed potential measurements of photosynthetic and methanotrophic primary production at 15 chalk rivers highlighted seasonal and geographical variation in the

ratio of methanotrophic to photosynthetic production in riverbed sediments (Chapter 2) but we have yet to link this with the depleted $\delta^{13}C$ signature in grazing invertebrates.

The gravel biofilm is a site of both photosynthetic and methanotrophic (chemosynthetic) primary production (Shelley *et al.*, 2014) and invertebrates grazing unselectively upon this as their sole source of nutrition would typically have very similar or slightly enriched $\delta^{13}C$ values (~+0.4‰) to the biofilm due to trophic fractionation during metabolism (Post, 2002). The $\delta^{13}C$ value of riverbed algal carbon, although very variable, generally falls with -35‰ to -20‰ (Finlay, 2001) whereas methanotrophic biomass has a much more depleted signature of -50‰ or lower (Summons *et al.*, 1994, Whiticar, 1999). Therefore, any change in the relative proportions of the two autotrophic carbon pools should result in a change in the overall $\delta^{13}C$ value of the biofilm which will be conserved as it passes up a trophic level into the grazers.

If shading decreases benthic photosynthetic production and methanotrophic production is unaffected we would expect an increase the relative contribution of MDC to the food web in shaded areas relative to open areas of riverbed. Consequently, we might expect the isotopically light methane signature to be more pronounced in organisms feeding in shaded stretches of a river compared to those feeding in well illuminated stretches. Moreover, the well characterised response of methanotrophs to raised methane concentrations (Bender & Conrad, 1992, Bogner *et al.*, 1997, Dunfield *et al.*, 1999) means the bulk biofilm carbon should be more ^{13}C-depleted in rivers with higher methane concentrations as has been shown in chironomids from lake sediments of varying methanogenic potentials (Deines & Grey, 2006). We would therefore predict the most depleted $\delta^{13}C$ signatures to be in invertebrates feeding in heavily shaded stretches of rivers with high methane concentrations.

To quantify the seasonal variation in the importance of MDC as a basal resource we sampled one river seven times over a year. Then, to assess the natural variation between rivers we

performed a field survey of 15 rivers, encompassing a broad range of methane concentrations. Within each river, we quantified photosynthetic and methanotrophic production in adjacent heavily shaded and well illuminated stretches to estimate the effect of shading on the ratio of chemosynthetic to photosynthetic basal resources. Additionally, we analysed the $\delta^{13}C$ of the main food web components to test our hypothesis regarding the changing contribution of methane derived carbon as a function of shade. We chose to analyse the key basal resources (leaf litter, the dominant aquatic macrophyte, *Ranunculus spp.*, and the riverbed biofilm) and the most abundant primary consumers (two species of cased caddis (*Agapetus fuscipes* and *Silo nigricornis)* and freshwater limpets (*Ancylus fluviatilis*)).

4.2 Methods

4.2.1 Study sites

To assess the intra-river effect of seasonal changes in light and methane concentration on the importance of MDC as a basal resource, we sampled bimonthly for one year at Bere Heath Farm on the Bere stream, in Dorset, from in February to December 2011 (n=6). To investigate the inter-river variation, fifteen chalk streams (Table 4.1) from across southern England were visited in August 2011 (15^{th}- 30^{th}) during maximum riparian shading and the end of the summer peak in river-water methane (Shelley *et al.*, 2014) both of which were hypothesised to affect the origin of carbon available to the river food web. At each site, adjacent shaded and unshaded stretches of river were sampled and, across the 15 sites, eight had the open areas upstream of the shaded and seven *vice versa* (Table 4.1). Each stretch was ~30m long and the two areas were always within 100m of each other except for the Granta were the shaded area was ~300m upstream of the open area due to access constraints.

4.2.2 Water CH_4, pCO_2 and ΣDIC concentration

Dissolved methane and carbon dioxide in the river water was quantified by taking water samples (n=12 for each river section at mid-depth and mid-channel) using polytetrafluoroethylene (PTFE) tubing attached to a 60 mL gas-tight syringe. Water was gently discharged into the bottom of a 12 mL gas-tight vial (Exetainer, Labco) and allowed to overflow (3 times) before half were fixed (100 µL of the bactericide $ZnCl_2$ 50% w/v in ultra-pure water) and all were then sealed. The samples for pCO_2 analysis could not be fixed as the bactericide also acidifies the sample but we know there is no change in pCO_2 within the first 24 hours if the vial is kept refrigerated. Upon return to the laboratory, within 3 hours, a 2mL headspace (analytical grade helium) was introduced using a two-way valve and a gas-tight

syringe (Hamilton). After equilibration (30 minutes), gas samples (100 µL) were withdrawn from the headspace and injected into a gas chromatograph equipped with a flame ionising detector and a hot-nickel catalyst to reduce the CO_2 to CH_4 (Agilent Technologies, (Sanders *et al.*, 2007)). The concentration of CH_4 or CO_2 in the headspace was calculated from peak areas calibrated against known standards (Scientific and Technical Gases) and the amount in the original river water sample was calculated using solubility co-efficients (Yamamoto *et al.*, 1976). To measure total dissolved inorganic carbon (ΣDIC), 100 µL of HCl (12.2 M) was injected through the septa of the fixed samples and, after equilibration, the concentration of CO_2 in the headspace was measured as above against a calibration series (0-10 mM) of sodium carbonate. Water temperature, pH (Hanna Instruments) and nutrient concentrations (nitrate, nitrite, ammonia and phosphate) were also measured at all sites.

4.2.3 Stable isotope analysis of dissolved pCO_2 and ΣDIC

The stable isotopic signatures of pCO_2 and ΣDIC in the water samples were measured with an elemental analyser (Flash EA 1112, Thermo-Finnigan), coupled to a continuous flow isotope ratio mass spectrometer (IRMS; Finnigan MAT $Delta_{Plus}$, Thermo-Finnigan). Gas samples from the headspace of either the acidified samples for ΣDIC (100µL) or the un-fixed samples for pCO_2 (500 µL) were injected into to the mass spectrometer with helium used as a carrier gas and certified CO_2 gas, calibrated against a secondary standard (Sucrose, -12.42‰ vs. VPDB, ref. 8542), was used as a reference gas.

$\delta^{13}C$ values were calculated using the following equation:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000 \qquad \text{Equation 1}$$

where R is the ratio of the heavy isotope to the light isotope and the units are parts per thousand (‰).

4.2.4 Potential for methane oxidation

To quantify the potential for methane oxidation, gravels from six discrete patches within each open and shaded area (*n*=6x2 for each river), were carefully kicked into a net and then transferred to zip-lock bags, and kept cold during transfer to the laboratory. Approximately 500 mL of river water was also collected. In the laboratory, approximately 1 g of gravel was placed into a gas-tight vial (as above) with 5 mL of river water (*n*=6 for each river). Once sealed, the air headspace was enriched with methane (300 µL of 10,000 ppm CH_4 in He) to give an initial concentration of 450 nmol CH_4 L^{-1} in the water. The concentration of methane in the headspace of each vial was measured immediately after spiking and then at 24 hour intervals for 4 to 5 days. Between measurements, samples were incubated on rollers (Denley, Spiramix 10), at 11°C, in the dark to mimic average riverbed temperature and prevent photosynthesis from removing CO_2 and raising the pH. The rate of methane oxidation was calculated from the negative slope of the relationship between the number of hours since the first measurements and the amount of methane at each time point, i.e. the nmol CH_4 consumed per hour during the linear phase. After the final measurement, gravels were dried and weighed and rates of methane oxidation were normalised to dry mass. Control vials were set up to test for any potential for methane oxidation in the river water used in the incubations.

4.2.5 Quantifying riverbed irradiances

Photosynthetically active radiation (PAR; 400 to 700nm) was measured (as µmol quanta m^{-2} s^{-1}) on each visit using a Skye Quantum sensor (Skye Instruments Limited). Measurements (*n*=6-12 depending on channel width) were taken on the river bed along transverse transects (*n*=3) which were spaced by approximately 10 m. Shaded and unshaded sections were measured within a 10 minute time frame, to minimise the effect of changing light conditions.

4.2.6 Photosynthetic production and chlorophyll

To quantify the potential for photosynthetic production in the riverbed, gravel samples (*n*=6x2 for each river) were collected, as above and oxygen evolution was measured over timed periods of light and dark. Approximately 30 g of sediment was placed into Perspex incubation chambers (internal diameter 13 cm) which were then fully submerged in a well-oxygenated, temperature-controlled water bath (45 L of river water, kept at 11°C). The chamber lids were fitted with a built-in rotating magnetic flea (200 rpm), driven by an external magnetic unit (Rank Brothers Ltd.) and a cable gland for holding an oxygen electrode. The oxygen concentration was logged at 1 minute intervals using four fast-response calibrated oxygen electrodes (50 µm tips fitted with stainless steel protective guards, Unisense), connected to in-line amplifiers and a four-channel data-logging meter (Unisense). A photon flux density of 55 µmol photons m^{-2} s^{-1} was generated at the gravel surface by placing a high intensity tungsten light source 50 cm above the tank. After 45 minutes of logging in the light, the water bath was made dark and logging continued for a further 45 minutes (Shelley *et al.*, 2014, Trimmer *et al.*, 2010). The chambers were then opened and sediment was transferred to 200 mL bottles (Nalgene) along with 20 mL of acetone (90% v/v with ultrapure water). They were left to extract for 24 hours in a dark refrigerator after which absorbance was measured at 750 nm to check for clarity, and 650 nm for chlorophyll extinction (Dalsgaard, 2000). Finally, the gravel samples were dried to a constant weight and both the rates of photosynthesis and chlorophyll content were normalised to dry mass.

4.2.7 Estimating carbon fixation

Daily methanotrophic production was estimated using the following equation:

$$Methanotrophic\ production = \left(\frac{R_{mo}}{C_i}\right) \times C_{amb} \times V \times CFE \times d \times h \quad \text{Equation 2}$$

Whereby, R_{mo} is the rate of methane oxidation, C_i is the initial methane concentration, C_{amb} is the ambient methane concentration at the site, V is the volume in cubic centimetres taken up by one gram of gravel (0.95), CFE is the carbon fixation efficiency (0.5, *see* Chapter 5), d is the depth over which we have integrated the methane oxidation (15 cm is the conservative estimate of riverbed depth over which methane oxidation occurs at a similar rate to that at the surface (Shelley *et al.*, 2014)) and h is the number of hours in one day (24).

For photosynthetic production, the measured rates of net photosynthesis were scaled using a photosynthesis-irradiance curve constructed with gravels from the River Lambourn. The scaling was dependant on the riverbed irradiance measured at our visit relative to that in the laboratory incubation chambers (55 µmol photons m^{-2} s^{-1}). After adjustment for riverbed irradiance, the rates were calculated using a similar equation:

$$Photosynthetic\ production = R_{NP} \times V \times CFE \times f \times h \qquad \text{Equation 3}$$

Whereby net photosynthesis (R_{NP}) was multiplied by 0.95 (V), 1 (CFE), 1 (depth) and 13 (the number of hours of sunlight during August at 51.5°N).

4.2.8 Stable isotope analysis of organisms

We collected three basal resources and three common grazing invertebrates to measure $\delta^{13}C$ and assess changes in the importance of MDC. Plants and leaf litter were collected by hand and stored in plastic zip-lock bags. Biofilm samples were collected by scrubbing cobbles (>4cm length) with a toothbrush, dislodging the biofilm into a tray filled with river water (<50 mL) which was then decanted into a plastic 50 mL centrifuge tube (Falcon). A standard kick-net sample was used to collect gravel (n=5 per area, 10 per river) that was tipped into trays with river water and individual invertebrates were manually picked out using forceps. They were stored in centrifuge tubes along with river water for return to the laboratory in a

portable refrigerator, left for 24 h to clear their guts (Feuchtmayr & Grey, 2003) before being frozen. Following defrosting, caddis-fly larvae and limpets were extracted from their cases/shells and then all of the samples were covered (2-5 mL) with hydrochloric acid (0.5 M) to remove inorganic carbon. After acidification (~24 h or until effervescence stopped even with additional acid) samples were rinsed with deionised water, dried to a constant weight (at 60 °C) and then homogenised with an agate pestle and mortar. Samples were weighed (~0.5 mg for invertebrates, ~0.8 mg for biofilm and plants) into ultra-clean tin capsules (Elemental Microanalysis, U.K.) and run through an elemental analyser (Flash EA 1112, Thermo-Finnigan), coupled to a continuous flow isotope ratio mass spectrometer (IRMS; Finnigan MAT $Delta_{Plus}$, Thermo-Finnigan). $\delta^{13}C$ values were calculated as in section 4.2.3.

4.2.9 Statistical analyses

To determine the significance of differences between the shaded and open areas of the rivers, paired t-tests (two tailed) were performed and the p-values are reported. The stable isotope value for the organisms were not available for every site and so two sample (unpaired) t-tests were used instead. To test for a significant correlation between two continuous variables, we used linear regression analysis. For the seasonal study, date was fitted as a random effect when a mixed effects model to determine whether the changing capacity for methane oxidation with changing methane concentration, was different in the shaded and open areas.

4.3 Results

4.3.1 Seasonal survey

At the Bere Stream, the concentration of methane in the river water (Figure 4.1a) and the capacity for methane oxidation varied seasonally; both at a maximum in late summer and were at their minimum in winter. The increase in methanotrophic capacity, that is, the rate of methane oxidation at a constant initial concentration of methane, was correlated with an increase in the ambient methane concentration (p=0.002). The riverbed gravels from the shaded area had a greater capacity to oxidise methane compared to the open area in the summer (Figure 4.1b) but over the whole year, the capacity was not significantly different between the two areas. Moreover, there was no difference in the response of methanotrophic capacity to ambient methane concentration between the shaded and open areas (p>0.05). The carbon fixed via methanotrophy displayed a similar seasonal pattern to the methane concentration (Figure 4.1c), not surprising as the calculation is driven by the strong kinetic response of methane oxidation to changing substrate concentration. Over the year, methanotrophic production was not different in the shaded and open areas (p=0.195) but if we exclude the winter times points, when methane concentrations were low and shading was minimal, then the methanotrophic production was greater in the shaded area than in the open area (p=0.043, Table 4.1 Figure 4.1c).

