A comparison of methane dynamics between wetlands constructed for wastewater treatment and a natural sedgeland in South Australia.

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Dedication

This thesis is dedicated to my late father and brother, whose untimely deaths are a constant reminder of the fragility of life and an inspiration to seize the day.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other University and, to the best of my knowledge, it contains no material previously published or written by any other person except where due reference and acknowledgement is made.

I give my consent to this thesis being made available for photocopying and loan.

Kerri Louise Muller
21st of June, 2001.

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Abstract

Rates of methane production, oxidation and emission were measured in summer and winter at two wetlands constructed to treat either secondary treated sewage (Willunga Wetlands) or urban/industrial stormwater (Greenfields Wetlands) and at a natural sedgeland (Bool Lagoon) in southern Australia between 1994 and 1998.

The water regime at Willunga Wetlands (3 ha) was stable with respect to inflow velocity (4 L s^{-1}) and water depth (c. 3m). The influent sewage was highly organic (DOC > 25 mg L^{-1}) and the water column was permanently stratified in summer. In contrast, stormwater inflow velocities (zero to > 1000 L s^{-1}) to Greenfields Wetlands (40 ha) were highly variable and low in organic content (DOC < 7 mg L^{-1}). Water depths (0 to 2.2m) varied seasonally, being deepest in winter, but remained too shallow to support persistent stratification even in the hot summer months. Freshwater inputs to Bool Lagoon (2500 ha) were also highly variable. Much of the system was dry, but where it was inundated, the water column was shallow (0 to 1.2m) and diurnally mixed.

Methane production rates (in vitro) varied over three orders of magnitude from 0.87 ± 0.1 to 605 ± 61 nmoles CH_4 mL^{-1} h^{-1} but, in all treatments, sediments from Willunga Wetlands yielded the highest rates of production. At both Willunga Wetlands and Greenfields Wetlands, rates of methanogenesis decreased by up to 76% between the inlet and the outlet. Methanogenesis was highest at the sediment surface, decreasing with increasing sediment depth at all sites, and was both temperature and season dependent. Additions of fermenter bacteria and alternative electron acceptors suggest that competition for methanogenic precursors occurred at all sites, being the least at the inlet to Willunga Wetlands.

Methanotrophy was an exclusively aerobic process at Greenfields Wetlands and Bool Lagoon, varying from 0.15 ± 0.05 to 489 ± 15 nmoles CH_4 mL^{-1} h^{-1}. At Willunga Wetlands, both aerobic and anaerobic methanotrophy was detected at rates up to 140 nmoles CH_4 mL^{-1} h^{-1}. Nevertheless, net methanogenesis was negative at Greenfields Wetlands and Bool Lagoon but strongly positive at Willunga Wetlands. Accordingly, methane emissions from Willunga Wetlands were the highest, ranging from 0.63 ± 0.09 to 8.91 ± 2.6 mmoles CH_4 m^{-2} h^{-1}. The inlet of Greenfields Wetlands

(1.15 ± 1 to 6.07 ± 1.3 mmoles CH_4 m^{-2} h^{-1}) and Bool Lagoon (0.6 ± 0.08 to 2.81 ± 0.7 mmoles CH_4 m^{-2} h^{-1}) had very similar rates of emission and the lowest rates were observed at the outlet to Greenfields Wetlands (0.06 ± 0.01 to 0.53 ± 0.11 mmoles CH_4 m^{-2} h^{-1}). However, estimates of whole wetland emissions suggest that Willunga Wetlands (7 t CH_4 y^{-1}) released significantly less methane than either Greenfields Wetlands (67 t CH_4 y^{-1}) or Bool Lagoon (38 to 1300 t CH_4 y^{-1}, depending on the level of inundation) on an annual basis.

Rates of both in vitro and in situ methane production strongly correlated with field estimates of methane emissions at all sites except at Greenfields Wetlands where winter emissions were higher than expected from rates of methane production. It is suggested that rapid winter inflows to Greenfields Wetlands physically scoured the sediments and released stored methane emissions effectively uncoupling emissions from seasonal rates of production. Water column mixing, associated with autumnal turnover, also had the potential to enhance emissions at Willunga Wetland above that expected from the rate of methanogenesis.

Chapter 1

Chapter 1: Introduction

1.1: Methane as a greenhouse gas

The greenhouse effect of the earth's atmosphere is an intrinsic part of the thermal regulation of the planet. Without it the earth's average temperature would be approximately 33°C lower (Ramanathan, 1998) and the planet would be uninhabitable by most known life forms. The greenhouse effect is caused by atmospheric trace gases absorbing long-wave, outgoing solar radiation reflected from the earth's surface that would otherwise escape and vent heat from the atmosphere. Numerous gases are transparent to short-wave radiation from the sun but absorb the reflected long-wave radiation, leading to a net warming of the atmosphere as more radiation is absorbed than is re-emitted.

A major concern is that large-scale anthropogenic processes have modified the gaseous composition of the atmosphere to the point that the absorption characteristics have changed and the earth is getting progressively warmer. In the 10,000 years between the last ice age and the industrial revolution, the concentrations of major greenhouse gases, such as CO_2, CH_4 and N_2O, were relatively stable. This has changed since industrialisation and the concentrations of these gases continue to increase: CO_2 at 0.5% y^{-1}, CH_4 at 1% y^{-1} and N_2O at 0.25 % y^{-1}. These data have been collected at modern field sites as well as from ancient gas samples trapped in glacial ice (Cicerone and Oremland, 1988; Matson and Harriss, 1995) and the evidence for human-forced changes in atmospheric composition is now indisputable.

Xenobiotic trace gases, such as SF_6 (50, 000 y), generally have longer atmospheric retention times than biogenic trace gases, CO_2 (50 - 200 y), CH_4 (12 y) and N_2O (200 y), and the source strengths of xenobiotic gases are relatively well-defined (IPCC, 1996). In contrast, understanding the relative source and sink strengths of biogenic gases requires an intimate knowledge of complex ecosystems that transverse the globe and that are simultaneously responding in a specific, but often unknown, way to the impacts of global warming itself.

Methane, the most abundant greenhouse gas in the atmosphere aside from water vapour and carbon dioxide (IPCC, 1995), has 20 times the warming potential of CO_2 on a per mole basis (IPCC, 1996). It is chemically active in the atmosphere, altering the concentrations of other greenhouse gases such as the $OH^{\bullet}$ radical which is the major

oxidising agent in the troposphere (Khalil and Rasmussen, 1983), and complicating predictions of future methane-related impacts. Biogenic processes contribute 395 Tg of methane, from a global total of 550 Tg, to the atmosphere each year. The rest arises primarily from fossil fuel-related activities (100 Tg y^{-1}) and biomass burning (40 Tg y^{-1}). Of the biogenic fraction, wetlands produce 115 Tg and rice paddies another 60 Tg (IPCC, 1996). Thus emissions from these types of wetlands comprise approximately 44% of the global biogenic methane flux. Important methane sinks are photochemical oxidation by tropospheric $OH^{\bullet}$ (Khalil and Rasmussen, 1983) and microbial oxidation in wetland and upland soils [see reviews by King (1992) and Segers (1997)]. Therefore methane dynamics in wetlands have the capacity to increase or decrease wetland-specific atmospheric emissions as well as modify local ambient concentrations, and as such the study of methane emissions from wetlands warrants further investigation.

1.2 Methane dynamics in wetlands

Methane is biologically generated in wetlands by strictly anaerobic methanogenic bacteria involved in the anaerobic breakdown of organic matter (Zeikus, 1977). The autecology of these organisms is reviewed in detail in Chapter 2 but suffice it here to say that because their activity is strongly controlled by the absence of oxygen and the amount of easily degradable organic carbon (Segers, 1997), methanogens are usually restricted to ostensibly anoxic regions within the sediment and water layers.

The process of methane consumption is also microbially-mediated, being primarily controlled by the presence of oxygen and methane (Amaral and Knowles, 1995; also see Chapter 2). The major site for methane oxidation in wetlands is the oxic:anoxic interface where oxygen and methane co-exist at favourable ratios (Amaral and Knowles, 1995; Segers, 1997). This interface typically occurs at or near the sediment:water interface (King, 1990), although in deep, eutrophic lakes, the oxic:anoxic interface may exist in the water column (Utsumi et al., 1998). Methane excess to the rate of oxidation accumulates in the soil and water profile and may be lost to the atmosphere via molecular diffusion or bubble ebullition. These emission processes are driven by the concentration gradient which develops between the sediment and the atmosphere (Conrad, 1996). Ebullition is often reported to be the dominant pathway in unvegetated sediments (Chanton and Martens, 1988; Landsdown et al., 1992; Devol et al., 1998) and in vegetated wetlands, methane may additionally be lost via plant-mediated transport (for review see Holzapfel-Pschorn et al, 1986; Chanton et al., 1993).

1.3 Wetlands constructed for wastewater treatment

For almost 30 years, wetlands have been constructed to treat a range of wastewaters, with the treatment of either domestic waste (sewage) or stormwater the two most common design criteria. Surface flow wetland designs have been the most common in North America where wastewater treatment wetlands numbered 600 nationwide in 1998 (Cole, 1998). In contrast, European systems are predominantly the more space-efficient, sub-surface flow designs which numbered more than 500 in 1998 (Cole, 1998). Constructed wetlands of both types have been constructed in Australia since the 1970s and although concise numbers are not currently available, they are expected to number several hundred nationwide.

Methane emissions from constructed wetlands, aside from rice paddies, have been omitted from global wetland inventories. For example, Prather et al. (1995), IPCC (1995) and Boeckx and Vancleemput (1996) separate all wetland areas into two categories; wetlands or rice paddies, with no category for wetlands constructed for wastewater treatment. Further, Mudge and Adger (1995) do not include wetlands constructed for wastewater treatment in their global appraisal of methane fluxes solely from artificial wetlands. However, rates of methane production, oxidation and emission from wetlands constructed for wastewater treatment are likely to differ from those observed in natural wetlands and rice paddies.

First, the carbon load entering a wetland constructed for wastewater treatment may be far greater than that entering natural wetlands or rice paddies. For example, secondary sewage typically contains 35 mg L^{-1} total organic carbon (Tebutt, 1998) whereas freshwater contains less than 10 mg L^{-1} (EPA, 1998). Second, the type of carbon entering a constructed wetland (e.g. sewage) may be more easily degraded than carbon originating from a natural autochthonous or allochthonous source (eg. plant-derived detritus) and this may have a downstream effect on the rate of methane emission.

Third, the water regime experienced in a wastewater treatment wetland may differ in terms of timing, duration and depth from that of a natural wetland or rice paddy in the same region due to both the wastewater inflow patterns and the constructed wetland basin design. For example, sewage treatment wetlands in Queensland remain permanent all year in climatic zones that otherwise only support seasonally inundated wetlands (Greenway and Simpson, 1996).

Fourth, the site chosen for wetland construction may not possess the geophysical characteristics (e.g. soil type, slope, elevation) that would naturally facilitate wetland

formation. Although the second and third points may also be true for rice paddies, methane dynamics in rice paddies are strongly affected by rice management (Cicerone and Shetter, 1981; Singh and Singh, 1995; Adachi et al., 1996; Cao et al., 1996; Neue et al., 1996; Minami and Takata, 1997), which is not a feature of wetlands constructed for wastewater treatment and therefore rates of methane emissions obtained for rice paddies should not be extrapolated to wetlands constructed for wastewater treatment. Furthermore, methane emissions from different classes of wastewater treatment wetlands may be different as source water characteristics are likely to strongly influence methane dynamics.

1.4 Study objectives

1. Estimate and compare relative rates of methane production and oxidation in open water areas within two constructed wetlands of similar age receiving either sewage or stormwater and within a natural wetland from a similar geographical location;
2. Provide estimates of atmospheric methane emissions from all three wetland classes; and
3. Investigate factors controlling methane production (source strength), oxidation (sink strength) and release routes to the atmosphere (molecular diffusion and bubble ebullition) from open water surfaces.

1.5 Study Approach

Three wetland systems were chosen from in or around the Adelaide region (South Australia) to represent three wetland classes: constructed wetlands receiving sewage, constructed wetlands receiving stormwater and natural wetlands receiving freshwater (Figure 1.1). The first system, Willunga Wetlands, was constructed in 1994 to ameliorate sewage effluent discharge from a population of c. 5,000 people in the Willunga region. The effluent is secondarily treated in traditional open oxidation lagoons before being gravity fed into the Willunga Wetland system. Once treated in the wetlands, the water is reclaimed to irrigate public green areas.

Approximately 70 km away from Willunga on the northern side of the Adelaide Metropolitan Area, the second system, Greenfields Wetlands, is located. These wetlands were built between 1991 and 1994 and receive stormwater from a predominately urban/light industrial catchment of 100 km^2. Greenfields Wetlands are considered a robust comparative site to Willunga Wetlands because they experience a

similar climate (Table 1.1) and are of similar age (Willunga Wetlands: constructed early 1993; Greenfields Wetlands: Stage II constructed in 1990 and Stage I in 1994). Major differences between the sites relate specifically to basin design (Chapter 3) and influent water quality and quantity (Chapter 4).

Choosing a suitable natural wetland for comparison was more difficult as few large, natural wetlands remain close to Adelaide. Bool Lagoon, although situated 450 km from Adelaide, was considered the best choice as a representative natural wetland. A range of University research projects had already taken place there (for example: Adcock, 1991; Adcock and Ganf, 1994; Rea and Ganf, 1994; Brownlow et al., 1994) and the management history was well known. In addition, rates of methane production and emission have already been estimated from this system (Muller, 1993; Muller et al., 1994). Bool Lagoon is in a region of higher average annual rainfall and lower average temperature than Willunga and Greenfields Wetlands (Table 1.1) and it receives agricultural run-off rather than run-off from a natural landscape, as is common for natural wetlands in south-eastern Australia (Boulton and Brock, 1999). However, it is an ancient, natural wetland and it receives relatively freshwater (EPA, 1998) in a relatively natural seasonal pattern and in these two critical aspects it provides a suitable natural wetland to contrast to Willunga and Greenfields Wetlands.

Intrawetland sites were restricted to open water areas experiencing permanent inundation to avoid the additional complication of choosing comparable vegetation beds and water regimes across all three wetlands. Areas adjacent to both the inlet and outlet structures of the constructed wetlands were chosen to assess the potential effect of flow path length on methane dynamics. At Bool Lagoon, a series of permanently inundated locations along the flow path was not available and investigations were restricted to the first two basins in series, Hacks Lagoon and Main Basin. Rates of methane production, oxidation and emission were determined in winter and summer on a range of spatial and temporal scales to allow comparison of their relative magnitudes across the sites and the development of a methane balance for each site.

1.6 Overall hypothesis

The wetland which provides conditions most favourable for methanogenesis will support the highest rates of methane production, oxidation and emission. Accordingly, the site-specific rates are expected to decrease in the following order:

Willunga inlet> Willunga outlet> Bool Lagoon > Greenfields inlet > Greenfields outlet

1.7 Thesis structure

Chapter 1: provides an overview of the research problem and states the objectives of the study;

Chapter 2: contains a review of the methanogenic and methanotrophic processes in wetlands. It does not discuss emission processes per se but instead discusses the highly complex and interdependent nature of syntrophic methanogenesis and investigates the various modes of methane oxidation, thereby providing background information for interpreting the experimental results;

Chapter 3: describes the study sites and outlines the experimental materials and methods;

Chapter 4: assesses the physico-chemical characteristics of the wetlands that are likely to affect the activities of methane producing and oxidising bacteria. These data were collected concurrent with the methane production, oxidation and emission data in Chapters 5 and 6 but are presented separately for clarity;

Chapter 5: reports and compares specific rates of methane production and oxidation and investigates the factors controlling these processes;

Chapter 6: provides estimates of atmospheric emissions for each wetland, relates these to the specific rates of methane production and oxidation obtained in Chapter 5 and investigates the factors controlling methane emissions; and

Chapter 7: presents the final comparative discussion on methane dynamics in the three wetlands, including an assessment of the impact wetlands constructed for wastewater treatment may have on the Australian wetland inventory and a series of future research and management directions.

Chapter 2

Chapter 2: A review of methanogenic and methanotrophic processes in wetlands

2.1: The Methanogens

2.1.1: Phylogeny

Methanogens belong to an ancient group of organisms, the Archaea, the discovery of which modified the fundamental classification of all life forms. Previously all organisms were defined as either prokaryotes or eukaryotes but the Archaea did not fit neatly into either Prokaryota or Eukaryota and a tripartite tree of life was proposed. Morphologically, Archaea resemble prokaryotes, exhibiting rod, spiral and spherical shapes and many grow as clusters or aggregates (Aravalli et al., 1998). However, the prokaryotes had originally been defined in terms of lacking eukaryotic traits and therefore were assumed to be monophyletic. As a consequence, the traits that came to be known as prokaryotic were based on few examples such as *Escherichia coli* and its relatives (Woese, 1993). The Archaea possess some characteristics that are like *E. coli* but also some that are more like the eukaryotes. For example, genome sequences analysed by Koonin et al. (1997) showed that 70% of the identified protein products of *Methanococcus jannaschii* are either like bacterial (44%) or eukaryotic (13%) products or are similar to both (13%). The tripartite tree shows three distinct, monophyletic groupings: archaeobacteria, eubacteria and eukaryotes.

Archaeal organisms; thermophiles, extreme halophiles and methanogens, are highly diverse and capable of living in the most extreme of niches. They have few similarities, but most Archaeal cellular envelopes contain S-layers with glycoprotein and cytoplasmic membranes with high levels of tetra-ether-lipids (Kandler and König, 1985). *Methanopyrus kandleri*, a thermophilic methanogen, is an exception as it has 2,3-di-O-geranylgeranyl-*sn*-glycerol as the dominant membrane lipid (Stetter, 1995) and is the only member of the Order *Methanopyrus* (Kurr et al., 1991). Traditionally, methanogens were thought to be restricted to specialised habitats but recent evidence reviewed by Aravalli et al. (1998) would suggest that they are far more widespread. New molecular approaches to ecological studies using 16S rRNA probes have found novel Archaea in a variety of temperate and cold environments, including freshwater sediments, as well as in the more extreme Archaeal habitats. These new techniques do not require the culturing of cells or the detection of cellular activity and thus have the potential to uncover new species and functional groups in niches previously considered well understood.

Before the Archaea were defined and the 8th Edition of Bergey's Manual was published, the methanogens were classified along with non-methanogens on the basis of morphological traits. Bryant (1974) first brought the methanogens together as a physiologically distinct and coherent group based on the ubiquitous trait of producing methane as a final catabolic product. Verification of this grouping by 16S rRNA sequencing followed (Balch et al., 1979; Woese, 1987) and the evolutionary link between methanogens and the other Archaea became evident. Methanogenic bacteria are highly diverse. The GC content of their DNA varies from 25-60% (Jones et al., 1987) across the functional group. This diversity, coupled with the rapid and ongoing discovery of new isolates and 16S rRNA sequences (see Aravalli et al., 1998), maintains the taxonomy of methanogens in a state of flux. Boone et al. (1993) present a recent taxonomy of methanogens based on the following species-level distinctions: DNA reassociation of less than 70%, change in hybrid DNA melting point greater than 5 °C, 16S rRNA similarity less than 98% and substantial phenotypic differences (also see Wayne et al., 1987). The proposed taxa classify methanogens into five orders within the kingdom, *Archaeobacteria; Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales* and *Methanopyrales*. No doubt this will be further modified as more species are identified and their genetic codes sequenced, thus the reader is advised to seek the most current literature for details on Archaeal taxonomy.

2.1.2: *Bioenergetics*

Methanogens share the physiological trait of producing methane as the end-product of their catabolism. Three catabolic pathways, CO_2-reducing, methylotrophic and aceticlastic, are recognised by which methanogens derive energy from a restricted range of substrates. Formate and H_2 + CO_2 are converted to methane via the CO_2-reduction pathway. Methanol and methylamines are converted via the methylotrophic pathway and only a limited number of methanogens employ the aceticlastic pathway to oxidise acetate (Deppenmeier et al., 1996). The electrons liberated during methanogenic catabolism are not fed into a classical cytochrome and quinone electron transport chain. Instead, redox reactions involved in these processes are partly catalysed by enzymes bound in the plasma membrane that generate or, in the case of endergonic reactions, use electrochemical ion gradients.

All the pathways have in common the two-electron reduction of methyl-coenzyme M to methane catalysed by methyl-coenzyme M reductase but they differ in the source of the methyl group to be transferred to coenzyme M (see Ferry, 1999 for details). Therefore,

the coenzyme M analogue, bromoethanesulphonic acid (BES) is considered a specific metabolic inhibitor of all methanogens (Zeikus, 1977). Coenzyme F_{420} is another unique and highly important electron carrier in the methanogens. It can be irreversibly dissociated by oxygen thereby conferring anoxic habitat requirements on the methanogens (Ferry, 1999). Other enzymes involved in methanogenic bioenergetics, such as F_{420}-reducing hydrogenase and CoM-S-S-HTP reductase, are also inactivated in the presence of oxygen (Ni and Boone, 1998). ATP synthesis occurs via one or two types of ATP synthase and the synthesis of cellular macromolecules begins with the central anabolic intermediate, acetyl-CoA (see Blaut, 1994). Energy coupling requires the establishment of a proton motive force and electron transport phosphorylation. Sodium ions play a key role in these processes as well as activating membrane-bound enzymes (Ni and Boone, 1998).

2.1.3 Substrate range

Methanogens, as a group, display extreme catabolic specialisation. Many methanogens can only use one or two substrates, primarily one-carbon organics (Blaut, 1994), and the most versatile strains, in the genus *Methanosarcina*, can only use seven substrates (Zinder, 1993). Consequently, methanogens are highly dependent on other organisms to supply suitable substrates (see Section 2.3). Most of the methane produced in nature is synthesised from the methyl group of acetate (Ferry, 1999), the acetate having been released from incomplete catabolism of larger organic compounds. For example, in freshwater systems, methanogenesis from acetate and H_2 dominates the methane pool, with other substrates accounting for < 5 % (Lovely and Klug, 1982; Goodwin and Zeikus, 1987; Yavitt and Lang, 1990; Peters and Conrad, 1996). Similarly, in digestors, acetate accounts for approximately 67% of all methane produced (Smith and Mah, 1966; Mountfort and Asher, 1978; Boone, 1982; Zinder et al., 1984). The exceptions are: in the rumen where the host utilises acetic acid, removing it from the substrate pool (Mah et al., 1977); in marine systems where competition with sulphate-reducing bacteria dictates the use of CO_2 and methylated substrates (Sowers and Ferry, 1983; Winfrey and Ward, 1983; King, 1984; Whiticar et al., 1986) and in temperate acidic peat bogs (pH=3.5) where methane is generated exclusively by CO_2-reduction (Landsdown et al., 1992).

Acetate utilisation may dominate methane production in freshwater systems but only members of *Methanosarcina* and *Methanothrix* are capable of using it as a substrate (Zinder, 1993). In contrast, most methanogens are capable of converting H_2 + CO_2 or formate to methane via the CO_2-reduction pathway alone or in conjunction with formate

dehydrogenase, in the latter case (Schauer and Ferry, 1980; Zinder, 1993). Formate may be present in methanogenic habitats as it is a fermentation product of many microbes and of the plant metabolite oxalic acid (Allison et al., 1985). Two methanogens, *Methanobacterium thermoautotrophicum* (Daniels et al., 1978) and *Methanosarcina barkeri* (O'Brien et al., 1984), have been shown capable of utilising CO as a substrate. They appear to extract electrons from CO with CO dehydrogenase but grow very slowly. In addition, some hydrogenotrophic methanogens can use short chain alcohols (Widdel, 1986; Zellner and Winter, 1987) and methanol has been well described as a methanogenic substrate (Bryant et al., 1967). Methanol is probably not a major methanogenic precursor in nature in modern times (Schink and Zeikus, 1980) but it may have been important in ancient earth ecosystems (Bockelée-Morvan et al., 1991).

2.1.4 Environmental tolerances

Salinity

The minimum salinity level that will support freshwater methanogens is 1 mM Na^+, as sodium is required for the inwardly directed sodium motive force involved in their bioenergetics (Müller et al., 1987; Kaesler and Schönheit, 1989). Above this threshold methanogens can be found at most salinity ranges. The freshwater and marine methanogens are highly diverse (Aravalli et al., 1998) but there are few extremely halophilic methanogens. Those that are extreme halophiles belong to the *Methanosarcinaceae*, employ methylotrophic catabolism (Zinder, 1993) and are the only methanogens to possess cytochromes. Cytochromes are also found in the extreme aerobic halophiles, suggesting a common ancestor. Freshwater methanogens, without shared halophilic traits, may become adapted to marine conditions by reducing the thickness of the 'methanocondroitin' sacculus that surrounds them, and provides resistance for internal turgor pressure maintenance when in freshwater (Zinder, 1993). In this adapted but reversible state, they are more sensitive to lysis by detergent, grow as single cells rather than clusters and have a lower temperature optimum (Kriesl and Kandler, 1986). In addition, methanogens can adapt to increased salinity by accumulating osmoregulatory compounds, such as α- and β-glutamate (Robertson et al., 1990). As a consequence methanogens are found in habitats ranging from fresh to hypersaline.

Temperature

In nature, methanogens can be found in habitats with ambient temperatures ranging from 2°C in marine sediments to 110°C in geothermal vents (Zinder, 1993). Mesophilic and thermophilic methanogens are highly diverse but thermophiles have the capacity to grow more rapidly than mesophiles (Jones et al., 1983; Zinder, 1990). The vast majority of methanogens have temperature optima of 30°C or greater and none of the species reviewed by Jones et al. (1987) could grow below 10°C. These pure culture studies are reflected in mixed samples from the field that often show temperature optima of c. 35°C and ambient temperatures below 15°C limit methanogenesis (Zeikus and Winfrey, 1976; Conrad et al., 1989). However, it is clear that methanogens can be active in habitats such as marine sediments at temperatures below 10°C (Warford et al., 1979) and in the profundal sediments of deep lakes, such as Lake Constance with a permanent temperature of 4°C (Schulz and Conrad, 1996). The effect of temperature on mixed anaerobic populations may be complex and the rate of methanogenesis can be indirectly affected by temperature. For example, Schulz and Conrad (1996) found that rates of methanogenesis responded to seasonal changes in the profundal zone of Lake Constance even though it remained at a constant 4°C. The seasonal changes were considered a product of the seasonal variation in detrital inputs from moribund phytoplankton cells.

pH

Generally, methanogens have pH optima for growth near neutrality (Jones et al., 1987). They do, however, exist in extremely acidic environments such as peat bogs (pH values of 4 or less). Peat samples and isolates with pH optima near 6 can produce methane at pH levels as low as 3 (Williams and Crawford, 1984; Williams and Crawford, 1985). Similarly, Goodwin and Zeikus (1987) observed CO_2-reduction and aceticlastic methanogenesis at pH levels as low as 4 in bog sediments with a pH optima of 5-6. In contrast, *Methanohalophilus zhilinae* and *Methanobacterium thermoalcaliphilum* have pH optima of 8 and 9.2, respectively (Blotevogel et al., 1985; Mathrani et al., 1988) and are considered to be moderately alkaliphilic.

Oxygen

All methanogens are obligately anaerobic (Zehnder, 1988) and require growth media with redox potentials more negative than -300 mV (Hungate, 1967). Therefore common methanogenic habitats are anoxic in the bulk phase. However, methanogens have

remarkable tolerances to oxygen exposure given that they are non-sporulating (Fetzer et al., 1993) and have been found in all soil types, even those considered oxic and that have never been inundated (Peters and Conrad, 1995). In addition, enumeration of methanogens during the annual cycle of flooding and drawdown in rice fields shows a relatively stable and high titre at all times of year (Mayer and Conrad, 1990; Schutz et al., 1990; Asakawa and Hayano, 1995).

It may be that methanogens are not as oxygen sensitive as previously thought from enzymatic studies which show a irreversible dissociation of coenzyme F_{420} upon oxygen exposure (Hausinger et al., 1985). Conversely, methanogens may be very oxygen sensitive and survive in habitats that are grossly oxic or not highly-reducing, either by residing in anoxic microsites or living intimately with other micro-organisms that can remove inhibitory reaction products (Zinder, 1993; Muller et al, 1994). Kiener and Lesinger (1983) demonstrated a range of tolerances to oxygen exposure in different methanogenic species grown in pure culture. *Methanosarcina barkeri* was the most tolerant, remaining viable for over 24h of oxygen exposure. This was attributed to its clump forming habit and supports the hypothesis that methanogens persist in anaerobic microsites within ostensibly aerobic environments. Furthermore, there is evidence that methanogens have adapted to modern aerobic habitats by the production of super oxide dismutase (Kirby et al., 1981) and modified F_{420} coenzymes (Hausinger et al., 1985) and so far unidentified methanogens may be present in soil (Hales et al., 1996) that are less oxygen sensitive or dominate in less reducing conditions than those methanogens currently known.

2.1.5 Methanogenic habitats

Anaerobic environments that support methanogenesis include: freshwater and marine sediments, rice paddies, geothermal springs, digestive tracts and faeces, landfills, anaerobic digestors, algal mats and the heart wood of living trees (Kiene, 1991; Zinder, 1993; Singh and Singh, 1995). Oxic environments, such as desert soils, contain methanogens as well (Peters and Conrad, 1995). This suggests that methanogens are more ubiquitous than thought from culture studies which indicated their strict necessity for anoxia. Anaerobic digestors and freshwater sediments provide similar habitats for methanogens except that the digestors generally have much higher organic carbon loads, permit organism washout and provide more consistent conditions of temperature and loading rate than freshwater sediments. For example, primary sludge contains c. 5% w/v organic matter which is fed semi-continuously or continuously into an incubation vessel at 35 °C and acetate concentrations in these systems are in the millimolar range.

In contrast, freshwater sediments contain micromolar acetate concentrations (Zinder, 1993) and have variable temperature, moisture and detrital supply regimes.

In both digestors and freshwater sediments, the majority of the carbon present is in the form of complex plant derived compounds. Consequently, the methanogens lie at the end of a food-chain comprising: fermentative anaerobes, homoacetogens and both H_2-oxidising and acetotrophic methanogens (Figure 2.1). These anaerobes form various functional groupings ranging in number from three (Wolfe and Higgins, 1979) to five (Schink, 1997) required for complete digestion of complex carbon. Acetotrophic methanogens usually account for ~ 70 to 95% of the methane produced in digestors and freshwater sediments (Smith and Mah, 1966; Mah et al., 1977; Mountfort and Asher, 1978; Boone, 1982; Lovley and Klug, 1982; Zinder et al., 1984; Thebrath et al., 1993). In marine systems sulphate is supplied to the sediment from the water column and sulphate-reducing bacteria dominate catabolism in the upper sediment layers. Here the methanogens utilise methlyated non-competitive substrates rather than acetate and a marine methanogen has been isolated that is incapable of using acetate (Oremland et al., 1982; Sowers and Ferry, 1983; Jones et al., 1987; King, 1994). Acetate may be a precursor to methane if the organic loading rate is high enough or the sulphate supply low enough to saturate the sulphate-reducing community with electrons and allow electron flow to methane. However, most of the methane produced in deep marine sediments is consumed as it diffuses through the sulphate-reducing zone (Martens and Berner, 1974; Reeburgh and Heggie, 1977; Iversen and Jorgensen, 1985) and marine systems are generally not considered a major source of atmospheric methane (Cicerone and Oremland, 1988). Details of the complex interactions between sulphate-reducing bacteria (SRBs) and methanogens (MBs) are presented in Section 2.5.

Microbial degradation of complex plant material in gastrointestinal tracts of animals provides the host with quality food from cellulose (Hungate, 1975). Fatty acids produced by microbial fermentation are not further degraded by microbes but accumulate to concentrations useable by the ruminant host (Miller, 1991). The host facilitates this acid accumulation by swallowing enough water and saliva to keep the retention time in the gut short enough to exclude the slow growth of acetotrophic methanogens (Zinder, 1993). Therefore, hydrogenotrophic methanogens dominate rumen methanogenesis, especially members of *Methanobrevibacter* (Miller, 1991), which are also associated with protozoa (Vogels et al., 1980).

Termite guts are also significant methanogenic habitats. It was thought that methane dynamics within them were analogous to those in the rumen; however, recent studies

suggest that the gut microbiota maintain steep O_2 and H_2 gradients within the gut lumen. Therefore only the centre provides an anoxic methanogenic habitat. The methanogens compete strongly with acetogens for H_2 but remain a major H_2 sink in the termite gut (Brune, 1998).

Geothermal vents are widespread on the terrestrial landscape and methanogenesis within them has been studied in New Zealand, the USA, Iceland and Italy (Zinder, 1993). Temperatures in these springs range from 40 to 100 °C and *Methanobacterium thermoautotrophicum* and *Methanethermus fervidus* have been isolated from them (Stetter et al., 1981; Zeikus et al., 1980). Marine systems provide thermophilic habitats near shore (temperature up to 100 °C) and in deep sea hydrothermal vents, where temperatures reach 350 °C due to the high hydrostatic pressure (Jannesch and Taylor, 1984). Other methanogenic habitats include the heartwood of trees (Zeikus, 1977) and methanogens have been implicated in the process of biocorrosion (Daniels et al., 1987).

2.2 The Methanotrophs

2.2.1 Phylogeny

Bacteria that oxidise methane are thought to be exclusively aerobic. They are a subset of the methylotrophs, organisms capable of utilising organic compounds more reduced than formic acid as energy and carbon sources and assimilating formaldehyde into cellular carbon (Hanson and Hanson, 1996). The first methanotroph was isolated in 1906 by Söhngen (as cited in Hanson and Hanson, 1996) but it was not until 1970 that a comprehensive study of 100 methane-utilising bacteria was published (Whittenbury et al., 1970). Whittenbury and co-workers proposed a separation of the methanotrophs into 5 groups (or genera); *Methylomonas, Methylobacter, Methylococcus, Methylocystis* and *Methylosinus* (Whittenbury and Dalton, 1981; Whittenbury and Krieg, 1984). Subsequently, a sixth genus has been added, *Methylomicrobium* (Bowman et al., 1993; Bowman et al., 1995).

Three types of methanotrophs have been distinguished based primarily upon their cell and spore morphologies and genetic and biochemical properties. Type I methanotrophs: *Methylococcus*, *Methylomicrobium*, *Methylobacter* and *Methylomonas*, are generally short, single rods or cocci with a G+C content of 49-60 mol % and some strains are capable of forming cysts. Type II, *Methylosinus* and *Methylocystis*, are generally crescent-shaped rods, rods or pear-shaped cells and have a G+C content of 62-67 mol %, with some strains forming cysts or exospores. The third group, Type X (for example, *Methylococcus capsulatus*) were distinguished from Type I methanotrophs

by their possession of low levels of enzymes also found in the Calvin-Benson cycle (Whittenbury and Dalton, 1981; Whittenbury and Krieg, 1984). Type X methanotrophs can also grow at higher temperatures than Types I and II and have a higher G+C content (59-65 mol %) than most Type I methanotrophs [see Hanson and Hanson (1996) for review].

As with the methanogens, the taxonomy of the methanotrophs is in a constant state of flux [see King (1992) for review]. They are now being exploited to consume xenobiotic compounds (see Fox et al., 1990) which, coupled with the recent development of specific oligonucleotide probes (Brusseau et al., 1994; Holmes et al., 1995) and understanding of specific methanotrophic fatty acid profiles (Guckert et al., 1991), will ensure the rapid discovery of new isolates from a variety of habitats. Further, not all methanotrophic organisms are bacteria; thus field investigations need to include various organisms such as yeasts (Wolf and Hanson, 1980). In addition, Jones and Nedwell (1993) have isolated *Trichoderma* and *Penicillium* species with the capacity to oxidise methane from landfills, suggesting that the capacity to oxidise methane is more widespread than previously considered.

2.2.2 Bioenergetics

The aerobic oxidation of methane is initiated by methane monoxygenase (MMO) enzymes which utilise two reducing equivalents to split the dimer bond in molecular oxygen (Lipscomb, 1994). One of the oxygen atoms is incorporated into CH_4 to form CH_3OH whilst the other is reduced to form H_2O. Two forms of MMO have been found in methanotrophs, a soluble form (sMMO) which was purified from types I and X and a particulate or membrane-bound form (pMMO) which can be formed in all known methanotrophs (Hanson and Hanson, 1996). The ecological consequences of this are that methanotrophs which contain pMMO have higher growth rates on methane and have higher affinity for methane than do cells that possess sMMO. This is due to 1) the co-requirement for NADH + H^+ which often limits growth on methane in the sMMO catalysed reaction and 2) the utilisation of a higher potential electron donor by pMMO (Prior and Dalton, 1985; Leak and Dalton, 1986a,b; Lipscomb, 1994). In general, Type I methanotrophs employ the ribulose monophosphate (RuMP) pathway for formaldehyde assimilation and Type II methanotrophs employ the serine pathway (King, 1992). Type X are similar to Type I in that they employ the RuMP pathway but are distinguished by the possession of serine pathway enzymes as well (Whittenbury and Dalton, 1981; Whittenbury and Krieg, 1984).

2.2.3 *Environmental tolerances*

O_2 and CH_4 requirements

In field conditions, methanotrophs may be exposed to micromolar concentrations of CH_4, or lower, and to suboxic to microaerophilic oxygen concentrations in habitats adjacent to a biogenic methane source (Kuivila et al., 1988; King et al., 1990; Ward and Kilpatrick, 1990; Frenzel et al., 1992). The apparent Km values for pure cultures, enrichments, cell-free extracts and purified MMO are within this range; from 1 to 92 µM for methane and 0.1 to 37 µM for oxygen [see King (1992) for review]. There does not appear to be any taxonomic basis to account for the spread in values and separate studies on the one species have returned very different Km values, suggesting environmental effects. Half-saturation constants (Kapp) for methane-uptake in natural unpurified samples are at the low end of this Km range, varying from 2 - 10 µM in freshwater wetland and lake sediments (Lidstrom and Somers, 1984; Kuivila et al., 1988; King, 1990b). Similarly, Lidstrom and Somers (1984) and Kuivila et al. (1988) report Kapp values for oxygen-uptake (10 - 20 µM) in Lake Washington sediments that compare well with those obtained in purified systems.

In counter-gradient and chemostat culture studies, type I methanotrophs dominate in conditions of low-methane and high-oxygen and type II methanotrophs dominate in high-methane, low-oxygen conditions (Amaral and Knowles, 1995). Specifically, Amaral and Knowles (1995) conducted an experiment in which cultures of pure methanotrophic strains and mixed soil samples were added to media-filled diffusion columns supplied with methane and oxygen at opposite ends. Bands of microbial cells developed in the columns at positions relative to O_2:CH_4 ratios of 1.57 to 1.97. Most of the bands contained type I methanotrophs and developed where CH_4 and O_2 supply rates were high. However, one band occurred where CH_4 concentrations were highest and O_2 was only available in trace amounts (<0.5%). This lower band only contained type II methanotrophs. These results suggest that Type I methanotrophs would be best suited to conditions of high O_2 and higher than ambient CH_4, as found in water overlying methanogenic sediment, and that type II methanotrophs would be more suited to oxic zones within methanogenic sediments where O_2 supply is very low but CH_4 concentrations are high. This is supported by studies in Cleveland Harbour, where type I methanotrophs were most abundant in the water column and type II were only found in the methanogenic, microaerophilic sediments (Reed and Dugan, 1987), and by studies of methanotrophs associated with the oxygenated rhizospheres of macrophytes growing in methanogenic sediment which were exclusively type II (King, 1994). Consequently, Type I and II methanotrophs appear to be functionally distinct.

Non-methanogenic soils in equilibrium with ambient air contain low nanomolar concentrations of methane (Conrad, 1996), thereby excluding methanotrophs with Km values as described above. Kinetic studies cannot account for the oxidation of atmospheric methane observed in oxic soils [Km < 200 nM (Conrad, 1996)], a process that is obviously accomplished by unknown methanotrophs (Bender and Conrad, 1992). Alternatively, the kinetic studies suggest that methanotrophy would be well-supported in niches adjacent to methanogenic habitats where methane concentrations are higher than ambient but oxygen is still available. In addition, the exclusively aerobic methanotrophs can survive long periods of exposure to anoxia (Bender and Conrad, 1995) and are able to initiate methane oxidation within hours of exposure to oxygen (Roslev and King, 1994). Most methanotrophs can also form resting stages to overcome drought or nutrient-poor conditions (Hanson and Hanson, 1996), rendering them very tolerant of changeable environmental conditions.

The effect of temperature and pH

The majority of methanotrophs are mesophiles with the exception of *Methylococcus capsulatus* and related isolates that can grow at temperatures up to 50 °C (Bowman et al., 1993; Hanson et al., 1991) and a psychrophilic tundra community described by Omel'chenko et al. (1992). Natural methanotrophic populations may adapt to extant temperature regimes because different soils exhibit different methane oxidation responses to temperature. For example, methane oxidation in peat soils is generally optimal at 25 °C (Bender and Conrad, 1995) whereas isolates from an Arctic bog hollow had temperature optima at 10 °C or lower (Omel'chenko et al., 1992 and 1993). However, Dunfield et al. (1993) did not report temperature adaptation by methanotrophs in the northern peatlands, thus generalisations about temperature-dependence across different methanotrophic habitats cannot be drawn.

The pH optima observed in soil samples are similar to those reported for pure culture studies and appear to reflect an adaptation to the pH characteristics of the field site. Optimum and minimum pH values for methanotrophy in acidic peat samples were lower than those for alkaline peats and soils, and rates of methane oxidation at pH = 4 -5 were higher in acidic than alkaline samples (Dunfield et al., 1993). In addition, Borne et al., (1990) observed equivalent rates of methane oxidation in a variety of soils with native pH levels ranging from 3.5 to 8. Methanotrophic bacteria capable of growing at pH levels below 5 have not been isolated. However, Wolf and Hanson (1980) describe a facultative methanotrophic yeast that grew best at pH values below 6 and could oxidise

methane after enrichment at a pH of 3.5, suggesting that as a functional group, methanogens can tolerate a wide range of pH conditions.

Effect of nitrogenous fertilisers

The addition of ammonium fertilisers has been shown to inhibit atmospheric methane uptake by soils in a range of pure culture and field-based studies (eg. Bedard and Knowles, 1989; Steudler et al., 1989; Bronson and Mosier, 1994; Crill et al., 1994; Schnell and King, 1994). However, Flessa et al. (1995) and Tate and Striegel (1993) observed no effect of nitrogenous fertiliser additions. When added to flooded sediments, which tend to be circumneutral (Lerman, 1988), the bulk of the nitrogen species will remain as ammonium as its equilibrium with ammonia is pH dependent. It is ammonia, as opposed to ammonium, that is thought to directly inhibit MMO (Megraw and Knowles, 1987), thereby reducing methane oxidation.

The situation in oxic soils is more complex as ammonia-oxidising bacteria can oxidise methane and methanotrophs can oxidise ammonia. Nitrite is formed when methanotrophs consume ammonia which may lead to inhibition of methane oxidation (King and Schnell, 1994). In addition, the methane-oxidising zone may exist deeper in the soil than the top dressed fertiliser can penetrate as ammonium-N. Therefore the methanotrophs may be afforded physical protection by the topsoil (Dunfield et al., 1995). The inhibitory effect is fertiliser specific as Willison et al. (1995) found that ammonium fertilisers decreased methane oxidation but nitrate fertiliser did not, even though the fertilisers had been applied for 138 years. This has implications for agricultural soils or wetlands receiving agricultural run-off as the inhibition may persist for long periods (King and Schnell, 1994).

2.2.4: Methanotrophic habitats

Most methanotrophs have Km values well-above methane concentrations observed in soils in equilibrium with the atmosphere (Conrad, 1996). Consequently, methanotrophs tend to live near methanogens, a ready source of methane. As methanotrophs are aerobes and methanogens are anaerobes, the methanotrophic niche is, therefore, often satisfied at the interface between oxic and anoxic environments. Habitats where this interface occurs include: freshwater sediments, rice paddies, landfills, ocean waters, terrestrial soils, sewage sludge, aquatic plant rhizospheres and invertebrate gills (Hanson, 1980; Strand and Lidstrom, 1984; Hanson et al., 1991; King, 1992; Holmes et al., 1995). Aerobic methanotrophs do not appear to inhabit hypersaline environments (Conrad et al., 1995), although anaerobic methane oxidation was observed in the water

of a hypersaline, alkaline soda lake (Iversen et al., 1987). Aerobic methane oxidation has not been reported in the rumen (Hanson and Hanson, 1996), rendering it the only major methanogenic habitat not also inhabited by methanotrophs.

Methanotrophic bacteria can significantly reduce potential methane emissions from wetlands and other methanogenic habitats because they tend to inhabit the oxic:anoxic interface which separates the methanogenic zone from the atmosphere (King, 1990a; King, 1990b; Amaral and Knowles, 1995; Segers, 1997; Utsumi et al., 1998). Furthermore, those methanotrophs that occur in non-methanogenic terrestrial soils have the potential to consume methane that has previously been emitted to the atmosphere. Although consumption of methane at atmospheric concentrations has not been confirmed in culture studies (Conrad, 1996), a detailed review by King (1992) shows that atmospheric methane consumption has been confirmed in field studies of various agricultural soils and grasslands, deciduous and coniferous forests and tundra soils. Given that methanotrophy in soils is highly sensitive to nitrogen fertilisers (Mosier et al., 1991), the rate of methanotrophy in terrestrial systems may have decreased substantially since deforestation and agricultural improvement in developed countries (Keller et al., 1990). The highest rates of methane oxidation in oxic soils are reported from landfill cover soils (Whalen et al., 1990). Oxidation proceeded with first-order kinetics from very low methane concentrations (1 ppm) and was influenced by the water content of the soils in this system. Water logged landfill soils consume methane at a similar rate to freshwater sediments (Whalen et al., 1990) but methane oxidation was significantly enhanced when the soil water content decreased to below 5% w/w (Adamsen and King, 1993), suggesting that landfill cover soils could be an effective methane sink.

2.2.5: Anaerobic methanotrophy

Anaerobic methanotrophy occurs in some methanogenic habitats, particularly marine and hypersaline sediments, but is far less well understood than the process of aerobic methanotrophy. Marine sediments alone are thought to consume 100 to 200 Tg y^{-1} of methane in anaerobic conditions (Reeburgh, 1980; Hanson and Hanson, 1996) and methane consumption has also been observed in anoxic marine waters and the sediments of soda lakes and freshwater systems (Reeburgh and Heggie, 1977; Panganiban et al., 1979; Reeburgh, 1980; Alperin and Reeburgh, 1985; Inversen and Jorgensen, 1985; Iversen et al., 1987; Alperin et al., 1988). The bulk of the evidence for anaerobic methanotrophy is based on the use of $^{14}CH_4$ as a sole substrate, *in situ* diffusive methane models or delta $^{13}CH_4$ profiles (Conrad, 1996). In general, anaerobic

methane oxidation is coincidental with sulphate-reduction or ferric iron reduction (Hoehler et al., 1994; Hanson and Hanson, 1996). Furthermore, not a single organism that can oxidise CH_4 in the absence of O_2 has ever been isolated (Conrad, 1996). It has been suggested that anaerobic methane oxidation is mediated by a reversal of the methanogenic pathway (Hoehler et al., 1994). This is thermodynamically possible if H_2 partial pressures are kept very low by nearby H_2 consumers and may explain the co-incidence of anaerobic methanogenesis with other hydrogenotrophs. It may also be that an as yet undescribed methylotrophic archaea is consuming methane in these environments. A more detailed discussion appears below in Section 2.5.2.

2.3: Catabolism in wetlands

In most freshwater systems, between 2 and 10 % of the carbon fixed in primary production is anaerobically converted to methane (Kuivila et al., 1988; Richey et al., 1988). In these studies the relative proportion of methane produced increased to 20% if the oxygen-poor sediments were considered alone. This highlights both the importance of anaerobic carbon fluxes in inundated sediments as well as the intrinsic limits to aerobic decomposition. Oxygen diffuses 10, 000 times more slowly through inundated sediments than through terrestrial soils (Ponnamperuma, 1972). Thus inundated soils are often characterised by a lack of free oxygen (Zehnder, 1988).

Although molecular oxygen is generally considered to be exhausted from a system at an arbitrary redox potential of 200 mV (Ponnamperuma, 1972), Revsbech et al. (1980) state that free O_2 may be entirely removed before the sediment becomes chemically reducing. Redox potential, or electron availability, is often measured in biological systems because electrons are essential reactants in catabolic reactions. Electron availability affects the oxidation states of a limited but critical range of elements; H, C, N, O, S, Mn, Fe, Co and Cu, as the limits are set by the stability of the water molecule. The oxidising potential is limited by the oxidation of water to molecular oxygen and the reducing potential is limited by the reduction of hydrogen ions to molecular hydrogen (Bohn, 1977). Redox potentials are pH dependent (Whitfield, 1974) and, in sediments, represent a combined potential, the weighted average of the electron exchange currents between all the reactive redox pairs in the system (Bohn, 1977). Natural systems rarely reach equilibrium as there is a constant exchange of electron donors and acceptors with other systems. Therefore strict relationships between the Nernst equation, equilibrium potentials and redox potentials are not rigorously defined for natural systems. As such, redox potentials have limited usefulness in studying natural systems but they are often measured as a means of comparing between and across heterogeneous systems.

Flooded soils tend to yield more stable redox potentials than other natural systems as they contain large amounts of organic matter (electron donors) and the redox potential is dominated by high H^+-H_2 exchange currents which override small perturbations from other reactive pairs (Bohn, 1977). As such, consistent patterns in redox potentials have been observed on a variety of spatial and temporal scales and models of microbial succession and competition have been developed based primarily on temporal and spatial redox potential gradients (see Conrad, 1996 for review). However, Brandl et al. (1993) observed that the sediment depth at which conditions switched from oxidising to reducing was affected by small scale changes in topography. In the flocculent sediment of the central basin of Lake Geneva, Switzerland, reducing conditions occurred at a few millimetres depth but 10 to 30cm away, oxidising conditions penetrated to 6cm depth in the sediment of 5 to 15cm deep trenches.

Although redox potentials have limited meaning in natural systems, the following section includes reference to threshold redox potentials for different groups of microbes because much of our understanding of anaerobic microbiology was generated in the laboratory in pure culture work. In these conditions, redox potentials are more robust and redox potential thresholds for various microbes were defined. The reader should not that these thresholds are not definitive in bulk sediment studies and that a myriad of microbial activities can occur across a range of bulk sediment redox potentials due to the provision of microniches within the sediment.

2.3.1: Aerobic processes

Aerobic respiration is the most thermodynamically favourable mode of microbial respiration, yielding 688 kcal $mole^{-1}$ (Poole and Wildish, 1979). It requires a redox potential of 400 mV or greater, where molecular oxygen can accept electrons during ATP production. Oxygen is utilised by a diverse community of decomposers; bacteria, fungi and protozoa, facilitating the initial breakdown of complex polymers such as cellulose, polysaccharides, proteins and lipids. Aerobic respiration directly produces CO_2 via the tricarboxylic acid cycle but polymer mineralisation is often incomplete, and smaller organic moieties are produced as well as CO_2. The initial lysis of polymers is often mediated by extracellular or membrane-bound enzymes which cleave complex polymers into soluble monomers that can be taken up by the aerobic cell (Madigan et al., 2000). Once the supply of oxygen is exhausted the remaining degradable carbon may be utilised by anaerobic bacteria.

Oxygen is supplied from the atmosphere and from photosynthesis within the illuminated areas of the wetland. Highly productive wetlands can have surface water layers

supersaturated with oxygen if the oxygen demand of the system is exceeded by photosynthetic or atmospheric supplies (Boulton and Brock, 1999). However, oxygen demand varies on a diel cycle. In the dark hours, photosynthesis ceases but the photosynthetic organisms continue to demand oxygen in respiratory processes; therefore the net oxygen demand increases. High carbon loads will also increase the oxygen demand of the system, thereby decreasing the depth oxygen penetrates into the sediment (Revsbech et al., 1980; Reimers and Smith, 1986).

Typically, oxygen entering the sediment is utilised rapidly by aerobes so that only the very surface layer of inundated wetland sediment will be oxic, whilst the bulk phase is anoxic. For example, King (1990b) reported sediment oxygen penetration depths of 1-2 mm in sandy silt deposits and < 5mm in stream-bed sands of a Danish wetland, and King et al. (1990b) reported depths of 8 mm in the Florida Everglades. However, in addition to the thin oxic layer on the surface, wetland sediments colonised by aquatic plants may contain deeper oxic patches where oxygen, excess to the plant's requirements, has leaked into the bulk sediment from the roots. The extent of subsurface oxygenation will depend on both the macrophyte species and the density of the roots (Armstrong and Armstrong, 1988; Flessa and Fischer, 1992; Flessa, 1994; Howes and Teal, 1994).

In general, plants that employ pressurised ventilation to aerate their submerged organs will supply the rhizosphere with more oxygen than plants that rely solely on diffusive flux for root aeration (Brix et al., 1992). Nevertheless, Flessa (1994) found that the aquatic herbs, *Myriophyllum verticullatum* and *Ranunculus circinatus*, can sustain an oxidising rhizosphere with redox potentials > 450 mV near the root tips, even though neither species employs pressurised flow-through for tissue aeration. Moreover, in regions where the root tips overlapped, redox potentials could be as high as $^{+}650$ mV, representing well-oxygenated microniches.

These oxic zones serve an important role in enhancing oxygen supplies for decomposition (and methane oxidation) and create a mosaic of aerobic and anaerobic microniches throughout the sediment profile. This mosaic tempers the extremity of the anaerobic habitat supported in wetland sediments with respect to the extremity of other habitats utilised by archaeobacteria that may be hypersaline, acidic or thermogenic (Holland et al., 1987).

Aerobic decomposition is limited by oxygen supply in wetland systems but is generally considered a more rapid and important process than anaerobic decomposition (Reddy and Graetz, 1988). This may, at least partially, be an artefact of the apparent rapid

decay of fresh, labile detritus before the onset of anoxia a theory that is supported by the importance of anaerobic catabolism to total catabolism in marine systems (Reeburgh, 1983; Ferdelman et al., 1988) and the work by Rogers and Breen (1982) on the comparative decay rates of naturally senescent and artificially dried detritus.

2.3.2: Anaerobic processes

The relative importance of aerobic and anaerobic catabolism in wetlands is affected by temporal and spatial variation in oxygen availability. In permanently flooded beds of *Eleocharis sphacelata*, an emergent macrophyte which employs pressurised ventilation (Brix et al., 1992), at least 60 % of the combined aerobic and methanogenic carbon fluxes were accounted for by methane production (Boon and Mitchell, 1995). In contrast, the less permanently inundated beds of *Myriophyllum* sp. and *Vallisneria gigantea*, neither of which employ pressurised flow-through, returned approximately 30 and 40 % of the combined carbon flux as methane, respectively. Regardless of the proportion of mineralisation they account for, aerobic microbial processes are often critical, initial steps in the complete breakdown of complex organic carbon to methane, a process which is finalised by anaerobic microbes.

Wolfe and Higgins (1979) proposed the differentiation of anaerobic microbes into three functionally distinct but interdependent groups (Figure 2.1). The first group, the hydrolytic microbes, cleave polymers and ferment the hydrolysis products into intermediates such as fatty acids and alcohols. The second group, the acetogens, metabolise these intermediates into predominantly acetate, H_2 and CO_2 which are used to reduce electron acceptors other than oxygen in the terminal stages of catabolism by the third group of diverse microbes. Furthermore, Schink (1997) emphasises the importance of syntrophic interactions between these groups in order to completely mineralise complex organic matter.

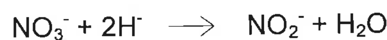
The fermenters

The extracellular hydrolytic enzymes used to convert polymers to oligomers and monomers are produced by primary fermentative bacteria which also ferment the resulting monomers to fatty acids, succinate, lactate, alcohols etc. (Figure 2.1). Some of these fermentation products, e.g. acetate, H_2 and CO_2, can be used directly by the methanogens. Other products e.g. fatty acids, are degraded to acetate, H_2, CO_2 and perhaps formate by secondary fermenters or obligate proton reducers, before use by the methanogens. Fermentation, in these cases, involves the oxidation of organic matter using an organic moiety as the electron acceptor as well (Wetzel, 1983). Organisms employing fermentation for energy conservation produce ATP via substrate-

level phosphorylation (Holland et al., 1987). As such they are not restricted by the redox potential requirements of electron transport phosphorylation and can tolerate a wide range of redox conditions within the sediment mosaic (Jones, 1982) where they can supply anaerobes with important metabolic precursors (Atlas and Bartha, 1993).

Nitrate-reducing bacteria

After oxygen is exhausted, the next most thermodynamically favourable electron acceptor exploited by anaerobic bacteria is nitrate. Furthermore, the dissimilatory reduction of nitrate to dinitrogen gas (denitrification) is more energetically favourable than the dissimilation of nitrate to ammonia (Krumbien, 1983; Kuhn et al., 1991). Nitrate will accept electrons from monomers such as amino acids and nucleotides at approximately $^{+}$250 mV and its reduction will yield approximately 649 kcal per mole of substrate.

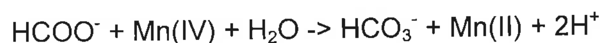


$$NO_3^- + 2H^- \longrightarrow NO_2^- + H_2O$$

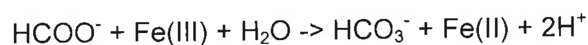
Most denitrifiers are facultatively anaerobic heterotrophs but they are taxonomically diverse and widespread in nature (Knowles, 1982). Their carbon and energy sources include a wide array of carbohydrates and organic acids and most are capable of completely reducing nitrate to dinitrogen gas, although some lack the necessary reductase enzymes (Knowles, 1982).

Metal-reducing bacteria

As the redox potential, the size of the organic moiety and hence its reducing power, continues to decrease, manganic compounds are transformed to manganous compounds (redox potential, $^{+}$225 mV) and ferric compounds to ferrous (redox potential, $^{+}$120 mV).



$$HCOO^- + Mn(IV) + H_2O \rightarrow HCO_3^- + Mn(II) + 2H^+$$



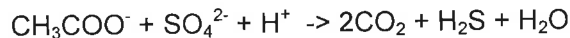
$$HCOO^- + Fe(III) + H_2O \rightarrow HCO_3^- + Fe(II) + 2H^+$$

Lovely (1991) and Nealson and Myers (1992) have demonstrated that Fe III and Mn IV reduction is predominantly enzymatic in nature, although Roden and Edmonds (1997) suggest that the production of sulphide by sulphate-reducing bacteria can provide substantial reducing power for these metals. The bioavailability of iron and manganese is strongly dependent on their oxidation state. Iron, especially, can be temporarily but effectively removed from the available electron acceptor pool by forming sulphide precipitates (Stumm and Morgan, 1970). Manganese and ferric iron reducers are

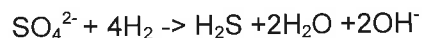
known to be physiologically and taxonomically diverse bacteria, although they have not been extensively studied. Both groups seem to oxidise organic acids (and aromatic compounds in the case of ferric iron reducers) and liberate acetate, H_2 and CO_2, important methanogenic precursors (Lovely, 1991).

Sulphate-reducing bacteria

Sulphate reduction is the next bacterial process in the thermodynamically predicted sequence and it occurs at redox potentials between -75 and -150 mV.



$CH_3COO^- + SO_4^{2-} + H^+ \rightarrow 2CO_2 + H_2S + H_2O$

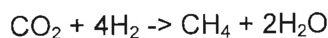


$SO_4^{2-} + 4H_2 \rightarrow H_2S + 2H_2O + 2OH^-$

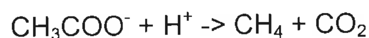
Sulphate-reducing bacteria (SRB) gain 9 kcal per mole of substrate oxidised and have long been considered the methanogens' major competitor. They are physiologically and taxonomically diverse and grow across a wide range of pH, salinity, temperature and pressure conditions, being responsible for the bulk of the hydrogen sulphide produced in anaerobic environments (Krumbein, 1983; Atlas and Bartha, 1993). The utilisation of organic compounds by SRB is complex. They can oxidise a range of substrates including succinate, malate and ethyl alcohol, although they apparently prefer pyruvate, lactate and acetate (Atlas and Bartha, 1993). However, acetate, H_2 and CO_2 can be produced as metabolic by-products. Therefore, the activity of SRB can reduce or increase the size of the substrate pools available for methanogenesis.

The methanogens

The least energetic microbial process is methanogenesis. According to thermodynamic theory, it occurs at the most reducing conditions (below -250 mV), where carbon becomes the terminal electron acceptor and methane is evolved. Methanogenesis yields 8-40 kcal per mole and methane can be formed from the same low molecular weight carbon compounds, eg. acetate, utilised by the more thermodynamically favoured microbial processes outlined above.



$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$



$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$

The methanogens have been reviewed in detail above, but here the reader is reminded that they have a highly restricted substrate range and are considered poor competitors if alternative electron acceptors are available.

This sequence of microbial processes can be observed over several spatial and temporal scales. Redox potentials in inundated sediments decrease rapidly with increasing sediment depth and as redox conditions govern potential electron flow between donor and acceptor, microbial species composition is closely related to sediment depth. Aerobic respiration occurs in the top 6-7 mm of the sediment (Kuivila et al., 1988), whilst the anaerobes are activated lower in the sediment profile where redox potentials are below $^{+}$250 mV. This spatial sequence is commonly reported in the literature although the respective depths at which each functional group is activated may differ across systems, presumably due to differences in relative oxygen penetration, organic loading and availability of alternative electron acceptors (Cappenberg, 1974a; Mah et al., 1977; Poole and Wildish, 1979; Gwynfryn-Jones, 1982; Brandl et al., 1990; Whalen and Reeburgh, 1992; Mitsch and Gosselink, 1993).

In addition, Gunnison et al. (1985) have shown that newly flooded sediments undergo chemical transformations over several years during the transition from being terrestrial to aquatic. They observed the same microbial sequence: aerobe to facultative anaerobe to anaerobe, through time and with increasing sediment depth. This is supported by evidence that methane emissions were not detected until 3 months after flooding an ephemeral system (Happell and Chanton, 1993). During the initial 3 months, organic matter accumulated in the sediment, highlighting the dependence methanogens have on the organisms which precede them in the microbial cascade for the supply of suitable substrates. Although generalisations can be made regarding the sequential occurrence of microbial functional groups or the dominance of one group over another, caution should be exercised because the redox potential measured in a system can only give the most gross habitat description.

2.4: Understanding anaerobic bacterial interactions

2.4.1: Syntrophic methanogenesis

Methanogenesis is the least exergonic microbial process compared with aerobic catabolism or the alternative anaerobic respiratory pathways. The energy released from one mole of hexose when anaerobically converted to methane and CO_2 is only 15 % of the energy that would be liberated in an aerobic conversion of hexose to CO_2 (Schink, 1997). The low amount of energy gained in methanogenesis forces the methanogens to be efficient and co-operative organisms. Their dependence on other microbes can be mutual and so intimate that neither partner can operate without the other. Also, together, they can display activities that neither partner could accomplish on their own. This type of mutual microbial dependence commonly is termed syntrophy.

The classical example of syntrophic methanogenesis is the modern interpretation of the 'Methanobacterium omelianskii ' culture described by Barker (1940). Originally considered to be one organism, it was later shown to be a co-culture of two organisms: the H_2-producing strain S and the H_2-consuming methanogen, strain M.o.H, which co-operated to convert ethanol to acetate and methane by the apparent mechanism of interspecies hydrogen transfer (Bryant et al., 1967). The fermentative organism, strain S, could not be grown on ethanol in pure culture because the bioenergetics were endergonic without the presence of a H_2-scavenging partner. When the M.o.H. organism consumed H_2 and the partial pressure dropped to below 100 Pa, the oxidation of ethanol became energetically favourable for strain S, which produced both acetate and H_2 for consumption by strain M.o.H.

The production of one ATP molecule requires the movement of 3 H^+ ions across the bacterial membrane. Therefore the lowest quantum of energy that can be metabolically conserved is that required to move one proton or -20 kJ mol^{-1} (4.8 kcal mol^{-1}). This is approximately the amount of energy released in syntrophic fermentations (Schink, 1997). Five different groups of bacteria co-operate to convert complex organic matter to methane; the latter members are dependent on the earlier members to provide suitable substrates and the earlier members benefit from the latter removing metabolic end-products (Figure 2.1).

Primary fermenters behave like strain S. They profit from their intimate associations with H_2-consuming bacteria, such as methanogens under low H_2 partial pressure, by conserving relatively more energy than if the H_2 were not removed. At very low H_2 concentrations the carbon and energy flux from catabolism is mainly directed through monomeric C1 compounds or acetate (Figure 2.1). But, if H_2 levels increase, the reduced intermediates such as other fatty acids, lactate and alcohols become more important and the fermenter conserves less energy. For example, when *Ruminococcus albus*, a carbohydrate-fermenting, H_2-producing bacterium is grown in pure culture acetate, ethanol and H_2 are detected as end products. But when it is grown in continuous culture with *Vibrio succinogenes*, only acetate and succinate are detected. In addition, a pure *R. albus* culture will produce 3.3 ATP for every mole of glucose oxidised but a mixed culture of *R.albus* and *V. succinogenes* will produce 4 ATP per mole of glucose (Iannotti et al., 1973).

To optimise their energy gain, H_2 scavengers need a close physical association with the H_2 producers, as the H_2 gradient that exists between the two microbes will dissipate the potential energy available to the consumer along the distance the H_2 molecule travels

(Dolfing, 1992). Close proximity will also induce efficient H_2 removal, thus enhancing the growth of the H_2 producer and increasing the chance that the methanogen will receive the H_2 over its competitors. Most anaerobes will outcompete MB for substrate utilisation (based on the thermodynamic theory outlined above) but homoacetogenic hydrogenotrophs gain less energy from H_2 consumption than MB under standard conditions. Therefore they are generally poor competitors (Schink, 1997).