Dependant variable	Independent variable	Test	n	*p*-value
MO capacity	Methane concentration	ANOVA linear regression	14	0.002
MO capacity	Open/Shade	T-test (paired)	14	0.195
MO capacity (date as random effect)	Methane concentration and open/shade	Mixed effect Model	58	>0.05
MOP	Open/Shade	T-test (paired)	14	0.195
MOP (excl. Feb and March)	Open/Shade	T-test (paired)	10	0.043
GPP	Open/Shade	T-test (paired)	12	0.017
Chrolophyll	Open/Shade	T-test (paired)	12	0.458
NPP	Open/Shade	T-test (paired)	12	0.082
MOP as % of (MOP+NPP)	Open/Shade (excl. August where no NPP)	T-test (paired)	8	0.046
δ^{13}C biofilm	Open/Shade	T-test (paired)	14	0.044
δ^{13}C Ranunculus	Open/Shade	T-test (paired)	12	0.004
δ^{13}C Agapetus	Open/Shade	T-test (paired)	14	0.766
δ^{13}C Silo	Open/Shade	T-test (paired)	13	0.153
δ^{13}C limpets	Open/Shade	T-test (unpaired)	11	0.03

Table 4.1: Key statistical results and test details for the seasonal study at the Bere Stream. MO=methane oxidation, MOP= methanotrophic production, NPP= net photosynthetic production. All t-tests were paired except for when data was missing for one area as was the case for limpets where none were founded in late August and September.

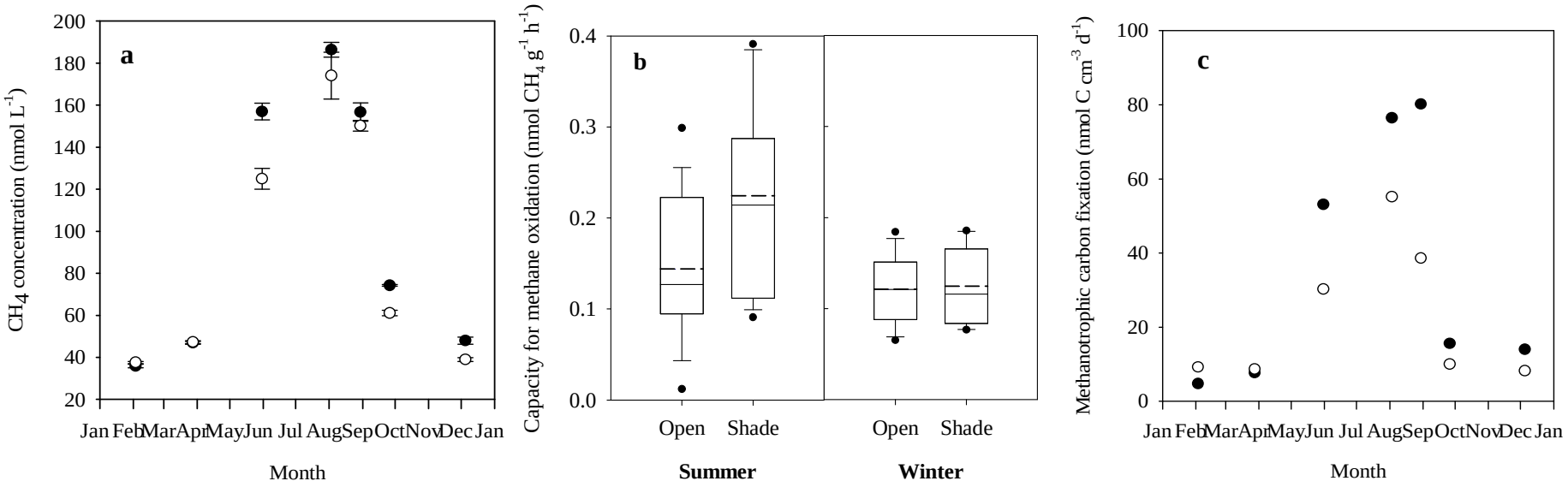

Figure 4.1: a) The dissolved concentration of methane in the river water in the shaded (filled circles) and open (open circles) stretches of the Bere Stream. Mean values (n=6) ±*SE*. b) The rate of methane oxidation when incubated at a constant initial methane concentration (100 nmol CH_4 L^{-1}) i.e. the capacity for methane oxidation in the summer (May, August and September) and winter (December, February and March). Median (solid line), mean (dashed line) and 25% and 75 % quartiles (box ends) are shown. c) Carbon fixed via methane oxidation in the shaded and open stretches of the Bere Stream.

Riverbed irradiance remained low throughout the year in the shaded area (Figure 4.2a) because the increased shading in the summer counteracted the seasonal increase in sunlight intensity. In contrast, in the open area, we measured over 20 times more light reaching the riverbed in August than in early February (Figure 4.2a). Gross photosynthesis was higher in the open gravels than in those from the shade (p=0.017) but there was no difference in the chlorophyll content (p=0.176) which averaged 3.2 µg g^{-1} dry sediment (Table 4.1). Net photosynthetic production (NPP) did not follow a smooth seasonal pattern because there was no net photosynthesis in either area in August, nor in the shaded area in March, August and December. In all but one month (August), there was more NPP in the open area than the shaded area but over the whole year, statistically there was no difference in NPP (p=0.082). The light intensity reaching the gravels in the laboratory incubations (55 µmol photons m^{-2} s^{-1}) was more intense than that measured at the riverbed in five out of the six visits in the open area and always more intense than riverbed irradiances in the shaded area. Also, the laboratory light was below saturation and therefore in the light limiting part of the PI curve (*see* Chapter 2), the normalisation for ambient light was a down-scaling for most data points and the adjustment was greater for the shaded area gravels (~23 times) than the open are gravels (~7 times). The highest estimate of photosynthetic production was 432 nmol C cm^{-3} d^{-1} in the open area in late May, when, at the same time, only 30 nmol C cm^{-3} d^{-1} was fixed in the shaded stretch (Figure 4.2b).

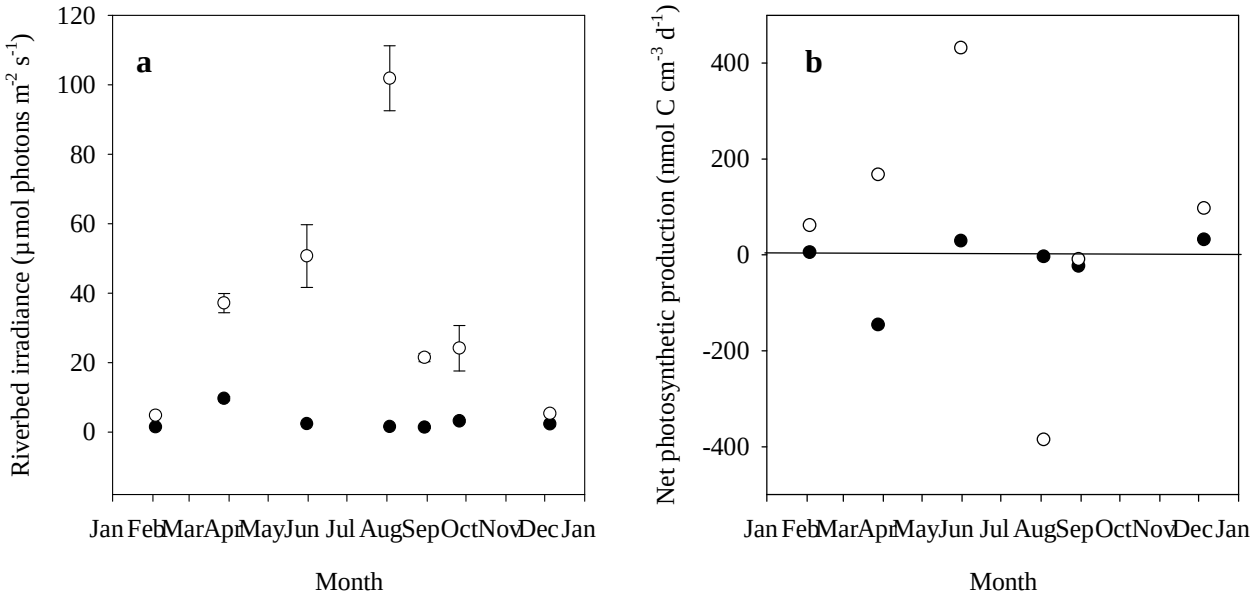

Figure 4.2: a) Riverbed irradiances at the Bere Stream measured within 5 cm of the gravels at noon (± 1 h) along three transects in the shaded area (filled circles) and open area (open circles). Mean values ±*SE*, *n*=6-18. b) Net photosynthetic production in the shaded and open stretches (filled and open circles, respectively) of the Bere Stream, modelled using laboratory oxygen evolution measurements, a photosynthesis-irradiance curve, the *in situ* light measurements and day length. The horizontal line indicates the compensation point, below which respiration outstrips photosynthesis and so the surface gravel layer is heterotrophic and there is no autochthonos carbon fixed that is then available to the food web.

Methanotrophic carbon fixation was calculated as a percentage of that fixed via both photosynthesis and methanotrophy. At the Bere Stream, in the months when there was NPP, methanotrophic carbon fixation accounted for the equivalent of 5-13 % of production in the open area (Figure 4.3) and 30-64 % in the shaded area where riverbed light was less intense which limited photosynthesis. When there was no NPP, methanotrophy contributed 100% of the fixed C.

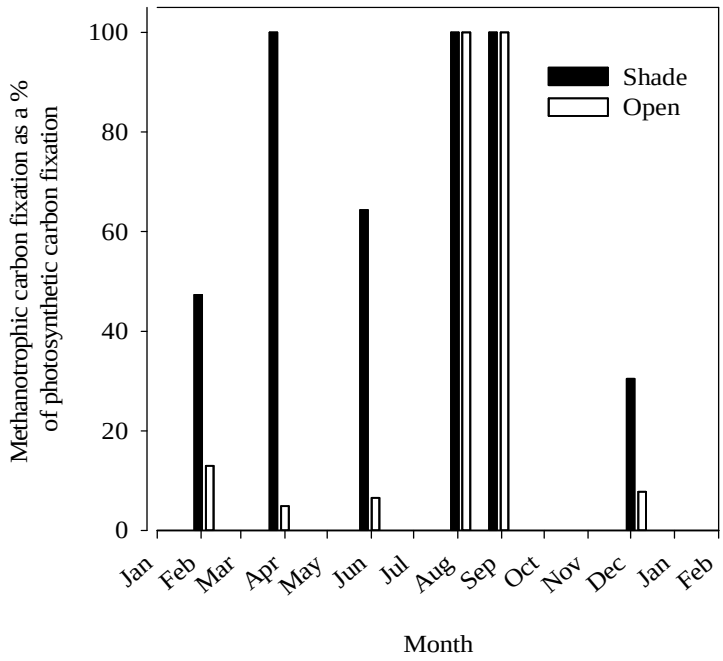

Figure 4.3: Methanotrophic carbon fixation over an annual cycle at the Bere Stream as a percentage of the sum of photosynthetic and methanotrophic production. Where there was no net photosynthetic production, methanotrophy contributes 100% of the ratio. Gaps represent periods not sampled.

The stable isotope analysis data did not show any strong seasonal trends but there were differences between the organisms from the open and shaded stretches (Figure 4.4). The biofilm (p=0.044) and *Ranunculus spp.* (p=0.004) were more ^{13}C-depleted in the shaded area than the open area (Table 4.1) and the biofilm was more depleted (in the shade) in summer when shading and methane concentration were greatest (Figure 4.4). However, there was no difference in the δ^{13}C of the caddis *Agapetus* (p=0.766) nor *Silo* (p=0.153) between the shaded and open areas. Limpets (p=0.03) were more depleted in the open area than in the

shade (Table 4.1) but they were not sampled in late August or September because they were too small and difficult to find.

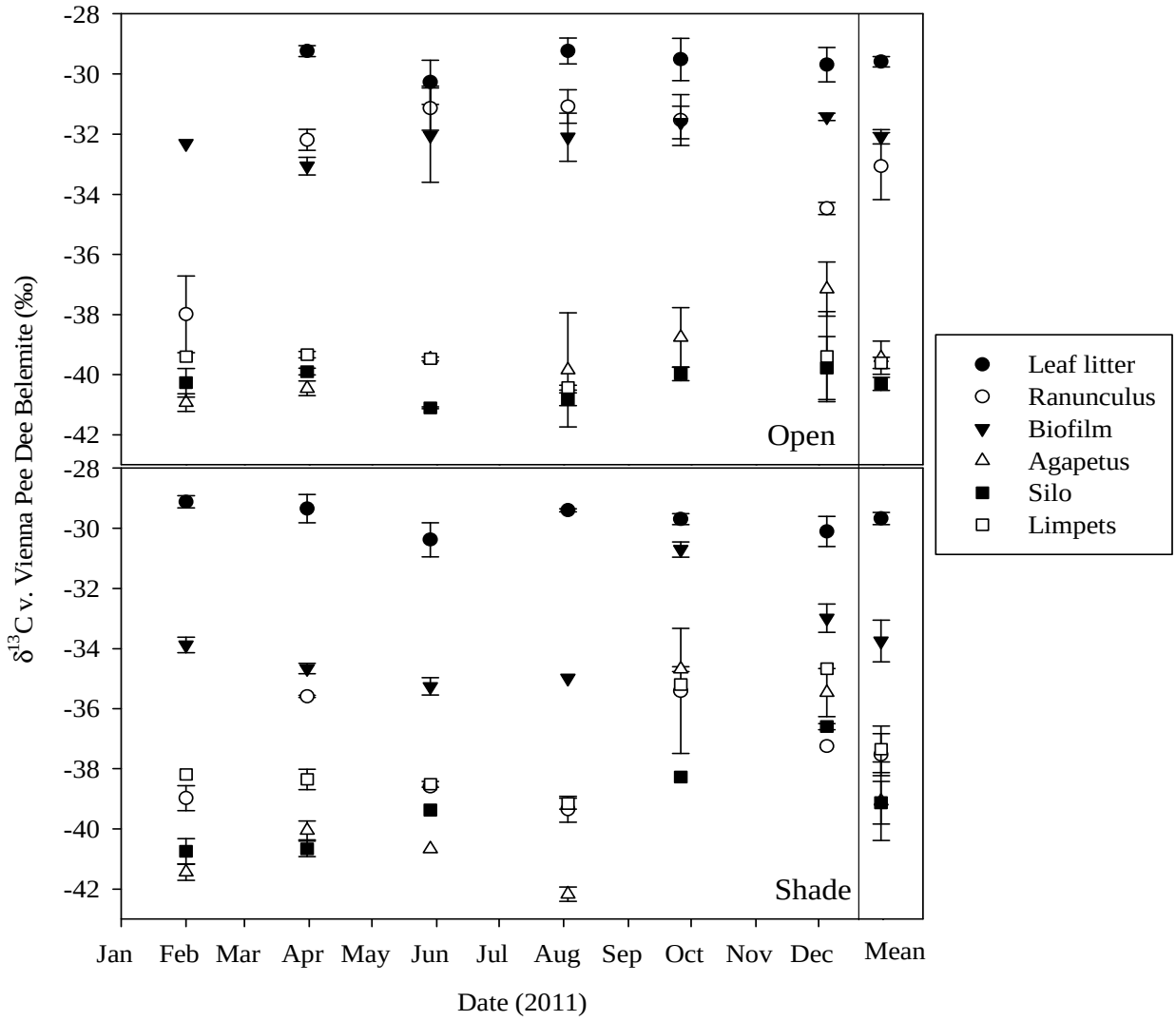

Figure 4.4: Results of the stable isotope analysis for three basal resources and three of the most abundant grazing invertebrates in the Bere Stream throughout 2011. Mean values ±*SE* (*n*=1-3). Mean values for the annual dataset are presented on the right of the graph.

4.3.2 Geographical Survey of 15 rivers

The rivers sampled covered much of the geographical extent of chalk in southern England, stretching from the Frome in west Dorset, to the Granta in Cambridgeshire and down to the Darenth in Kent (Figure 4.5). All 15 rivers were sampled within 15 days in August 2011 yet still displayed a wide range of methane concentrations, ranging from 35 nmol L^{-1} at the Misbourne in Buckinghamshire to 218 nmol L^{-1} at the Piddle in Dorset (Figure 4.5). As with the seasonal study at the Bere Stream, the methane concentration was higher in the shaded area than in the open area (average of 90% higher in the shade) (Figure 4.5, Table 2, p=0.025).

The reduction in riverbed irradiances in the shaded areas relative to the open areas was quantified as a percentage reduction in riverbed irradiance and the mean average reduction was 89% (Table 4.2). The nutrient concentrations across the survey sites covered a wide range, particularly with regards to nitrate (from 196 to 1716 µmol L^{-1} at the Darenth and Stort, respectively) and phosphate (from 0.2 to 82.6 µmol L^{-1} at the Allen and Stort, respectively) which is driven by proximity sewage treatment plant outlets and the prevailing land use in the catchment. ΣDIC (2.5-4.6 mmol L^{-1}), pCO_2 (83-512 µmol L^{-1}, 5-43 times air equilibration) and pH (7.8-8.8) were high across all sites as is common in chalk rivers (Table 4.2).

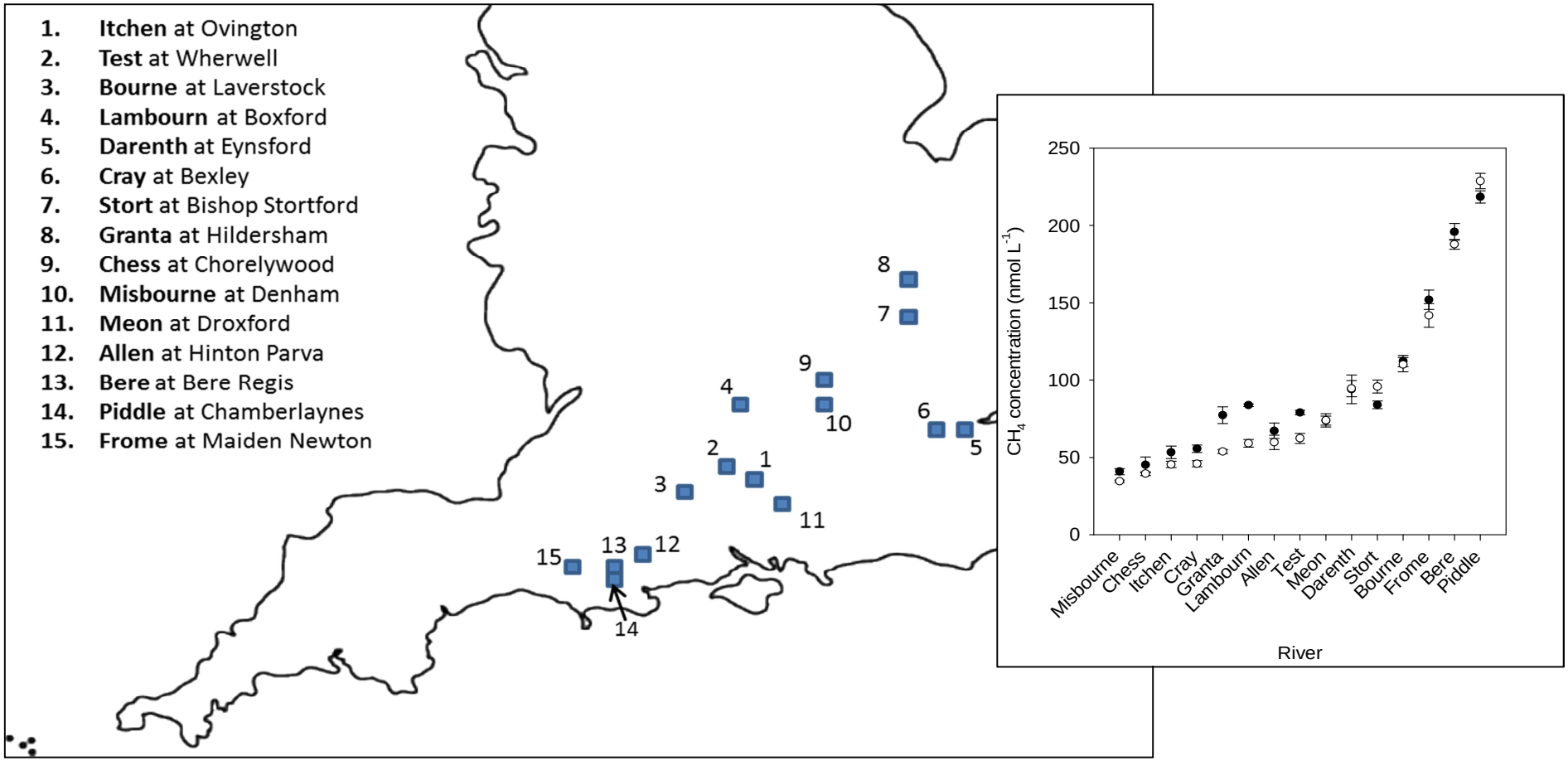

Figure 4.5: a) Annotated map showing the geographical distribution of the study sites on 15 chalk rivers. b) The concentration of methane in both the shaded (filled circles) and open (open circles) stretches of river at the study sites in August 2011. Mean values ±*SE*, *n*=6.

River	Date sampled	Upstream section	Water temperature (°C)	pH	Irradiance (% difference)	ΣDIC (mmol L^{-1})	pCO_2 (µmol L^{-1})	CH_4 (nmol L^{-1})	NO_3 (µmol L^{-1})	NO_2 (µmol L^{-1})	NH_4 (µmol L^{-1})	PO_4 (µmol L^{-1})
Itchen	15/08/2011	Open	14.5	7.84	92	4.1	274	49	688	2.3	6.5	0.5
Test	15/08/2011	Open	15.3	7.99	92	3.9	83	71	774	1.2	6.1	0.4
Bourne	16/08/2011	Open	16.3	8.14	88	3.7	131	111	880	1.3	5.5	1.3
Lambourn	16/08/2011	Open	14.5	8.30	91	4.2	120	71	923	1.4	3.5	1.0
Darenth	20/08/2011	Shade	17.8	8.10	74	2.5	346	94	196	0.7	4.0	0.5
Cray	20/08/2011	Shade	20.0	8.15	98	3.8	512	51	245	2.2	7.1	0.6
Stort	23/08/2011	Open	15.6	7.80	90	3.1	286	90	1716	1.9	10.3	82.4
Granta	23/08/2011	Shade	17.5	8.11	96	3.8	150	66	1221	2.4	15.6	36.4
Chess	24/08/2011	Shade	16.5	8.19	80	4.2	148	42	785	4.1	13.1	5.4
Misbourne	24/08/2011	Shade	18.5	8.26	68	4.2	117	38	866	1.7	10.1	4.2
Meon	29/08/2011	Shade	14.0	8.75	91	3.7	102	74	732	0.9	4.4	0.4
Allen	29/08/2011	Open	16.3	8.21	89	3.7	117	63	994	0.8	3.5	0.2
Bere	29/08/2011	Open	16.2	8.11	95	4.2	202	192	946	1.8	5.5	0.5
Piddle	30/08/2011	Open	14.3	8.12	97	4.6	219	224	835	1.1	8.5	0.3
Frome	30/08/2011	Shade	14.4	8.27	93	4.1	151	147	407	0.7	4.3	1.4

Table 4.2: Site sampling details. Water temperature and pH were measured at mid-channel and mid-depth and average concentrations for water gases and chemistry are reported. Surface irradiances are reported as the percent reduction in the shade compared to the open stretches at each river and are averages of at least 20 measurements. Nutrient concentrations are an average of three replicate water samples which were filtered and frozen on site and measured on a Skalar San++ continuous flow analyser in the laboratory.

The capacity for methane oxidation was significantly higher in the shaded areas than in the open areas (Figure 4.6a, Table 4.3, p=0.027). The shaded area of the Piddle had, by far, the highest capacity for methane oxidation and was also the site with the highest concentration of dissolved methane. Using a gross carbon fixation efficiency of 50 % (Chapter 5) and conservatively integrating over the top 15 cm of the riverbed (Shelley *et al.*, 2014) methanotrophic production in August 2011 accounted for between 18 nmol C cm^{-3} d^{-1} (open area of the Cray) and 794 nmol C cm^{-3} d^{-1} (shaded area of the Piddle) and over the annual cycle this ranged from 5-80 nmol C cm^{-3} d^{-1} in the Bere Stream.

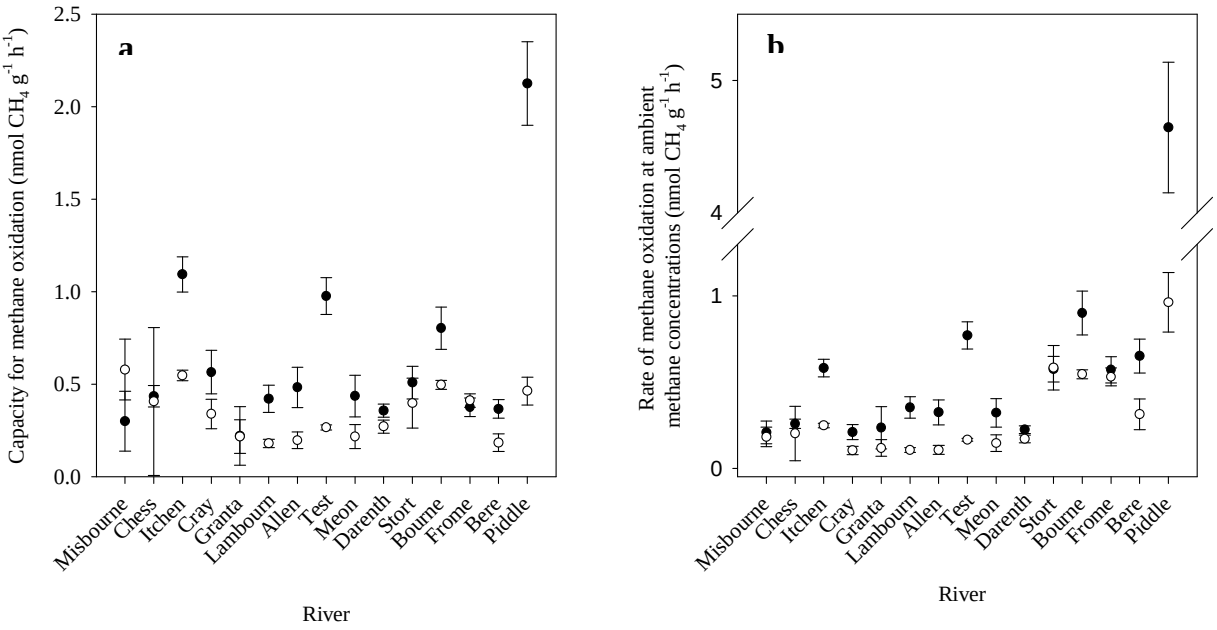

Figure 4.6: a) The capacity for methane oxidation in the riverbed gravels from 15 different chalk rivers. Means values (n=6 ±SE) for the shaded (filled circles) and open (open circles) for each river. All rates were calculated from a constant (100 nmol L^{-1}) initial concentration of methane in order to eliminate the kinetic effect of substrate availability and determine any true differences in methanotrophic capacity. b) Rates of methane oxidation in the riverbed gravels at the methane concentration at each specific river in August 2011. The data are ordered in ascending methane concentration order from left to right.