The flow of electrons to homoacetogens in Figure 2.1 is not clear. Having a reliance on H_2, homoacetogens must receive electrons from fermentation (Schink, 1997). Presumably, they take advantage of their high metabolic flexibility, compared with the methanogens, to utilise resources under competitive conditions. For instance, homoacetogens can utilise two or more substrates simultaneously, a process which allows for resource competition with several functional groups at once and has the potential to increase the energy conservation from any one substrate (Schink, 1997). However, in conditions which are sub-optimal for hydrogenotrophic MB and sulphate-limited, homoacetogens can become the dominant H_2 sink. In one such habitat, the slightly acidic sediment (pH = 6.1) of Lake Knaack, USA, all electrons flow through the acetate pool and no methane is produced via CO_2-reduction. It appears that aceticlastic MB maintain the acetate concentrations low enough for homoacetogenesis to become thermodynamically favourable (Phelps and Zeikus, 1984); therefore methane is still produced, but not from CO_2-reduction because the homoacetogens are utilising the H_2. Similarly, hydrogenotrophic MB are outcompeted in low temperature environments where the energy yield for homoacetogenesis exceeds that for hydrogenotrophic methanogenesis. The opposite occurs at high temperatures where aceticlastic methanogenesis becomes thermodynamically less favourable than homoacetogenesis. In such conditions, the homoacetogenic process occurs in the opposite direction and electrons flow through the C1 pool and H_2 towards methane (see Schink, 1997 for review). Thus the schema in Figure 2.1 represents an intermediate situation which probably describes the bioenergetics of moderate habitats such as sewage digestors and temperate wetlands well but would need to be modified for habitats at either temperature extreme.

Hydrogen appears to be the ideal electron carrier in syntrophic interactions due to its size and diffusivity properties (Schink, 1997) but formate could also act in a similar manner as proposed by Bryant et al. (1967). The detection of interspecies formate transfer is currently hindered by methodological techniques lacking in sensitivity. Although the microbial electron transfer systems can be explained based purely on H_2 exchange, the role of formate should not be discarded as it has potential to be an

important electron carrier as well. Syntrophic fermenters also excrete acetate which can be metabolised by MB and Schink (1997) reviews the influence acetate removal has on the overall energetics of syntrophic fatty acid or benzoate degradation. Regardless of the form, bioenergetic interactions between the four bacterial groups responsible for syntrophic methanogenesis are an intimate and integral factor controlling carbon and energy flow paths during anaerobic catabolism.

2.4.2: Interactions between methanogens and sulphate-reducers

In anoxic, sulphate-rich marine sediments, primary fermentation products can be completely mineralised to CO_2 in just two steps, terminating with sulphate-reducing bacteria (SRB) operating independently of syntrophic processes. In freshwater, complete mineralisation requires at least three steps and generally terminates in methane production (Schink, 1997). Traditionally, it was considered that the interactions between SRB and MB were those of simple competition for common resources. Studies on aquatic sediments from a variety of habitats (marine, estuarine, lake, reservoir, freshwater wetland and saltmarsh) have shown that catabolism tends to be dominated either by methanogens or sulphate reducers, which supports this suggestion (for example: Cappenberg, 1974a; Claypool and Kaplan, 1974; Martens and Berner, 1974; Reeburgh and Heggie, 1977; Mountfort et al., 1980; Senior et al., 1982; Ward and Winfrey, 1985; Crill and Martens, 1986; Hines et al., 1993). A spatial separation between net H_2S and CH_4 production is evident in most marine sediments and along other salinity gradients, although they are not obligately mutually exclusive,.

The spatial separation between MB and SRB may be caused by:

- direct competition for substrates
- inhibition of methanogenesis by SO_4^{2-} or HS^- toxicity
- maintenance of very stable redox conditions above the threshold for methanogenesis, or
- consumption of the end-products of either type of microbe, thus that microbe evades field detection e.g. anaerobic methane oxidation (Reeburgh and Heggie, 1977; Mitsch and Gosselink, 1993).

Both MB and SRB can utilise acetate, H_2 and CO_2 and it appears from extensive field evidence that the major factor controlling their relative distributions is competition for these substrates. If organic matter availability is not limited, electron flow can occur through both pathways without interference but if organic carbon availability is limiting the pathways must compete for electron flow.

The pioneering work of Cappenberg (1974a, b) showed for the first time substrate interactions between MB and SRB in a German freshwater lake, Lake Vechten. He concluded that as the sulphate-reducers oxidised lactate to acetate they provided aceticlastic methanogens with their primary substrate. The physical separation of the two microbes was explained by methanogenic sensitivity to hydrogen sulphide, the toxic by-product of sulphate-reduction. Although sulphide may be toxic to methanogens in certain conditions, there is little evidence that it is toxic at levels measured in natural systems (Mountfort et al., 1980; Senior et al., 1982). As the summer progressed, microbial activity increased, the inorganic sulphate pool was depleted and methanogenesis became an increasingly more important terminus for catabolism (Cappenberg, 1974b). Thus it was apparent that hydrogen sulphide toxicity was not the major factor controlling the physical separation of the microbes.

Winfrey and Zeikus (1977) observed that methanogenesis could be inhibited by as little as 0.2 mM SO_4^{2-} and that this inhibition was reversible by either H_2 or acetate addition. This suggested that the two microbial groups competed for both substrates, not just one or the other. Laboratory experiments by Abram and Nedwell, (1978a) confirmed this proposal when addition of a H_2-utilising sulphate-reducer to a syntrophic culture, actively producing methane, inhibited methanogenesis. The suggestion was that SRB could outcompete MB for H_2 consumption. These workers also showed that addition of excess H_2 simultaneously increased sulphate reduction and methanogenesis (Abram and Nedwell, 1978b), suggesting that although the two microbes are not inherently antagonistic, competition for common substrates primarily controls their relative distributions.

The competition model was supported by observed changes in relative activities along environmental SO_4^{2-} gradients. For example, Martens and Goldhaber (1978) found a trend from SRB-dominated to MB-dominated catabolism along an decreasing salinity gradient from marine to freshwater conditions. Also, when Brandl et al. (1990) added gypsum to a freshwater lake sediment and created a temporal SO_4^{2-} gradient the sediment changed from being ostensibly methanogenic to sulphate-reducing. Therefore, when electron flow is limited SRB dominate over MB unless sulphate supplies also become limiting.

In the 1980's, two models for the distribution of MBs and SRBs were published and the relationships between the two microbes became more defined. First, Lovely and Klug (1986) developed a model based on the *in situ* physiological characteristics of the microbes which predicted their relative importance in lakes of various trophic status and

generated profiles of sulphate, acetate, methanogenesis and sulphate-reduction comparable to that observed in field trials. They found that 30 μM SO_4^{2-} was the critical concentration above which a sulphate-reducing zone could develop and maintain an acetate concentration below the methanogenic threshold. At lower sulphate concentrations, the dual limitations of low sulphate availability and methanogenic acetate consumption support a methanogenic niche. However, they do state that within the reported ranges of salinity, the rate of organic matter deposition was the main factor controlling the relative importance of the two microbes, presumably because electron availability superseded sulphate availability as a controlling factor.

In the second model Capone and Kiene (1988) compared freshwater and marine communities and found similar results; SO_4^{2-} availability and the higher affinity displayed by the sulphate-reducers for H_2 controlled the inter-relationship. In addition, the freshwater sulphur pool was dominated by organic species, unlike the marine pool which was dominated by inorganic sulphur, readily useable by SRB. They concluded that terminal catabolism in marine and freshwater systems is distinctly different and depends on the type of sulphur as well as the quantity.

In essence, the basis for the competitive advantage of SRBs over MBs appears to be kinetic. Kristjansson and Schönheit (1983) reported that across phylogenetically distant strains of MB (5 strains) and SRB (4 strains), SRB had lower Ks values for both H_2 (2 μM for SRB vs. 6-20 μM for MB) and acetate (0.2 μM for SRB vs. 3 μM for MB). In addition, Schönheit et al. (1982) report that a sulphate-reducing *Desulfobacter* species was able to utilise acetate 15 times faster than a methanogenic *Methanosarcina* species. Therefore sulphate-reduction can dominate catabolism by maintaining H_2 and acetate levels below the methanogenic thresholds.

There are several reasons why sulphate-reducers have higher affinities for these substrates. First, sulphate-reducers have a periplasmic hydrogenase; therefore they can achieve a proton motive force across the periplasmic membrane coupled to ATP synthesis. Methanogens in contrast have a cytoplasmic hydrogenase and require H_2 to move across the cytoplasmic membrane, thereby requiring a longer substrate passage (Kristjansson and Schönheit, 1983). Second, the Ks of a reaction in part reflects the free energy exchange of the reaction, therefore the lower Ks for sulphate-reduction would imply a greater energy yield from the reaction (Kristjansson and Schönheit, 1983). And third, sulphate-reducers and methanogens use different enzyme pathways to achieve cell growth and division and no doubt there are inherent differences which

will be reflected in the Ks values determined for each bacterial group (Kristjansson and Schönheit, 1983; Sparling and Gottschalk, 1990).

However, MBs and SRBs are not always directly competing for common substrates. For example, Ueki and Ueki (1990) did not find SRB and MB to be competing for acetate in the organic-rich environment of an anaerobic digestor. Instead they found that adding SO_4^{2-} or specific SRB metabolic inhibitors did not change the rate of methanogenesis but that inhibiting MB increased sulphate-reduction rates. This implies that competition between SRB and MB was affected not by increased acetate concentrations but by the availability of a range of non-competitive substrates, such as other volatile fatty acids, that permitted the simultaneous production of aceticlastic methane and hydrogen sulphide from alternative substrates. Furthermore, SRB may produce acetate, H_2 and CO_2 as metabolic by-products (Krumbein, 1983) and potentially increase the carbon flow to methanogenesis. Therefore, the interactions between SRB and MB are not simply competitive and the recent interpretations of their interactions with respect to anaerobic methane oxidation furthers their complexity (see Section 2.4.4).

2.4.3: Interactions between methanogens and denitrifiers

Another well-studied anaerobic interaction is that between methanogenic and denitrifying bacteria, particularly with respect to their effects on nitrogen cycling in rice paddies and the potential for denitrifiers to ameliorate methane emissions from these systems. Most researchers have used similar sampling techniques involving the initial air-drying of sediment before incubation (for example; Bollag and Czlonkowski, 1973; Balderstone and Payne, 1976; Wang et al., 1993). This process is known to affect the physico-chemical properties of sediments as well as to kill, or impede the activity of, large numbers of bacteria ill-adapted to rapid changes in moisture regime (Boon et al., 1997; Haynes and Swift, 1989; Qui and McComb, 1994; Mitchell and Baldwin, 1998; Mitchell, in prep.;).

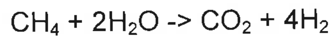
Typically in these experiments a lag phase was seen between the time nitrogenous compounds were added to the sediment slurries and the time taken for methane to evolve. This was interpreted as a direct inhibitory effect of nitrogenous compounds on methanogenic enzymes rather than a competitive effect due to common substrate utilization by most researchers. A more recent paper by Kluber and Conrad (1998) suggests that nitrate additions inhibit methane production reversibly. They measured more parameters than Bollag and Czlonkowski (1973), Balderstone and Payne (1976) or Wang et al. (1992) and as such could draw more advanced conclusions. Kluber and

Conrad (1998) suggest that nitrate additions can inhibit methanogenesis in three ways: 1) direct toxicity, 2) enhanced competition for H_2 with denitrifiers and 3) enhanced reoxidation of iron and sulphate resulting in enhanced competition with iron and sulphate-reducing bacteria. However, the possibility that anaerobic methane oxidation may be affected by nitrate additions was not considered and if it were occurring then the calculations of free energy exchange made by Kluber and Conrad (1998) would be incorrectly based on net methane production rates rather than gross production (Mitchell, in prep.). The direct effect that any one nitrogenous compound may have on the competing processes of methane production and oxidation makes interpretation of the interactions between MB and denitrifiers highly complex.

2.4.4: Anaerobic methane oxidation revisited

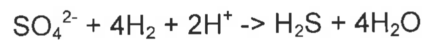
Although it is thermodynamically possible to oxidise methane anaerobically with the alternative electron acceptors known to inhibit methanogenesis (eg. NO_3^-, SO_4^{2-}), organisms that can oxidise methane in the absence of oxygen have never been isolated (Conrad, 1996). There is, however, strong evidence to suggest that methane is oxidised under anaerobic conditions where other anaerobes, particularly SRB, are active. The first line of evidence is that methane produced in marine sediments rarely reaches the water column because it is oxidised to carbon dioxide in the anoxic, sulphate-rich sediment layers overlying the sulphate-poor methane production zone (Martens and Berner, 1974; Reeburgh, 1976; Iversen and Jorgensen, 1985). Second, $^{14}CH_4$ tracer experiments (Reeburgh, 1980; Iversen and Blackburn, 1981) and studies of CH_4 and CO_2 stable carbon isotope ratios (Blair et al., 1993; Popp et al., 1995) have shown that anaerobic methane oxidation is mediated by microbes but did not isolate them. Third, maximum rates of methane oxidation and sulphate-reduction coincide in the narrow transitional band between the sulphate-rich and methane-rich marine sediment zones (Devol, 1983; Devol et al., 1984; Alperin and Reeburgh, 1985; Iversen and Jorgensen, 1985). From these types of observations, several authors have concluded that SRB are involved either directly or indirectly in the anaerobic oxidation of methane (Martens and Berner, 1974; Iversen and Blackburn, 1981; Devol, 1983; Devol et al., 1984; Alperin and Reeburgh, 1985; Iversen and Jorgensen, 1985; Hoehler et al., 1994).

In 1994, Hoehler et al. (1994) postulated that methane is oxidised anaerobically by a consortium of SRB and MB in Cape Bight Lookout, USA. Initially, methane is oxidised to $CO_2 + H_2$ via a reversal of the CO_2-reduction pathway termed 'reverse methanogenesis':



$CH_4 + 2H_2O \rightarrow CO_2 + 4H_2$

Following this, the H_2 is consumed by the SRB:



$SO_4^{2-} + 4H_2 + 2H^+ \rightarrow H_2S + 4H_2O$

Thermodynamically this process is only favourable at H_2 concentrations below 0.29 nM. Therefore the presence of the SRB, or presumably any H_2-scavenger, is critical. Furthermore, BES (a specific MB inhibitor) only partially inhibited methane oxidation, leading Hoehler et al. (1994) to propose the induction of 'reverse methanogenesis' in MB living in a MB-SRB consortium.

The complex interactions between MB and SRB are depicted in Figure 2.2 including the reverse methanogenesis postulate which has been supported by studies other than Hoehler et al. (1994). First, culture studies indicate the presence of enzymatic pathways capable of oxidising methane in MB (Zehnder and Brock, 1979). Second, *Desulfovibrio desulfuricans* co-oxidises methane in the presence of another electron donor (eg. pyruvate, Davis and Yaraborough, 1966) but is unable to grow on methane alone (Postgate, 1969). Third, Iversen (1984, as cited in Hoehler et al., 1994) has shown that the growth of three *Desulfovibrio* strains on pyruvate and methane can be accounted for by methane oxidation. Fourth, Hansen et al. (1998) have confirmed the postulate in a series of sulphate and molybdate (a specific SRB inhibitor) addition experiments. The addition of sulphate increased sulphate-reduction in sulphate-depleted sediments, terminated net methane production immediately and decreased anaerobic methane oxidation to 38% of the control. Molybdate additions did not affect net methane production but uncoupled methane oxidation from methane concentration. This effect was delayed by three days in sulphate-depleted sediments but was immediate, although incomplete, in sulphate-rich sediments, suggesting to the authors that SRB may only be directly involved in methane oxidation at high sulphate concentrations. The strongest inhibitory effect on methane oxidation was the combined effect of sulphate and molybdate additions.

In summary, many field and laboratory studies provide evidence that suggests that anaerobic methane oxidation is microbially mediated by methanogenic bacteria which reverse the CO_2-reduction pathway, a process facilitated by the activity of hydrogenotrophic bacteria maintaining very low H_2 partial pressures. The energetic pathways responsible for anaerobic methane oxidation are currently unclear and, as Schink (1997) suggests, the energy gain from this pathway is so small as to be only available to one of the participating syntrophic organisms. Most of the studies have

concentrated on MB and SRB, presumably due to their 'competitive' interactions along salinity concentration gradients, but there is no evidence to suggest that this role is limited to SRB. Presumably, any hydrogenotrophic bacteria may suffice, thereby potentially enabling anaerobic methane oxidation to occur in most, if not all, methanogenic habitats.

However, the consumption of methane by as yet undescribed anaerobes cannot be discounted. Evidence for novel archaea, which are predominantly or exclusively methanotrophic, has been found in marine sedimentary methane seeps (Hinrichs et al., 1999). This suggests that anaerobic methanotrophy is more widespread than previously thought. The organisms detected by Hinrichs et al (1999) appear to be peripherally related to *Methanobacteriales* and *Methanomicrobiales* and from ^{13}C biomarker studies they appear to utilise methane as a carbon source. These microbes have not been found in any other freshwater or marine surveys but the study by Hinrichs et al (1999) opens new perspectives on archael ecology and evolution and future studies may reveal the environmental conditions suitable for their growth and explain their ecological function and distribution controls.

Chapter 3

Chapter 3: Methods

3.1 Study period

The data presented in this thesis were collected between July 1994 and January 1998. The most intensive periods were the summers of 1994/1995, 1995/1996 and 1996/1997 and the winters of 1996 and 1997. Seasons were considered to change at the solstice or equinox rather than with conventional calendar months. Therefore summer periods of any year extended from 21 December to 20 March, autumn from 21 March to 20 June, winter from 21 June to 20 September and spring periods from 21 September to 20 December.

3.2 Study sites

The following section describes the three wetland systems; Willunga Wetlands, Greenfields Wetlands and Bool Lagoon. Field and laboratory investigations were conducted at the inlets (the sites of source water inflow) and outlets (the sites of treated water outflow) of the two constructed wetlands and comparable sites were chosen at Bool Lagoon. The inlet and outlet areas of each wetland were chosen on the basis that the extremes in methane production, oxidation and emission rates for a given wetland would be detected by monitoring at the extremes of the flow path. All three wetlands are located in south-eastern South Australia between the 400 mm and 600 mm rainfall isopleths (Figure 1.1).

3.2.1: Willunga Wetlands

A series of 3 wetland basins was constructed at Willunga, a semi-rural township located 40 km south of Adelaide CBD (Lat. 35°16'S, Long. 138°35'E), in early 1993, to ameliorate secondary treated sewage from a population of, then, ca. 5,000 people. Three treatment basins were constructed on the southern side of Port Willunga Creek, approximately 300 m from the municipal sewage oxidation ponds, and a winter storage pond was constructed on the northern side of the creek to hold treated water prior to summer irrigation of the local golf course and public green areas (Figure 3.1). The treatment ponds covered 3 ha and wastewater flow through the whole system, from the municipal sewage plant to the storage lagoon, was continuous and gravity fed.

The three treatment basins were of similar bathometric design and were serially fed secondary sewage from the nearby municipal oxidation pond. Incoming effluent was discharged continuously into each wetland via a deep entry pool, approximately 3m

deep and 40m in diameter (Figure 3.1). The wastewater then flowed across a 40-60 cm deep, vegetated shelf before being discharged to the next treatment basin in series, or the storage pond in the case of the third wetland. The shallow shelves in each wetland basin contained two gravel baffles, 65 cm high by 3 m wide, set perpendicular to the flow path which divided the shelves into three bays (Figures 3.1 and 3.2). These shelves and the perimeters of the basins were planted with a range of emergent and semi-emergent macrophytes, including; *Triglochin procerum, Phragmites australis, Typha domingensis*, *Bolboschoenus medianus* and *Baumea articulata* (Figure 3.2).

The major study sites at Willunga Wetlands were chosen in the 3 m deep, unvegetated entry pools of the first and third wetlands and termed WILLUNGA-INLET and WILLUNGA-OUTLET, respectively (Figure 3.1). The study sites did not extend to within 4 m of the surrounding vegetation. Additional sites in the 3m deep area of the second pond (Site 2) and in the shallow, vegetated areas of each pond (sites 3, 4 and 6) were used for specific short-term investigations (Figure 3.1).

3.2.2: Greenfields Wetlands

The Salisbury City Council constructed Greenfields Wetlands (Long. 34°52'S, Lat. 138°35'E) in the Adelaide suburb of Greenfields on a 40 ha site adjacent to Dry Creek Channel (Figure 3.3). This channel drains stormwater from the 100 km^2 Dry Creek catchment area within which the predominant land uses are light industry and residential housing. Only 1-2 % (Jenkins, 1996) of the stormwater flow along Dry Creek enters Greenfields Wetlands for treatment; the rest is transported directly to sea.

The wetlands were built in two stages; Stage I in 1990 and Stage II in 1994 (Figure 3.3). Stage I was designed primarily as avifaunal habitat and thus incorporated a number of islands and temporary wetland habitats interconnected with channels and covered with diverse vegetation (Figures 3.3 and 3.4). Silt from the stormwater inputs collected at the entrance to Stage I and impeded flow into the wetlands such that the efficiency of the system was reduced and this prompted the construction of Stage II. Stage II was constructed as a long, U-shaped channel upstream of Stage I to increase the flow path length, thereby trapping silt and providing cleaner water to Stage I (Figure 3.3).

Since 1994, water has entered Stage II from Dry Creek and travelled along the U-shaped channel and into either Stage I or a separate wetland system, the Connector Wetlands. Water entering Stage I continues along the channel to the outlet where it flows back into Dry Creek (Figure 3.3). The mean flow path length through Greenfields Wetlands is 3 km (Jenkins, 1996) and most of the channels were free of vegetation

during the study period. Greenfields Wetlands were planted with a range of macrophytes but these plants tended to inhabit the banks of the channels and the temporary wetland areas rather than the channels themselves, particularly in Stage II (Figure 3.4).

The major study sites at the Greenfields Wetlands were at the locations where water enters Stage I from Dry Creek and exits Stage II back into Dry Creek (Figure 3.3). These sites were termed, GREENFIELDS-INLET and GREENFIELDS-OUTLET, respectively and are situated in channel areas, less than 50m wide and 2.5m deep, with steeply sloping sides and not within 4 m of surrounding vegetation. Additional sites along the length of the flow path (sites 2-5) were used for specific short-term investigations (Figure 3.3).

3.2.3: Bool Lagoon

Situated in the south-east agricultural district of South Australia, approximately 350 km S-E of Adelaide (Figure 1.1), Bool Lagoon Game Reserve (Lat. 37°5'S, 140°42'E) is a natural wetland comprising a series of linked basins, including Hack's Lagoon Conservation Park (Figure 3.5). In total the wetlands cover 2883 ha. Water flows into the system from Mosquito Creek, entering Hack's Lagoon before flowing into the Main Basin of Bool Lagoon. The only exit is via Drain M, in the Western Basin, which is controlled by a regulator.

Mosquito Creek catchment is c. 100 km^2, extending to the east of Bool Lagoon into land that is primarily used for agriculture and irrigated horticulture. The mean average rainfall in the ten years preceding this study was 590 mm (Bureau of Meteorology, pers. comm.). The rainfall is highest in winter and lowest in summer (Cawood, 1996). The wetland basins have flat bottoms and gently sloping sides. This bathymetry allows for high rates of evapotranspiration, which, coupled with the variable rainfall, leads to a large variation in seasonal water levels. The basins, beginning with Hack's Lagoon, generally fill between August and September and gradually draw down over summer to reach minimal levels in autumn (see Figure 3.3 for flow path details and Chapter 4 for water level data).

Most of the wetland area was vegetated with macrophytes, including: *Triglochin procerum, Baumea arthrophylla, Melaleauca halmatuorum, Phragmites australis* and *Typha domingensis* (Figure 3.6). The whole system was rarely inundated during the study period. Consequently, sites that were suitable for comparison with sites at Willunga and Greenfields Wetlands (i.e. those that were permanently inundated and

unvegetated) were restricted to Hack's Lagoon and the Main Basin of Bool Lagoon. A site was chosen in each basin and termed either BOOL-HACKS or BOOL-MAIN, accordingly (Figure 3.5). BOOL-HACKS was used throughout the study period, but BOOL-MAIN was abandoned after winter 1996 because it was no longer permanently inundated.

3.3: Water column analyses

3.3.1: Water levels

The water depth was measured with either a 1 m or 3.5 m long, graduated pole each time the sites were visited throughout the study period. At Willunga and Greenfields, measurements were always made from the rear of an aluminium dinghy, whereas at Bool Lagoon measurements were made from a convenient boardwalk or by wading to the site.

Additional water level data were obtained from Salisbury City Council and Jenkins (1996) for Greenfields Wetlands, and the South Eastern Water Conservation and Drainage Board (now the South East Catchment Water Management Board) for Bool Lagoon. The Salisbury City Council measured water levels at the outlet weir of Greenfields Wetlands on a daily to weekly basis between June 1994 and December 1997. These data were collated and appear in Chapter 4. Water regimes differ between the basins of Bool and Hack's Lagoons, therefore depths from the gauging board in Hack's Lagoon were used, which are calibrated to the Australian Height Datum (mAHD). The gauging board is situated at 47.62 mAHD in Hack's Lagoon, whereas the site BOOL-HACKS is situated at 47.6 m AHD. The height of BOOL-HACKS relative to the gauging board was determined in winter 1995 when the water level was 48.6m AHD.

3.3.2: Temperature and dissolved oxygen

Dissolved oxygen and temperature were determined either with a YSI 51B Dissolved Oxygen Meter or a YSI 3800 Water Quality Meter (Yellow Springs Instrument Company, Yellow Springs, USA). In either case, the attached probe was lowered to various depths within the water column from the aluminium dinghy or by carefully wading to the site and lowering the probe by hand as far away from any disturbed sediment as possible.

Water temperature profiles at WILLUNGA-INLET were measured with a datalogger between December 1996 and March 1997. Temperature readings from a chain of 8 thermocouples suspended from a raft were taken every 30 minutes with a Squirrel datalogger (Grant Instruments, Cambridge, England). This apparatus was also used to

monitor water column temperatures during the planktonic methanogenesis and methanotrophy trials in January 1998.

3.3.3: *Water sampling techniques*

Three methods were used, according to water depth. The first method was used to collect water from the top 20 cm of the water column. In these cases, water was drawn directly into 60 mL polyethylene syringes held at the appropriate water depth.

The second method was used at sites where the water was shallow, less than 1.5m deep. Water samples were collected from various depths using a multiple syringe and pole apparatus, constructed of a series of 60 mL polyethylene syringes attached at 5 or 10cm intervals along two sides of a 1.5m long, graduated polyvinyl chloride (PVC) pole. The PVC pole comprised of two pieces. One piece held the syringe bodies firmly and vertically onto the pole and the other piece, a sliding central pole with pegs that lodged under the rim of the syringe plungers, allowed the syringe plungers to be remotely operated when it was slid upwards. The syringes were loaded on to the apparatus in a fully closed position and lowered into the water. When the pole was at the desired depth the central pole was slowly pulled to fill the syringes with water. In this way, a simultaneous collection of water samples down the water column was conducted.

The third method was employed in water deeper than 1.5 m. Water samples were collected from various depths with 60 mL syringes attached via a stopcock to a PVC tube (5 mm ID) strapped to a 3.5 m long, graduated pole. Care was taken to prevent bubble formation in the tube while the water was being drawn into the syringe. Flocculent sediment samples (ie. samples of the semi-liquid sediment at the sediment:water interface) were also collected with this tube and pole apparatus by replacing the 5mm internal diameter tube with a 10mm internal diameter tube.

3.3.4: *Dissolved methane*

Water samples were collected in 60 mL syringes at various water depths via the techniques described above. Half the water sample was discarded from the syringe and a quantity of air equivalent to the remaining water volume (usually 30 mL of each) was drawn into the syringe. The water sample was then air-equilibrated by vigorously shaking the capped syringe for 2 minutes. Full details are in Ross et al. (1997). The degassed water was discarded and the headspace gas transferred into a pre-evacuated vacutainer (Becton-Dickinson, Dublin, Ireland), which was kept on ice during transport back to the laboratory. Methane concentrations of the headspace were analysed by gas

chromatography. Details regarding vacutainer evacuation and gas chromatography appear below.

3.3.5: Dissolved organic carbon

Water samples were collected by the techniques described above at Willunga and Greenfields Wetlands and kept on ice during transport to the laboratory where they were filtered under vacuum through a 0.22 μm polycarbonate membrane (Gelman Sciences, Lane Cove, Australia). The filtrate was refrigerated or frozen until being analysed for organic carbon content on an ANATOC automatic carbon analyser (SGE, Melbourne, Australia). Aliquots (200 μL) of benzoic acid were used to calibrate the ANATOC analyser to standard quantities of carbon (μg).

3.3.6: Total organic carbon

Water samples were collected in triplicate at various water depths at Willunga and Greenfields Wetlands. Total organic carbon analyses were performed on these samples by the Australian Water Quality Centre (AWQC), a business unit of the South Australian Water Corporation, Bolivar, South Australia. All samples were delivered to the AWQC on the same day that they were taken.

3.3.7: Redox potential

Water and flocculent sediment samples were collected in triplicate at various water depths at WILLUNGA-INLET and WILLUNGA-OUTLET. Only three syringes were filled at any one time and immediately after they were brought out of the water, the plungers were removed and a redox probe (Hanna Instruments, Woonsocket, RI, USA) was inserted approximately half way into the full syringe barrel to minimise atmospheric contamination.

3.4: Sediment analyses

3.4.1: Sediment sampling techniques

Two methods were used, according to water depth. The first method sediment was used in water less than 1.5 m deep. Cores up to 25 cm depth were removed with a metal corer fitted with a removable PVC liner (8 cm I.D.). The desired amount of sediment (generally the top 1 or 5 cm) was removed from the PVC liner and transferred to plastic bags in the open air or the PVC liners were wrapped in plastic and the sediment transferred to incubation vessels under anaerobic conditions. The PVC liners were split in half lengthways to enable rapid sediment removal.

The second method involved a longer corer and was used in water up to 3.2 m deep. The corer consisted of a transparent Perspex tube mounted on a 3.6 m long, retractable aluminium pole. A tap, which could be remotely operated by pulling on a cord running the length of the pole, was fixed between the Perspex tube and the pole. When the corer was lowered the tap was kept open but when the corer had been pushed a suitable distance into the sediment, the tap was closed to form a suction above the sediment and thus minimise the amount of sediment lost from the corer as it was drawn up through the water column. The sediment samples were released into plastic bags held over the open end of the Perspex tube by opening the tap and immediately the samples were placed on ice for transport back to the laboratory or placed within an anaerobic chamber located on the wetland banks.

3.4.2: Redox potential

Sediment redox potential was measured in millivolts (mV) using a redox probe (Hanna Instruments, Woonsocket, RI, USA) connected to a multimeter (Finest Instruments, 201 Multimeter, Melbourne, Australia). The probe was directly inserted 2 cm into the sediment surface at sites where the water was less than 1.5m deep because the cord attached to the probe was 1.6m long. In deeper water, sediment samples were taken with the Perspex tube corer and the redox probe was inserted at 2cm depth into the intact core immediately that the core was collected. Refer to Section 2.3 for discussion on the limitations of redox potentials in the bulk sediment phase.

3.4.3: Organic carbon content

Sediment samples were taken from Willunga Wetlands, Greenfields Wetlands and Bool Lagoon using the techniques described above. The sediment samples were kept on ice until they were returned to the laboratory where they were air-dried at 30 °C for up to three days. Once dry the sediment samples were crushed with a mortar and pestle or ground in an electric coffee grinder and sent to laboratories in the Department of Soil Science at The University of Adelaide for organic carbon analysis by Walkley and Black rapid titration procedure.

3.5: Methanogenesis and methanotrophy

3.5.1: In vitro methanogenesis

The following procedure for determining rates of in vitro methanogenesis was adapted from Boon and Sorrell (1991) and Boon and Mitchell (1995). Sediment samples were taken using the techniques described above and placed into an anaerobic chamber either at The University of Adelaide laboratories (*in vitro* incubations) or on the banks of

the wetland (*in situ* incubations and Bool Lagoon *in vitro* incubations) within 1 to 4 hours of sampling. The anaerobic chamber was constructed of two rigid, open topped Perspex boxes that fitted flush together and were sealed with a rubber gasket and a series of metal clips. Gloves were attached to the front of the chamber to allow handling of the samples. The chamber was sealed with all necessary equipment inside and flushed with ultra high purity (UHP) N_2 gas (BOC Gases, Adelaide, Australia) until anaerobic indicator strips (Oxoid, West Heidelberg, Australia) showed that conditions were anaerobic.

At this stage, three replicate 5mL aliquots of sediment were removed with the barrel of a 5mL plastic syringe from the top 1 or 5cm of sediment, according to experimental procedure (see Chapter 5). Each aliquot was placed into a 25mL vacutainer along with 2 mL of treatment solution made up in wetland water filtered through 0.2μm membrane filters (P/N 60301, Gelman Sciences, Lane Cove, Australia) that had been deoxygenated by bubbling overnight with UHP N_2 gas and adding L-cysteine (final concentration 0.03 % w/v). Similarly, L-cysteine was routinely added to all samples to ensure anaerobic conditions (after Boon and Sorrell, 1991).

Treatment solutions included L-cysteine (Sigma, St Louis, USA), Na_2MoO_4 (Ajax Chemicals, Sydney, Australia), Na_2SO_4 (Ajax Chemicals, Sydney, Australia), $NaNO_3$ (Ajax Chemicals, Sydney, Australia), sodium acetate (BDH Chemicals, Victoria, Australia), cellobiose (Sigma, St Louis, USA) and ANOX, a mixed anaerobic fermenter culture. The ANOX culture (a mixture of *Bacillus* species, DMS3005, supplied by ARDA-Tek, Darwin, Australia), used to increase numbers of fermenter bacteria, was activated by the addition of TSB broth and incubation at 20°C for 2 hours prior to addition to the vacutainers as a treatment solution. ANOX is a commercial product used in the aquaculture industry to enhance detritus degradation. The effect of the live ANOX treatments was controlled for by the incorporation of vacutainers treated with microwave-killed ANOX cultures in the experimental design.

Once treated with the various solutions, the samples were bubbled with UHP N_2 (BOC Gases, Adelaide, Australia) for 2 minutes to minimise contamination from any residual oxygen in the chamber and to reduce the headspace methane concentration to less than 30 ppm (after Boon and Mitchell, 1995). The vacutainers were capped inside the anaerobic chamber and transferred to either the water column, a water bath or a constant temperature chamber.

The incubations occurred at various temperatures for 24 hours with gentle shaking. Headspace methane concentrations were routinely determined at 0, 2, 4, 8, 12 and 24h by removing three replicate 500 µL aliquots with a gas tight syringe (SGE, Melbourne, Australia) and injecting the contents directly into a gas chromatograph. Methanogenic rates were calculated from the slope of the regression plotted between elapsed time and headspace methane concentration.

3.5.2: In situ methanogenesis and methanotrophy

Concurrent rates of methanogenesis and methanotrophy were determined *in situ* at each wetland site in order that samples underwent natural perturbations in light and temperature. Clay sediments were sampled as described in Section 3.4.1 and processed in an anaerobic chamber situated on the banks of the wetland. Triplicate subsamples were treated with ether L-cysteine (final concentration 30 mM) or BES (final concentration 120 mM). The tubes were wrapped in plastic bubble wrap and replaced on the sediment surface or in the holes left by the corer in the case of Bool Lagoon. Headspace methane concentrations were extracted into evacuated vacutainers at 0, 4, 8, 12 and 24h and kept on ice until the samples were analysed for methane concentration by gas chromatography.

Flocculent sediment and water samples were collected using the tube and pole apparatus described in Section 3.3.3. The treatments imposed differed at the three wetlands depending on the results of the *in vitro* methanogenesis trials. L-cysteine (final concentration 30 mM) was used in the anaerobic treatments with and without the addition of BES (final concentration 120 mM) at all the wetlands. Similarly, at all the wetlands $HgCl_2$ was added to three replicate syringes to determine whether methane was being lost from the syringes abiologically.

At Greenfields Wetlands and Bool Lagoon, an aerobic BES amended treatment was also performed. These samples were made aerobic by adding 7mL of filtered wetland water that had been bubbled with industrial air (BOC Gases, Adelaide, Australia) overnight. Oxygen saturation was tested with the 51B YSI oxygen probe before the water was transferred to the vacutainers. At saturation, 7mL of water would contain 56µg of O_2, enough to support aerobic methanotrophy at 30 nmol mL^{-1} h^{-1} for 5 hours, assuming a CH_4:O_2 ratio of 2 (Amaral and Knowles, 1995). This rate is equivalent to the rate of *in vitro* methanogenesis observed in sediment samples from Bool Lagoon and Greenfields Wetlands.

Once the treatment solutions were added to the 60 mL syringes they were suspended at the appropriate water depth or on the sediment surface. The tubes did not contain a headspace and a 2 mL subsample of the contained water was removed from each syringe at 0, 1, 2,4, 6, 12 and 24 h via the one-way valve into a series of 5 mL syringes. Immediately before the subsamples were removed, the 60 mL syringes gently shaken to evenly distribute the contents. The 60 mL syringes were replaced onto the water as soon as possible, whilst the subsamples were air-equilibrated and the headspace transferred to a 2 mL vacutainer. The gas samples were stored on ice or under refrigeration until analysis by chromatography within 7 days. The change in dissolved methane concentration over time from the anaerobic controls was linear for the first 24 h and the slope from this period was used for rate calculation. Only the linear portion of the plots were used for BES treated samples.

3.6: Methane emissions

3.6.1: Total atmospheric flux

The total flux of methane across the water-air interface was measured using six static chambers ranging in volume from 375 to 700 L (see Figures 3.2, 3.4, 3.6 and 3.7). Each chamber was collapsible and consisted of two square PVC frames separated by four removable aluminium poles. Transparent PVC film (0.5 mm thickness, DuPont, USA) was permanently glued to the edges of the upper and lower PVC frames. The aluminium poles were fitted by lodging the ends into PVC bases attached to the corners of the upper and lower frames and fastening the pole with a nut and bolt. When the poles were in place the film stretched over the taut, cuboid frame (Figure 3.7). Upon removal of the poles the chambers would collapse to a final height of ca. 30 cm with the folded PVC film protected inside the frame.

A 10 cm diameter, 6V brushless fan (General Electric, Adelaide, Australia) was mounted near the top of one pole inside each chamber. The fans were powered by rechargeable lead batteries (Yuasa, Tokyo, Japan) mounted in a central position on the top of the chamber. This battery location aided chamber stability in windy conditions. Horizontal floats were attached to the lower frame such that the lower 10 cm of the chamber remained below the water surface forming a gas-tight seal.

The chambers were deployed from either an aluminium dinghy or by wading to the site (see Figures 3.2, 3.4 and 3.6). When using the boat, ropes were strung across the water surface and attached to star droppers on opposite banks of the wetland. The boat was manoeuvred into position by using the ropes rather than oars which were considered to cause too much disturbance to the water column structure.

The chambers covered an area of water between 0.75 and 1 m^2 and were deployed for various time periods from 20 minutes to 24 hours. Samples of the internal atmosphere were collected in triplicate at 2 minute to 24 hour intervals by attaching pre-evacuated vacutainers to a hypodermic needle fixed directly to the chamber (Figure 3.7). Gas samples were kept in the vacutainers at less than 10°C for no more than 7 days before analysis on a gas chromatograph (see Section 3.7). Methane was released from the water surface if the concentration of methane in the internal chamber atmosphere increased over time. The detection limit was approximately 0.05 mmol CH_4 m^{-2} h^{-1} and only those linear relationships with r^2 values greater than 0.95 were utilised.

3.6.2: Ebullition

The rate of bubble loss from the sediment-water interface was only monitored at Greenfields wetlands during this project. Inverted funnels (19.5 cm diameter at base) standing on metal tripods (height 20 cm) were placed onto the sediment surface with a minimum of physical disturbance to measure the volume of gas being released from the sediment. The funnel opening was covered with a graduated 60 mL polyethylene syringe barrel (Becton-Dickinson, Dublin, Ireland) to collect gas bubbles leaving the sediment. A lag time of ten minutes was allowed between deploying the funnels and fitting the syringes to compensate for enhanced flux directly after funnel deployment. Funnels were typically deployed for 24 h, at the end of which time the volume of gas contained within the syringes was recorded. The funnels were designed after Boon and Sorrell (1991), who modified the design of Durako et al. (1982).

The volume of gas was recorded using the inverted funnels but the gas contained within the syringe barrels could not be used for gas composition analysis because it was collected over a 24 h period and compositional changes may have occurred. Instead, gas bubbles were released from the sediment by disturbing it with a long wooden pole and collected in a funnel and syringe. The gas was transferred to vacutainers and stored on ice until being analysed by gas chromatography for methane concentration. The combined measurements of volume and composition were then used to calculate an ebullitive flux in mmol CH_4 m^{-2} h^{-1}, assuming STP conditions.

3.7: Gas storage and analysis

Gas samples were drawn into pre-evacuated 7 mL vacutainers in the field for storage and transport to the laboratory. The vacutainers were evacuated for 30 seconds via a 22G hypodermic needle (Becton-Dickinson, Dublin, Ireland) attached to a vacuum pump (General Electric, Adelaide, Australia). Checks for the efficiency of the evacuation procedure were performed by connecting 10 randomly selected vacutainers from each

set of 100 to a supply of water and measuring the volume of water drawn up. If the volume was greater than 6.5 mL then the vacutainers were considered adequately evacuated. Calibration of the gas transfer procedure was conducted by sampling standard gases containing 10, 100 and 1000 ppm methane (Scotty Gases, Plumsteadville, PA, USA) in the field. These tests showed that the procedure decreased the methane concentration in the samples by less than 2%.

The methane content of the sample gases was determined by gas chromotography. Gas samples were directly injected into a Perkin-Elmer (Connecticut, USA) Autosystem gas chromatograph with a 250 µL gas-tight syringe (SGE, Melbourne, Australia). The majority of the gas samples contained less than 1000 ppm and were analysed with a flame ionisation detector (FID) at an oven temperature of 60°C using UHP H_2 (BOC Gases, Adelaide, Australia) flowing at 20-25 mL min^{-1} as a carrier gas. The H_2 was passed through an oxygen trap (Alltech, Deerfield, IL, USA) and a moisture trap (Alltech, Deerfield, IL, USA) before entering either a CTR or Poropak Q packed column (Alltech, Deerfield, IL, USA). More methane-rich gases were analysed with a thermal conductivity detector (TCD) on the same chromatograph at an oven temperature of 100°C.

3.8 Statistical analyses

The results were analysed for statistical significance using seven different statistical tests. First, the data were checked for normality with Shapiro-Wilk tests at a significance level of 0.05 using the computer package JMP (SAS Institute, USA) on a MacIntosh computer. Second, if the data cells failed the normality test they were transformed using the equation, $\log_{10}(x+1)$, on the computer spreadsheet package Excel (Microsoft, USA). Third, the equality of variance in the data cells was tested using one or all of the following tests in the computer program, JMP: Levene, Bartlett, O'Brien and Brown-Forsythe. Fourth, the significance of the variation in the data was determined with either a one-way or two-way ANOVA at a significance level of 0.05 using either Excel or JMP computer programs. Results of the ANOVA analyses for Chapters 4, 5 and 6 are presented sequentially in Appendices 1.1 to 1.3.

Chapter 4

Chapter 4: Physico-chemical description of the wetland habitats

4.1 Introduction

This chapter describes the physico-chemical characteristics of the 5 wetland sites. The parameters chosen describe the seasonal water regime and thermal stratification patterns of the water column; carbon content of the surface waters and sediments; redox potential of the sediments; and dissolved oxygen and methane concentrations, because these were considered the most relevant to the activities of methanogenic and methanotrophic bacteria across the variety of wetland conditions presented in the three chosen wetlands.

Water regime is a fundamental descriptor of wetland habitat as it describes the frequency, timing, extent, depth and duration of wetland inundation (Boulton and Brock, 1999). In particular to methane dynamics, it is the major controller of the amount of oxygen in the system. When a sediment is exposed it is directly supplied with oxygen from the atmosphere but when it is inundated the rate at which oxygen is supplied to the sediment is dependent on the water depth, the extent of mixing in the water column (thermal stratification) and the rate of oxygen supply and demand within the water column (Revsbech et al., 1980; Reimers and Smith, 1986; Lerman, 1988).

Oxygen enters the water column from the atmosphere extremely slowly and although a limited amount is produced in the water column by photosynthetic organisms (Boulton and Brock, 1999), oxygen also diffuses slowly in water (2.30×10^{-5} cm^2 sec^{-1}; Lerman, 1988) and therefore its vertical distribution is strongly affected by physical mixing of the water column which is consequently affected by the extent of thermal stratification in the water column.

Thermal stratification occurs when a water body absorbs heat from the sun. The energy is absorbed rapidly with depth and the upper water layers can become relatively warmer than the lower layers. Wind acts to mix the upper water layers and distributes the absorbed heat further down the water column (Mitsch and Gosselink, 1993). If the water body is shallow, mixing may occur down the whole depth of the water column and oxygen may be uniformly distributed (Boulton and Brock, 1999), supplying the sediments with more oxygen than in stratified conditions. The higher the density gradient between the upper and lower water layers the greater the energy needed to

mix the layers and the more stable the temperature, dissolved gas concentrations and redox potentials are in the benthic environment (Williams and Beresford, 1998).

Below the euphotic zone (the upper water layers illuminated with sufficient light for photosynthesis) in a stratified wetland, the deeper water is effectively isolated from oxygen supplies; respiration and decomposition (methane production) predominate and oxygen is consumed. Therefore the depth that oxygen penetrates the water column is will also depend on the amount of organic carbon available for decomposition. In a natural wetland, most of the detritus is complex-plant derived polysaccharides (Capone and Kiene, 1988). These compounds are part of the refractile detritus pool and decompose slowly and rarely to completion before being buried in the organic carbon fraction of the sediment. The alternative detrital pool comprises more simple organics, such as sugars and amino acids that are labile and degrade very quickly (Godschalk and Wetzel, 1978a, b and c; Carpenter, 1981). Most natural freshwaters contain an order of magnitude more labile, dissolved organic carbon (DOC) than refractile, particulate organic carbon (POC) with a DOC concentration less than 10 mg L^{-1} (EPA, 1998) and a BOD less than 5 mg L^{-1} (EPA, 1999). However, wastewaters such as sewage and stormwater can demand up to 45-56 mg O_2 L^{-1} (Table 4.1) suggesting that the distribution of oxygen may be more limited in constructed wetlands receiving wastewater than in natural, freshwater wetlands. In addition, high concentrations of other nutrients such as nitrogen and phosphorous may be present (Table 4.1) which can support large phytoplankton populations that ultimately add to the detrital load on the wetland sediment (Franken, 1992).

The competing processes of oxygen supply and demand will dictate the oxygen profile in the water column. Coupled with the rate of supply of simple organic carbon compounds, the oxygen profile will also determine the suitability of the habitat for methanogenic bacteria. Provided that methanogens are or have been active in the wetland, methane may be available and methanotrophs can occur where the dissolved methane and oxygen concentrations satisfy their requirements.

It is predicted that constructed wetlands receiving wastewater have the potential to produce more methane per unit time than natural wetlands on the basis that the load of carbon entering the system may be higher than that experienced by most natural wetlands, the carbon entering the constructed wetland may be more labile and that the water regime may be more conducive to methane production for a longer annual period (see Chapter 1). Considering that methanotrophic activity is to some extent dependent

on methanogenic activity (Segers, 1997), it is expected that the methanotrophic habitat will be most favourable in the constructed wetlands as well.

Therefore it is predicted that the suitability of the methanogenic and methanotrophic habitats provided at the 5 wetland sites will decrease in the following order:

WILLUNGA-INLET > WILLUNGA-OUTLET > Bool Lagoon > GREENFIELDS-INLET > GREENFIELDS-OUTLET.

4.2: Methods

4.2.1: Experimental design

The water level at WILLUNGA-INLET was measured each time the site was visited between June 1994 and January 1998. Water level data for Greenfields Wetlands was collated from daily to weekly readings taken by Salisbury City Council at the outlet weir from June 1994 to December 1997. Similarly, the water level data for Bool Lagoon was compiled from data collected by the South-East Drainage Board from June 1994 to January 1998.

The carbon content of the surface water (0.1 m depth) was analysed for both total organic carbon (TOC) and dissolved organic carbon (DOC) content as described in Chapter 3. At Willunga, triplicate water samples were taken from WILLUNGA-INLET and WILLUNGA-OUTLET during November 1994, July 1996 and January 1999. Algal populations at Willunga Wetlands were very dense and their organic exudates and dead cells were considered a significant additional source of methanogenic carbon. Chlorophyll a concentrations were determined at 0.1, 1 and 2m depth in WILLUNGA-INLET and WILLUNGA-OUTLET in summer 1996/1997. Triplicate water samples were taken from GREENFIELDS-INLET and GREENFIELDS-OUTLET in November 1994 for direct comparison to the TOC and DOC data generated at Willunga Wetlands at that time. In May 1995, the surface water was sampled twice from GREENFIELDS-INLET and GREENFIELDS-OUTLET to address temporal and/or spatial variability in organic carbon inputs induced by intermittent winter storms. Two sampling dates (4 and 22 May 1995) were chosen to coincide with winter extremes in turbidity levels as determined concurrently by Jenkins (1996) at GREENFIELDS-OUTLET. Samples were not taken from Greenfields Wetlands in January 1998 as the inlet area had been recently and extensively dredged. Water samples were not taken from Bool Lagoon and data collected by the South Australian EPA between June 1995 and December 1997 were used instead (EPA, 1998).

The top 5 cm of sediment at WILLUNGA-INLET and WILLUNGA-OUTLET were sampled for organic carbon content in October 1995 and January 1998. In October 1995, triplicate sediment cores were taken to 5 cm depth and analysed *in toto*. In January 1998, six replicate cores were taken to 5 cm, three of which were analysed *in toto* whilst only the top 1 cm layer of sediment was analysed from the remaining three cores. The sediments at GREENFIELDS-INLET and GREENFIELDS-OUTLET were sampled by the same procedure except that the second set of cores were taken in October 1998 before the inlet area was dredged. Organic carbon content of the sediments at BOOL-HACKS and BOOL-MAIN was measured in triplicate (to 5 cm depth) once in January 1996.

Intensive surveys of sediment redox potentials (in the top 5 cm of sediment) were conducted at GREENFIELDS-INLET and GREENFIELDS-OUTLET to assess the extent of spatial heterogeneity. Redox potentials were measured in the top 2 cm of sediment on a 1 m^2 grid across the channel along the first (GREENFIELDS-INLET) and last 30 m (GREENFIELDS-OUTLET) of flow path length. At WILLUNGA-INLET and WILLUNGA-OUTLET, depth profiles of the redox potential of the water (at 50 cm intervals) and flocculent sediment layers were generated on a single day in both summer (18 January 1996) and winter (26 July 1996). In addition, spatial heterogeneity in redox potentials was addressed at Willunga Wetlands in a similar manner to at Greenfields Wetlands but on a 5 m^2 grid. It should be noted that redox potential surveys were conducted at Willunga and Greenfields Wetlands for the qualitative assessment of spatial heterogeneity and not as a definitive determinant of the potential to support methanogenic bacteria. Redox potentials were not measured at Bool Lagoon during this study.

The extent of thermal stratification and the depth of oxygen penetration in each wetland were determined in the afternoon in summer and winter. Single profiles of temperature and dissolved oxygen were taken on 4 randomly-chosen days in the middle month of both seasons (ie. in January and July 1996) during methane emission determinations. For consistency only profiles taken between 14:00 and 16:00h at the inlets and outlets of Willunga and Greenfields Wetlands and in Hack's and Bool Lagoons are presented in this chapter.

At Willunga Wetlands, measurements were taken from the inlet and outlet at 8 depths: 0.1, 0.5, 0.75, 1, 1.5, 2, 2.5 m and in the flocculent sediment layer (floc), which was at 2.9 m depth in summer and 3.1 m in winter. At Greenfields Wetlands and Bool Lagoon the water depth was more variable (0 to 2.2 m). Accordingly, profiles were generated at c. 10 cm intervals down the water column in summer and at c. 20 cm intervals in winter

to yield a total of 5 depths in each profile. At all times of year surface samples were taken at 0.1 m depth and the deepest samples in the floc layer. Similar profiles were generated at Bool Lagoon over three consecutive days in winter (10-13 July 1996) and summer (6-9 January 1996) with a vertical sampling interval of 10 cm in both seasons. Nocturnal profiles were made at Greenfields Wetlands and Bool Lagoon with the portable meter, whilst at Willunga Wetlands a chain of thermocouples was suspended in the water column of WILLUNGA-INLET from January to July 1997 attached to a floating datalogger which recorded thermal data every 30 minutes. The intensive sampling at Willunga Wetlands was to monitor the more complicated patterns of diel destratification, through the change in seasons, in the deeper water column.

Profiles of dissolved methane concentrations down the water column were taken at WILLUNGA-INLET, WILLUNGA-OUTLET, GREENFIELDS-INLET, GREENFIELDS-OUTLET, BOOL-HACKS and BOOL-MAIN. The sampling interval varied with season and water depth, but in all cases the most shallow sample was taken 18 mm below the air-water interface, the deepest within the flocculent sediment and at least two water samples were taken at intermediate depths. Water samples were replicated in triplicate.

4.2.2: Statistical Analyses

Data were checked for normality of distribution with a Shapiro-Wilk test. Values greater than W=0.05 were accepted as being normally distributed. Non-parametric data sets were transformed with the equation $\log_{10}(x+1)$. A one-way ANOVA was performed to test the effect of *wetland site* on the transformed TOC and DOC data from November 1994. This was followed by a Tukey-Kramer test to determine significant groupings (α= 0.05). Similarly, the combined effects of *site-day-organic carbon fraction* were tested on the transformed TOC and DOC data from Greenfields Wetlands on 4 and 22 May 1995, the effect of *wetland site* was tested on the organic C content of the top 5 cm of sediment; the effect of *intra-wetland site* was tested on the organic carbon content data from the 1 and 5 cm sediment layers and the effect of *water depth* was tested on the dissolved methane concentrations. The relevant ANOVA tables appear in Appendix 1.1 and summaries of data presented in the results text are mean values ± one standard deviation.

4.3: Results

4.3.1: *Water regime*

The average water depth at WILLUNGA-INLET was 3.1 m in winter and 2.9 m in summer (Figure 4.1a) with an average flow rate of 4.3 L s^{-1} (Piranti, 1995). In contrast, inflows into Greenfields Wetlands were highly variable. For example, during the period February to August 1995, monthly inflow volumes ranged from 11 ML in March to 579 ML in July (Jenkins, 1996). This was reflected in the more variable water levels at GREENFIELDS-OUTLET which ranged from 1.4 to 2.2 m at the outlet weir (Figure 4.1b). The water regime at Hack's Lagoon was the most variable (Figure 4.1c). In winter, the maximum water depth was 1.4 m at the gauging post. Over the summer months large areas of Bool Lagoon dried out, leaving only one persistent pool in the centre of the Hack's Lagoon which was consistently c. 20 cm lower than the gauging post. This permanent pool was used as the BOOL-HACKS site.

4.3.2: *Carbon content of the surface waters and sediments*

The surface waters of Willunga Wetlands were higher in organic carbon content than the surface waters of Greenfields Wetlands. In November 1994, the DOC and TOC values were 6 to 8 times greater at Willunga Wetlands than Greenfields Wetlands irrespective of intra-wetland location ($P<0.05$; Table 4.2). At this time there was no evidence of an effect of flow path length on TOC content at either wetland. However, the DOC content at GREENFIELDS-INLET was significantly higher than at GREENFIELDS-OUTLET ($P<0.05$). Organic carbon concentrations at Willunga Wetlands were very similar each time they were sampled; average DOC content ranged from 28.3 to 37.6 mg L^{-1} and TOC ranged from 32.7 to 39.4 mg L^{-1}, across both sites and all sampling times (Table 4.2). At Greenfields, winter storms increased the carbon content at the inlet but the concentrations were still only a quarter of those at Willunga Wetlands. The samples taken on 4 May 1995 followed three days of inflow velocities greater than 950 L s^{-1} when turbidity at GREENFIELDS-OUTLET was 120 NTU (Figure 4.2). The inflows then decreased and remained less than 50 L s^{-1} until 25 May 1995 (except for two brief storms which temporarily increased the inflow velocities to c. 100 L s^{-1} on 12 and 13 May).

The second set of samples were taken on 22 May 1995 after 8 days of inlet velocities less than 50 L s^{-1} when the outlet turbidity levels had decreased to 60 NTU. Despite the large differences in turbidity readings between the two dates, the organic carbon content remained less than 6 mg L^{-1}. The highest concentrations were seen on 4 May 1995 at GREENFIELDS-INLET, indicating an slight increase in organic carbon concentration

induced by the winter storms. By 22 May the concentrations had decreased at GREENFIELDS-INLET and there was no difference in concentrations from inlet to outlet. Coefficients of variance for DOC (10 to 13%) and TOC (7 to 8 %) content were generally low at all the wetland sites and DOC represented between 83 and 87% of the TOC content, irrespective of the source water (Table 4.2). The average DOC concentration in Mosquito Creek during 1995 to 1997 was 11.7 ± 11 mg L^{-1} (EPA, 1998). These readings may be slightly higher than those measured at Greenfields Wetlands but they were highly variable.

The sediments of Bool Lagoon were significantly (P= 0.001) more organic in the upper 5 cm than those of the constructed wetlands (Table 4.3). The site with the highest carbon content was BOOL-MAIN containing 10.4 ± 0.7 % organic carbon, being significantly higher than BOOL-HACKS containing 8.6 ± 0.4 % organic carbon. In contrast, all the sites in the constructed wetlands contained less than 5% organic carbon (0.25 to 3.6 %) in the top 5 cm. At WILLUNGA-INLET, the highest organic carbon content, 5.6 ± 0.8 %, was in the top 1 cm of sediment (Tables 4.3 and 4.4). However, at WILLUNGA-OUTLET there was no significant difference between the organic content of the surface 1 cm and the top 5 cm of sediment. A more definite trend was seen at Greenfields Wetlands (Tables 4.3 and 4.4) where the top 1 cm of sediment was significantly more organic than the top 5 cm at both sites. The highest value, 6.6 ± 0.8 % organic carbon, was measured in the sediment from the top 1 cm of GREENFIELDS-INLET, which appears to be similar to that in the top 1 cm of WILLUNGA-INLET, 5.6 ± 0.8 %. No statistical tests were performed across the wetlands for the 1 cm sediment depth data due to the two wetlands being sampled in different seasons.

4.3.3: Redox status

The surface water layers at WILLUNGA-INLET and WILLUNGA-OUTLET were highly oxidising (+220 to +350 mV) in both summer and winter (Figure 4.3). The redox potential decreased with increasing depth reaching highly reducing conditions in the benthic water and flocculent sediment layers (-300 to -450 mV). In summer, the redox potential decreased with increasing depth, dropping to negative values below 1 m depth in both ponds, whilst positive values persisted to below 1.5 m depth in winter. At all times of year (and in both ponds), the water layers overlying the sediment were highly reducing, -280 to -420 mV. The survey of sediment redox potentials at Willunga Wetlands showed a very low level of spatial heterogeneity within the flocculent sediments. The redox potentials across the 3 m deep entry pools were -450 ± 10 mV in WILLUNGA-INLET and -360 ± 15 mV in WILLUNGA-OUTLET with no discernible spatial trends. Redox potentials in the sediment of Greenfields Wetlands (Figure 4.4) were

much more spatially variable than at Willunga. Patches of differing redox potentials were apparent at GREENFIELDS-INLET with the most highly reducing (-250 to -380 mV) areas associated with vegetation (ie. along the banks). Unvegetated areas were very variable ranging from -50 to –280 mV although areas within the channels that were scoured consistently had the least reducing conditions. A similar pattern emerged at GREENFIELDS-OUTLET although the magnitude of the potentials was less extreme varying from -75 to -260 mV.

4.3.4: Oxygen and temperature profiles

Willunga Wetlands

The thermal structure of the water column in WILLUNGA-INLET was relatively consistent across the four summer days (Table 4.5a). Surface water temperatures ranged from 28.8 to 32.2 °C and were strongly attenuated with depth. For example, on 2 January 1996 (Day 1) the temperature decreased from 31.8 to 22.5 °C in the surface metre. Benthic temperatures were very stable (c. 19°C) over the four days in the floc layer. Surface concentrations of oxygen were very high, reaching 22.35 mg L^{-1} at 30.8 °C (300 % saturation). Oxygen concentrations decreased rapidly with depth and only 0.95 to 2.16 mg L^{-1} oxygen remained at 1 m depth. Below 1.5 m, oxygen concentrations were at or below the limit of detection (<1 mg L^{-1}).

The thermal structure of the water column in WILLUNGA-OUTLET was very similar to that in WILLUNGA-INLET on these four summer days. Surface temperatures ranged from 28.4 to 32.4 °C and benthic temperatures were stable around 19 °C, only changing by 0.4 °C over the four days. Although the oxygen concentrations at the surface of WILLUNGA-OUTLET were lower than in WILLUNGA-INLET, oxygen penetrated deeper into the water column of WILLUNGA-OUTLET (Table 4.5b).

At 1 m depth, oxygen concentrations ranged from 4.73 to 5.89 mg L^{-1} in WILLUNGA-OUTLET, approximately 3 times the amount available at 1 m depth in WILLUNGA-INLET. Similarly, oxygen concentrations at 1.5 m in WILLUNGA-OUTLET ranged from 1.98 to 2.31 mg L^{-1}, whilst in WILLUNGA-INLET concentrations were below the limit of detection (<1 mg L^{-1}). Oxygen concentrations didn't fall below this threshold until 2 m depth in WILLUNGA-OUTLET. Therefore in WILLUNGA-INLET two thirds of the water column was hypoxic (>1 mg L^{-1}) whilst in WILLUNGA-OUTLET, approximately half the depth of the water column was. In both WILLUNGA-INLET and WILLUNGA-OUTLET, chlorophyll a concentrations were highest in the surface waters, where oxygen concentrations were super-saturating, and decreased with depth coincidently with decreasing oxygen concentrations (Table 4.6).

The surface temperatures in winter ranged from 14.8 to 15.6 °C over the four days in WILLUNGA-INLET, approximately half the summer temperatures which ranged from 28.8 to 32.2 °C (Table 4.7a). The water column exhibited thermal stratification and benthic temperatures consistently remained below 13 °C. Surface oxygen concentrations were slightly higher than in summer, ranging from 22.48 to 28.49 mg L^{-1}, however, oxygen penetrated less deeply into the water column than in summer. At 0.75 m the water was only 25% saturated, with concentrations ranging from 1.6 to 2.48 mg L^{-1}. By 1 m depth, concentrations were below the limit of detection. By contrast, in summer, water at 1m contained 1.57 ± 0.53 mg L^{-1} oxygen. Surface temperatures at WILLUNGA-OUTLET ranged from 14.6 to 15.2 °C in winter. Thermal stratification was also evident in WILLUNGA-OUTLET as temperatures decreased to 12.2 to 12.5 °C in the floc (Table 4.7b). Oxygen concentrations in the surface waters of WILLUNGA-OUTLET (10.9 to 12.5 mg L^{-1}) were approximately half that in WILLUNGA-INLET. However, oxygen penetrated deeper into the water column with concentrations of 1.55 ± 0.26 mg L^{-1} reaching 1 m depth before dropping below 1 mg L^{-1}.

Diel thermal data over the summer of 1995/1996 showed that the strong thermal stratification which built up over the daylight hours in WILLUNGA-INLET was largely persistent during the night (Figure 4.5a). The diel mixing depth varied from 0.5 to 1 m over the four days with the deepest mix occurring at dawn on the first day. The following day, thermal stratification was distinct between 0.1 and 0.5 m water layers, then mixing to 0.5 m occurred on Days 2 and 3. As oxygen concentrations in the surface 0.5 m of WILLUNGA-INLET were always greater than 10 mg L^{-1} in the afternoons, diurnal mixing in summer usually only incorporated the well-oxygenated layers. The diel cycles followed this pattern from mid-December until late summer when the water column significantly cooled (Appendix 2). Over autumn and winter the mixing depth increased to 2.25 m incorporating both oxic and anoxic water layers (Figure 4.5b). Appreciable diurnal stratification developed in the upper water layers in winter but this was generally broken down overnight. Oxygen penetration was also lower in winter than summer indicating that in winter the water column would be more anoxic over the diel cycle than in summer.

Greenfields Wetlands and Bool Lagoon

Summer water temperatures at GREENFIELDS-INLET ranged from 21.5 to 26.3 °C at 0.1 m over four afternoons (Table 4.8a), with benthic temperatures ranging from 20.1 to 24.3 °C. Despite the thermal stratification, oxygen penetrated the c. 55 cm water column to the sediment surface (c. 4 mg L^{-1}) but was rapidly attenuated in the floc layers. The thermal structure at GREENFIELDS-OUTLET was very similar (Table 4.8b).

The slight increases in temperature and oxygen concentrations at the outlet were likely to be a function of the sampling order rather than site specific differences as GREENFIELDS-OUTLET was routinely sampled 1 hour after GREENFIELDS-INLET. Oxygen penetration throughout the water column at GREENFIELDS-OUTLET was similar to GREENFIELDS-INLET and again concentrations decreased markedly in the floc.