Test variable	Open average	Shaded average	Statistical test	p-value	n
Riverbed irradiance	243	35	T test (paired)	**0.000**	30
Chlorophyll content	6.1	6.0	T test (paired)	0.958	30
GPP	349	426	T test (paired)	0.133	30
BSPP	64	100	T test (paired)	**0.005**	30
NPP	200	116	T test (paired)	0.054	30
Methane concentration	89	96	T test (paired)	**0.025**	30
MO capacity	0.4	0.6	T test (paired)	**0.027**	30
MO rate	0.3	0.7	T test (paired)	0.096	30
MOP as % of (MOP+NPP)	48	83	T test (paired)	**0.025**	30
δ^{13}C biofilm	-32.4	-33.0	T test (2 sample)	0.542	29
δ^{13}C Ranunculus	-29.9	-33.2	T test (2 sample)	0.066	19
δ^{13}C Agapetus	-37.1	-38.9	T test (2 sample)	0.065	22
δ^{13}C Silo	-37.0	-33.0	T test (2 sample)	0.101	17
δ^{13}C limpets	-37.6	-34.3	T test (2 sample)	0.125	8

Table 4.3: Statistical results for the comparison of photosynthetic and methanotrophic production parameters and stable isotope analyses of organisms. GPP= gross photosynthetic production, BSPP= biomass specific photosynthetic production (units of O_2 produced per unit chlorophyll), NPP= net photosynthetic production, MO= methane oxidation, and MOP= methanotrophic production.Significant p-values are shown in bold.

Given the short period over which the sampling took place and the small range of latitudes covered, the average riverbed irradiances in the open areas should be very similar. Therefore, it makes sense that the lower values displayed in Figure 4.8a are those that were obtained at dusk (Test and Stort) or on overcast days (Frome, Bere and Piddle) which was unavoidable on our schedule. Nonetheless, the riverbed in the shaded areas consistently received less light than the riverbed in the open areas (Figure 4.7a, Table 3, p=0.000) and at 13 out of the 15 sites, photosynthesis was light limited at the time of sampling in the shaded areas and in all of the open areas it was light saturated (estimated from our single P-I curve with a K_m of 38

µmol photons m^{-2} s^{-1}). There was no difference in the chlorophyll content of the gravels in the shaded and open areas (p=0.958) but photosynthesis was more efficient in the shaded areas (p=0.005), averaging 100 nmol O_2 µg Chl h^{-1} and only 64 nmol O_2 µg Chl h^{-1} in the open areas under laboratory light conditions (Table 4.3). NPP was measured in all of the gravels from the open areas and in 12 of the 15 from the shaded areas (no NPP in the Cray, Bere and Frome) (Figure 4.7b). In most rivers, NPP was higher in the open area relative to the shaded area with the only anomaly being the River Chess where NPP was almost twice as high in the shaded area (Figure 4.7b). Overall, NPP was not significantly different between the two areas (p=0.054).

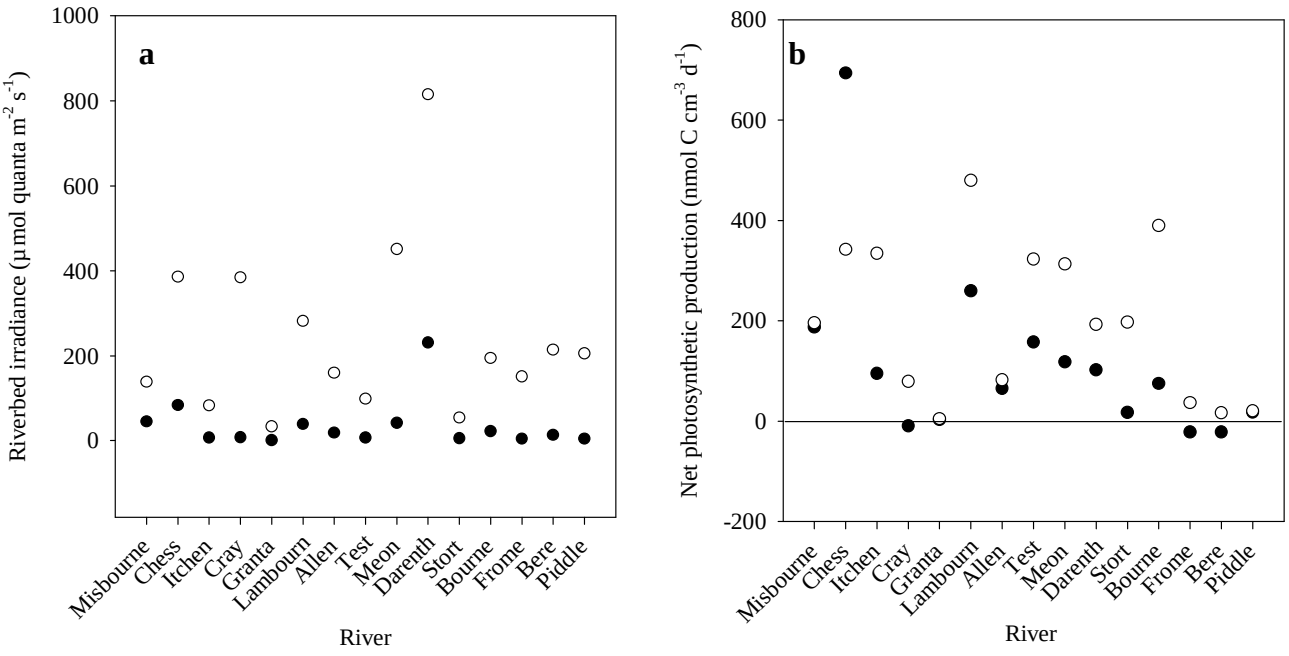

Figure 4.7: a) Riverbed irradiances as measured when sampling each river. b) Net photosynthetic production at each site in August 2011, modelled from laboratory oxygen evolution measurements, a PI curve and *in situ* light measurements. Filled circles are shaded areas and open circles are open areas.

As with the seasonal study, we have expressed methanotrophic carbon fixation as a percentage of the sum of that fixed via methanotrophy and photosynthesis. Where there was NPP, MDC was least important in the Lambourn (open area, 3.7%) and most important in the Piddle (shaded area, 97.8%) with a median value of 18.2% in the open areas and 51.3% in the shaded areas and when there was no NPP (net oxygen consumption in light incubations), methanotrophy accounted for 100% of production. There was a significant positive correlation between the importance of MDC as a basal resource and the ambient methane concentration in both the open (p=0.004) and shaded areas (p=0.043, Figure 4.8).

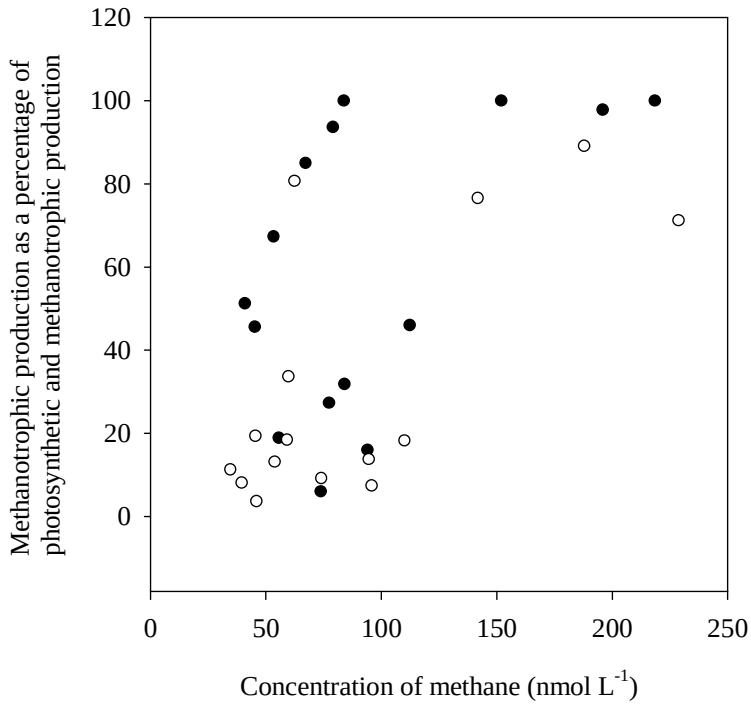

Figure 4.8: Ambient methane concentration scattered against the importance of methane derived carbon as a percentage of net photosynthetic and methanotrophic production in open (open circles) and shaded (filled circles) areas. ANOVA of linear regression returned significant p-values for both open (0.004) and shaded (0.043) datasets.

In the wider survey (August 2011), all of the grazing invertebrates sampled were ^{13}C-depleted relative to the putative photosynthetic (autotrophic and allochthonous) basal resources (Figure 4.9) but there was no difference in the δ^{13}C of the resources or invertebrates

between the shaded and open areas (Table 4.3). The $\delta^{13}C$ of the biofilm was negatively correlated with ambient methane concentration (p=0.006) with the most depleted biofilms at the sites with the highest methane concentration but there was no correlation with capacity for methane oxidation.

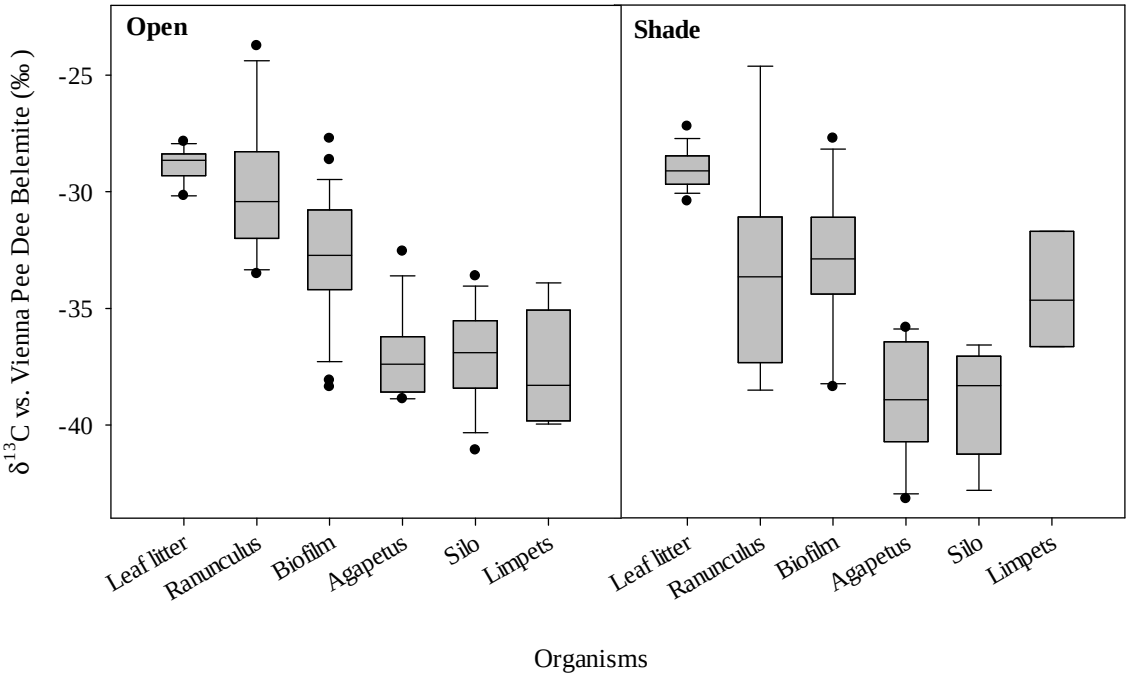

Figure 4.9: The $\delta^{13}C$ of the major photosynthetic basal resources and grazing invertebrates from 15 rivers. The median line crosses the box formed of the 25th and 75th percentiles. The whiskers show the 10th and 90th percentiles. Two-tailed, t-tests found no difference between the open and shaded samples (Table 4.2).

4.4 Discussion

Here, we have quantified the contribution of photosynthetic and methanotrophic carbon fixation to autotrophic production in 15 major chalk rivers across southern England. As hypothesised, we measured more methanotrophic production in the shaded areas than in the open areas and the difference between the two was greatest in summer when the difference in riverbed irradiances was at its maximum. At all sites, the grazing invertebrates were ^{13}C-depleted relative to the biofilm, leaf litter and plants which is generally regarded by stable isotope ecologists to indicate significant contribution of methane derived carbon to their diet (Jones & Grey, 2011). However, the quantified significance of methanotrophy to autotrophic production in the riverbeds (as calculated from the process data) did not correlate with any of the carbon stable isotope data.

The seasonal pattern in dissolved methane concentration in the river water at the Bere Stream was very similar to those previously published for other groundwater fed streams (Sanders *et al.*, 2007, Shelley *et al.*, 2014) with a five-fold increase in summer relative to winter, but the range of methane concentrations across the 15 rivers in August 2011 was even greater, displaying more than a six-fold increase (35-229 nmol CH_4 L^{-1}). The strong kinetic response of methanotrophs at riverine methane concentrations has previously been described (Shelley *et al.*, 2014), but we have also shown a changing capacity for methanotrophy in response to methane concentration which, to our knowledge, has not previously been seen in riverbed sediments. This relationship did not hold true across the 15 rivers which suggests the inter-site variation in other variables swamped the effect of substrate availability on the development of the methanotrophic community.

Photosynthetic production was calculated from NPP (rather than GPP as explained in Chapter 2) because it is the true equivalent of methanotrophic production as both quantify the carbon fixed into biomass and therefore available to higher trophic levels. The use of a single P-I

curve for both sections of all fifteen rivers was not ideal but was the only practical solution within the time frame. Stream biofilm communities do not respond to changing irradiances in the same way as phytoplankton do, and chlorophyll content is thought to be a better indicator of biomass specific photosynthetic production (BSPP) than ambient light intensity when comparing sediments from different rivers of varying light regimes (Boston & Hill, 1991). Given this finding, as the chlorophyll content was equal in the shaded and unshaded areas, the use of a single PI curve to normalise the laboratory photosynthesis measurements, should not cause significant skew to the data. Indeed, the riverbed irradiances were so much lower in the shaded stretches than in the open stretches (>90%), the leaving NPP unadjusted for ambient light would have led to a massive overestimate of NPP in the shade.

The importance of MDC relative to NPP was correlated with ambient methane concentration. Indeed, both seasonally and geographically, changing methane concentrations drove variations in methanotrophic production and the changes in NPP (driven by riverbed irradiances) were of secondary importance. Within sites, the differing contributions of MDC to the basal resource pool was driven by concurrent reduced NPP and increased capacity for methane oxidation in the shade relative to the open. The reduction in NPP can be explained by the light limitation of photosynthesis but the increased capacity for methane oxidation requires more thought. It could be a response to the higher concentrations of methane in the shade relative to the open (Table 4.3) and the consequential increased methanotrophic biomass in the shade, as was measured across the seasons in the Bere Stream. But the difference in methanotrophic capacity (45% lower in open) is much greater than that in methane concentration (7%) and so it is does not fully explain the differences measured. Competition for space on the gravel particles in the open is likely to be greater than in the shade because NPP is greater. There is also some evidence that methanotrophs are inhibited

under high irradiances (Murase & Sugimoto, 2005) but this has never been shown in sediments.

The significance of MDC in the open areas was of a similar magnitude to that previously calculated at another site on the River Lambourn (median= 18%, 11% at the Lambourn, *see* Chapter 2). However, in the shaded areas, the relative contribution of MDC rose substantially (58.4%) which highlights the value of adding this extra layer of complexity to the survey.

The data presented here suggest that methanotrophy should be much more important in the shaded areas, relative to the open areas, and particularly in summer, but our stable isotope data for invertebrates does not offer these hypotheses any support. The biofilm was more depleted in ^{13}C in the shade than the open, in line with the changing importance of MDC and therefore predicted relative size of the methanotrophic biomass relative to the photosynthetic biomass. However, this was not a pattern conserved across sites, or at least not strong enough to be significant at the 0.05 level of confidence. We know that grazing caddis larvae and limpets feed directly upon sediment biofilms (Alvarez & Pardo, 2005, Becker, 2005, Hunter, 1953) and that they are capable of assimilating (indirectly, via biolfilm) methane in laboratory incubations (unpublished data), yet we do not see any change in the $\delta^{13}C$ of the *Agapetus* with increasing importance of MDC in the biofilm. One explanation may be that the methanotrophic biomass is not sufficiently isotopically distinct from the rest of the biofilm to be traced into invertebrate biomass. This is possible when the pool of methane is relatively small (as it is in riverbed gravels, compared with stratifying water bodies) because as the lighter isotope is preferentially assimilated, the remaining methane pool becomes isotopically enriched (Fry, 2006) and so methanotrophs fixing this methane would also be less depleted relative to those in high methane environments. Biogenic methane in rivers is typically ~-60‰ but when a high proportion of the substrate is exhausted, the $\delta^{13}C$ of the remaining

methane will approach that of the original organic matter (Whiticar, 1999), which in rivers, is almost always photosynthetic (typically -25 to -30‰). Furthermore, methane that has spent many years undergoing oxidation in fully closed systems such as groundwater aquifers, could be more enriched (-20‰ in Dorset groundwater, Trimmer *et al.* manuscript in prep.) than photosynthetic carbon which is particularly important to note as our study systems are groundwater fed rivers.

In short, there are large obstacles with using stable isotope analyses to infer carbon sources to primary consumers in rivers; first, we cannot isolate the end members in order to quantify their $\delta^{13}C$ value because they are present in a mixed community biofilm, secondly, it is likely that the two resources are not isotopically distinct and finally, the grazing community are unselective in their feeding behaviour. It may be that the isotopic signal is only strong enough to be traced up a trophic level when methanotrophs constitute a much higher percentage of the organic carbon within the biofilm community. Besides photoautotrophs, there are many other non-methanotrophic members of the mixed community, which will further dilute any isotopically light carbon signal, provided by the methanotrophs, in the bulk biofilm. Nonetheless, we have presented evidence of the importance of MDC in rivers as a basal resource even in the presence of NPP and we have begun to untangle the drivers of variation in its relative importance across sites and seasons.

1

4.5 References

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Chapter 5: Sustained carbon fixation efficiency of riverbed methanotrophy

*This work was done in collaboration with Susanna Maanoja (MRes student), Dr. Myrsini Chronopoulou (Post Doc.) and Prof. Mark Trimmer and has been submitted to ISME Journal for publication. SM performed the initial step of tracing the $^{13}CH_4$ into both the organic and inorganic C fractions with laboratory and field support from me. I have marked the sections carried out by SM with a * but have left them in for completeness of the scientific story. The molecular analyses performed by MC have been removed as I had no part in them nor are they integral to my part of the project.*

5.1 Introduction

It is now well established that the majority of methane produced *in situ* under anoxia in aquatic ecosystems does not escape to the atmosphere. Indeed, up to 91% (wetland sediments), 97% (ombrotrophic peats), and ~50% to 100% (lake water columns) of that methane is aerobically oxidised to CO_2 (Bastviken *et al.*, 2002, King *et al.*, 1990, Nedwell & Watson, 1995) by methanotrophic bacteria at the oxycline (He *et al.*, 2012). Rivers are commonly oversaturated with methane with respect to the atmosphere and in the last few years we have furthered our understanding of how this carbon can be harnessed through riverbed methanotrophy to supplement photosynthetic primary production (Shelley *et al.*, 2014, Trimmer *et al.*, 2010). However, we are still unsure about the amount of methane that is oxidised en route through the broader landscape, which is thought to be considerable, and the overall proportion transformed within a river itself, before the remainder outgases to the atmosphere (Cole *et al.*, 2007, De Angelis & Scranton, 1993, Melack *et al.*, 2004).

Clearly methane oxidation will alter the balance of carbon gases (CO_2 + CH_4) released from any aquatic ecosystem to the atmosphere but, in addition, the methanotrophic bacteria responsible produce chemosynthetic carbon (Hanson & Hanson, 1996). Such chemosynthetic

production is now known to be significant in many lakes (Jones & Grey, 2011) across the globe and recently we highlighted its widespread potential throughout the chalk rivers of southern England (Shelley *et al.*, 2014, Trimmer *et al.*, 2009a, Trimmer *et al.*, 2010). The groundwater percolating through the chalk that dominates the flow in these productive rivers is oversaturated with methane (Darling & Gooddy, 2006) and the riverbed community of methanotrophs acts as a sink for some of that methane. To date, most freshwater ecologists would argue that riverine food webs and production are based firmly on allochthonous detrital resources and authochthonous photosynthetic production, with only the balance between the two contentious. Hence, our previous finding that the highly abundant grazing macroinvertebrates (*Agapetus fuscipes* and *Silo nigricornis*) in these chalk rivers appeared to derive up to 30% of their carbon from metabolised methane was surprising (Trimmer *et al.*, 2009a, Trimmer *et al.*, 2010).

Now, in order to more fully assess the significance of methanotrophy as a source of primary production in rivers, we need to better quantify its carbon fixation efficiency; i.e. how much carbon is fixed per mole of methane oxidised? Methanotrophs derive both their carbon for assimilation and energy from the oxidation of methane and the fraction assimilated (x) can be represented as (Urmann *et al.*, 2009):

$$CH_4 + (2\text{-}x)O_2 \rightarrow (1\text{-}x)CO_2 + xCH_2O + (2\text{-}x)H_2O \qquad (1)$$

While it is comparatively easy to measure an amount of methane oxidised by a sample of water or substratum (sands, muds, gravels), actually converting this to an amount of carbon assimilated or fixed (x in equation 1) has proved to be a non-trivial task (Bastviken *et al.*, 2003, King *et al.*, 1990, Maxfield *et al.*, 2012). The recent development of a stable isotope switching technique ($^{13}CH_4/^{12}CH_4$), in combination with sophisticated multi-compound-

specific mass spectrometry, has begun to probe the true dynamics of carbon assimilation, turnover and decay in soils, but it cannot give a genuine measure of the efficiency of organic carbon production via methanotrophy (Maxfield *et al.*, 2012). Here we propose a simple alternative approach: first we used mass balance in a series of repeat batch incubations of riverbed gravel and water to quantify the partitioning of $^{13}C\text{-}CH_4$ during methanotrophy into either organic or inorganic carbon ($\sum$DIC: CO_2, HCO_3^-, CO_3^{2-}); then we show that the same estimate of carbon fixation efficiency can be derived more simply by just quantifying the fraction $^{13}C\text{-}CH_4$ recovered as ^{13}C-DIC. We have already characterised a gradient in the potential for methanotrophy across thirty-two chalk rivers in southern England (Shelley *et al.*, 2014); we now examine the efficiency of carbon fixation by methanotrophy across that gradient.

5.2 Materials and Methods

5.2.1 Study site and sediment collection

To trace ^{13}C-CH_4 into ^{13}C-organic and ^{13}C-inorganic carbon, we collected gravels from the River Mimram (51.80524N – 0.151212W, Hertfordshire) for the repeat batch incubations and then, due to site access restrictions, for the follow-up set of ^{13}C-DIC yield experiments, we used gravels from the River Lambourn (51.438585N – 1.384889W, Berkshire), a chalk stream of similar size and water chemistry. Both are typical chalk rivers with coarse gravel beds and dense growths of macrophytes (predominantly *Ranunculus* spp.) (Pretty *et al.*, 2006). Surface layer gravels (n=6) were sampled using a kick-net and stored in a portable refrigerator for return to the laboratory where they were pooled, mixed, and sieved (1.4 mm $\leq x <$ 5.66 mm).

5.2.2 Tracing ^{13}C-CH_4 into ^{13}C-organic and ^{13}C-inorganic carbons*

Sub-samples (8 × 8 g) of gravel were weighed into serum bottles (20 mL) along with river water (10 mL) and then sealed (butyl and tear-off aluminium seals), leaving an air headspace. The vials were enriched with ^{13}C-CH_4 (99 atom%) to give 613 nM (± 50 nM, *SE*, n=8) in the water phase and additional control vials were setup with just river water before all of the vials were incubated on a tipper in the dark at 8°C (to mimic average river water temperature). The headspace was then measured repeatedly (~ every 8h-12h) for methane by GC/FID (Sanders *et al.*, 2007) and in order to calculate the rate of methane oxidation, the change in methane over the first two or three time points was divided by the number of hours, and then normalised to the dry mass of gravel in each vial. Once > 90% of the methane had been oxidised in each batch, the headspace was analysed for ^{13}C-CO_2 (pCO_2) by continuous flow isotope ratio mass spectrometry (CF/IRMS), and then the water was removed, acidified (50 μl HCl 12.2M), and

measured for ^{13}C as DIC (Σ CO_2, HCO_3^- and CO_3^{2-}) by CF/IRMS (Miyajima *et al.*, 1995, Trimmer *et al.*, 2009a). Summation of $p^{13}CO_2$ plus ^{13}C-DIC equalled the total amount of ^{13}C-CH_4 metabolised to inorganic carbon. Following the end of each incubation, gravel (2 g) was harvested, freeze-dried and stored at -18°C for analysis of ^{13}C in either the lipid or bulk organic carbon (TOC) fraction (see below). New water was added and the vials were enriched again with CH_4 and the process was repeated seven more times to test the effect of repeat incubations and track isotopic labelling of the biofilm over time. Thus, the labelling of TOC was a cumulative measure and the ΣDIC was discrete for each incubation (as both the headspace and the water were replaced).

5.2.3 Quantifying the yield of bulk ^{13}C-organic carbon by wet oxidation to CO_2*

An adaptation of a standard wet-oxidation method (no. 505C; (APHA, 1995)) was used to recover and quantify the yield of bulk ^{13}C-organic carbon from the harvested gravels. A subsample (~300 mg) of freeze-dried gravels was transferred into a serum bottle (20 mL), which was then acidified to remove carbonates (4 mL H_3PO_4 6% v/v), before the addition of 5 mL of 0.15 M potassium persulfate. After sealing, all of the vials were autoclaved (121°C, 3 h) and the headspace subsequently analysed for CO_2, first by GC/FID (as above but after catalytic reduction to CH_4 over hot nickel) to quantify the TOC and then by CF/IRMS for abundance of ^{13}C-CO_2 to quantify the labelled fraction.