The majority of inflows into Greenfields Wetlands occur in autumn and winter (see Jenkins data in Chapter 1) thus the water level in winter was approximately 30 cm deeper than summer (Table 4.9a and b). Thermal stratification did not develop at GREENFIELDS-INLET or GREENFIELDS-OUTLET in winter. Winter temperatures ranged from 7.9 to 8.2 °C and oxygen concentrations were 7.36 to 11.2 mg L^{-1}. In general, GREENFIELDS-INLET contained more oxygen than GREENFIELDS-OUTLET which was considered a function of differences in wind exposure at the two sites; GREENFIELDS-OUTLET has more heavily vegetated banks than GREENFIELDS-INLET and the vegetation stands much higher than the water level. GREENFIELDS-INLET, by contrast is very exposed. The surface water was c. 70% saturated with oxygen. Oxygen concentrations at the sediment surface of GREENFIELDS-INLET in winter were approximately twice that in summer and the floc layers did not attenuate oxygen as greatly as in summer. Oxygen attenuation was greater in the sediment at GREENFIELDS-OUTLET.

Temperature and oxygen data were gathered over three consecutive days per season at BOOL-HACKS. The summer data were collected in December 1995 when water levels were c. 45 cm, similar to those in the channel at Greenfields Wetlands in summer. Thermal stratification developed during the day with surface temperatures ranging from 25.2 to 28.6 °C and benthic temperatures of 23.8 to 25.1 °C (Table 4.10). The surface oxygen concentrations were lower than at Willunga Wetlands but similar to Greenfields Wetlands although oxygen concentrations in the floc layer were higher, only dropping to c. 1.8 mg L^{-1}. Both Greenfields Wetlands and Bool Lagoon underwent diel cycles of stratification-destratification (Table 4.11). As described above appreciable thermal stratification developed in the water columns of both wetlands during the warm summer days. However at night, the thermal layers were broken down and the whole water column was mixed. Complete mixing occurred throughout the diel cycle in winter at Bool Lagoon and the water temperature did not exceed 12 °C.

4.3.5: Vertical dissolved methane profiles

Vertical profiles show methane concentrations greater than that expected for water at equilibrium with ambient air (0.04 µg L^{-1} at 20 °C), indicating net methane production at

all of the wetland sites. Surface concentrations (0.1 m depth) in WILLUNGA-INLET were 279.6 ± 11.9 μg CH_4 L^{-1} and increased with depth to 2824.6 ± 117.8 μg CH_4 L^{-1} at 2.9 m in summer (Table 4.12a). The dissolved methane concentration in the water entering WILLUNGA-INLET contained less than 25 μg L^{-1}.

The rate of increase in methane concentrations changed with increasing depth. In the oxic zone (0.1 to 1 m depth) the gradient was 248 μg CH_4 L^{-1} m^{-1} and in the anoxic zone (1 to 2.9 m) it was 600 μg CH_4 L^{-1} m^{-1}. Summer methane concentrations in WILLUNGA-OUTLET were much lower than in WILLUNGA-INLET, being only 35.8 ± 7.4 μg L^{-1} at 0.1 m and 1486.5 ± 275.9 μg L^{-1} at 2.9 m. The vertical gradients in both the oxic (0.1 to 1.5 m) and anoxic (1.5 to 2.9 m) zones were very similar to those in WILLUNGA-INLET, being 240 and 727 μg CH_4 L^{-1} m^{-1}, respectively, even though the absolute concentrations were different. In winter, the dissolved methane concentrations decreased by at least an order of magnitude in both ponds at all depths (Table 4.12a). In WILLUNGA-INLET and WILLUNGA-OUTLET, there was no evidence of a concentration gradient within the oxic zone, which extended to 0.75 m in WILLUNGA- INLET and 1m in WILLUNGA-OUTLET. However, a significant gradient was observed in the anoxic zones, being 122 μg CH_4 L^{-1} m^{-1} in WILLUNGA-INLET and 57.5 μg CH_4 L^{-1} m^{-1} in WILLUNGA-INLET.

In summer, the water column at GREENFIELDS-INLET was stratified with respect to methane. Dissolved methane concentrations were 32.3 ± 7.9 μg L^{-1} at 10 cm and increased steadily to 93.5 ± 15.2 μg L^{-1} at 45 cm depth ($P<0.05$; Table 4.12b), resulting in a vertical gradient of 135 μg CH_4 L^{-1} m^{-1}. This was in contrast to the profile of methane concentrations at GREENFIELDS-OUTLET, where dissolved concentrations in the lower water layers (17.8 ± 2.3 μg L^{-1}) were similar to those at the surface (11.3 ± 1.4 μg L^{-1}). Methane concentrations did not significantly increase ($P<0.05$) until the flocculent sediment at 45 cm depth (17.8 ± 2.3 μg L^{-1}). In winter, there was no vertical methane concentration gradient, probably as a result of the strong and persistent thermal mixing of the water column (see Table 4.9). Methane concentrations decreased in winter but the concentrations at GREENFIELDS-INLET remained 2-3 times that at GREENFIELDS-OUTLET.

The profiles of dissolved methane concentrations at Bool Lagoon were very similar to those at GREENFIELDS-INLET. Surface concentrations were 28.7 ± 9.3 at BOOL-HACKS and 27.4 ± 8.7 μg L^{-1} at BOOL-MAIN and increased to 95.9 ± 31.6 and 93.3 ± 26.7 μg L^{-1} in the flocculent sediments, respectively (Table 4.13). In winter, there

was no increase in dissolved methane concentrations with depth (P= 0.99771 and 0.9813), presumably due to the complete mixing of the water column.

4.3.6: O_2:CH_4 ratios

Counter gradients of methane and oxygen developed in the wetlands during periods of thermal stratification. The summer O_2:CH_4 ratios in WILLUNGA-INLET were 21 to 79 in the top 0.5 m of the water column (Table 4.14). At 1 m depth, the ratio had dropped to 1.26 based on mean values of the dissolved gas concentrations but if one standard deviation is added to the mean values the ratio increases to 1.57. Oxygen penetrated deeper into WILLUNGA-OUTLET, generating very high O_2:CH_4 ratios (563 to 15.6) down to 1.5 m depth below which oxygen concentrations dropped to below 1 mg L^{-1}. Over winter, ratios of greater than 560 were observed in the surface 0.5 m of WILLUNGA-INLET, whilst in WILLUNGA-OUTLET ratios greater than 250 persisted to a depth of 1 m. However, the surface water temperatures dropped from >26°C in summer to c. 13 °C in winter which may also affect rates of methane consumption (See Chapter 2).

The two sites at Greenfields Wetlands showed very similar changes in temperature and dissolved gas concentrations with increasing water depth across the seasons. Methane concentrations at the air-water interface of GREENFIELDS-INLET were c. 35% of the benthic values whilst at GREENFIELDS-OUTLET they were >60% of the benthic value. The O_2:CH_4 ratios at GREENFIELDS-INLET did not exceed 300 whereas at GREENFIELDS-OUTLET the ratios were >700 throughout the water column (Table 4.14). In winter the water column was completely mixed at both sites and the ratios were generally greater than 1000. At the inlet, oxygen concentrations decreased in the floc layer but the other parameters did not change with increasing depth. At GREENFIELDS-OUTLET, the water column was thoroughly mixed, nevertheless, the concentration of methane decreased at the air-water interface.

The water column dynamics at BOOL-HACKS were very similar to that at GREENFIELDS-INLET (Table 4.14). The rate of change in dissolved methane concentrations did not change with increasing depth and surface values were c. 30% of those in the floc. In winter, the water column was completely mixed and there were no physico-chemical gradients in the water column. Oxygen concentrations dropped in the floc layer indicating a consumption of oxygen. O_2:CH_4 ratios were 19 in the surface waters and increased to 350 in the floc. Winter ratios were much higher (>1500 in the water column) because the concentration of methane in the water column had decreased markedly since summer.

4.3: Discussion

It is evident from the physico-chemical data presented here that the three wetlands provide contrasting habitats for methane producing and consuming bacteria. To begin with, the water regimes experienced by each wetland were very different. The water level at Willunga Wetlands was relatively static at c. 3 m depth and persistent thermal stratification developed in the water column over the summer months. In addition, the benthic water and the underlying sediment were anoxic (<1 mg L^{-1}) and had redox potentials less than -300 mV, optimal conditions for methanogenesis (Hungate, 1967). By contrast, the water level at Greenfields Wetlands was more variable, ranging over the annual cycle from 1.4 to 2.2 m at the outlet weir. The water levels measured at the sites GREENFIELDS-INLET and GREENFIELDS-OUTLET were lower, dropping to c. 45 cm in summer. Complete drawdown was not experienced at these sites because the water regime was dampened by the channellised design of Greenfields Wetlands. However, much of the higher lying land adjacent to the channels became exposed over summer as the water table dropped (Jenkins, 1996).

Like Greenfields, Bool Lagoon is a winter-fed system but unlike Greenfields, Bool Lagoon is a wide (c. 15 x 9 km) relatively flat series of basins. The amplitude of the seasonal drawdown experienced at Bool Lagoon was far greater and more widespread than that experienced at the Greenfields Wetlands sites. Across the majority of the Bool Lagoon complex the water table dropped to below the surface in late summer, rising again in the cooler months. Only one area of Hack's Lagoon (the BOOL-HACKS site) remained permanently inundated over summer and the water level in that pool dropped to less than 10 cm in autumn 1997.

Water regime strongly affects methanogenesis in wetlands (Bubier and Moore, 1994) and water table position alone can explain 62 % of variation observed in methane production rates (Dise, 1993). Permanently flooded rice paddies produce more methane than those that are intermittently inundated (Mishra, 1997) and this has been linked to the provision of a constantly reducing habitat (Trolldenier, 1995). Furthermore, the distance to the oxic-anoxic interface is a major factor controlling the relative activities of methanogenic and methanotrophic bacteria (Roslev and King, 1996; Segers, 1997). Therefore the contrasting water regimes of these three wetlands may indicate major differences in methanogenic and methanotrophic habitat provision.

The whole of the water column at Greenfields Wetlands was well oxygenated. Anoxia, as indicated by reducing conditions, only became apparent in the sediments where redox potentials ranged from less than -250 mV near vegetated areas to -50 mV in more

scoured parts of the channel. To have the more negative redox potentials associated with the vegetation is contrary to those commonly reported in the literature for both pressurising and non-pressurising macrophytes beds (Flessa, 1994; Grosse, 1996) and may indicate that the plants were providing a valuable source of carbon to an otherwise carbon-limited system, and only in these vegetated areas was the rate of decomposition high enough to induce reducing conditions within the bulk sediment.

Although methanogens are generally considered to be strict anaerobes, field observations show that the initiation redox potential may not be very reducing; -150 mV in rice paddies, for example (Masscheleyn, 1993; Wang, 1993). Furthermore, in natural Australian wetlands significant methane production has been observed in sediments that were ostensibly oxidising or only poorly reducing. For example, in 1994, significant rates of potential methane production (140 mmol m^{-2} h^{-1}) were measured in sediment cores taken from *Triglochin procerum* and *Baumea arthrophylla* beds in Bool Lagoon. These sediments had redox potentials ranging from -50 to +280 mV (Muller, 1993). Similar rates of methane production have been reported for other Australian wetlands including a permanent wetland with sediment redox potentials of c. +100mV (Boon and Sorell, 1991) and an ephemeral floodplain system with sediment redox potentials of +176 to +243 mV (Boon et al., 1997).

These observations may be explained by considering that 5 mm diameter platinum probes were used to measure redox potential. First, these probes do not indicate true sediment Eh (as discussed in Boon and Sorrell, 1991) and second, the spatial scale across which these measurements were made is many times greater than the size of a methanogen or methanotroph and thus confounds the redox potentials of many potential niches. Very small-scale variations are important and ubiquitous controlling factors of aquatic microbial processes (Brandl et al., 1993). In addition, methanogens can actively occupy anaerobic microsites with sediments that are aerobic in the bulk phase, e.g. forest soils (Sexstone and Mains, 1990). Although examination of methanogenic and methanotrophic processes at these microscales is beyond the scope of this project, it should be recognised that redox potentials represent a coarse habitat descriptor, particularly in systems with variable water regimes and it is only at this coarse, qualitative scale that these measurements are considered meaningful in this thesis.

Once anaerobiosis has been established, the major factor controlling methanogenesis is availability of suitable organic substrates. This has been shown *in vitro* by the enhancement of methanogenesis following the addition of substrates, such as H_2,

acetate, cellulose or leaf extracts (Williams and Crawford, 1984; Valentine et al., 1994; Amaral and Knowles, 1994; Boon and Mitchell, 1995). Both of the constructed wetlands received organic carbon from their source waters of which c. 90 % was DOC and therefore readily available for cellular respiration (Fenchel and Blackburn, 1979).

Water entering Willunga Wetlands was very carbon-rich containing > 25 mg L^{-1} DOC and > 30 mg L^{-1} TOC. The values obtained were similar to those reported by Tebutt (1998) for secondary sewage; mean 35 mg L^{-1} TOC. In contrast, the DOC and TOC concentrations at Greenfields Wetlands and Bool Lagoon were 16 to 36% of those at Willunga Wetlands and were towards the low end of the range reported in the literature. Graham (1989) reports that the average TOC content of urban runoff in Australian cities is 25 mg L^{-1} from a range of 6 to 130 mg L^{-1}, whereas the water at GREENFIELDS-INLET contained 4 to 7 mg L^{-1} DOC and TOC at all sampling dates. In addition, the TOC inputs were more variable at Greenfields Wetlands, increasing after winter storm inputs (ie. 4 May 1995). Over the 7 month period, February to August 1995, Greenfields Wetlands received c. 10 kg of TOC [assuming a constant concentration of 6 mg L^{-1} and summing the monthly inflow volumes reported by Jenkins (1996)]. In the same period Willunga Wetlands received 2.7 tonnes of TOC in, assuming a constant concentration of 35 mg L^{-1} TOC and flow rate of 4.3 L s^{-1} (Piranti, 1995).

In addition, the water column at Willunga Wetlands supported a large population of phytoplankton. The maximum chlorophyll a readings exceeded 900 µg L^{-1} in WILLUNGA-INLET and 650 µg L^{-1} in WILLUNGA-OUTLET. If an average specific attenuation coefficient for chlorophyll a of 0.021 ln (mg chla m^{-3}) is assumed (Ganf pers. com.), then light sufficient for net photosynthesis would be exhausted at 25 cm in WILLUNGA-INLET and at 35 cm in WILLUNGA-OUTLET. However, chlorophyll a was detected at 2 m where light availability would not sustain growth. This suggests that the phytoplankton were dying off in the upper water layers and sinking down the water column. These moribund cells would provide labile methanogenic action to the benthos (Franken et al., 1992) additional to the DOC content measured in the surface waters. This further highlights the differences in the organic content of the surface waters of the two constructed wetlands.

Despite the large organic carbon loads entering Willunga Wetlands, the sediments contained less than 6 % organic carbon even in the surface 1 cm deep layer (which would have included minimal quantities of the clay used for construction of the basin). Greenfields Wetlands had very similar organic carbon content in the sediment to Willunga Wetlands even though the influent carbon concentrations were so much lower.

The sediment at Bool Lagoon was more organic (c. 10% organic C) than the sediment in the constructed wetlands. The organic content obtained here for Bool Lagoon is very similar to the range (4.51 to 12.37 % organic C) reported by Brownlow (1997) for the same system. However, wetland soil organic matter content usually varies from 15 to 75% (Mitsch and Gooselink, 1993) and much higher values (45%) have been reported for another Australian wetland, Ryan's Billabong (Boon and Sorrell, 1991). It is therefore likely that methane production in all the sediments of these wetlands will be carbon limited as even natural wetland sediments that contain 45 % organic carbon display methanogenic substrate limitation (Boon and Sorrell, 1995). However, source water carbon concentrations may over ride sediment carbon content as the main source of methanogenic action in these constructed wetlands.

Consistent, with seasonal fluctuations in solar irradiance, the water temperatures in the three wetlands were much higher in summer than winter. Although, methanogenesis can occur from 5 to 110 °C (Ferry, 1993), field measurements suggest that methanogenesis is inhibited by temperatures lower than 15 °C (Zeikus and Winfrey, 1976; Conrad et al., 1987). Winter water temperatures at all the wetlands were below this threshold and were likely to inhibit methanogenesis, but near-optimal temperatures (25 °C as defined by studies in Zeikus and Winfrey, 1976 and Conrad et al, 1987) occurred in the benthos in summer.

Although most freshwater bodies in the world are supersaturated with methane relative to atmospheric concentrations (Lerman, 1988), methanotrophs require methane concentrations above population-specific threshold values of 1 to 92 µM, or 16 to 1472 µg L^{-1} (Conrad, 1996). Moreover, methane concentrations below 5 to 10 µM CH_4 (80 to 160 µg L^{-1}) limit methanotrophy in lake systems (Rudd and Hamilton, 1975; Harrits and Hanson, 1980) thus the distribution of methanotrophs is primarily controlled by dissolved methane and oxygen profiles (Utsumi et al., 1998). In order to satisfy the O_2:CH_4 ratios suitable for methanotrophy (Amaral and Knowles, 1995), oxygen concentrations of > 125 µg L^{-1} would be required when non-limiting methane concentrations were present. Therefore in methanogenic sediments inundated with oxygenated water, methane oxidation is generally restricted to the oxic/anoxic interfaces where dissolved methane concentrations are high, such as the sediment surface or the rhizospheres of ventilating aquatic plants (King, 1990b; Gerard, 1993; Bender and Amaral, 1994; Buchholz, 1995; Krumholz, 1995; Boon et al., 1997; Calhoun, 1997). Similarly, in eutrophic lakes the major site of methanotrophy is at the interface between the oxic, methane-poor epilimnion and the anoxic, methane-rich hypolimnion (Rudd and Hamilton, 1975; Harrits and Hanson, 1980).

The oxygen status of the benthic water and flocculent sediment layers at Willunga Wetlands was consistently low. Oxygen did not penetrate below 1.5 m at any time of year, although it did penetrate more deeply into the water column of WILLUNGA-OUTLET, which established anoxic hypolimnia in both ponds. Conditions conducive to aerobic methanotrophy (>80 µg L^{-1} CH_4 and > 125 µg L^{-1} O_2) were found only in the top 1 m of WILLUNGA-INLET and between 75 and 150 cm depth in WILLUNGA-OUTLET in summer. The superficial water layers of WILLUNGA-OUTLET were oxygen-rich but methane limited (< 80 mg L^{-1}). This is reflected in the steep vertical gradient in O_2:CH_4 ratios observed. Alternatively, the benthic waters were oxygen limited and methane-rich. It appears that Willunga Wetlands would provide productive habitat for aerobic methanotrophs at the interface between the aerobic and anaerobic water layers but not in the surface sediments.

In contrast to Willunga, oxygen penetrated the whole of the water column at Greenfields Wetlands and Bool Lagoon, reaching the sediment surface. However, the surface waters at GREENFIELDS-INLET and BOOL-HACKS were methane-poor. Dissolved methane concentrations were above the threshold for methanotrophy (28 to 68 µg L^{-1}) but were, nevertheless, limiting. At Ryan's Billabong in eastern Australia, rates of planktonic methylotrophy were undetectable at similar methane concentrations (Boon, pers. comm.) but methyltrophs were detected by 16s rRNA oligonucleotide probes (Ross et al., in press). This would indicate that although the rates of methanotrophy were too low to measure, sub-optimal methanotrophic habitat was provided in the water column.

Conditions in the flocculent sediments GREENFIELDS-INLET were more conducive to methanotrophy, providing an oxic/anoxic interface and methane concentrations > 80 mg L^{-1}. Similar conditions were observed at Ryan's Billabong where sediment samples have been shown to aerobically consume methane (Boon and Lee, 1997). The environment at GREENFIELDS-OUTLET was generally poor habitat for methanotrophs. The methane concentrations in the water were very low and although the concentrations increased in the flocculent sediment, only threshold levels were observed (18 µg L^{-1}). Lidstrom and Somers (1984) could not detect planktonic methanotrophy at methane concentrations of 8 mg L^{-1}. Thus the waters at GREENFIELDS-OUTLET are considered to be poor methanotrophic habitat and even the flocculent sediment can only be considered to have marginal methanotrophic habitat value.

In contrast to the effect that variable water level has on methane production, methanotrophy is likely to be enhanced in drawdown conditions, especially if drawdown

occurs in summer when temperatures are high and favour rapid microbial activity. Optimal soil moisture for methanotrophy in organic sediments (14% organic carbon) is c. 30 %, above which additional moisture causes a decrease in methanotrophic rates (Bender, 1993; Czepiel, 1993). At optimal soil moisture, the spatial variability in rates of methane oxidation can be explained by organic carbon content (Czepiel, 1993), presumably as organic carbon is a strong predictor of methanogenic activity.

Methane concentrations at all sites were much lower in winter, being generally below the threshold for methanotrophy (16 µg L^{-1}) in the surface oxygenated water layers. In addition, water temperatures had dropped to 8 - 15 °C from summer temperatures of > 25 °C. The effect of temperature *per se* on rates of methane oxidation differs across wetland locations and types but in a range of peat systems optimal temperatures of c. 25°C have been observed (Bedard and Knowles, 1989; King, 1992; Whalen and Reeburgh, 1992; Dunfield et al., 1993; Bender and Conrad, 1995). However, as rates of methane consumption are also dependent on methane concentrations, an interaction occurs between seasonal changes in methane concentrations and temperature. For example, in a Japanese dimictic lake, peak seasonal methane oxidation was observed in winter following autumnal turnover when water temperatures were only 6-9 °C but dissolved methane concentrations were at a seasonal high (Utsumi et al., 1998). Therefore although it is clear that the wetlands provide suitable summer habitat for methanotrophs it is less clear as to whether methanotrophic activity will be detectable in winter. This will be related to the specific temperature responses of the extant populations as well as being influenced by seasonal methane availability.

In conclusion, the habitats available for methanogens and methanotrophs differed across the three wetlands. Soil carbon content is a strong predictor of methane production rates in natural wetlands (Williams and Crawford, 1984; Yavitt et al., 1987; Yavitt and Lang, 1990; Nilsson and Bohlin, 1993; Sundh et al., 1994; Crozier et al., 1995; Denier van der Gon and Neue, 1995) and the quality and quantity of organic carbon in these wetlands was variable and site-specific. Methanogens have a restricted range of substrates and as such flourish in habitats with high levels of DOC (Boone, 1982; Zinder et al., 1984; Zinder, 1993). The sewage effluent in Willunga Wetlands contained more than 25 mg L^{-1} DOC and supported high phytoplankton biomass and a very reducing benthos. The stormwater feeding Greenfields Wetlands was far less organic and the redox potentials at Greenfields Wetlands were more spatially heterogeneous and less reducing than at Willunga. This would imply that the carbon supply to the sediments in Greenfields Wetlands was less than that in Willunga Wetlands, even though the organic carbon contents of the sediments at the two

constructed wetlands were similar. In contrast, methanogens at Bool Lagoon were totally reliant on sediment carbon for substrates as the inflows ceased and the water table dropped over summer. Sediment carbon in this ancient vegetated wetland would tend to be refractile, according to models of the decomposition of complex plant material (Godschalk and Wetzel, 1978a,b and c).

Aside from organic status, the wetlands provided contrasting habitats in term of water regime. In summer when temperatures were most conducive to microbial activity, Bool Lagoon experienced extensive drawdown that exposed the sediments to atmospheric oxygen thereby reducing the methanogenic habitat and increasing the methanotrophic habitat (Roslev and King, 1996). Greenfields Wetlands also experienced a variable water regime although the study sites remained inundated. As summer inflows to Greenfields Wetlands were infrequent, the supply of methanogenic substrates may become limiting over summer especially considering the spatial variability in redox potentials and the young age of the vegetation on the channel banks. In contrast, Willunga Wetlands were flooded to c. 3 m depth in summer and continuously received fresh organic inputs, providing an ideal habitat for methanogens and sub-optimal conditions for methanotrophs.

Chapter 5

Chapter 5: Methanogenesis and methanotrophy

5.1 Introduction

It is evident from the data presented in Chapter 4 that the three wetlands vary with respect to the physico-chemical characteristics that are thought to effect the activity of methanogens and methanotrophs.

Willunga Wetlands provided the most suitable environment for methanogens. The benthic water and the sediments were highly reducing and rich in organic carbon and the temperature regime was optimal in the summer months. In addition, it is likely that methanogenic and fermenter organisms were entering Willunga Wetlands in the inflowing sewage. It is expected, however, that the environment was less favourable for aerobic methanotrophs which would have been restricted to the surface oxygenated water layers where their requirements for oxygen and methane were satisfied.

Greenfields Wetlands were less favourable for methanogens than Willunga Wetlands. The sediments contained similar organic carbon contents but they were less reducing than at Willunga Wetlands and the incoming water was less organic rich. Therefore it is likely that the methanogens at Greenfields Wetlands will be less active than those at Willunga Wetlands. However, Greenfields Wetlands provided more favourable conditions for methanotrophs than did Willunga Wetlands, particularly in the sediments.

Conditions at Bool Lagoon were similar to those at Greenfields Wetlands except that the methanogens were reliant on sediment organic carbon rather than allochthonous carbon because inflows ceased over summer and the water table dropped. In these conditions, the methanogenic habitat would be restricted to the surface sediments which were also likely to provide suitable habitat for methanotrophs.

With respect to their suitability as habitats for methanogens and methanotrophs the wetland sites were ranked in the order predicted in Chapter 1. That is:

WILLUNGA-INLET>WILLUNGA-OUTLET>BOOL-HACKS>GREENFIELDS-INLET
>GREENFIELDS-OUTLET

The experiments presented in the following chapter were designed to test if the rates of methane production and oxidation measured at each wetland site were also ranked in this order.

In addition these experiments were designed to determine:

1. what factors controlled methanogenesis and methanotrophy in the three wetlands
2. whether the controls were the same at each site; and
3. what proportion of the methane produced was consumed by methanotrophy.

5.2: Methods

5.2.1: In vitro experiments

The effect of temperature on *in vitro* methanogenesis was tested on samples taken in summer and winter from the inlets and outlets of Willunga and Greenfields Wetlands and from BOOL-HACKS and BOOL-MAIN. Five cores were taken at each site in July and December 1996 and three replicate subsamples were incubated at 5, 10, 20, 30 and 50 °C in the laboratory by the protocol described in Sections 3.4 and 3.5. The only amendment to the sediment samples was L-cysteine to maintain anoxic conditions.

The second *in vitro* experiment was performed on flocculent sediment collected from Willunga and Greenfields Wetlands in spring 1996 and from Bool Lagoon in autumn 1997. Two batches of 5 cores were collected from the constructed wetlands during the middle of spring (21 October to 20 November, see note in Chapter 3) and returned to the laboratory for incubation at 20 °C.

The sediment treatments were:

1.	anaerobic controls:	2 mL of deoxygenated wetland water (L-cysteine at a final concentration of 0.03 % w/v)
2.	alternative electron acceptors:	$NaNO_3$ (10 mM); Na_2SO_4 (10 mM)
3.	specific inhibitor:	Na_2MoO_4 (5 mM)
4.	potential substrates:	sodium acetate (10 mM); cellobiose (10 mM)
5.	cultured fermenters:	ANOX culture; ANOX culture + cellobiose (10 mM)
6.	fermenter control:	killed ANOX culture.

The cultured fermenters (DMS 3005) were a mixed culture of anaerobic fermenter bacteria, from the *Bacillus* genera (see Section 3.5.1). This culture was used to lyse complex organic moieties into acetate and H_2 to determine whether the methanogens were substrate limited (see Figure 2.1). Cultures of sulphate-reducing bacteria, which may have also supplied the methanogens with acetate and H_2 (see Figure 2.2), were

not used because the experiment was designed to determine whether the methanogens were competing with sulphate-reducers in the wetland sediments.

Three replicate vacutainers were prepared for each treatment and controls were run each time a batch of 5 cores were collected, thus there were six replicate controls for each site. All the incubations were performed within a single ten day period in autumn 1997 at Bool Lagoon. A make-shift laboratory was set up in a shed on-site to avoid the time delays apparent in transporting sediment back to Adelaide. The mobile water bath used was rudimentary and unable to maintain 20 °C, but it could maintain 25 °C within ± 0.5 °C; thus the incubation temperature at BOOL-HACKS was 5 °C higher than that used for laboratory incubations of sediments from Willunga and Greenfields Wetlands.

5.2.2: In situ experiments

In December 1996 (summer) and July 1997 (winter), concurrent rates of *in situ* methane production and oxidation were determined in the surface water and sediments. Each wetland site was studied on a separate day during this period. Water and sediment samples were incubated for 24 hours *in situ*, submersed in the wetland water, under the various treatments outlined below and summarised in Table 5.1.

Clay sediment samples were incubated with L-cysteine in 20 mL vacutainers on the sediment surface. Triplicate samples were processed from six cores per site in an anaerobic chamber situated on the wetland banks.

The flocculent sediment (floc) was collected and incubated in 60 mL syringes with no headspace. At Willunga Wetlands, half the syringes filled with floc received L-cysteine solution (anaerobic control) and half received BES solution (methanogenic inhibitor). The BES treatment was intended to show whether methane was consumed at *in situ* sediment oxygen tension, therefore L-cysteine was not introduced. The same treatments were conducted at Greenfields Wetlands and Bool Lagoon but a third treatment was included for the floc samples, in which oxygen and BES were added together to stimulate aerobic methanotrophy and inhibit methane production, simultaneously. L-cysteine was not added to this third treatment and care was taken in sampling to avoid altering the extant dissolved gas status of the water during sampling. Three replicate syringes were prepared for each floc treatment and incubated on the sediment surface for 24 hours. Incubations (minimum of 5 hours) began at 12 Noon to allow for the determination of methanotrophy rates during the warmest part of the day.

Oxygen was added to Greenfields Wetlands and Bool Lagoon sediment samples because the floc at these two wetlands was in direct contact with oxygenated water at

all times of year and was not consistently reducing. By contrast, at Willunga Wetlands the floc was permanently overlain by anoxic water and was highly reducing. In addition, *in vitro* methanogenesis rates in Willunga Wetland samples were up to 400 nmol mL^{-1} h^{-1}, therefore not enough oxygen could be added to the syringes to accommodate equivalent rates of methane oxidation without diluting the samples by more than half. Such a large dilution was considered unacceptable, therefore an aerobic treatment was not performed at Willunga Wetlands during this experiment. An alternative experiment was designed to address the rates of aerobic methanotrophy at WILLUNGA-INLET (see below).

In the same 24 hour period, water samples were incubated to determine rates of methane consumption in the oxygenated water column. The water levels at Greenfields Wetlands and Bool Lagoon varied over the seasons. To account for this variation, samples were taken at 10 cm below the air/water interface and 10 cm above the water/sediment interface in both seasons. In summer, the methane concentrations and temperatures at the two depths were typically different, as the water column was stratified, although they may have only been separated by 10 cm of water. In winter, the water column was typically well-mixed at both wetlands and the samples were very similar. However, at both times of year the water was oxygenated. The water depth at Willunga Wetlands was relatively static (c. 3 m) and only the top 1 to 1.5 cm was typically well-oxygenated at any time of year. Therefore in order to compare relative rates of aerobic methanotrophy across the wetlands, water samples were taken 10 and 50 cm below the air/water interface at Willunga Wetlands in the well-oxygenated epilimnion.

Three replicate samples were prepared for each water depth at each wetland site by the protocol described in Chapter 3. The syringes were incubated at the depth from which they were sampled for 5 hours in summer and 24 hours in winter to determine rates of methanotrophy. Temperature and dissolved oxygen in the bulk water and floc phases were measured at the beginning of each incubation and temperature was taken every hour during the incubation period. The initial dissolved methane concentration was determined from three replicate water and floc samples taken at the beginning of each set of incubations and immediately air-equilibrated.

5.2.3: Methanogenesis and Methanotrophy at WILLUNGA-INLET

On 1 March 1997, nine 60 mL syringes were filled with water sampled from each of six depths (0.1, 0.5, 1, 1.5, 2 and 2.5 m) and another nine were filled with floc from WILLUNGA-INLET.

BES was added to one third of the samples to inhibit methane production, $HgCl_2$ (30mM final concentration) to a third to inhibit all biological activity and a third were amended with N_2-purged, filtered wetland water to account for dilution in the other treatments. The syringes were replaced at the appropriate water depths and incubated for 24 hours beginning at 1400 h. Water samples were extracted from the syringes at 0, 1, 2, 4, 8, 21 and 27 h and dissolved oxygen concentrations were measured five times during the incubation. Rates of methanogenesis or methanotrophy were determined by plotting the dissolved methane concentration against time (see Chapter 3).

Temperature was recorded automatically every 30 minutes at various depths in WILLUNGA-INLET by the thermocouple chain and data logger. At approximately 1700 h on 2 March 1997, three more water samples (60 mL) were taken at each water depth for TOC and DOC analysis. These were kept on ice for 4 hours until filtration (0.2 µm membrane filter) before routine analysis (see Chapter 3).