5.2.4 Quantifying the carbon fixation efficiency (CFE) by the yield of ^{13}C-DIC.

After establishing that we could recover 100% of the ^{13}C-CH_4 as either ^{13}C-DIC or ^{13}C-organic carbon, we could use the ^{13}C-DIC fraction to more simply quantify the efficiency of organic carbon production, without the need to quantitatively extract and purify the organic fractions. If the production of ^{13}C-DIC is linear over time, with an origin through zero, then all of that

^{13}C-DIC must be due solely to methanotrophic metabolism and not reworking of the ^{13}C-CO_2 within the biofilm. Consequently, the proportion of ^{13}C-CH_4 recovered as ^{13}C-DIC yields a direct measure of the CFE e.g. $1 - (\Delta\ ^{13}\text{C-DIC}/\Delta^{13}\text{C-CH}_4) = x$. Here, we take the total amount of ^{13}C-CH_4 oxidised to represent gross methanotrophy, while our measure of CFE is equivalent to net methanotrophy i.e. the amount of fixed carbon that would potentially be available to higher trophic levels and which, as such, is synonymous with net photosynthetic production (Shelley *et al.*, 2014).

Accordingly, we followed the oxidation of ^{13}C-CH_4 and parallel evolution of ^{13}C-DIC during short (60 h) incubations. Independent aliquots (55 to give 5 replicates at 11 time points) of prepared gravels were enriched with 99 atom% ^{13}C-CH_4 to give 1900 ppm in the headspace and 2400 nM in the water (± 28 *SE*, *n*=55), incubated as above and then sacrificed (5 vials ~every 5 h) for quantification of CH_4 and ^{13}C-DIC (as above). Next, we tested the effect of methane concentration on the oxidation kinetics and fixation efficiency at 37 different concentrations (~10 nM to 7000 nM CH_4 in the water), spanning the seasonal range in the river water and gravel porewater (~30 nM-150 nM) and far beyond (Shelley *et al.*, 2014, Trimmer *et al.*, 2009a). The headspace was sampled repeatedly over time (as above) and analysed for CH_4 and ^{13}C-DIC to calculate the CFE (as above).

Finally, although the use of 99 atom% ^{13}C-CH_4 assures detectable labelling of products during short incubations (<5h), fractionation can occur during methanotrophy (Whiticar, 1999). So to test for any effect of fractionation prepared gravels were incubated under 11 different mixtures of $^{13}CH_4/^{12}CH_4$: from natural abundance (here simply $^{12}CH_4$) to 99 atom% $^{13}CH_4$ in 10% increments, and at a final concentration in the water phase of 635 nM (± 32nM, *SE*, *n*=11).

5.2.4 Carbon fixation efficiency across multiple rivers

Having established a simple routine assay for estimating the CFE of methanotrophy, we now wanted to estimate that efficiency using a larger sample of rivers and examine whether that efficiency was consistent across a natural geographical gradient of riverbed sediments. We previously characterised a gradient in the potential for methanotrophy across 32 chalk rivers in southern England (Shelley *et al.*, 2014) and here, we selected eight of those rivers covering the range of oxidation potentials (Figure 5.1). The GCF of methane oxidation for each gravel bed was quantified as above (tracing ^{13}C-CH_4 into ^{13}C-DIC).

5.2.5 Potential interference from copper in the methane oxidation experiments

Given the potential sensitivity of methane mono-oxygenase to copper (Leak and Dalton, 1986a), we set-up replicate (n = 10) incubations of gravel plus river water (as above), gravel plus UHP water, river water only and UHP only, all in the acid-washed (0.1M HCl) gas-tight vials which we use routinely for this type of incubation and let them incubate at 8°C, as above, for 4 days. After, each vial was opened, the water phase filtered (pre-rinsed 0.2µm Mini-Sart) and analysed for copper by furnace atomic absorption spectrometry (Varian GTA 110 & 220FS, Victoria, Australia) calibrated between 0 and 21 µg Cu L^{-1}.

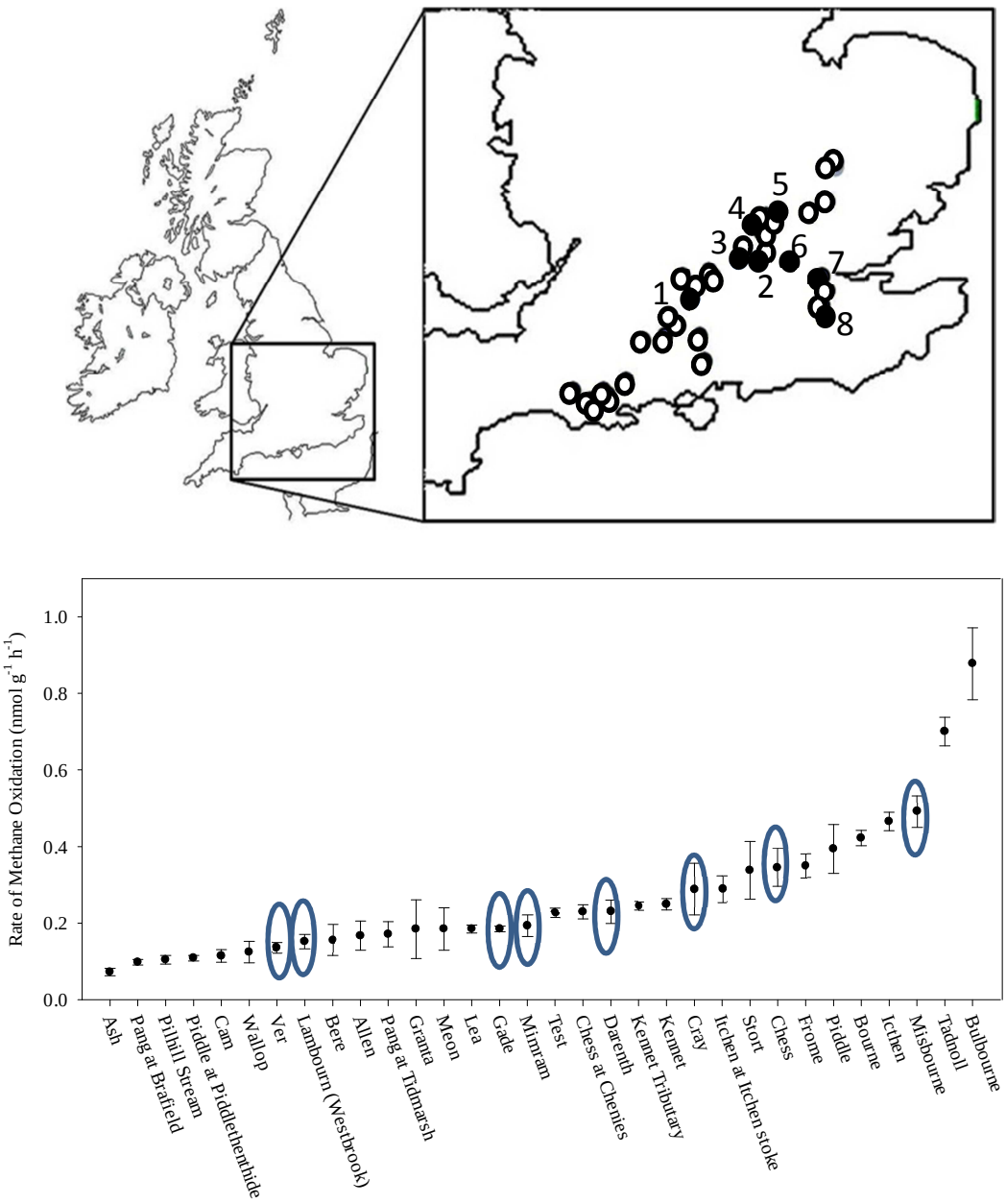

Figure 5.1: Distribution of the 32 (open and closed circles) Chalk rivers in southern England that have all proved positive for methane oxidation (*see* (Shelley *et al.*, 2014) for full details). Closed circles indicate the location of the eight rivers used in this study, where the total distance west to east was approximately 110 km. 1. Lambourn 2. Misbourne, 3. Chess, 4. Ver, 5. Gade, 6. Mimram, 7. Cray, 8. Darenth. The graph shows the changing methane oxidation rate (means ± *SE*, *n*=6) across 32 rivers with those revisited for this study circled. All started at a constant methane concentration and so variation in rate represents a changing capacity for methane oxidation across the sites.

5.3 Results

5.3.1 Recovery of ^{13}C-CH_4 in ^{13}C-organic and ^{13}C-inorganic carbon fractions*

Methane was oxidised rapidly over the first 20 to 30 hours in each of the eight batch incubations (Figure 5.2) and, although there was some variation in the rates of oxidation, there was no significant change over the entire 17 days of incubation, with an average rate of 1.8 nmol CH_4 g^{-1} h^{-1} (± 0.2, *SE*, *n*=8). We subdivided the ^{13}C assimilated into a crude lipid extract and bulk organic carbon fraction. Our crude lipid extract approached an isotopic steady-state (i.e., an approximately constant $\delta^{13}C$) after approximately 9 days, while the bulk organic carbon fraction took a little longer. Importantly for our approach we were able to recover all of the ^{13}C-CH_4 introduced to the vials (105% ± 6 *SE*, *n*=8; one-sample t-test, $t = 0.83$, $P > 0.05$) as either ^{13}C-DIC ($\Sigma CO_2+HCO_3^-+CO_3^{2-}$) or as total ^{13}C-organic carbon (bulk plus lipid fractions), with the proportion being evenly split (~50% each) between the two (Table 1). Finally, the percentage of ^{13}C-CH_4 recovered as ^{13}C-organic carbon did not vary significantly between the repeat batch incubations (ANOVA, $F_{7,7} = 1.49$, $P > 0.05$) and was 52% (± 6 *SE*, *n*=8) on average (Table 5.1).

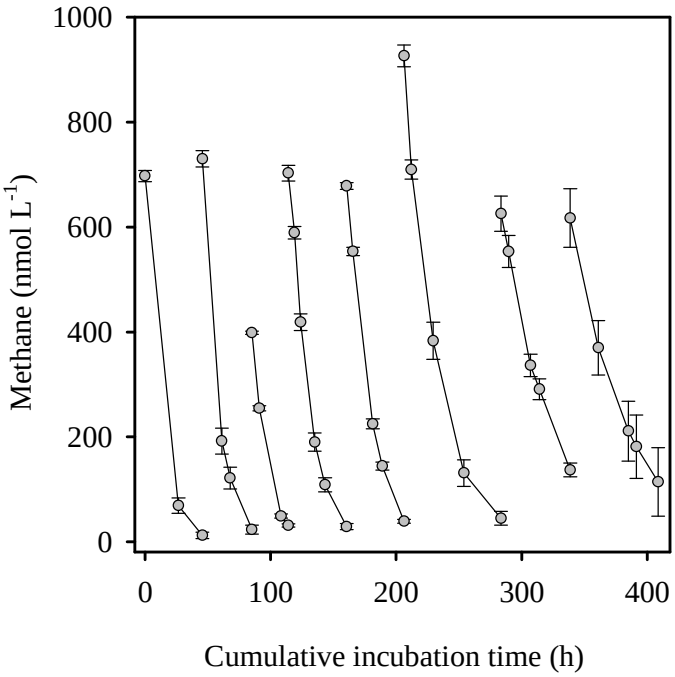

Figure 5.2: Oxidation and incorporation of ^{13}C-CH_4 during repeat batch incubations with small amounts of riverbed gravel. Rapid initial rates of methane oxidation following each enrichment of the headspace with CH_4.The sequential enrichment approach was to check whether the labelling (and therefore the assimilation of MDC) was linear over time.

Table 5.1: Summary budget for the recovery of oxidised $^{13}CH_4$ as either dissolved inorganic carbon ($\sum DI^{13}C = CO_2$, HCO_3^- and CO_3^{2-}) or total organic carbon in the first eight repeat batch incubations. Note, there was no significant difference between the recovery in either fraction and both were indistinguishable from 50% and, overall, the total recovery of $^{13}CH_4$ was ~ 100%. Methane oxidised was calculated from time series gas chromatography measurements. DIC was calculated from 3 mL sub-samples of the water after each batch had finished and TOC from the wet oxidation assay performed on a subsample of the gravel after each batch incubation.

Batch	1	2	3	4	5	6	7	8	
Time (hours)	51	90	119	166	212	289	344	414	
$^{13}CH_4$ oxidised (nmol g^{-1})	25	31	20	47	53	127	88	87	
Cumulative $^{13}CH_4$ oxidised (nmol g^{-1})	25	56	75	122	176	302	390	477	
$TO^{13}C$ (nmol g^{-1})	15	34	49	62	86	57	274	183	
$\sum DI^{13}C$ (nmol g^{-1})	12	13	9	33	32	63	49	49	
									mean
Recovered as TOC (%)	61	61	65	51	49	19	70	38	52 ± 6
Recovered as DIC (%)	46	42	46	69	60	49	56	57	53 ± 3
Total (%)	107	103	111	120	109	68	126	95	105±6

5.3.2 Quantifying the carbon fixation efficiency by the yield of ^{13}C-DIC.

In our follow-up short (<60 h) incubation the evolution of ^{13}C-DIC was linear over the first 28 hours, after which the small remaining pool of ^{13}C-CH_4 limited the rate of production of ^{13}C-DIC (Figure 5.3a). We then used the ratio of ^{13}C-DIC produced per ^{13}C-CH_4 oxidised (0.48 ± 0.02 *SE*, *n*=30; Figure 5.3b) during the first 25 hours (when linearity was greatest) to estimate carbon fixation efficiency through methanotrophy to be 0.52 (i.e., 1-0.48 x 100 = 52% efficient ± 2%). Note that this estimate of 52% was indistinguishable from that measured directly as fixed ^{13}C-organic carbon over the previous 17 days of the repeat batch incubations (52 ± 6 *SE*, *n*=8, as above).

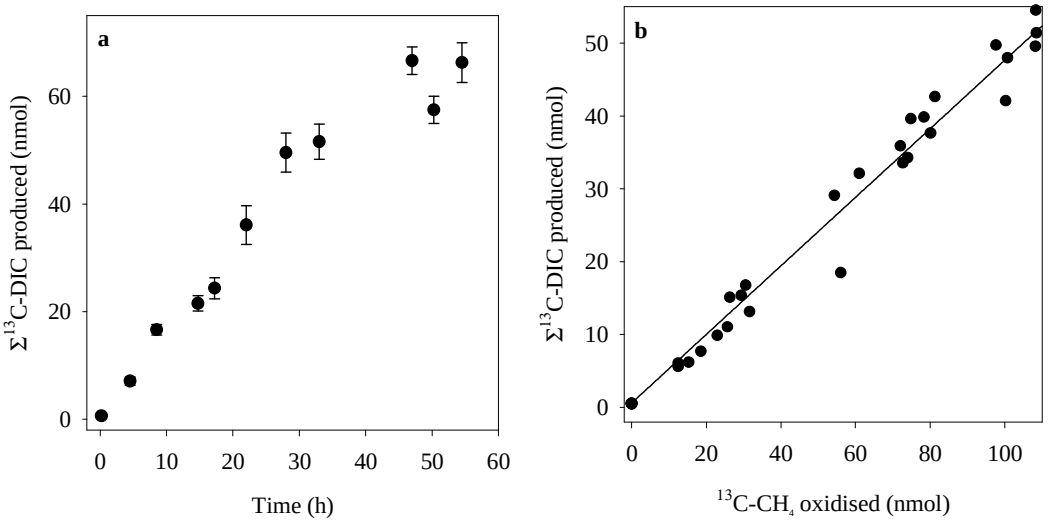

Figure 5.3. The production of ^{13}C-DIC over time used as a measure of carbon fixation efficiency by riverbed gravel methanotrophs during short-term incubations. (**a**) Production of ^{13}C-DIC from the oxidation of ^{13}C-CH_4 over ~ 60 hour to the point of CH_4 limitation. Mean ± *SE*, *n*=5 (**b**) the ^{13}C-DIC as a function of the amount of ^{13}C-CH_4 oxidised during the first 15h, when the linearity of DIC production was strongest (r^2=0.93, *P*<0.001) and each datum is the result of a single incubation. The slope (0.48) is equivalent to the ratio of DIC to CH_4 and 1 – 0.48 is a measure of carbon fixation efficiency *i.e.*, 0.52 x 100 = 52% fixed.

5.3.3 The effect of methane concentration on the oxidation kinetics and fixation efficiency

We measured a clear kinetic effect of methane concentration, with the rate of oxidation increasing some 10 fold over the local seasonal range in river-water methane concentration (e.g. ~ 20 nM to 150 nM, inset Figure 5.4) and even beyond towards a potential plateau at approximately 7000 nM CH_4 (Figure 5.4), indicating a high capacity for methane oxidation in these riverbed gravels. In contrast to the marked kinetic effect, there was no significant relationship between the fraction of ^{13}C-CH_4 recovered as DIC and the initial concentration of methane (Figure 5.4b) and over this range, the average carbon fixation efficiency was 53% (± 0.01 *SE*, n = 40) and in good agreement with the previous trials which both yielded 52%. There was no effect on the rate of methane oxidation as a function of the proportion of ^{13}C atom % within the CH_4 with gravels exposed to the eleven different mixing ratios (Figure 5.5, p=0.185).

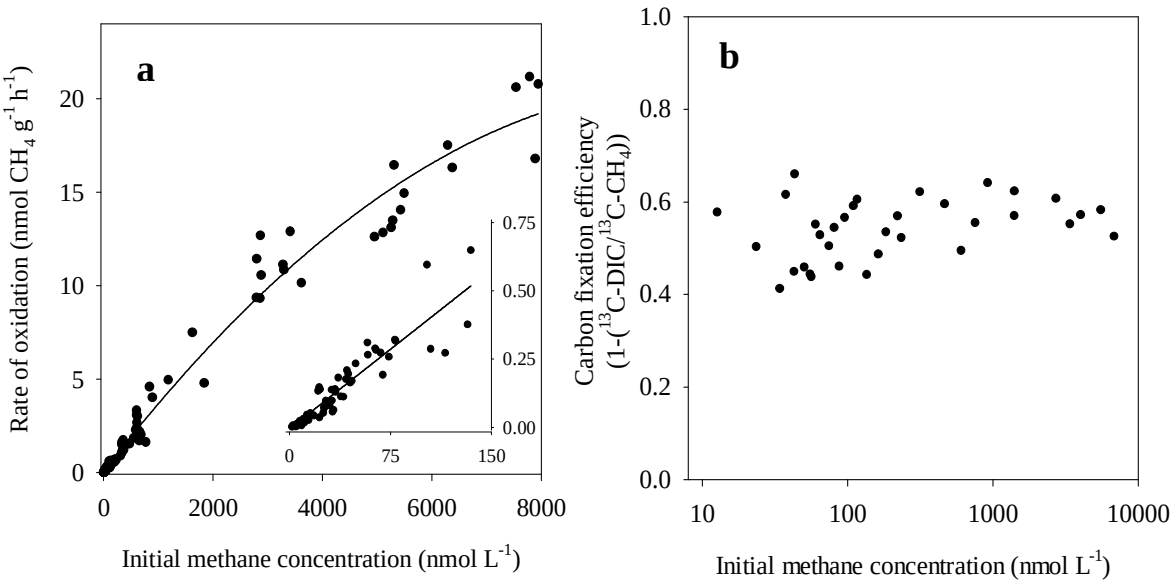

Figure 5.4. Kinetic effects of methane on its rate of oxidation and the efficiency of carbon fixation. (**a**) Rate of methane oxidation as a function of methane concentration from below, within and far beyond ambient river concentrations (insert, 1st order linear regression r^2=0.90 within chalk river annual methane concentrations). (**b**) Carbon fixation efficiency exhibiting

no relationship with methane concentration from ~ 10 nM CH_4 to 9000 nM CH_4. The mean value estimated for efficiency of 53% (± 1) was indistinguishable to those determined either in the repeat batch incubations (52%, ± 6) or that from ^{13}DIC over 15h (52%, ± 2, *see* Figure 5.3). Each datum point is the result of a single incubation.

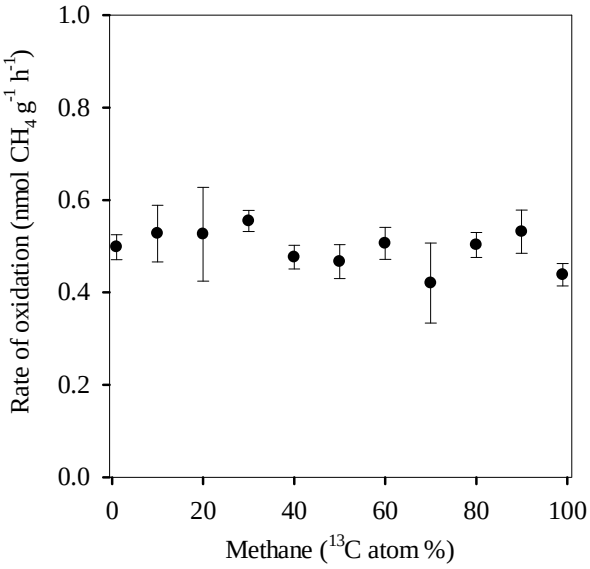

Figure 5.5. Methane oxidation rate at eleven different atom percent mixing ratios. There was no effect of using ^{13}C-CH_4 as a tracer on the rate of methane oxidation.

5.3.4 Carbon fixation efficiency across multiple rivers and potential interference from copper

The gravels collected from the eight rivers in our broader survey all oxidised methane with varying degrees of activity (Figure 5.6a) with the slowest rate measured in the Ver gravels (1.6 nmol CH_4 g^{-1} h^{-1}) and the fastest were those from the River Gade (10.3 nmol CH_4 g^{-1} h^{-1}). Using the ratio of ^{13}C-CH_4 oxidised to ^{13}C-DIC produced (as described above), we calculated the CFE for each river and they ranged from 0.4 (±0.14, *SE*) at the River Chess to 0.60 (±0.13, *SE*) at the River Ver. There was no relationship between the capacity for methane oxidation and the efficiency of the process (*P*=0.105) The difference between samples was not very strong (ANCOVA for rate x river *P*=0.015) and so to account for this moderate 'river effect' we treated 'river' as a random effect in a mixed-effects model and used that to derive an overall population estimate of the ratio of ^{13}C-DIC produced per ^{13}C-CH_4 oxidised (ß 0.55, ±0.055, *SE* d.f. 83). Accordingly, the model estimated the carbon fixation efficiency via methanotrophy across all eight rivers to be 45% (i.e. (1-0.55) x 100) (Figure 5.6b).

The highest concentrations of copper were measured in the gravel plus UHP incubations (7.5 µg Cu L^{-1}), probably as a consequence of the dissolution of chalk in the mildly acidic UHP (~pH5.5). Gravels plus river water yielded a final concentration of 5.1 µg Cu L^{-1} which was greater than that for river water only (4.0 µg Cu L^{-1}) and the UHP (2.3 µg L^{-1}). Hence, we would conclude that the gravels are a natural source of copper to their associated biofilms.

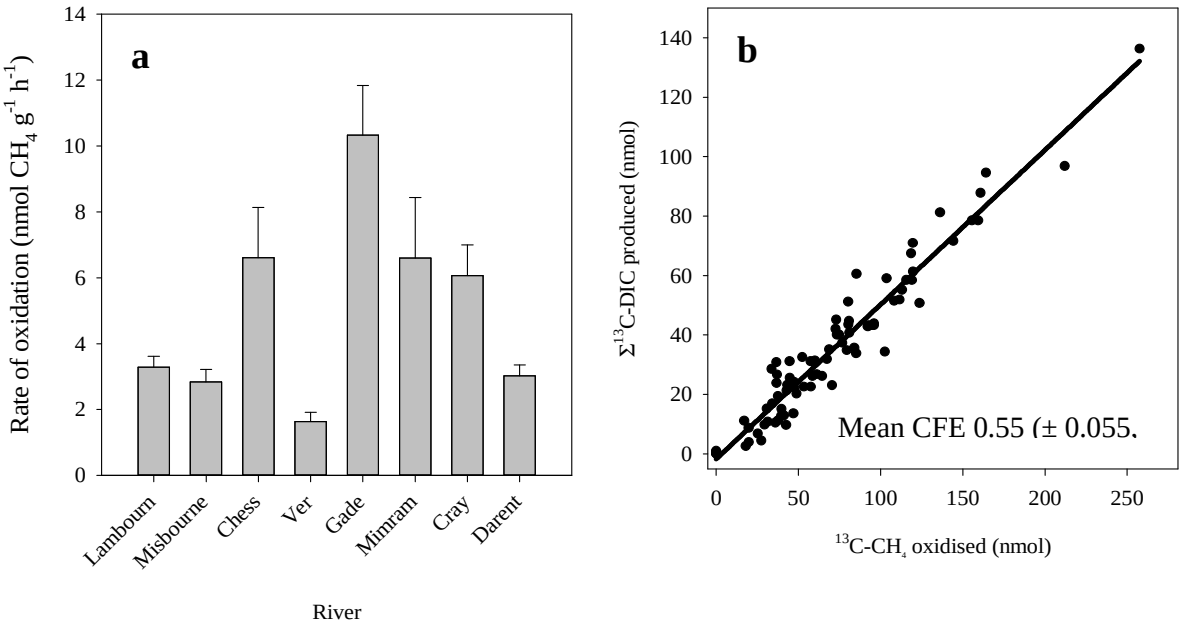

Figure 5.6: Carbon fixation efficiency of methane oxidation in the riverbed gravels from eight chalk rivers. (**a**) Rate of methane oxidation as measured in the ^{13}C-CH_4 incubations used to calculate the CFE. Mean values, ±*SE*, *n*=10. (**b**) Pooled data for the ^{13}C-CH_4 time series incubations for all eight rivers.

5.4 Discussion

Recently we demonstrated that chemosynthetic production coupled to the oxidation of methane is widespread throughout the 32 major chalk rivers of southern England (Shelley *et al.*, 2014) and here we have clearly shown that the fraction of carbon fixed via that widespread methanotrophy is indeed high. In addition, the efficiency with which the riverbed methanotrophs fix carbon is independent of the methane concentration. The concentration of methane in the rivers studied here varies with the season, as indeed it does for many others, reflecting both greater production within the river and its wider catchment, plus changes in lateral import and oxidation in the river itself (Bouillon *et al.*, 2012, De Angelis & Lilley, 1987, De Angelis & Scranton, 1993, Kone *et al.*, 2010, Sanders *et al.*, 2007, Trimmer *et al.*, 2009a). Such efficiency, coupled to the methanotrophs' high dynamic range for methane, will enable this chemosynthetic production to track the seasonal range of methane in the river water but also to exploit the much higher concentrations found in the depositional sediments; both in the channel margins and trapped beneath the luxuriant growths of *Ranunculus* spp. and *Berula* spp. (Sanders *et al.*, 2007, Trimmer *et al.*, 2009b).

Here we have used the fraction of ^{13}C recovered as ^{13}C-DIC to more simply and directly quantify the efficiency of methanotrophic production, without the need to quantitatively extract and purify the organic fractions (Maxfield *et al.*, 2012). We know that there was no significant loss of ^{13}C-methanol (or other intermediate metabolite) through cell leakage or lysis during our initial phase of ^{13}C-DIC production (that was then incorporated by non-methanotrophs) because the production was linear and went through zero. Eventually, some of the ^{13}C assimilated by the methanotrophs will be reworked and shared amongst other members of the gravel community, otherwise it would be of no greater ecological significance, but this process has been shown to de detectable only after some 2 to 3 weeks in soils (Maxfield *et al.*,

2012). During this latter phase the ^{13}C-DIC respired would no longer represent pure methanotrophy. However, we are confident that such an effect was negligible in our, much shorter incubations, for the following reasons.