5.2.4: Statistical analyses

Data cells were checked for normality (W>0.05) with Shapiro-Wilk tests and as all the cells failed, the data were transformed with the equation $\log_{10}(x+1)$ before being analysed by either one-way or two-way ANOVA. Four tests were used to test for equality in variance; O'Brien, Brown-Forsythe, Levene and Bartlett, but of these, only Levene was used routinely. In most instances, the one-way ANOVAs were used to test the effect of *site* on individual data sets in summer and winter. These were followed by Tukey-Kramer tests to determine the wetland site order. The interactive effects of *site* and *season* were tested by two-way ANOVA. The exceptions were the two one-way ANOVAs performed separately on the DOC and TOC concentration data to test the effect of *water depth*. The results of the relevant ANOVAs are shown in Appendix 1.2.

5.3: Results

5.3.1: In vitro methanogenesis

Temperature

Rates of *in vitro* methanogenesis varied from 0.5 to 575 nmol mL^{-1} h^{-1} across all wetland sites and were highest in samples incubated at 30 °C and lowest in samples incubated at 5 °C (Figure 5.1). The rates for each site doubled with each increase in incubation temperature from 5 °C to 30 °C. However, at 50 °C the rates decreased to only 40-60 % of those at 30 °C. Two-way ANOVA on data from the 30 °C incubation indicated the individual effects of both *site* and *season* were highly significant (P = 0.0000). However, all the samples responded to the change in seasons with a similar increase from winter

(July 1996) to summer (December 1996) and the order of the wetland sites remained constant (P= 0.9363). Rates from WILLUNGA-INLET samples were always highest, followed sequentially by WILLUNGA-OUTLET, GREENFIELDS-INLET, BOOL-HACKS and GREENFIELDS-OUTLET. The site order in the 30 °C incubations was verified for summer and winter by the highly significant Tukey-Kramer groupings shown in Figure 5.1.

5.3.2: Substrate and fermenter additions

Control rates of *in vitro* methanogenesis in this experiment were in the same site order and were of similar magnitude to those determined in the above experiment (Figure 5.1 and Table 5.2). In all the treatments, the samples from WILLUNGA-INLET yielded the highest rates and GREENFIELDS-OUTLET samples yielded the lowest rates (Figure 5.1 and Table 5.2). Nitrate additions had the strongest and most consistent inhibitory effect across the wetland sites but WILLUNGA-INLET and WILLUNGA-OUTLET samples were the least affected (88 ± 3 and 71 ± 11 % of the control, respectively). At Greenfields Wetlands, the effect was more marked at the inlet (25 ± 2 %) than the outlet (59 ± 14 %) whilst samples from BOOL-HACKS were the most strongly affected, being reduced to 12 ± 3% of the control. During these incubations the redox potential did not change more than ± 20 mV with respect to the control, indicating that the inhibitory effect of nitrate was not due to changes in redox potential.

Sulphate inhibition was less widespread and was only significant at Greenfields Wetlands where it reduced rates to 76 ± 2 and 81 ± 6 % of the controls at the inlet and outlet, respectively. The inhibitory effect of sulphate additions at Greenfields Wetlands, although less strong than the effect of nitrate additions, suggests that sulphate-reducers were competing with methanogens for substrates in a sulphate-limited environment. Furthermore, additions of molybdate (a specific inhibitor of sulphate-reduction) increased rates of methanogenesis to 220 ± 15 % of the control at GREENFIELDS-INLET and 241 ± 31 % at GREENFIELDS-OUTLET. However, molybdate additions also accelerated methanogenesis in WILLUNGA-INLET samples where no inhibitory effect of sulphate additions had been observed. Similarly, marginal increases in rate were observed in molybdate-amended samples from BOOL and WILLUNGA-OUTLET, neither of which had shown significant sulphate inhibition.

Acetate and cellobiose additions only slightly increased the rate of methanogenesis in the WILLUNGA-INLET samples but increased rates by 2- to 4-fold in samples from the other wetland sites. The stimulation was greatest at Greenfields Wetlands with no distinction in response between the inlet and outlet sites. Addition of the ANOX culture increased rates in all the samples by at least 50 to 100 % but BOOL-HACKS samples

were most strongly affected, increasing to 429 ± 78 % of the control. When cellobiose was added along with the ANOX broth, the rates at BOOL-HACKS were equivalent to those when fermenters (ANOX) were added alone but were higher than if cellobiose were added alone. The combined treatments yielded rates 472 ± 63 % of the control at the inlet and 431 ± 88 % at the outlet of Greenfields wetlands; higher than those for the fermenter additions alone but comparable to those for cellobiose additions alone. By contrast, the rates at Willunga wetlands were not more affected by the combined treatments than either cellobiose or ANOX additions alone. The increases seen by adding broth containing fermentative bacteria were attributable to biological activity as the killed samples increased control rates by < 10%. Table 5.2 also shows the results of a similar experiment conducted at Ryan's Billabong (NSW, Australia) for comparison (see Discussion).

5.3.3: *In situ methanogenesis and methanotrophy*

The temperature and dissolved oxygen and methane data collected during the *in situ* incubations are presented in Tables 5.3 to 5.5. The initial dissolved gas concentrations and the maximum and minimum hourly temperatures during each 24 h period are shown. In most of the summer incubations temperatures did not vary more than 5 °C in 24 h, although in the surface waters of Willunga Wetlands, temperature fluctuations during one incubation could be as large as 8 °C (Table 5.3). In winter, incubation temperatures fluctuated by less than 3 °C in all the samples and less than 1 °C in the sediment samples (Tables 5.3 to 5.5).

Temperatures in the surface water (10 and 50 cm depth) at Willunga Wetlands varied more across the seasons than did temperatures in the benthos (5 cm above the sediment surface; Table 5.3). The surface waters were approximately 10 to 15 °C warmer in summer than winter whilst the seasonal temperature change in the benthos was only 7 °C. In contrast, the sediment temperature regime closely followed the temperature regime observed in the surface waters at the more shallow Greenfields Wetlands and Bool Lagoon. The whole water column was approximately 15 to 18 °C warmer in summer than winter at Greenfields Wetlands and 11 to 14 °C warmer in summer at Bool Lagoon.

The concentration of dissolved methane increased with increasing water depths at all the wetland sites, ranging over three orders of magnitude (Tables 5.3 to 5.5). In all cases, summer dissolved methane concentrations were greater than in winter ($P<0.05$). The highest concentrations (2784 ± 124 µg L^{-1}) were measured in the benthos of WILLUNGA-INLET in summer, whilst in winter the surface water layers of all the wetland

sites contained less than 10 μg L^{-1}. WILLUNGA-INLET also experienced the largest seasonal change with the surface waters undergoing a 54-fold increase in concentration from winter to summer. By contrast, the seasonal increase in surface water concentrations at Greenfields Wetlands and Bool Lagoon was only 4-fold. Benthic concentrations at all the wetland sites, except GREENFIELDS-OUTLET, underwent a 13-fold increase from winter to summer. At GREENFIELDS-OUTLET the increase was only 4-fold, the same as at the surface. Overall, conditions in the wetlands during these incubations were similar to that described for each site in Chapter 4.

Sediments

The effect of $HgCl_2$ was consistent across the sites resulting in no significant change (as determined by one-way ANOVA) between the initial and final headspace methane concentrations in the treated samples. The anaerobic incubation of clay and floc samples in control conditions resulted in an increase in methane concentration over time and the rates appear in Table 5.6. The rates from the BES treated floc samples represent methanotrophy in the sediment and are shown in Table 5.7, whilst the results from the surface water incubations are shown in Table 5.8 as rates of surface water methanotrophy. All the data cells passed Shapiro-Wilk tests for normality (W>0.3) but not all the data cells passed all four of the tests (see Methods above) for equal variance. Thus the $\log_{10}$ transformed data were normal but on the borderline for equality of variance. In addition, the sample sizes were small (n=3) reducing the power of the variance tests. ANOVA is generally more robust to a lack of normality than to data with unequal variance but the resultant P values were highly significant (P=0.0000) in all of the tests, thus the differences in data cells detected by the ANOVAs are taken to be bona fide.

Anaerobic rates of methane production from the 5 wetland sites varied over three orders of magnitude from, 0.13 nmol mL^{-1} h^{-1} in winter clay samples from GREENFIELDS-OUTLET to 605 nmol mL^{-1} h^{-1} in summer floc samples from WILLUNGA-INLET (Table 5.6). The effects of *site* and *season* were highly significant (P=0.0000) and these effects strongly interacted in both the floc and clay layers (P=0.0000). Methanogenic rates were higher in summer than winter at all the sites but the magnitude of those differences varied. At WILLUNGA-INLET summer rates were 13 times higher than the respective winter rates whilst at GREENFIELDS-INLET the summer rates were only 6 times those in winter. One-way ANOVA and subsequent Tukey-Kramer tests across the s*ites* for each *season* and *sediment depth* showed that the relative order of the sites changed across treatments (Table 5.6). In the floc in summer, the rate of methane production from WILLUNGA-INLET samples was higher than that in samples from the other wetland sites,

which decreased in the following order: WILLUNGA-OUTLET, GREENFIELDS-INLET, BOOL-HACKS and GREENFIELDS-OUTLET. Across *site* and *sediment depth*, WILLUNGA-INLET samples were always in the highest ranked group and GREENFIELDS-OUTLET samples were always in the lowest ranked group but the magnitude of the differences between the sites changed. In the clay layers in summer, rates of methane production at GREENFIELDS-INLET and BOOL-HACKS were similar to each other but the rest of the sites retained the same order as above. In winter, rates at all the sites became more homogeneous. For example, there were no significant differences between WILLUNGA-INLET and WILLUNGA-OUTLET samples at either sediment depth and rates of methane production from GREENFIELDS-INLET samples were higher than those from BOOL-HACKS in the floc layer but not in the clay.

Only the flocculent sediment samples were examined for methanotrophic activity using BES additions. Absolute rates of methanotrophy, irrespective of whether O_2 was added or not, varied over two orders of magnitude from 8.7 nmol mL^{-1} h^{-1} at GREENFIELDS-OUTLET in winter to 562 nmol mL^{-1} h^{-1} at WILLUNGA-INLET in summer (Table 5.7). These were more homogeneous across the sites than relative rates of methanogenesis, but major differences occurred in the type of methanotrophy present in the benthos of the three wetlands. At Willunga wetlands, methanotrophy occurred in all water layers, even those that were ostensibly anaerobic (<1 mg O_2 L^{-1}). However, at Greenfields Wetlands and Bool Lagoon methanotrophy was an exclusively aerobic process, only occurring in those vacutainers amended with oxygen.

Disregarding whether the process was aerobic or anaerobic, two-way ANOVA showed that the methanotrophic rates were affected by *site* (P=0.000) and *season* (P=0.000) and the interaction between them (P=0.000). Rates were higher in summer than winter at all sites. However the magnitude of change over the seasons varied across the wetland sites from a 33-fold increase at GREENFIELDS-OUTLET to only a 5-fold increase at GREENFIELDS-INLET. One-way ANOVA and Tukey-Kramer tests showed that the order of the wetland sites differed from the order observed for methane production at the 5 sites (Table 5.7). WILLUNGA-INLET was not always in the highest ranked group and GREENFIELDS-OUTLET was not always in the lowest ranked group, as had been the case for methanogenesis. In summer, rates from GREENFIELDS-INLET were higher than expected, being similar to those from WILLUNGA-INLET. Methanotrophic rates at WILLUNGA-OUTLET were relatively low, being less than those from GREENFIELDS - INLET and -OUTLET. The site order in winter deviated further from the order observed for methane production, with methanotrophic rates at GREENFIELDS-INLET and BOOL-HACKS being the highest. The rates at WILLUNGA-INLET and WILLUNGA-OUTLET were

intermediate and GREENFIELDS-OUTLET displayed the lowest rates of methanotrophy in winter.

Surface waters

The rate of dissolved methane loss in the surface water samples was linear over time for at least the first 4-5 hours of the summer incubations and for the whole 24 hours in winter. Only the linear portions of the regressions were used to calculate the rates of methanotrophy. Average temperature changes during the incubations were 2.5 °C in summer and <1 °C in winter.

In both summer and winter, methanotrophic activity increased with increasing water depth. At 10 cm depth in summer, rates ranged from -0.15 to -1.41 nmol mL^{-1} h^{-1} (Table 5.8) across the wetland sites, but in winter all rates at this depth were below the level of detection (-0.03 nmol mL^{-1} h^{-1} in 24 hour incubation). Summer rates of methanotrophy at 10 cm depth were very similar across all the sites, with the exception of GREENFIELDS-OUTLET (P=0.000) which supported much lower rates (Table 5.8). At 50 cm, the highest rates observed were from the summer WILLUNGA-INLET samples (-7.92 nmol mL^{-1} h^{-1}) and the lowest detectable rates (-0.049 nmol mL^{-1} h^{-1}) were from GREENFIELDS-OUTLET winter samples. The independent effects of *site* (P=0.0001), *season* (P=0.0000) and the interaction between them, *site by season* (P =0.0000) were highly significant on methanotrophy at 50 cm depth. The site order at 50 cm depth in summer was the same as the order for summer methane production in the flocculent sediment. However, in winter, the differences between most of the sites were lost with only GREENFIELDS-OUTLET being lower than the rest.

5.3.4: Methanogenesis and methanotrophy at WILLUNGA-INLET

Rates of planktonic methane production and consumption were determined during the 27 hour period beginning at 1400 h on 1.3.97. Strong summer thermal stratification of the water layers was evident and nocturnal mixing only occurred to a depth of 1m the night before the experiment and to a depth of 0.5 m on the night of the experiment (Figure 5.2). Dissolved oxygen data, taken on 5 occasions during the 27 h period, showed that the oxygen concentration in the surface water layer (1m depth) was greater than 1 mg L^{-1} O_2 at all times and peaked at over 15 mg L^{-1} (Table 5.9). In the metalimnion (between 1 and 2 m), oxygen concentrations fluctuated between 2 and <0.05 mg L^{-1}, and the hypolimnion (2 to 3m depth) remained anoxic, with O_2 concentrations < 1 mg L^{-1} except at 2100 h when 1.2 mg L^{-1} O_2 penetrated to 2.5 m.

In addition to vertical changes in oxygen status, the concentrations of dissolved and total organic carbon in the benthos were higher than at the surface (Figure 5.3). In the top 2 m of the water column, dissolved organic carbon concentrations remained below 32 mg L^{-1} in both WILLUNGA-INLET and WILLUNGA-OUTLET. At 3 m depth, in the floc, DOC concentrations had increased to 44.2 ± 5.2 mg L^{-1} in WILLUNGA-INLET and 35.5 ± 7.1 mg L^{-1} in WILLUNGA-OUTLET. The TOC concentration increased more dramatically with depth, from ≤ 35 mg L^{-1} in the top 2m of both ponds to 491 ± 250 mg L^{-1} in the floc of WILLUNGA-INLET and 250 ± 35 mg L^{-1} in WILLUNGA-OUTLET floc samples.

Methane oxidation was detected at all water depths via loss in dissolved methane concentration over time in the BES-amended samples. At 0.1, 0.5 and 1m depths, control samples (-BES) yielded the same rates of methanotrophy as the +BES samples (see Figure 5.4a for the plot of dissolved methane concentration against time at 0.1 m). At 1.5 m depth, where oxygen concentrations were low but diurnally variable, rates of methane consumption in the control and BES-amended samples were equivalent but much more rapid than in the more shallow layers (refer, Figure 5.4b). However, in the deeper water layers (>1.5 m) the treatments had different effects; control samples produced methane and BES-amended samples consumed methane (see Figure 5.4c for rates in the floc). In water samples from all depths, $HgCl_2$ treatment resulted in no change in dissolved methane concentration over time.

As the water depth increased, rates of both methane production and oxidation increased but so did the rate of net methane production (Table 5.10). Methane oxidation was evident at all water depths, ranging over 4 orders of magnitude, from -0.05 to -140 nmol mL^{-1} h^{-1}. The upper water layers (< 1.5 m) were aerobic yet supported less methane oxidation per unit time and volume than the lower water layers (>1.5 m) that were poorly oxygenated (<1 mg L^{-1}). The rates of methane oxidation were calculated from the first 8 h of the incubations when the rates were linear (r^2 >0.95) and the extant methane supplies were not exhausted.

Methanogenesis became evident in control samples collected from 1.5 m depth and below. These rates also varied over 4 orders of magnitude, from 2 to 1003 nmol mL^{-1} h^{-1}. At 1.5 m depth, the relative rates of methane production and oxidation were very similar (15 vs -13.7 nmol mL^{-1} h^{-1}). Both processes increased in rate with increasing water depth, but the rate of methane production increased more dramatically such that in the floc, the rate of methane production was an order of magnitude higher than the rate of oxidation.

Overall, the water column at WILLUNGA-INLET produced 106 mmol m^{-2} h^{-1} more methane than it consumed. Comparative studies were not performed at WILLUNGA-OUTLET but an estimate of the net methane production can be made from the relative strengths of methane production observed in the two ponds in other experiments. Summer rates of methane production and oxidation in the sediment of WILLUNGA-OUTLET were consistently c. 30% of the relative rates observed in WILLUNGA-INLET. Therefore, if WILLUNGA-INLET produced 106 nmol CH_4 m^{-2} h^{-1} then the net methane production at WILLUNGA-OUTLET would be estimated at 36 nmol CH_4 m^{-2} h^{-1}.

5.4: Discussion

Rates of methane production

In all the field and laboratory trials, the highest rates of methane production were from the WILLUNGA-INLET samples. The second ranked site, WILLUNGA-OUTLET, had rates higher than those from GREENFIELDS-INLET and BOOL-HACKS, which were intermediate, and the lowest rates were from GREENFIELDS-OUTLET. The wetland sites were ranked in the order predicted in Section 1.6, except that rates from GREENFIELDS-INLET were high enough to be similar to or greater than those from BOOL-HACKS. Site differences were more apparent in summer than winter and in the flocculent sediment than the clay but in all cases, methane production rates at Willunga Wetlands exceeded those from Greenfields Wetlands and Bool Lagoon.

In vitro rates of methane production in the wetland sediments varied over two orders of magnitude from 5.3 ± 1.2 to 382 ± 36 nmol mL^{-1} h^{-1}, in control conditions. These data are much higher than the typical values reported for potential methane production in northern hemisphere wetlands and rice paddies which range from 10^{-2} to 101 pmol mL^{-1} h^{-1} (see review in Segers, 1997). However, Bachoon and Jones (1992) report higher than typical rates (1 nmol mL^{-1} h^{-1}) from the surface sediment of a subtropical minerotrophic wetland incubated at 30 °C, and Casper (1996) found rates exceeding 2 nmol mL^{-1} h^{-1} in the profundal zone of an eutrophic lake. Rates of methane production in GREENFIELDS-OUTLET samples (5.3 ± 1.2 nmol mL^{-1} h^{-1}) were the most similar to the those in Bachoon and Jones (1992) and Casper (1996) but rates from the other study sites were up to two orders of magnitude higher (382 ± 36 nmol mL^{-1} h^{-1} at WILLUNGA-INLET, for example). The sediments from Bool Lagoon, GREENFIELDS-INLET and Willunga Wetlands were incubated at 25 °C, 5 °C lower than the incubations in Bachoon and Jones (1992). Therefore differences in incubation temperature are unlikely to explain the differences in methanogenic rate. In addition, Bachoon and Jones (1992)

sampled only the top 2 cm of sediment which is comparable to the flocculent sediment samples taken here, thereby negating sediment depth as an influencing factor.

Potential rates of methane production determined at Ryan's Billabong (18 ± 0.1 nmol mL^{-1} h^{-1}) in spring 1993 (Boon and Mitchell, 1995) were approximately half those reported here for BOOL-HACKS and GREENFIELDS-INLET (sampled in autumn 1997 and spring 1996, respectively). However, Boon and Sorrell (1991) report much higher potential methane production rates (69 to 79 nmol gDW^{-1} h^{-1}) from Ryan's Billabong when sampled in summer. To some extent the differences between rates determined for Australian wetlands and those reported elsewhere may be due to methodological factors (Segers, 1997). In particular, both this study and those conducted at Ryan's Billabong (Boon and Sorrell, 1991; Boon and Mitchell, 1995) routinely amended sediment samples with L-cysteine to ensure anaerobic conditions. It is now known that the L-cysteine additions may have provided an additional methanogenic carbon source and that L-cysteine has been implicated in the cycling of sulphur in anaerobic environments (MacCrehan and Shea, 1995). However, those incubations conducted by Muller et al (1994) at Bool Lagoon did not contain L-cysteine and the measured rates of methane ebullition and total emissions measured at Ryan's Billabong from March 1993 to May 1994 (Boon and Sorrell, 1995) and at Bool Lagoon in summer 1992/1993 (Muller et al., 1994) are among the highest found for any freshwater wetland. This suggests that methane production rates in temperate Australian wetlands are high on a global scale and that any methodological factors are likely to be insignificant.

Even accounting for enhanced methane production rates in southern Australia, globally-speaking, *in vitro* methane production rates at Willunga Wetlands were extremely high; 380 times that reported by Bachoon and Jones (1992) and up to ten times higher than rates observed at Ryan's Billabong, GREENFIELDS-INLET and Bool Lagoon (refer Table 5.2). Methanogenesis at Willunga Wetlands may be more akin to that observed in sewage reactors than in freshwater wetlands. If it were assumed that a) the secondary sewage entering Willunga Wetlands contained 30 mg L^{-1} TSS, which is typical of such wastewaters (Kadlec and Knight, 1996) and b) 80 % of the TSS were volatile (primary sludge data, Li et al., 1996) then based on: methane production rates from anaerobic digestors (Salminen and Rintala, 1999); a flow rate of 4.3 L s^{-1} (Piranti, 1995) and an entry pool area of c. 700 m^2, approximately 50 mmol CH_4 m^{-2} h^{-1} would be produced in the conditions present at Willunga Wetlands. This estimate is very similar to the net rates of methane production calculated for WILLUNGA-INLET and WILLUNGA-OUTLET in Section 4.3.x (106 and 36 mmol m^{-2} h^{-1}, respectively), supporting the suggestion that

methane dynamics in Willunga Wetlands resemble those in sewage reactors, particularly in the highly organic floc of WILLUNGA-INLET.

Temperature had a significant effect on methane production. *In vitro* rates were maximal at 30 °C, substantially lower at 10, 20 and 50 °C and minimal at 5 °C and this response was independent of wetland site and the time of year at which the sediments were sampled. Similar temperature responses have been observed in field samples from Ryan's Billabong (Boon and Mitchell, 1995) and northern hemisphere lake sediments (Winfrey and Zeikus, 1977). However, temperature alone was not responsible for the seasonal changes in rates of methanogenesis. Samples collected in July had lower rates of methane production than their December counterparts when incubated at 30 °C and *in situ* methanogenesis was 13 times higher in summer than winter, when sediment temperatures had increased by 7-10 °C. These data suggest that either the number of methanogens was lower in winter than summer or that the methanogens were limited *in situ* by another temperature-dependent process, such as fermenter activity, in winter. Enumeration of methanogenic populations would assist in answering this question.

Evidence for microbial competition

Methanogenic populations sampled from each of the wetland sites responded differently to the addition of alternative electron acceptors and metabolic inhibitors. The addition of nitrate had the greatest inhibitory effect on all the sediment samples. Inhibition in sediments from Bool Lagoon was strong with nitrate-amended sediments yielding rates only 12 ± 3% of the controls. Sediment samples from Greenfields Wetlands showed a more variable response. At the inlet, nitrate reduced methanogenesis to 25 ± 2 % of the control but at the outlet inhibition was much less marked (59 ± 14 % of the control). Willunga Wetland samples showed only a marginal response to nitrate additions, 88 ± 3 and 71 ± 11 % of the control at WILLUNGA-INLET and WILLUNGA-OUTLET, respectively.

Considering that a resource is defined as being limiting if additional supplies lead to an increase in activity (Begon et al., 1990), these data suggest that the methanogens at Bool Lagoon and Greenfields Wetlands were competing with nitrate-reducing bacteria for limiting substrate supplies in a nitrate-limited environment. Redox potential shifts were not observed in the nitrate-amended samples [as was the case for Wang et al. (1992)] making unfavourable redox conditions an unlikely inhibitory factor. Conversely, the data from Willunga Wetlands would suggest that either the methanogens were not in competition with nitrate-reducers for substrates or that nitrate supply was not limiting nitrate-reduction to the same extent as in the other two wetlands.

In a similar experiment conducted at Ryan's Billabong, Boon and Mitchell (1995) observed strong inhibition in nitrate-amended samples with rates only 8 ± 3 % of the controls (mean ± standard error). In addition, Bollag and Czlonkowski (1973) and Balderston and Payne (1976) found that various nitrogen oxides inhibited methanogenesis in sediments and whole cell suspensions, although they disagreed on the whether oxidation state was an important factor. More recent evidence would suggest that nitrate can be a powerful inhibitor of methanogenesis by stimulating denitrification and reducing the organic substrate and/or H_2 pools for acetoclastic methanogens (Ram et al., 1993; Akunna et al., 1994; Achtnich et al., 1995; Scholten and Stams, 1995; Banik et al., 1996; Drake et al., 1996). This proposal is strengthened by the transient nature of the nitrate inhibition which can be relieved after nitrate depletion or H_2 addition (Akunna et al., 1994; Achtnich et al., 1995). Denitrifiers, as a functional group, are able to consume a wide range of substrates (Delwiche and Bryan, 1976) but it appears that in the above studies, denitrifiers were utilising substrates that otherwise may have been available for methane production. Willunga Wetland samples were the exception, displaying only marginal nitrate inhibition. This may be due to a lack of substrate competition in this very organic-rich, sewage-fed system, akin to the anaerobic digestors studied by Ueki and Ueki (1990).

Alternative explanations may be that 1) the added nitrate was consumed in non-biological redox reactions or 2) nitrate additions stimulated anaerobic methanotrophy. Aerobic methanotrophs require an external source of nitrogen (Kiene, 1991). If this were also true for anaerobic methanotrophs then the added nitrate may have increased the rate of methanotrophy rather than decreased the rate of methanogenesis. The competing processes of methane production and consumption were confounded in the experimental design employed in this project because changes in dissolved methane concentration over time were the only measure of both methane production and consumption rates. A different approach in which a suite of methods including i) ion-selective sensors for core and *in situ* measurements; ii) *in situ* incubation techniques with isotopic tracers and benthic chambers; and iii) molecular genetic methods such as polymerase chain reaction of DNA and microbial activity measured with mRNA were employed is required to determine independent rates of methane production and consumption.

In a similar way, the effects of sulphate addition on methanogenesis were far less apparent at Willunga Wetlands than in samples from the other two wetlands. However, molybdate was very effective at Willunga Wetlands, enhancing methane production in WILLUNGA-INLET samples to 158 ± 56 % of the control. At WILLUNGA-OUTLET a similar

pattern emerged but the effect was less marked. Sulphate additions did not significantly affect the rates of methane production (91 ± 9 % of the control), even though SO_4^{2-} concentrations were 10mM, much greater than the threshold reportedly required for maintenance of sulphate-reducing zone (Lovely and Klug, 1986). Accordingly, molybdate only increased methanogenesis to 122 ± 23 % of the control. These data suggest that at Willunga Wetlands, the methanogens were competing with sulphate-reducing bacteria at non-limiting sulphate concentrations, as sulphate additions were ineffectual but molybdate appeared to relieve some competition. Competition between sulphate-reducers and methanogens in sewage sludge appears to be for H_2 utilisation as the two functional groups utilise different organic substrates (Ueki and Ueki, 1990). This may also be the case at Willunga Wetlands. However, the interactions between sulphate-reducers and methanogens can influence the rate of methane oxidation (Hoehler et al., 1996) as well as the rate of methane production, and sulphate-reducers can both produce and consume acetate (Krumbien, 1983). Therefore the interpretation of these data is more complex than a simple model of competition for limiting substrates, especially if anaerobic oxidation were also occurring in these samples (see below).

At Greenfields Wetlands, the sulphate and molybdate treatments clearly indicated competition between methanogens and sulphate-reducing bacteria. Sulphate additions reduced rates to c. 80% and molybdate increased rates to 220% or more of the control. The larger effect of molybdate suggests that the extant sulphate concentrations at Greenfields Wetlands were supporting some sulphate-reduction. In summer, electrical conductivity readings at Greenfields Wetlands could exceed 5000 mg L^{-1} (Cox, 1993). It is uncertain how much of the conductivity is due to sulphate ions *per se* but the experimental evidence above suggests that there is enough sulphate to support substantial sulphate-reduction. The results from Bool Lagoon were less clear. Molybdate additions stimulated methane production but so did sulphate additions, albeit at a lower level. These results are similar to those reported for incubations of sediment from Ryan's Billabong, where both sulphate and molybdate enhanced methane production rates. It is likely that in these natural wetlands, sulphate reducers were competing with the methanogens, as this has been observed in similar freshwater sediments and rice paddies (Winfrey and Zeikus, 1977; Lovley and Klug, 1986; Brandl et al., 1990; Delwiche and Cicerone, 1993; Achtich et al., 1995) but these treatments do not unequivocally support this hypothesis.

Substrate limitation

Methane production in the flocculent sediment was always higher than comparable rates in the clay sediment layers as was the case for Ryan's Billabong as well (see Boon and

Sorrell, 1991). Considering that the water column supplies the sediment with organic substrates, this observation suggests that the depth dependence was due to substrate limitation *per se* or limiting rates of substrate diffusion. Furthermore, organic substrate additions, either directly as acetate or cellobiose or indirectly via increased numbers of fermenters, enhanced methane production in flocculent sediment samples from all the sites, suggesting that even methanogens in the very surface sediment were substrate-limited.

Willunga Wetlands were the least affected by organic substrate additions. At WILLUNGA-INLET, acetate and cellobiose both increased methanogenesis to 125 – 130 % of the control, whilst addition of fermenters with or without added cellobiose yielded rates c. 150 % of the control. The higher rates observed when fermenters were added regardless of additional cellobiose, would suggest that the methanogens in WILLUNGA-INLET were limited by substrate supply from the fermenters and not carbon content *per se*. This is supported by the very high incoming carbon loads and the accumulation of TOC in the benthos (incoming concentration of 35 mg L^{-1} vs floc concentrations > 500 mg L^{-1}). WILLUNGA-OUTLET samples responded more strongly to the addition of substrates or fermenters. These amended samples yielded rates 190% or greater than the control.

Rates of methane production in WILLUNGA-OUTLET samples could be increased to that observed for WILLUNGA-INLET control samples when both cellobiose and fermenters were added together, indicating a limitation for carbon *per se*. These data suggest that the methanogens at the inlet to the sewage treatment wetland were limited by the supply of substrates from fermentative bacteria rather than the supply of organic carbon. As flow path length increased so did the apparent limitation by carbon content *per se* even though a concurrent decrease in surface water DOC and TOC content was not detected (see Chapter 3). However, the DOC and TOC concentrations in the floc of WILLUNGA-OUTLET were lower than in WILLUNGA-INLET, suggesting that the carbon supply was limiting the wetland system as a whole.

The methanogenic population from Greenfields Wetlands also showed carbon limitation. The addition of acetate and cellobiose increased methanogenesis by more than 360 % of the control at both the inlet and the outlet. Fermenters when added alone stimulated methane production less than the addition of direct substrates but the greatest stimulation was observed when both fermentative bacteria and cellobiose were added. In contrast to the situation at Willunga Wetlands, the carbon limitation at Greenfields Wetlands seems to be due to a lack of sediment carbon content *per se*, because

increasing fermenter activity without increasing the carbon content did not stimulate methane production as greatly as adding both fermenters and cellobiose or as adding just organic substrates alone.

The methanogenic response at Bool Lagoon differed from the response at both Willunga and Greenfields Wetlands. The addition of acetate or cellobiose to sediment samples increased methane production by more than 200% of the control. However, the addition of fermenters increased methane production to c. 430 % of the control whether or not cellobiose was also added. This indicates that the organic sediment at Bool Lagoon supplied adequate carbon for the additional fermenters. The studies at Bool Lagoon were performed in autumn when leaf drop at Bool Lagoon would have been at its annual peak (Rea and Ganf, 1994) supplying fresh plant detritus to the sediment. This further supports the suggestion that it was fermenter activity rather than organic carbon content *per se* that limited methane production at Bool Lagoon.

The magnitude of the response to fermenter and substrate additions at Bool Lagoon was greater than that at Willunga Wetlands even though the sediment carbon content at Bool Lagoon was much higher than at Willunga Wetlands. This suggests that the organic carbon reaching the sediment at Bool Lagoon may have been more refractile than the carbon at Willunga Wetlands, rendering it less readily available for methanogenesis. This is supported by evidence that the organic carbon at Willunga Wetlands was highly labile (DOC comprised 95 % of the TOC) and by the models of complex plant detrital decomposition in wetlands (Godschalk and Wetzel, 1978 a, b and c) that would suggest that the bulk of the extant sedimentary organic carbon at Bool Lagoon would be refractile. Fermenter additions were not made at Ryan's Billabong, so direct comparison cannot be made. However, methane production at Ryan's Billabong was enhanced by acetate and starch additions indicating that the methanogens in this natural wetland were also carbon limited (Boon and Mitchell, 1995). Whether this is due to fermenter activity or organic carbon content *per se* is unclear.