First, the ^{13}C-DIC produced in our experiments was strongly diluted by the ^{12}C-DIC produced by total community respiration, only making-up some 1.8% of the total DIC pool (^{12}C+^{13}C-DIC). The chances, therefore, of ^{13}C-DIC being fixed by any non-methanotrophic metabolism would have been negligible. Second, there was no increase in the rate of methane oxidation over the 17 days of repeat batch incubations, which indicated no net growth in the population of methanotrophs during this time. If this steady state was due to any growth being balanced by cell death, then those cells undergoing lysis would only have played a minor role in the metabolism and potential loss of any ^{13}C-methane, especially during the short ^{13}C-DIC experiments. If, however, there was only significant death, with no cell renewal, then not only would the rates of methanotrophy have systematically declined with time, but an isotopic steady-state would not have been reached during our initial batch incubations.

It has been known for a long time now that the efficiency of carbon fixation via methanotrophy is heavily NAD(P)H dependent and that this dependency can be modulated by the form of nitrogen being assimilated for growth (Anthony, 1978, Leak & Dalton, 1986b). For example, growth on nitrate would further exacerbate limitation by NAD(P)H because some of the NAD(P)H generated by the oxidation of CH_4 would need to be expended reducing NO_3^- to NH_2 during assimilation, whereas this metabolic cost would be less for growth on NH_4^+. The availability of copper also affects the efficiency of carbon fixation by regulating the synthesis of pMMO, which, compared to the soluble form (sMMO), has a lower overall demand for NAD(P)H (Leak & Dalton, 1986a). Accordingly, with suffient copper enabling synthesis of pMMO, the thoretical carbon fixation efficiencies for growth are: 45% to 47% on NO_3^- and higher at 59% to 62% on NH_4^+ (Leak & Dalton, 1986a).

Original work with ^{14}C-CH_4 in high-methane, land-fill cover soils, reported that 69% of methane oxidised was recovered from soils in the bulk organic carbon fraction, or a lesser fraction of 31% to 43%, in low-methane, forest soils (Roslev *et al.*, 1997, Whalen *et al.*, 1990). Here we directly determined the carbon fixation efficiency to be 45% on average (CI 36% to 54%), for a sample of eight chalk rivers in southern England. Having shown that the riverbed is a natural source of copper, we assumed that any expression of sMMO would have been neglible and that any methane oxidation would be driven by pMMO. In addition, the presence of sMMO appears rare, with it being absent from the vast majority of methanotrophs in pure culture (Chen and Murrell 2010). Note though, that these isolates fall into the group of low affinity methanotrophs (>40ppm~1.8µmol CH_4 L^{-1}), whereas there are no pure cultures of the high affinity methanotrophs which these river-types would belong to (down to 0.02 µmol CH_4 L^{-1}).

With the good agreement between our carbon fixation efficiency of 45% and the original theorectical predictions for growth on NO_3^-, we would infer that the riverbed methanotroph community is largely dependent on NO_3^- for its N requirements. Nitrate is abundant (800 µmol L^{-1} on average) in chalk rivers, both in the overlying water and at depth in the riverbed (Pretty *et al.*, 2006, Sanders *et al.*, 2007). The upper limit of our efficiency estimate (54%) implies that some of the methanotroph communties maybe assimilating NH_4^+ or, alternatively, that they are supplementing their growth by the co-metabolism of methanol (Chen and Murrell 2010). While we have no data for methanol, we do know that these riverbed sediments are areas of intense mineralisation and NH_4^+ is freely available in the porewater, albeit at concentrations orders of magnitude below those for NO_3^- (Pretty *et al.*, 2006, Triska *et al.*, 1994). In turn, the lower end of our estimate may reflect patchiness in the availabilty of copper or other restraints on growth (Leak and Dalton, 1986b). Overall, our findings suggest that chalk riverbeds provide a favourable habitat for methanotrophs.

The role of inland freshwaters in the global methane cycle is attracting renewed interest (Bastviken *et al.*, 2011) but whereas the potential for methane oxidation is both high and well characterised in wetlands and lakes (King *et al.*, 1990, Tranvik *et al.*, 2009), data from rivers have been lacking. Here we have demonstrated that riverbed methanotrophs have a high capacity to oxidise methane as well as fix carbon very efficiently. Not only does the riverbed attenuate some of the potential efflux of methane before it outgases from the river but methanotrophy provides an alternative chemosynthetic source of energy, in parallel to the well documented route of photosynthesis.

5.5 References

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Chapter 6: Conclusion and suggestions for future work

6.1 Overview

The data presented in the preceding chapters highlight widespread, efficient and ecologically important methanotrophic production in rivers. The activity of methanotrophs was driven by changes in the ambient methane concentration (i.e. substrate availability) which displayed seasonal and inter- and intra-river variation. Photosynthesis was also active on the surface layer of the riverbed, but there was not always *net* photosynthetic production (NPP). In the subsurface hyporheic gravels, where it is dark, methanotrophy continues to fix new carbon and allochthonous ingress of fresh chlorophyll from the surface provides a supplement to autotrophic carbon. The natural (and in some cases anthropogenic e.g. influence of sewage treatment plants) variation in field sites adds strength to my findings because it means the general patterns found in the research have prevailed in spite of broad changes in catchment land-use, nutrient loadings and discharges. As such, the results presented here can be used, with confidence, when up-scaled to apply to groundwater fed rivers worldwide. Moreover, many of the conclusions are independent of the groundwater influence and, particularly those from chapter 3, can be applied to patches of oxic gravel riverbeds and/or anoxic fine sediment patches of riverbed irrespective of the wider hydrological patterns.

6.2 The effect of methane concentration on the importance of MDC as a basal resource

Perhaps the clearest outcome from this body of work is the substrate limitation of methanotrophy at riverine methane concentrations. This forms an integral part of the calculations and conclusions in all four data chapters. In chapter two I characterised the dose response of methanotrophy and used the linear relationship to normalise measurements of methanotrophic capacity to monthly riverbed methanotrophic production. I used the same principals to model the data from our laboratory measurements in chapter four. Interestingly, I found variation in the methanotrophic capacity across 15 rivers, between the shaded and open

stretches of those rivers and over time in the same stretch at the Bere Stream. The ambient methane concentration did not fully explain the changing capacity, which suggested that other factors influence the size of the methanotrophic community.

Substrate availability played an integral part in shaping chapter three as the extent of substrate limitation determined the temperature dependence of methane oxidation. Moreover, the kinetic response to substrate was much stronger than the response to temperature. Methanogenesis has a strong temperature dependency but the high capacity for methane oxidation in the fine sediments, where methane is produced, combined with the kinetic response of methanotrophy, meant that methanotrophs will be capable of oxidising all of the methane produced in riverbeds even with a warming climate. However, even with this ability to oxidise methane over four orders of magnitude (2 nM – 20 µM), physical bypassing of the methanotrophs in the oxic sediment layer (either through ebullition or via plant stems) resulted in some of the gas escaping to the main channel water and then, ultimately, to the atmosphere. The relationship between microbial methane production and consumption with increasing temperature is such that the ratio of CH_4 to CO_2 emitted would remain constant. Thus, any change in the ratio of carbon gas emissions from aquatic systems as a function of temperature is likely due to the bypassing of methanotrophy rather than its inability to deal with rising methane concentrations.

Finally, converting rates of methane oxidation to estimates of methanotrophic production would not have been possible without knowing the carbon fixation efficiency (CFE) of the riverbed methanotrophs (chapter five). Given the response to raised methane concentrations, it was important to test whether the CFE was affected by initial methane concentration, and it was not. CFE was not significantly related to methane concentration and remained constant from 10 nM - 10 µM at 53% (±1). Further, the CFE was constant across different rivers spanning a range of methane concentrations (22 -126 nM) and capacities for methane

oxidation (0.30-1.05 nmol CH_4 g^{-1} h^{-1}) which was crucial for validating the rest of the work in this thesis. Without a constant CFE, calculation of methanotrophic production would have been extremely imprecise as the previously reported CFEs from the literature ranged from 6-80% (Bastviken *et al.*, 2003, King, 1992).

6.3 The effect of light availability on the importance of MDC as a basal resource

Light availability can affect the importance of MDC to riverine food webs in two ways: first, the direct effect of light on the activity of methanotrophic bacteria, and second, the effect on photosynthetic production which ultimately reduces or increases the relative importance of MDC as it is the competing basal resource within the gravels. With regards to the first approach, there are no solid conclusions to be drawn from this thesis. Preliminary attempts to incubate small volumes of riverbed sediments (<5 g) with methane under varying light intensities suffered from typical batch incubation problems. The mixed biofilm contains photosynthetic organisms as well as methanotrophs and a whole host of other micro-organisms. Even under low intensity lighting, photosynthesis drew the carbon dioxide concentration down to zero which pushed the pH up to 10, which inhibited methane oxidation. This is also likely to be the explanation for the photo-inhibition of methane oxidation observed in mixed cultures from a reservoir (Dumestre *et al.*, 1999). The use of pH buffers could allow these laboratory incubations to progress but field measurements using the benthic chamber and conservative tracer method would perhaps offer a better solution.

Chapters two and four address the question of how light affects the contribution of MDC by carefully modelling photosynthetic production. The study sites are famed for their high photosynthetic production but the dense stands of macrophytes, which can completely fill the channel in summer, heavily shade the riverbed and inhibit benthic NPP as a consequence. The detailed modelling presented in chapter two used published irradiance decay coefficients (from

surface to riverbed as a function of macrophyte cover patch type) and long term light data (Trimmer *et al.,* 2010), combined with a newly constructed photosynthesis-irradiance curve, chlorophyll content and oxygen evolution measurements (for GPP and NP) to model whole riverbed photosynthesis for each month. The strong seasonal trend in macrophyte cover had a larger impact on benthic NPP than the seasonal trend in sunlight intensity, resulting in dip in NPP in mid-summer. This chapter did not consider higher level, riparian shading, which can also drastically reduce riverbed irradiances in summer, limiting NPP and giving methanotrophic production a proportional increase in importance but this was addressed in chapter four.

An unexpected finding in chapter four was the significantly greater capacity for methane oxidation in the shaded stretches of stream relative to the more open areas. The NPP was reduced in the shaded areas, but chlorophyll content was unchanged. No single explanation for the increased MDC in the shade was found but the marginally higher methane concentrations here are likely to play a part, and preference for low light conditions and the photo-inhibition argument (Murase & Sugimoto, 2005) cannot be ruled out. Irrespective of the precise explanation for this pattern, methanotrophic production is higher in shaded areas and NPP is lower resulting in very high (median 51.3%) contribution of MDC to primary production in the shade. As with the changing methane concentration, the riparian (mainly deciduous trees) and in-stream shading peak in mid-summer creating optimum conditions for methanotrophic production and poor conditions for NPP, results in MDC always being most significant in the summer.

6.4 Wider ramifications and upscaling of findings

This body of work has shown that all riverbed sediments have the potential to oxidise methane even when the ambient methane concentrations are only marginally oversaturated relative to the atmosphere. Indeed, this in not unexpected as there is a plethora of research from soil scientists who have measured oxidation of atmospheric methane in surface soils (King *et al.*, 1989, Smith *et al.*, 2000). Moreover, the riverbed methanotrophs increase their rate of oxidation linearly, in response to raised methane concentrations over at least four orders of magnitude (2-20,000 nmol L^{-1}) which is a larger range than has been reported for wetlands, soils and lakes. These two characteristics lead me to conclude that this is a very plastic group of bacteria, but also crucially, they allow the upscaling of the results presented in the preceding four chapters to most freshwater systems.

The techniques applied to calculate the methane oxidation rates, capacities and carbon fixation efficiencies can be applied to any type of aquatic sediment as long as the ambient methane concentration, oxygen saturation and temperature are known. The magnitude (i.e. the slope) of the linear kinetic response of methanotrophs (as shown in chapters 2, 3 and 5) will vary depending on the density of the methanotrophic population in a given sediment sample, but once this is calculated, using a dose-response experiment, the rates can be used to estimate seasonal and spatial patterns in methanotrophic production within that environment. As such, the next logical step would be to continue to build the library of methane oxidation potentials across rivers, broadening out to other geologies and stream orders to build a global model for riverbed methane cycling. Further, the results in this thesis show methanotrophy can oxidise all of the methane produced under anoxic conditions and yet rivers are net sources of methane to the atmosphere indicating that some is escaping the sediment. Therefore, further work is needed to quantify the alternative pathways of methane transport e.g. ebullition and plant stem transport, so that methane cycling in rivers can be accurately modelled.

6.5 Parallel research projects

Aside from the research presented within this thesis, I have been involved with a number of other experiments, the most prominent of which centres on a suite of artificial channels and a larger NERC grant. Over two years myself and a post-doctoral researcher have used experimental channels to expose gravels and invertebrates to a range of methane and light treatments in order to disentangle the drivers of low $\delta^{13}C$ values in grazing caddis fly larvae (as published in Trimmer *et al.* (2009)). Ambient methane concentration consistently dictated methanotrophic capacity and shading reduced chlorophyll but no discernable effect on the $\delta^{13}C$ values of the grazers was observed. This research is ongoing and I hope it will result in publication in the near future.

Further afield, I spent six weeks on the RSS James Cook looking at carbon cycling in the North-Eastern Tropical Pacific oxygen minimum zone just off the Guatemalan coastline. I used isotope labelling techniques to measure aerobic and anaerobic methane oxidation and my water column profiling work indicated pelagic and benthic sources of methane. Sediment slurries showed high potential for methanogenesis but no potential for methane oxidation. I hope to combine my data with molecular results and publish the findings very soon.

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