Methanotrophy in the sediments

Methane consumption varied from 285 ± 34 to 562 ± 47 nmol mL^{-1} h^{-1} in summer to 8.7 ± 1.9 to 92.6 ± 18 nmol mL^{-1} h^{-1} in winter in the sediments of the three wetlands. Methane was consumed most rapidly at the inlets to Willunga and Greenfields Wetlands in summer and at GREENFIELDS-INLET and Bool Lagoon in winter, although the overall variation in rates of methanotrophy was much lower than the variation in *in situ* methanogenesis in the floc samples (0.87 ± 0.1 to 605 ± 61 nmol mL^{-1} h^{-1}). *In situ* rates of methanotrophy have not been measured for the sediment at Ryan's Billabong but

potential (*in vitro*) rates ranged from 50 to 450 nmol mL^{-1} h^{-1} (Boon and Lee, 1997). Unlike rates of methane production in Australian wetlands, the rates of sediment methanotrophy observed at the study sites and Ryan's Billabong are similar to those reported for other wetlands. In the Florida Everglades rates of methane consumption were 300 to 700 nmol gDW^{-1} h^{-1} (King et al, 1990b), in subarctic and temperate wetlands in Canada rates reached 19 nmol g^{-1} h^{-1}, Bender and Conrad (1994) found rates of 100 to 220 nmol gDW^{-1} h^{-1} in rice paddy soils amended with 20 % methane and Roslev and King (1996) report rates of 90 to 760 nmol mL^{-1} h^{-1} in a freshwater marsh. However, in many systems the potential rates of methane consumption are higher than the rates of production.

In two papers covering a range of Canadian wetlands, including very rich fens and temperate swamps, Moore and Knowles (1987 and 1990) found the rate of methanogenesis to vary from 1 to 182 nmol g^{-1} d^{-1} and the rate of methanotrophy, from non-detectable levels to 16 µmol g^{-1} d^{-1}. Similarly, Nedwell and Watson (1995) and Krumholz et al. (1995) report methane consumption rates one to two orders of magnitude higher than the comparable rates of methane production. The situation was the same for Bool Lagoon and Greenfields Wetlands, where sediment consumption was potentially far more rapid than production and the researchers at Ryan's Billabong report the same (Boon and Mitchell, 1995; Boon and Lee, 1997). Willunga Wetlands was the exception, with rates of methane production in the floc layers equivalent to rates of methane consumption in the floc during the interwetland *in situ* trials and evidence that methane production may be an order of magnitude higher in the floc layers during the whole water column study at WILLUNGA-INLET.

The studies by Moore and Knowles (1987 and 1990) and Boon and Lee (1997) compared *in vitro* methanogenic and methanotrophic activities. Determining whether the rates of methane oxidation would be higher or lower *in situ* is difficult as, in an undisturbed sediment, the methanotrophs would probably experience lower oxygen concentrations but higher methane concentrations, thereby respectively, decreasing and increasing methane consumption rates. Other studies determining the percentage of methane consumed in the sediments have compared atmospheric methane emissions under oxic and anoxic conditions. Variously, they report methane consumption to intercept 33 to 81 % of the methane produced (Conrad and Rothfuss, 1991; Happell and Chanton, 1993; Bosse and Frenzel, 1998). The studies comparing oxic and anoxic fluxes will always return a methane consumption rate less than 100% of methane production by the nature of their design. Further, all of these methods assume that methane production is an exclusively anaerobic process and methane consumption is

exclusively aerobic. However, this may not be the case for Willunga Wetlands, where significant methane consumption occurred in the sediment and benthic water layers under highly reducing and oxygen-poor conditions, suggesting that methane consumption was also an anaerobic process. Anaerobic methanotrophy may be more prevalent than commonly thought and may occur in most methanogenic habitats, effectively undetected by current methodologies that are typically looking for methanogenesis in anaerobic conditions by observing changes in methane concentrations over time.

Evidence for anaerobic methanotrophy at Willunga Wetlands

In designing the experiments to test which factors controlled *in vitro* methanogenesis (eg. sulphate and acetate additions), it was assumed that methanotrophy would be exclusively aerobic and therefore would be inhibited in the strictly anaerobic incubation conditions and would occur at negligible rates. Therefore an increase in headspace methane concentration over time would represent gross methanogenesis and an increase in rate above the control would represent an increase in methanogenic activity due to the treatment. This may have been the case for sediment samples from Greenfields Wetlands and Bool Lagoon where the floc layers were directly supplied with oxygen from the shallow oxic water and there was no evidence for methanotrophy in floc samples that had not been amended with oxygen. However in WILLUNGA-INLET samples, methanotrophy was evident at very low extant oxygen concentrations (less than 1 mg L^{-1} oxygen) and in highly reducing conditions (see Chapter 3).

It is thermodynamically possible to oxidise methane anaerobically with the alternative electron acceptors considered to inhibit methanogenesis but anaerobes capable of doing this have never been isolated (Conrad, 1996; Hinrichs et al., 1999) and the mechanism by which the very stable methane molecule is activated is not known. Strong evidence is available to suggest that in marine systems, anaerobic methanotrophy is co-incident with sulphate-reduction (Devol, 1983; Devol et al., 1984; Alperin and Reeburgh, 1985; Iversen and Jorgensen, 1985). Other studies suggest that novel, methanotrophic archaea exist near marine, methane seeps (Hinrichs et al., 1999). In freshwater systems, anaerobic oxidation has been detected at 0.5 mM SO_4^{2-} but not at concentrations below 0.2 mM (Panganiban et al., 1979; Yavitt et al., 1988; Nedwell and Watson, 1995). Panganiban et al. (1979) could not find evidence for anaerobic consumption at any nitrate concentration in lake water but ferric iron may also be involved in anaerobic methanotrophy in rice paddy soil (Miura et al., 1992).

Hoehler et al., (1994) postulated that methane consumption in anoxic marine sediments occurs via a methanogen-sulphate reducer consortium. The model suggests that methanogens oxidise methane and produce hydrogen by reversing the direction of the CO_2 reduction pathway (reverse methanogenesis, see Chapter 2.4.4 for more details). This occurs when hydrogen is sufficiently removed by the sulphate-reducers to a partial pressure where oxidation of methane is favourable for growth. This model can work for other electron acceptors (eg. Fe^{3+} and NO_3^-) and may explain the operation of the denitrifier-methanogen consortium observed by Thalasso (1997) in batch reactors.

Generally though, anaerobic methanotrophy is not considered a major route for methane in freshwater wetlands, although electron acceptors for the syntrophic processes can be available and may induce apparent methane inhibition. In rice paddy soil, Murase and Kimura(1996) found that anaerobic methane oxidation accounted for < 5% of methane emissions during the whole growing season. This may be the case for freshwater wetlands (eg. Greenfields Wetlands, Bool Lagoon and Ryan's Billabong) but if the oxidation observed in the anoxic waters of Willunga Wetlands is a truly anaerobic process, it appears to account for more methane than that observed by Murase and Kimura (1996) considering that the rates of anaerobic methane oxidation were up to two orders of magnitude greater than the rates of aerobic methane oxidation at WILLUNGA-INLET.

The evidence for anaerobic methanotrophy at WILLUNGA-INLET came from *in situ* incubations of anoxic, highly reducing (c. -350 to -400 mV) sediment and water samples in the presence of BES. BES is an analogue of the CoM enzyme and inhibits the reductive demethylation of methyl CoM (Kaesler and Schönheit, 1989b). It is thought to be a specific metabolic inhibitor of methane-producing bacteria, the only known carriers of CoM (Zeikus, 1977). Therefore if anaerobic methane oxidation is facilitated by reverse methanogenesis, it should also be inhibited by BES. However, the Willunga Wetlands samples exhibited methane consumption in the presence of BES, suggesting that the oxidation was facilitated by aerobic methanotrophs. This is unlikely. The redox potentials in the hypolimnion and sediments was less than -350 mV at all times during the year (Chapter 3) and as molecular oxygen is considered to be exhausted at redox potential of +200mV (Ponnamperuma, 1972), this environment could be considered anoxic although the sensitivity of the oxygen probe used was lost below c. 1.5 mg L^{-1}. It could be that the redox probe was too insensitive to detect aerobic microsites in the benthos within which aerobic methanotrophs were active or that benthic photosynthesis were occurring, supplying the aerobic methanotrophs with oxygen. However, the water column at Willunga Wetlands was permanently stratified during summer and oxygen

concentrations >1 mg L^{-1} rarely penetrated the surface 2 m of water. In addition, benthic photosynthesis was an unlikely source of oxygen as the Kd values calculated in Chapter 3 would suggest that photosynthesis was inactive below 35 cm even though chlorophyll was detected down to 2 m. Contamination during sample preparation may have been a source of oxygen but this is also unlikely as the water never came into contact with an aerobic atmosphere during sample preparation and the treatment solutions were prepared with deoxygenated water. Considering also that methane was not lost by non-biological processes (see $HgCl_2$ treatments), it appears that the methane oxidation observed in the presence of BES was anaerobic in nature.

There is evidence for competition between methanogens and other anaerobes at Willunga Wetlands, therefore potential exists for the formation of syntrophic partnerships as described by Hoehler et al. (1994) and Thalasso (1997). Anaerobic methane oxidation may be detected in the presence of BES if the inhibition were incomplete or had a differential effect on specific methanogens. Both Zehnder and Brock (1979) and Hoehler et al. (1994) found that BES only partially inhibited methane oxidation at concentrations of 60 mM or less. Complete inhibition was seen at BES concentrations of 120 mM (Hoehler et l., 1998). Methanogens in nature require these high levels of BES perhaps due to differential membrane permeability (Oremland and Capone, 1988) or sequestering of BES in the sediment environment.

The incubations at Willunga Wetlands were performed *in situ*, without continuous shaking and at a final BES concentration of 80 mM. If the anaerobes had formed aggregates these would still be intact during the incubation. Such aggregates would have a predictable spatial organisation of anaerobic functional groups based on cascading redox potentials (see Section 2.3). The facultative anaerobes, such as the fermenters, would inhabit the outer regions of the aggregate and the obligate anaerobes would reside in the interior, sequestering substrates from syntrophic associations with the fermenters. It follows then that the acetotrophs/hydrogentrophs will occur on a spatial gradient, with the most competitive strains nearer the outside of the aggregate and the least competitive strains in the centre of the aggregate. This same pattern may be inferred on the methanogenic species as well. Given that very low H_2 concentrations are the key factor inducing reverse methanogenesis (Thalasso, 1997) and that the methanogens in the centre of the aggregate are more likely to experience very low H_2 partial pressures, the central methanogens are more likely to be consuming methane than producing it. If BES were added to water or floc samples containing intact aggregates, it may only penetrate the outer layers or its concentration may be dramatically reduced within the aggregate. As such, it would preferentially inhibit the

methanogens towards the outside, those that are more likely to be undergoing methanogenesis than reverse methanogenesis. The result, when only monitoring dissolved methane concentrations over time, would be a net decrease in methane concentration as was observed in the BES-amended samples from the anoxic zones of Willunga Wetlands.

The anaerobic methanotrophy rate data were calculated from the first 8 hours of the 27 hour incubations. If the BES were acting to preferentially inhibit the methanogens employing methanogenesis on the outer edge of the aggregate, it would be expected that the H_2 concentration inside the aggregate would increase during the course of the incubation. As such the methanogens in the centre of the aggregate may switch from reverse methanogenesis to methanogenesis during the incubation thereby producing rather than consuming methane. This was not observed. It may be that novel archaea, occur in Willunga Wetlands that are able to consume methane in the presence of BES but this hypothesis awaits further investigation.

If syntrophic methanotrophy were occurring via reverse methanogenesis in the anaerobic incubations, the earlier interpretations of the effects of alternative electron acceptor and substrate additions are severely complicated. An increase in rate above the control may indicate that the treatment caused either an increase in the rate of methane production or a decrease in the rate of methane oxidation. The reverse is true for a decrease in rate relative to the control. Therefore the effect of the various treatments separately on methane production and oxidation cannot be determined from this experimental design, hence its reliance on the anoxic inhibition of exclusively aerobic methanotrophy. To understand the interactions between the anaerobes at Willunga Wetlands would require thorough autecological studies and the application of radiolabelled tracer techniques as described in the interpretation of the nitrate-amended rate data above.

Methanotrophy in the surface waters

Although the rates of benthic methane consumption were equal to or greater than the rates of benthic production, methane was released into the water column and was also consumed in the water column. Aerobic methanotrophy in the surface waters (to 50 cm depth for Willunga Wetlands sites) ranged from 0.049 ± 0.01 to 7.92 ± 0.67 nmol mL^{-1} h^{-1}, increasing in summer and with water depth. In summer, methanotrophy at 10 cm depth was similar at all sites except GREENFIELDS-OUTLET where it was approximately an order of magnitude lower than at the other sites. However, by 50 cm depth the observed rates of methanotrophy were in the same relative site order as that

determined in the benthic methanogenesis trials. Once again the overall variation in the rates of methanotrophy were much lower than the variation in methanogenic rates.

The methanotrophic activities in the aerobic surface waters were very similar across the wetland sites but the water column at Willunga Wetlands was much deeper than at the other two wetlands and an anoxic hypolimnion developed. The daily mixing depth at Willunga Wetlands was 1 to 1.5 m depth over summer. Oxygen concentrations decreased from supersaturated levels at the air-water interface to hypoxic levels at approximately 1 to 1.5 m depth (see Chapter 3 for detailed discussion). However, methanotrophy was evident at all water depths in WILLUNGA-INLET at the end of summer and instantaneous rates were highest in the anoxic water layers.

The thermal character of WILLUNGA-INLET was similar to that of shallow lakes which generally show a stratification of methanotrophic activity peaking at the oxic/anoxic interface where methane and oxygen co-exist (see Hanson and Hanson, 1996 for review). The profiles of methane oxidation differ across lakes depending on the oxygen status of the hypolimnion. Most studies have been performed on eutrophic lakes with either permanent or seasonally anoxic hypolimnions (Rudd et al., 1976; Harrits and Hanson, 1980; Iversen et al., 1987). In these systems, methane that is produced in the benthos is liberated into anoxic water and mostly consumed at the oxic/anoxic interface within the water column (Jannasch, 1975; Rudd and Hamilton, 1975; Harrits and Hanson, 1980; Scranton et al., 1993). Conversely, the methane produced in a lake without an anoxic hypolimnion is liberated into the oxic surface sediment layers where it is partially consumed by aerobic methanotrophs (Lidstrom and Somers, 1984; Kuivila et al., 1988; Frenzel et al., 1990; King et al., 1990b). Only the residual methane enters the water column where methanotrophy is controlled by the co-existence of oxygen and methane (Utsumi et al., 1998).

WILLUNGA-INLET appears to behave like an eutrophic lake with an anoxic hypolimnion. However, unlike other such systems (Iversen et al., 1987), significant methane oxidation was evident in the surface oxic water of Willunga Wetlands. Iversen et al. (1987) found that methane consumption in the anaerobic zones of Big Soda Lake dominated methane dynamics causing a net consumption in the hypolimnion. At WILLUNGA-INLET, methane production exceeded anaerobic oxidation measured in the BES-amended samples. As discussed above, this technique is not adequate for determining rates of anaerobic methane oxidation, thus anaerobic oxidation rates at Willunga Wetlands may be considerably higher. However, methane concentrations just below the air/water interface could reach 280 µg L^{-1} in WILLUNGA-INLET and surface rates of methanotrophy

exceeded 7 nmol mL^{-1} h^{-1}. This suggests that there was a net production of methane in the whole water column as the rates of methane oxidation were high, yet methane accumulated in the surface waters. By comparison, Iversen et al. (1987) report surface methane concentrations of 0.16 µg L^{-1} and very low rates of methane oxidation in the oxic waters of Big Soda Lake.

5.5: Conclusions

- Methane production rates ranged over four orders of magnitude and were ranked in the wetland order predicted. Rates from GREENFIELDS-INLET were higher than predicted and equalled those from Bool Lagoon, therefore the observed wetland site order was:

  WILLUNGA-INLET>WILLUNGA-OULET>BOOL-HACKS=GREENFIELDS-INLET>GREENFIELDS-OUTLET

  It is likely that the relatively high rates of methanogenesis observed at GREENFIELDS-INLET was supported by the incoming organic carbon rather than organic carbon in the sediments. This is supported by the relatively low rates of methanogenesis at GREENFIELDS-OUTLET and the relatively low organic carbon content of the sediments compared to the sediments at Bool Lagoon (Chapter 4).

- Methanogenesis occurred more rapidly at these wetlands than has been commonly observed in other freshwater wetlands. However, rates from GREENFIELDS-INLET and BOOL-HACKS were similar to those measured in another Australian wetland, Ryan's Billabong, suggesting that observed methane production rates in Australian wetlands are higher than those for other global regions.

- Methane production rates were higher in summer than winter and in samples of flocculent sediment than clay, irrespective of treatment or wetland site. There was also a decrease in the rate of methane production with increasing flow path length in the constructed wetlands. These data suggest that methane production was limited by organic carbon supplies on a whole wetland perspective.

- *In vitro* incubations suggest that the methanogens at Greenfields Wetlands were competing with denitrifiers and sulphate-reducers in an environment limited by alternative electron acceptor availability. However, the experimental techniques were unable to differentiate between anaerobic methanogenesis and methanotrophy which may affect this interpretation. In addition, methanogenesis

was limited by the amount of organic carbon in the sediment. Nevertheless, methane production rates at the inlet were higher than expected and were similar to those from an ancient natural wetland, Bool Lagoon.

- Competition between methanogens and denitrifiers was evident at Bool Lagoon but competition with sulphate-reducers was not clearly indicated. Methanogens at BOOL-HACKS were limited by fermenter activity rather than the organic carbon supply *per se*.

- Additional supply of alternative electron acceptors did not affect methanogenesis at Willunga Wetlands but net methane production was greater in the presence of molybdate, a specific inhibitor of sulphate-reducing bacteria. At the inlet, organic carbon was sufficient to support additional fermenter activity but the extant rate of fermentation limited methane production. As flow path length increased, limitation by organic carbon *per se* became evident. However, the apparent oxidation of methane under anoxic conditions renders these findings insubstantial. Without being able to differentiate between the competing processes of methane production and oxidation in anoxic incubations no further comment on the factors controlling net methane production can be made.

- Methane dynamics at Greenfields Wetlands and Bool Lagoon followed the generally accepted model for freshwater wetlands. Methane was produced in the sediments and was consumed aerobically at the sediment surface and in the water columns. No evidence for anaerobic oxidation was detected at Greenfields Wetlands or Bool Lagoon with the available techniques.

- At Willunga Wetlands, methane was produced and consumed in the sediment and water layers. Methane production only occurred in the anoxic zones but methane was consumed in oxic and anoxic water samples in the presence of BES. The results of the competition experiment would suggest that anaerobic methanotrophy was facilitated by either a sulphate-reducer-methanogen or denitrifier-methanogen consortium or both. The presence of novel, anaerobic methanotrophs may be an alternative explanation. Autecological studies and incubations with radiotracers would be required to advance these investigations.

- The potential for methane oxidation exceeded the potential for methane production in the sediments of Greenfields Wetlands and Bool Lagoon. Therefore at these two wetlands, the mass balance for methane production and oxidation was negative. Nevertheless, methane concentrations in the water column were greater

than expected from air equilibration and significant aerobic methanotrophy was detected in the water column.

- Willunga Wetlands produced more methane than was consumed within the water and sediment layers. Net methane production was estimated at 106 and 36 mmol m^{-1} h^{-1} for WILLUNGA-INLET and WILLUNGA-OUTLET, respectively. However, these calculations assumed that the *bona fide* rates of anaerobic methanotrophy were observed when in actuality the rates may be well underestimated and the net methane gain may be considerably lower.

Chapter 6

Chapter 6: Methane emissions

6.1: Introduction

The rates of methane production reported in Chapter 5 varied across the five wetland sites. Methanogenic activity decreased in the site order predicted in Chapter 1 except that GREENFIELDS-INLET had methane production rates equivalent to those from Bool Lagoon and was, therefore, advanced in the sequence, which otherwise agreed with the erected hypothesis (see Section 1.6). Given that methanogens are the source of methane for atmospheric emissions from wetlands (Segers, 1997), it follows that rates of methane emission from these five wetland sites will be ranked in the same site order as the rates of methanogenesis. Similarly, it is expected that summer emissions will be greater than winter emissions because of the differential rates observed in seasonal methanogenesis.

The relative rates of methanogenesis and methanotrophy are also likely to influence the rate of methane emission. At Willunga Wetlands, the potential for methane production far exceeded that for methane oxidation, whilst at Greenfields Wetlands and Bool Lagoon, the opposite was true. This further strengthens the hypothesis that methane emissions from Willunga Wetlands will be the highest of the three wetlands. Greenfields Wetlands and Bool Lagoon are expected to release less methane than Willunga Wetlands but the emissions are expected to be significant even though potential methanotrophy exceeded potential methanogenesis because:

1) methane emissions ranging from 0.42 to 1.05 mmol m^{-2} h^{-1} were observed from vegetated and unvegetated areas of the Bool Lagoon system in summer 1993/1994 near site BOOL-HACKS (Muller et al., 1994);

2) potential methane oxidation has been reported to exceed potential methane production in a range of wetland systems that have net methane emissions to the atmosphere (Moore and Knowles, 1987; Krumholz et al., 1995; Nedwell and Watson, 1995); and

3) up to 95% of total methane emissions from natural open water areas is via ebullition (Sorrell and Boon, 1992; Weyhenmeyer, 1999) and methane trapped in bubbles is effectively unavailable for methanotrophy.

Therefore it is expected that some methane will be lost to the atmosphere from inundated methanogenic wetlands regardless of the relative rates of methanogenesis and methanotrophy.

With respect to their potential for methanogenesis the five wetland sites were ranked as follows in both summer and winter:

WILLUNGA-INLET > WILLUNGA-OUTLET > GREENFIELDS-INLET > BOOL-HACKS > GREENFIELDS-OUTLET

The experiments presented in the chapter below were designed to test if the observed rates of methane emission were also ranked in this site order. Spring and autumn emissions were also included in the design to aid formulation of methane inventories as requested by the granting body (National Greenhouse Gas Inventory Commission, Department of Sport and Territories, hereafter NGGIC). It was expected that the spring and autumn emissions would lie between the respective summer and winter emission estimates, primarily as a function of the effect of temperature on methanogenesis (Section 2.1.4). In addition, these experiments were designed to determine:

- whether the static chamber technique was sufficiently robust;
- what level of temporal and spatial variation existed in the emissions at a range of scales;
- which parameters best correlated with methane emissions; and
- what factors controlled methane emissions at each site.

6.2: Methods

6.2.1: Robustness of the static chamber technique

The first test was to determine if the chambers were gas-tight. All six chambers were floated on an unvegetated constructed pond filled with aged tap water at The University of Adelaide, South Australia. A known volume of pure methane was added to each chamber to increase the internal methane concentration above ambient (1.7 ppm) to approximately 600 ppm in the first trial and 3000 ppm in the second trial. These target concentrations were chosen to reflect the expected final methane concentrations after short (less than 3 hours) and long (up to 24 hours) deployment periods in the field. Triplicate gas samples were taken from the chambers at 0, 2, 8, 12, 24, 48 and 72 hours. The samples were analysed immediately by gas chromatography in the adjacent laboratory.

One-way ANOVAs were performed to test the effect of *time* on the concentration of methane in the chambers. Water samples were taken from the surface of the pond (0.1 m depth) at 0 and 72 hours. Dissolved gases were extracted from the water and analysed for methane content (as described in Chapter 3) as a further test of whether the chambers had leaked. One-way ANOVAs were performed to test the effect of *time* on concentrations of methane in the surface waters. The ANOVAs for the gas and water samples were performed using JMP Version 3.2.

The second test was designed to determine whether the vacutainers used to collect and store gas samples leaked during typical storage periods of up to 10 days. Ten replicate vacutainers were filled with gas to target methane concentrations of 1.7, 100 and 3000 ppm. The first ten were filled with atmospheric air (approximately 1.7 ppm), the second ten with 100 ppm methane and the third set with 3000 ppm methane. The vacutainers were kept on ice for the first 24 hours in a field esky (insulated plastic box) under the shade of a tree before being transferred to the laboratory refrigerator for the next 9 days storage. This programme was designed to simulate routine field storage conditions. Gas samples were taken directly from the vacutainers with a 2 mL gas-tight syringe at 0, 0.5, 4 and 10 days and analysed for methane content by gas chromatography. The data cells were tested for normality with Shapiro-Wilk tests ($W>0.05$) before the effect of *storage time* was analysed by one-way ANOVA on the data sets for each target concentration using JMP Version 3.2.

In the third test, the rate of methane release into the chamber from a methanogenic water body was examined for linearity over time. High resolution data sets were generated by taking samples every two minutes for 70 minutes from 3 chambers deployed adjacent to each other at WILLUNGA-INLET in spring 1994. These samples were analysed for methane content by gas chromatography. Plots of methane concentration by time were generated and regression analyses performed using Excel Version 5.0 and JMP Version 3.2.

Fourth, the effects of physically disturbing the chambers were also examined. In one trial, three chambers were deployed 5 m apart for 250 minutes in the shallow area (62 cm water depth) of Pond 1 at Willunga Wetlands, adjacent to the WILLUNGA-INLET site. One of the three chambers was physically rocked back and forth for 2 minute periods beginning at 100 and 165 minutes. Another chamber was disturbed by turning the fan, which circulates the air inside the chamber, off at 120 minutes and on again at 190 minutes. The third chamber was left undisturbed with its internal fan running for the entire period. Triplicate gas samples were taken at 0, 90, 160 and 250 minutes and

analysed for methane content by gas chromatography. Plots of methane concentration by time were generated and regression analyses performed using Excel Version 5.0.

In another trial, two chambers were deployed 5 m apart and the sediment surrounding one of the chambers was trampled. The trampling was sustained for 3 minutes beginning at the 45 minute mark of a 120 minute deployment. Triplicate gas samples were taken at 0, 20, 40, 60, 80, 100 and 120 minutes and analysed for methane content by gas chromatography. Plots of methane concentration by time were generated and regression analyses performed using Excel Version 5.0.

The objective of the fifth test was to determine how many replicate chambers were needed to adequately describe the bona fide methane emissions and their intrinsic variability. Six chambers were deployed at WILLUNGA-INLET in spring 1994 for three 90 minute periods in one day beginning at 1000, 1145 and 1430 h. Eighteen separate emission estimates were thus generated and randomly chosen to create sets of data with increasing numbers of replicates from one to 18. The mean and standard deviation values obtained for each data set were plotted against the number of replicates used (using Excel Version 5.0) and the plots were visually examined to determine the minimum number of replicates required.

The sixth test was designed to assess the effect of increasing deployment time on methane emission estimates. Six chambers were deployed concurrently at WILLUNGA-INLET on 14 and 15 August 1996 for a total period of 25 hours. Triplicate gas samples were taken from the chambers at 0, 20, 40, 60, 80 and 100 minutes to generate a plot of methane concentration over time for the first 100 minutes. Subsequent samples were taken at 4, 18, 21 and 24 hours to generate plots of methane concentration over time for the whole 24 hour period to compare with those from the first 100 minutes. The effect of increasing deployment time was statistically analysed by comparing the ranges, means, standard deviations and coefficients of variance associated with the rates (methane concentration by time) generated after 100 minutes and 24 hours of deployment (using JMP Version 3.2). The relevant ANOVA results appear in Appendix 1.3.

6.2.2: Temporal and spatial variations in emissions

Temporal and spatial variations in methane emissions were examined at the five wetland sites at a range of scales between August 1994 and March 1997. A total of four hundred and eighty-two emission estimates were generated using the static chambers. The majority of these were conducted as part of the regular monitoring programme designed to satisfy both the experimental requirements to meet the objectives stated

above and the granting requirements of NGGIC. For these dual purposes, routine monitoring of methane emissions was conducted at each wetland site on five randomly selected days in winter and summer and on three randomly selected days in spring and autumn. Emissions were estimated in nine consecutive seasons from summer 1994/1995 to autumn 1997.

In 1996 and 1997, the days randomly selected for emission estimates in summer and winter were also used to conduct methane production and oxidation measurements (see Chapter 5). Emission estimates made during spring and autumn were often used for specific investigations such as determining the robustness of the chamber technique or the effect of water level on emissions at Willunga Wetlands (see below). A minimum of six chamber deployments was made at each site in the constructed wetlands in each season but more commonly nine to twenty-one deployments were conducted. Unfortunately, the logistical difficulties associated with travelling to Bool Lagoon restricted the number of trips that could be made to five seasons within the 28 month period, namely: summer 1994/1995, winter 1995, summer 1995/1996, autumn 1996 and autumn 1997.

During the period August 1994 to August 1996, the chambers were deployed for less than 2 hours each time (six replicates). After August 1996, when the studies on the effect of increasing deployment time were conducted and the time increases found to be robust, the chambers were routinely deployed for 24 hour periods (1-3 replicates). Chambers that were deployed for two hours or less were only deployed between 1000 and 1200 h or between 1430 and 1630 h to avoid monitoring at midday when pulses of methane may have been released in association with diurnal changes in atmospheric pressure (Boon, pers. com.).

Specific investigations

Day-to-day variations were examined at Willunga Wetlands and Greenfields Wetlands in summer, winter and spring 1995. Six chambers were concurrently deployed, for two hours or less, at the inlets in the mornings and at the outlets in the afternoons over two sets of three consecutive days. The site order was reversed each time such that by the end of all the investigations there were equal proportions of morning and afternoon deployments at each site. One-way ANOVAs were performed on each data set using JMP Version 3.2 to test the effect of *day* on methane emissions and the coefficients of variance (mean/standard deviation) were determined using Excel Version 5.0.

Intrasite variation was examined in spring 1994 by deploying six chambers either directly adjacent to one another or at 4 m intervals and comparing the variation

associated with the two resultant mean values. These trials were conducted at WILLUNGA-INLET, WILLUNGA-OUTLET, GREENFIELDS-INLET and GREENFIELDS-OUTLET. One-way ANOVAs were performed using JMP Version 3.2 to test the effect of *interchamber distance* on methane emissions and coefficients of variance (mean/one standard deviation) were determined using Excel Version 5.0. The relevant ANOVA results appear in Appendix 1.3.

6.2.3: Effect of flow path on methane production, oxidation and emission

In spring 1996, a series of investigations were conducted at six sites within Willunga Wetlands and six sites within Greenfields Wetlands to determine concurrent rates of methane production, oxidation and emission and to relate these to organic carbon content and methane concentrations in the sediments.

At Willunga Wetlands, two sites were chosen in each of the three ponds with respective water depths of 0.6 m and 3 m. The 3 m deep sites in Pond 1 and Pond 3 were the same areas as WILLUNGA-INLET and WILLUNGA-OUTLET. A comparable 3m deep site was chosen in Pond 2 and the shallow sites in each pond were chosen in the most downstream bay near the outlet pipes (Figure 3.1). At Greenfields Wetlands the six sites were chosen at approximately equal distances along a nominated flow path approximately 3 km in length. GREENFIELDS-INLET and GREENFIELDS-OUTLET were used as the two sites at the extreme ends of the flow path (Figure 3.2).

One chamber was deployed at each site for three consecutive 24 hour periods whilst rates of methane production and oxidation were determined. Three sediment cores were taken at each site from which three 5 mL subsamples were taken and incubated *in situ* with L-cysteine for methanogenic rate determinations. Rates of methanotrophy were determined by taking three replicate water samples in 60 mL syringes from 10 cm depth and incubating them *in situ* for 24 hours. An additional five sediment cores were taken from each site and the surface 2 cm layers were removed and analysed for organic carbon content. Finally, sediment methane concentrations were determined by collecting three replicate gas samples at each site released by agitating the sediment with a pole (see Chapters 3 and 5 for detailed methodology).

6.2.4: *Site-specific controlling factors - Willunga Wetlands*

The effect of water depth

In spring 1995, summer 1995/1996 and spring 1996, a series of three experiments investigating the effect of water depth on methane emissions at Willunga Wetlands were undertaken. Over three consecutive days, two chambers were deployed at each of three locations. A total of six locations were used, representing water depths of 0.6, 1.2 and 3 m in each of the three ponds. WILLUNGA-INLET and WILLUNGA-OUTLET were used to represent the 3 m deep areas of ponds 1 and 3 and the more shallow sites were located on the slope leading to the vegetated bays in each pond.

Emission estimates in spring 1995 were generated by deploying the chambers for 2 hours or less in the mornings (1000 to 1200 h) and afternoons (1430 to 1630 h), alternating between sites such that there was no bias towards certain *water depth/time of day* combinations. In summer 1995/1996 and spring 1996, the chambers were deployed for 24 hour periods typically commencing at 1400 h. One-way ANOVAs were performed to test the effect of *water depth* on methane emissions using JMP Version 3.2. The relevant ANOVA results appear in Appendix 1.3.

The effect of water column stability

Chambers were deployed for 24 hour periods at times when the water column at WILLUNGA-INLET was expected to turnover in autumn 1997 (i.e. destratify due to seasonal climatic changes). These times were chosen by assessing long term weather forecasts and attempting to predict when the first major turnover event after the long summer stratification period would occur. Six chambers were deployed at each attempt on WILLUNGA-INLET at 1700 h. The chambers were monitored at 1900, 2100, 2300 h on the first day and at 0100, 0300, 0500, 0700 and 1700 h on the second day as the turnover was expected to occur either around midnight or dawn. Dissolved oxygen and dissolved methane concentrations (three replicates) were determined at 0.1, 0.5, 1.5, 2, 2.5 and 3 m water depth at each sampling event. Water temperature data at a range of depths were automatically recorded every 30 minutes by the chain of thermocouples permanently deployed in WILLUNGA-INLET (see Chapters 3 and 4) to determine the time of turnover.

6.2.5: *Site-specific controlling factors - Greenfields Wetlands*

The effect of flow rate on methane emissions at GREENFIELDS-INLET was determined by estimating the total and ebullitive fluxes of methane during high (Winter 1996) and low (January 1997) flow periods. The inflow rate was measured by floating an orange

on the water surface near GREENFIELDS-INLET and measuring the time it took to travel a given distance in metres per second. Total methane emissions were estimated using six chambers deployed adjacent to each other for 2 hours or less in the mornings (1000 to 1200 h) or in the afternoons (1430 to 1630 h). Five separate estimates of flow rate were made each time the chambers were deployed.

The rate of methane ebullition was estimated by placing 20 inverted funnels on the sediment surface in areas adjacent to and at a similar distance from the inlet to where the chambers were deployed. The funnels were left in place for 24 h before the volume of gas collected was recorded. Ten sediment gas samples were taken each time the funnels were deployed in order to calculate the *bona fide* methane content of the released gas and to determine whether there had been any significant change in sediment methane concentration between the winter and summer readings. One-way ANOVAs were performed, using JMP Version 3.2, to test the effect of *season* on rates of methane ebullition, total methane emission and the methane concentration in the sediment gas samples. The relevant ANOVA results appear in Appendix 1.3.

6.2.6: Site-specific controlling factors - Bool Lagoon

The effect of aquatic vegetation on methane emissions was examined at Bool Lagoon, the most densely vegetated of the three study systems. Chambers were deployed in unvegetated areas and in *Phragmites australis* and *Triglochin procerum* beds at BOOL-HACKS for a maximum of 20 minutes in summer and 1 hour in winter. The relatively short deployment times were used to reduce any impact that enclosure may have had on pressurised flow through the plants.

In summer 1994/1995, six estimates of emissions were made at each of the three sites. In winter 1995 and summer 1995/1996, the number of replicates was reduced to three and the number of sites to two, due to logistical constraints. *Phragmites australis* was used to compare to the unvegetated areas in winter 1995 and *Triglochin procerum* was used in summer 1995/1996. One-way ANOVAs were performed on the data sets generated at each time using JMP Version 3.2.

In a second experiment, the effect of drawdown and the presence of aquatic vegetation was examined at BOOL-HACKS. Sites were chosen along an elevation gradient to represent unvegetated areas and areas covered with *Triglochin procerum* at three water depths: 15 cm (shallow), 35 cm (deep) and where the water table was less than 10 cm below the sediment surface (exposed). Three chambers were deployed at each site in the mornings (1000 to 1200 h) or afternoons (1430 to 1630 h) for a maximum period of

20 minutes to minimise the effects the chamber may have on flow rates through *Triglochin procerum*. One-way ANOVAs were performed on the resultant emission data to determine the effect of *vegetation type and water depth*. The relevant ANOVA results appear in Appendix 1.3.

6.2.7: Whole wetland inventories

Estimates of methane emissions from the entire wetland surfaces were determined by interpolating between measurements made at the inlets and outlets of the constructed wetlands. The rates obtained for BOOL-HACKS were assumed to represent rates from all inundated areas of Bool Lagoon.

First, the wetland areas assumed to be homogeneous with respect to methane emissions were determined. At Willunga Wetlands, this was based on the calculated surface areas of 0.6, 1.2 or 3m deep water in each of the three ponds. The areal data for Greenfields Wetlands were generated by determining the inundated area in winter/spring and summer/autumn that lay downstream of each of the sites in Stages I and II. The channellised design of Stage II dampened the seasonal variations in inundated area and water depths observed in Stage I. The emission rate determined for each site was then applied to the intersite area downstream of that site. For Bool Lagoon a series of seasonal inundation areas was generated assuming either a 'wet' winter in which the basins filled to 100% or a 'dry' winter in which the basins only filled to 20% capacity.

Second, spatial interpolation factors were generated to allow for the calculation of emissions estimates for wetland areas lying between the inlets and outlets of Willunga Wetlands and Greenfields Wetlands. These were calculated on a pro-rata basis and were based on the observed percentage differences in emission data collected at the inlets and outlets to test the effects of:

- water depth at Willunga Wetlands (Section 6.3.5) ; and
- of flow path on methane production, oxidation and emission at Willunga Wetlands and Greenfields Wetlands (Section 6.3.3).

Third, the individual emission estimates were converted from their original units (mmol m^{-2} h^{-1}) to kg ha^{-1} $season^{-1}$. Individual emission estimates were used to generate mean seasonal estimates with associated standard deviations for each of the nine seasons investigated. These estimates were then multiplied by the relevant spatial interpolation factors and wetland surface areas, thereby generating estimates of the total methane emission per hectare per annum. The final estimates of seasonal and

annual emissions in kg per wetland are shown as mean values rounded up to the nearest 100 kg.

6.3: Results

6.3.1: Robustness of the static chamber technique

Gas-tightness of chambers and vacutainers

There were no significant losses of methane over time from any of the six chambers as determined by one-way ANOVA ($P>0.05$). Methane concentrations in chambers set with a target concentration of 600 ppm ranged from 597 ± 31 ppm (n=18) at the beginning to 608 ± 25 ppm (n=18) at the end of the 72 hour trial in the six chambers. Similarly, methane concentrations in the six chambers with a target concentration of 3000 ppm did not changes significantly over time: 2989 ± 102 ppm (n=18) at the beginning and 3031 ± 98 ppm (n=18) at the end of the 72 hour trial. The dissolved methane concentrations in the pond water varied from 12 ± 1.3 μg L^{-1} (n=12) at the beginning to 15 ± 1.8 μg L^{-1} (n=12) at the end of the trials.

There were also no significant losses of methane over a ten day period from the 7 mL vacutainers used to store gas samples as determined by one-way ANOVA ($P>0.05$; Table 6.1). Storage temperatures during the experiment ranged from 0 to 4 °C in the esky and laboratory refrigerator. All practicable care was taken to ensure field samples were routinely stored within this temperature range.

Linearity of methane release over time

The increase in methane concentrations inside the static chambers was highly linear ($r^2>0.98$) in the three chambers which were sampled every two minutes for 70 minutes at WILLUNGA-INLET. Figure 6.1a shows the plot of methane concentration by time for one of these chambers. Note the 'rippling' effect which indicates a rapid and continuous release of small bubbles from the water column. All the chamber data presented in Section 6.3.2 and below were obtained from highly linear plots ($r^2>0.95$) generated with sampling intervals of 15 minutes or more. An example is shown in Figure 6.1b.

Effects of physical disturbance

Despite indications of a short, initial lag phase, the time courses for increasing methane concentration were highly linear ($r^2>0.97$) in all the chambers subject to physical disturbance. The effects of rocking the chamber and turning the fan on and off were minimal and resulted in an increase or decrease of less than 8% of the undisturbed chamber estimates (Figure 6.2a). Trampling of the sediment surrounding the chamber

immediately increased the rate of methane release (Figure 6.2b) but the effect was transitory and after 15 minutes the rate of methane release returned to a rate similar to that determined previous to the trampling.

Replication requirements

The variability associated with estimating mean and standard deviation values for emission data decreased as the number of replicate chambers increased (Figure 6.3). These data indicate that, ideally, 6-7 chambers should be deployed simultaneously in order to obtain *bona fide* estimates of emission rates and their variability. They also indicate that even with 18 replicate chambers, the coefficient of variance about the mean was 16%.

Effect of increasing deployment time

Rates of methane release into six chambers deployed simultaneously at Willunga Wetlands varied from 50 to 97 ppm h^{-1} after 100 minutes of deployment (Figure 6.4a and Table 6.2). The mean rate was 70 ppm h^{-1} and the coefficient of variance was 30%. After 24 hours, the range had narrowed (65-72 ppm h^{-1}; Figure 6.4b and Table 6.2), the mean was conserved at 69 ppm h^{-1} and the coefficient of variance had dropped to only 4.3%.

6.3.2: *Temporal Variation*

Intraseasonal

No consistent diel variation was observed in any of the emission data from these unvegetated wetland sites. However, in order to ensure emission data were comparable between sites and seasons the only emission estimates that appear below were generated with either:

a) replicate chambers deployed for less than 2 hours in the morning (between 1000 h and 1200 h) or in the afternoon (between 1430 h and 1630 h); or
b) replicate chambers deployed for 24 hours or more and therefore encompassing a full diel cycle.

Day-to-day variation at Willunga Wetlands and Greenfields Wetlands was not significant ($P<0.05$) on any of the occasions it was assessed. A typical result for each wetland site in spring 1995 is shown in Table 6.3. Coefficients of variance ranged from 10 to 27% for Willunga Wetlands and GREENFIELDS-INLET, which were of a similar magnitude to those observed within a single day. The coefficients of variance at GREENFIELDS-OUTLET were higher and ranged from 25 to 43%.

Interseasonal Variation

At most of the wetland sites, summer rates of methane emission were higher than those from winter, autumn and spring (Table 6.4). At Willunga Wetlands, summer emissions were twice those observed in spring and up to 4 times those observed in winter and autumn. At GREENFIELDS-OUTLET, a similar pattern was observed with summer emissions being 5 times the spring and autumn emissions and up to an order of magnitude higher than those in winter. BOOL-HACKS also emitted an order of magnitude more methane in summer than winter. The exceptional site was GREENFIELDS-INLET where the winter emissions were greater than or equal to those observed in summer and approximately three times higher than those observed in spring and autumn.

Interannual variation

The sampling period for methane emissions extended for 28 months, from December 1994 to March 1997. The emissions measured were remarkably similar from year-to-year (Table 6.4). For example, emissions measured from WILLUNGA-INLET in the summers of 1994/1995, 1995/1996 and 1996/1997 were 8.52 ± 5.7, 8.91 ± 2.6 and 7.78 ± 0.67 mmol m^{-2} h^{-1}, respectively. Emissions over the same summer periods from GREENFIELDS-INLET were 3.29 ± 0.48, 3.28 ± 1.4 and 3.87 ± 1.25 mmol m^{-2} h^{-1}, respectively. Unfortunately, the logistical difficulties associated with measuring at Bool Lagoon restricted emissions sampling to five occasions during the 28 month period. Summer emissions were monitored twice in 1994/1995 and 1995/1996 at which times the rates were 2.13 ± 1.61 and 1.26 ± 0.13 mmol m^{-2} h^{-1}, less than or equal to those from GREENFIELDS-INLET.

6.3.3: Spatial Variation

Intrasite

Emission estimates were not affected ($P > 0.05$) by whether the chambers were set adjacent to each other or at 4 m intervals at WILLUNGA-INLET, WILLUNGA-OUTLET and GREENFIELDS-OUTLET in spring 1995 (Table 6.5). At GREENFIELDS-INLET, however, the effect was significant ($P < 0.05$). Emissions estimated with the chambers deployed 4 metres apart were significantly higher and less variable than those generated with the chambers deployed adjacent to one another. Generally, the coefficients of variance at Greenfields Wetlands (12 to 54%), and particularly GREENFIELDS-OUTLET, were higher than those at Willunga Wetlands (12 to 19%).

Effect of flow path length in constructed wetlands

On all occasions, emissions from the inlets were greater than those from the outlets of the two constructed wetlands, Willunga Wetlands and Greenfields Wetlands (Table 6.4). The greatest differences were at Greenfields Wetlands where winter emissions at the outlet dropped to 1% of those at the inlet and the smallest differences were at Willunga Wetlands in spring where emissions dropped by only 55% between the inlet and the outlet (Table 6.6).

In spring 1996 a similar effect was observed in emissions from six sites along the flow paths of the two constructed wetlands. Rates of methane emission, production and oxidation and the organic carbon content and concentration of methane in the sediments all decreased with increasing flow path length (Tables 6.7 and 6.8). In addition, there was an effect of water depth at Willunga Wetlands (Table 6.7) that will be examined further in Section 6.3.5.

Sediment organic carbon content showed the weakest correlation with methane emission at Willunga Wetlands (r^2=0.619) and at Greenfields Wetlands (r^2=0.63; Table 6.9). The other parameters correlated more strongly with the rate of emission yielding r^2 values of 0.889 or greater. The most strongly correlated factor was benthic methane production at both Willunga Wetlands (r^2 = 0.992) and Greenfields Wetlands (r^2 = 0.976).

6.3.4: Interwetland comparisons

Methane emissions from the five wetland sites ranged from 0.06 ± 0.01 to 8.91 ± 2.6 mmol m^{-2} h^{-1} (Table 6.4). Summer emissions from WILLUNGA-INLET were the highest and winter emissions from GREENFIELDS-OUTLET the lowest of all emissions estimated. The site ranking generally adhered to that predicted in Section 6.1, except that emissions from GREENFIELDS-INLET were higher than predicted, being equivalent to those from WILLUNGA-OUTLET at most times of year.

In winter, emissions from GREENFIELDS-INLET were the highest of all, approximately double those from WILLUNGA-INLET, the second highest ranked site. It was predicted in Section 6.1 that emissions from Greenfields Wetlands would be less than those from Willunga Wetlands, but emissions from GREENFIELDS-INLET were observed to be greater than those from WILLUNGA-OUTLET in all seasons except spring when emissions from GREENFIELDS-INLET were low but highly variable. Emissions from GREENFIELDS-OUTLET were consistently the lowest rates observed, as predicted in Section 6.1. It was expected that emissions from Bool Lagoon would be lower than

those from Willunga Wetlands but higher than those from Greenfields Wetlands. That was not the case because emissions from GREENFIELDS-INLET were higher than those from Bool Lagoon as well as those from WILLUNGA-OUTLET.

6.3.5: Site-specific controlling factors

Water column depth and stability at Willunga Wetlands

Rates of methane emission increased as water depth increased in both Pond 1 (inlet pond) and Pond 3 (outlet pond) of Willunga Wetlands ($P<0.05$; Table 6.10). Each increase in water depth from 0.6 m to 1.2 m to 3 m led to a doubling or greater in the rate of emission. Therefore emissions from the 3 m deep areas were up to an order of magnitude greater than those from the shallow areas (60 cm).

The deeper areas at Willunga Wetlands developed a strong thermal gradient in summer 1996/1997 with surface water temperatures exceeding 30 °C and benthic temperatures rarely greater than 20 °C (Figure 6.5 and Appendix 2). This thermal stratification persisted over the summer months and the diel mixing depth was consistently less than 0.75 m. On the nights of February 24 and 25 1997, a storm passed over the Willunga Wetlands and the water column mixed down to 1.5 m depth (Figure 6.5). The surface waters cooled and the daily maximum temperatures dropped to values of 25 to 27 °C during the period 25 February to 4 March 1997.

Chambers were not set the night of the 'turnover' but impacts on the methane dynamics can be interpreted from data collected on 20 February and 2 March 1997 (Table 6.11). Previous to the turnover (eg. 20.2.97), the water column was thermally stratified and dissolved oxygen concentrations ranged from supersaturated (c.15 mg L^{-1}) at 0.1 m depth to <0.2 mg L^{-1} at 3 m depth. Dissolved methane concentrations were very high in the benthos (44354 ± 1036 µg L^{-1}) and the rate of methane emission to the atmosphere was 4.07 ± 1.1 mmol m^{-2} h^{-1}.

After the storm, the thermal gradient was much weaker and the thermal stratification which built up during the daylight hours was broken down every night by mixing which extended to 2.25 m depth (Figure 6.5). The dissolved oxygen profiles before and after the storm were apparently similar; however, the amount of methane stored in the water column had been markedly reduced as indicated by the lower dissolved methane concentrations (Table 6.11). The rate of methane emission had also decreased by approximately 50%, from 4.07 ± 1.1 mmol m^{-2} h^{-1} before the storm to 1.95 ± 0.71 mmol m^{-2} h^{-1} after the storm.

Influent flow rate to Greenfields Wetlands

The flow rate of water entering GREENFIELDS-INLET from Dry Creek during this experiment ranged from 0 to 7.3 m s^{-1}, peaking in the winter sampling period (Table 6.12). Total and ebullitive emissions also peaked in winter ($P<0.05$) at rates that were almost double those recorded in summer.

Ebullition was the dominant route for release in both winter and summer, accounting for 93 and 91% of the total emissions, respectively. However, the high rates of emission in winter did not lead to a significant change in the methane concentration of sediment gas bubbles over the period May 1996 to January 1997 ($P>0.05$; Table 6.13), although gas concentrations were more variable in August 1996 than in May 1996 or January 1997.

The effect of vegetation and drawdown at Bool Lagoon

The presence of aquatic vegetation did not significantly increase the rate of methane emission from BOOL-HACKS in summer 1994/95 when emissions were measured in unvegetated areas, *Phragmites australis* beds and *Triglochin procerum* beds ($P=0.0735$; Table 6.14). However, in summer 1995/1996, the presence of *Triglochin procerum* significantly increased methane emissions above that for unvegetated areas ($P = 0.0089$). Emissions from both the unvegetated areas and the *Phragmites australis* beds decreased in winter by up to an order of magnitude.

The effect of increasing water depth was to significantly ($P < 0.0001$) increase emissions at BOOL-HACKS in autumn 1997, irrespective of whether the site was vegetated with *Triglochin procerum* or not (Table 6.15). In exposed conditions, the unvegetated sediment consumed methane from the atmosphere within the chamber at approximately the same rate as adjacent sites inundated to 35 cm were emitting it.

6.3.7: Whole wetland emission estimates

Spatial interpolation factors were calculated from the results presented in Sections 6.3.3 and 6.3.5 in order to calculate methane emissions from the whole wetland surfaces. These factors were calculated for Willunga Wetlands and Greenfields Wetlands (using the methods in Section 6.2.5) and appear in Table 6.16a and b as multiplication factors to be applied to emission data from the four routine sampling sites in each season in order to estimate whole wetland emissions. The wetland surface areas assigned to each interpolation factor also appear in Table 6.16, along with the areal data used for Bool Lagoon (Table 6.16c).

Emission estimates for each of the nine seasons sampled (assumed to be 90 days each) appear in Table 6.17 (converted to units of kg ha^{-1} $season^{-1}$) and the averages calculated from these for summer, autumn, winter and spring appear in Table 6.18.

On a whole wetland basis, the lowest methane emissions were from Willunga Wetlands, which were estimated to emit 7,110 kg of methane to the atmosphere per annum (Table 6.19). Greenfields Wetlands emitted 67,400 kg of methane per annum which was 9.5 times that emitted from Willunga Wetlands. In dry winter conditions, emission estimates from Bool Lagoon (37,700 kg) were only 56% of those estimated for Greenfields Wetlands but if winter conditions at Bool Lagoon were wet, emissions were far greater (1,326,600 kg) than those from Willunga Wetlands and Greenfields Wetlands combined. These calculations do not account for the presence of aquatic vegetation nor the consumption of methane by exposed sediments areas.

6.4: Discussion

Methane emissions from Willunga Wetlands, Greenfields Wetlands and Bool Lagoon varied on a range of temporal and spatial scales from 0.06 ± 0.01 to 8.91 ± 2.6 mmol m^{-2} h^{-1}. The static chamber technique was found to be suitably robust for determining the factors which controlled the variation in these emissions provided that estimates were generated from deployments of either a) six or more chambers over a period of 2 hours or less or b) less than six chambers over a 24 hour period. Highly linear (r^2>0.95) time courses for methane concentration increases in the chambers were easily and regularly obtained under these conditions.

A certain level of variation was evident at each wetland location on any one sampling occasion even at the shortest time and space intervals. The coefficients of variation observed between chambers deployed adjacent to each other for periods of less than 2 hours ranged between 12 and 27%. The variation associated with estimates taken over consecutive days was of similar magnitude (10 to 43%), suggesting that day-to-day variation was no more significant than the variation observed between simultaneously deployed chambers. On a spatial scale, variation observed between chambers deployed simultaneously at WILLUNGA-INLET, WILLUNGA-OUTLET and GREENFIELDS-OUTLET was not affected by whether the chambers were adjacent to each other or not, suggesting that the variation occurred on a spatial scale smaller than the surface area of a chamber and that, therefore, intrasite location was not a significant variable. GREENFIELDS-INLET was the exceptional site. Here, the variation in emissions was greater in chambers deployed adjacent to one another than in those deployed at 4 m

intervals, suggesting that spatial variation at GREENFIELDS-INLET was on a more coarse scale than that at the other wetland sites.

Some of the observed variation may be accounted for by the process of ebullition. The loss of methane via this route is intrinsically variable because individual bubbles range in size and methane concentration (Chanton et al., 1989; Sorrell and Boon, 1992). Significant rates of ebullition were or have been detected at all three wetlands (Sections 6.3.1 and 6.3.5; Muller, 1993; Muller et al., 1994). Ebullition was not directly measured at Willunga Wetlands but the 'rippling' effect observed in the plot of methane concentration over time, monitored every two minutes (Figure 6.1a), would suggest that bubbles were continuously breaking on the surface of WILLUNGA-INLET. On still days it was evident from looking at the surface of the pond that small bubbles were constantly released across the surface. At GREENFIELDS-INLET, rates of ebullition ranged from 5.64 ± 1.2 mmol m^{-2} h^{-1} in winter to 3.45 ± 1.07 mmol m^{-2} h^{-1} in summer. These rates of ebullition are higher than those reported for Ryan's Billabong in south-eastern Australia (Sorrell and Boon, 1992) and are among the highest reported for any freshwater system (Ward and Frea, 1979; Cicerone and Shetter, 1981; Cappenberg et al., 1984; Devol et al., 1988).

To allow for the variation observed within one sampling period, variation in emissions across sites and seasons would have to be greater than approximately 30 to 40% to be reliably detected with chambers deployed for short periods. The variation associated with chamber estimates from longer deployment periods was much reduced (4%) and therefore intersite variation was better estimated with long deployment periods as occurred later in the sampling programme. Inventories are inherently limited by variations in emissions in both space and time (Cao et al., 1996) and therefore estimates with reduced errors are desirable. However, this technique is limited to unvegetated areas because the process of plant-mediated flux would be strongly impaired by chamber deployments of this length due to its reliance on humidity and temperature gradients between the atmosphere and the internal environment of the leaf (Knapp and Yavitt, 1992; Chanton et al., 1993; Boon and Sorrell, 1995).

Notwithstanding the high levels of intrasite variation, there were clear differences in the emissions from the different study sites and between the seasons. On all occasions, emissions from the inlet sites were higher than from the outlet sites at the two constructed wetlands, reflecting the concurrent decrease in the rates of methane production and the organic carbon content of the sediment.

Mean methane emissions from WILLUNGA-INLET exceeded 8 mmol CH_4 m^{-2} h^{-1} in the summer months and individual emission estimates were as high as 14.2 mmol CH_4 m^{-2} h^{-1}. These were the highest emissions recorded during the study period. These emissions are also high when compared to emissions reported in the literature. For example, emissions from a polluted freshwater canal in India were reported to be 1.93 mmol CH_4 m^{-2} h^{-1} (Verma et al., 1999), approximately four times lower than those from Willunga Wetlands. Relative high rates have been reported by Kiene (1991) for peatlands (5 mmol CH_4 m^{-2} h^{-1}) but these are still lower than those observed at WILLUNGA-INLET. Boon and Sorrell (1995) have reported rates up to 2.75 mmol CH_4 m^{-2} h^{-1} for a temperate wetland in south-eastern Australia (Ryans Billabong) but temperate wetlands tend to have much lower emissions (0.39 mmol CH_4 m^{-2} h^{-1}, Cao et al., 1996).

Emissions from WILLUNGA-OUTLET were lower than expected at all times of year but particularly in summer 1994/1995 and autumn 1996. During these periods emissions from WILLUNGA-OUTLET were similar to those from BOOL-HACKS and tended to be similar to emissions reported for Ryan's Billabong (Boon and Sorrell, 1995). Emissions from GREENFIELDS-INLET were generally higher than expected. They were consistently greater than or equal to emissions from WILLUNGA-OUTLET and during winter, were even higher than those from WILLUNGA-INLET. Winter emissions from GREENFIELDS-INLET were approximately 6 mmol CH_4 m^{-2} h^{-1}, equivalent to summer rates reported for peatlands by Kiene (1991). Emissions from GREENFIELDS-OUTLET were, as predicted, lower than those from any of the other sites, reflecting the relatively low rates of methane production and sediment organic carbon content (see Chapters 4 and 5). Emissions from GREENFIELDS-OUTLET in summer were similar to those reported for a Belgian freshwater wetland (Boeckx and Vancleemput, 1997) and for temperate wetlands in Cao et al. (1996).

The rate of methanogenesis was the factor that correlated most strongly with emissions from the study sites. The variation in methanogenesis explained more than 97% of the variation in emissions. Winter emissions from GREENFIELDS-INLET did not agree well with the concurrent rates methanogenesis because the high winter emissions were caused by physical rather than biological driving forces (see below). The relative amounts of organic carbon present in the wetland sediments only explained 62-63% of the variation in emissions at Willunga Wetlands and Greenfields Wetlands. Methane oxidation rates also correlated strongly with emissions (approximately 90% in both systems) but this may be more a function of the methanotrophs' reliance on

methanogens as a source of methane rather than a causal relationship between methanotrophic rates and emissions.

The rate at which emissions decreased with increasing flow path length was greatest at Greenfields Wetlands. This may reflect the overall lower organic status of this wetland system caused by: lower quantities of influent organic carbon compared with Willunga Wetlands, the relatively young and sparse vegetation compared with Bool Lagoon and the relatively long flow path. The lowest rates of emission found at any of the sites were at GREENFIELDS-OUTLET, which also had the lowest organic carbon load and was the most distant of all the constructed wetland sites from an inlet. The availability of organic carbon affects the rate of methanogenesis which in turn affects the rate of methane emission via the concentration gradient that exists between the sediment and the atmosphere (Conrad, 1996; Segers, 1997; Vandernat and Midelburg, 1998). These effects were evident in the methane dynamics along the flow paths of both Willunga Wetlands and Greenfields Wetlands.

At the majority of the sites, emissions from the summer periods were higher than those from the winter periods, as was predicted in Section 1.6 given the positive effect of temperature on methanogenesis (see Section 2.1.4) and the dependence of emissions on rates of methanogenesis. This type of seasonal pattern is consistent with that observed in a number of other studies in temperate regions and is likely to co-incide with seasonal patterns of wetland vegetation growth and decline and run-off from the catchment (King and Wiebe, 1978; Chanton and Martens, 1988; Chanton et al., 1989; Boon and Sorrell, 1992). At Greenfields Wetlands the seasonal pattern was inverted from the normal temperate pattern. Winter emissions were higher than or equal to those in summer even though the rates of methanogenesis were an order of magnitude lower in winter than summer. This was not expected. The most likely cause of the unusual seasonal pattern at Greenfields Wetlands was the seasonal inflow patterns. Being in a Mediterranean climate, inflows to Greenfields Wetlands peaked during winter and spring (Figure 4.2), the seasons of maximum runoff.

It is hypothesised that the high winter flow rates caused the high winter emissions by enhancing the rate of ebullition through physical disturbance of the sediments. The physical disturbance caused by rapid inflows is likely to have increased the rates of ebullition in winter, in much the same way that wave action causes episodic increases in ebullition in estuaries (Chanton et al., 1989) and changes in atmospheric pressure affect ebullition rates in Amazonian beaver ponds (Weyhenmeyer, 1999). This hypothesis is substantiated by emission data collected in winter 1995 concurrent with the TOC/DOC

analyses in high and low flow conditions. On 4 May 1995, when inflow rates were c. 1000 L s^{-1}, total methane emissions were 6.24 ± 0.8 mmol m^{-2} h^{-1} (n=6), whilst on 22 May 1995 when the inflow rate was less than 10 L s^{-1}, emissions had dropped to 4.13 ± 1.1 mmol m^{-2} h^{-1} (n=6). These data are collated in Figure 4.2 and Table 6.12.

This effect was not seen at Willunga Wetlands where the inflow rate was steady all year at 4 L s^{-1} (Piranti, 1995). Emissions from Willunga Wetlands were controlled by seasonal temperatures through the relative rates of methanogenesis and methanotrophy and the development and breakdown of thermally stratified water layers. Strong and persistent thermal stratification developed in the deep areas of Willunga Wetlands over the warm summer months. This stratification promoted anaerobic and highly reducing conditions in the benthic water layers and sediments and large stores of methane accumulated in the benthos.

The thermal stratification that broke down in autumn 1997 had persisted since December 1996 (see Appendix 2). Although emissions were not directly measured when the turnover event occurred, it can be inferred from the dissolved methane concentration profiles and the methane emissions before and after the event that large amounts of methane were released in a short time. Prior to the turnover, the hypolimnion contained between 1,800 and 44,400 μg CH_4 L^{-1}. After the event, the concentration had dropped to between 600 and 3,300 μg CH_4 L^{-1}, suggesting that approximately 125 g of methane per m^2, or 500 kg from the surface area of WILLUNGA-INLET, was lost from the water column during the intervening 12 days. The methane had two possible fates; loss to the atmosphere or oxidation by methanotrophs. If the methane was lost to the atmosphere, the bulk was probably rapidly released during the turnover event.

Short-term releases of methane are considered important in systems such as Greenfields Wetlands and Willunga Wetlands because 1) these types of emissions may account for a significant proportion of the annual methane emissions and 2) they are difficult to predict and short-lived and therefore are unlikely to be picked up in routine monitoring programmes. A similar conclusion was drawn by Weyhenmeyer (1999), who found that low sampling frequencies were inadequate to describe emissions from Amazonian beaver ponds which were intrinsically variable in space and time due to total emissions comprising 65% ebullition. However, the methane may have been oxidised by rapid methanotrophy in the surface water layers in which case it would not have been lost to the atmosphere.

Such was the case in the dimictic Lake Nojiri, Japan. Surface dissolved methane concentrations reached their seasonal high during the autumnal turnover period (Utsumi et al., 1998). Methane that had accumulated in the hypolimnion during the stratification period mixed rapidly through the water column and then decreased rapidly as a result of enhanced methanotrophic activity rather than enhanced emissions. Similar processes may be occurring at Willunga Wetlands although Lake Nojiri was more than 40 m deep and therefore the transport time from the hypolimnion to the atmosphere was much greater and more conducive to methanotrophic interception. It would be profitable to further investigate methane dynamics at Willunga Wetlands during the autumnal turnover period in order to ascertain the fate of the methane which has accumulated over summer (see Section 7.3).

On a unit time by unit area basis, Willunga Wetlands consistently had the highest rates of methane emissions, often exceeding 8 mmol CH_4 m^{-2} h^{-1}. However, the whole wetland site covers only 3 ha, resulting in lower annual emissions on a whole wetland basis than either Greenfields Wetlands or Bool Lagoon. The relative rankings of Bool Lagoon and Greenfields Wetlands were dependent on the area of Bool Lagoon that was assumed to be inundated in any given winter period. Following wet winters, Bool Lagoon was predicted to release up to 20 times more methane than the whole of the Greenfields Wetlands complex and 187 times that from Willunga Wetlands. Alternatively, if winter conditions were assumed to be dry, annual emissions from Bool Lagoon were only 56% of those from Greenfields Wetlands and 5 times those from Willunga Wetlands. Furthermore, if it were assumed that the exposed areas of Bool Lagoon were consuming methane at 0.8 mmol m^{-2} h^{-1} then the annual methane emissions from Bool Lagoon would be reduced from 37,700 to 30,910 kg per annum. Emissions of this order would still be 45% of those from Greenfields Wetlands and approximately four times those from Willunga Wetlands. These emission estimates appear to differ to those presented in Ganf et al. (1998). However, it is now known (Ganf pers. com.) that the calculations in Ganf et al. (1998) were not based on an inundated surface area of 2,500 ha for Bool Lagoon as stated in the text but rather on an area of 10 ha. If these spatial differences are accounted for, the two estimates converge.

If methane emissions through the vegetation at Bool Lagoon were included in these whole wetland emissions, the annual estimates may increase markedly. Emissions through aquatic vegetation often exceed that from comparable open water areas. Previous studies at Bool Lagoon (Muller, 1993; Muller et al., 1994) indicate that emissions from *Triglochin procerum* and *Baumea arthrophylla* beds, the two most

dominant plant species in the system, were 40-75% and 80-90% greater than those from adjacent open water areas, respectively. Investigations in this study yielded less clear results. Nevertheless, it is reasonable to expect that emissions from Bool Lagoon may exceed 2,000,000 kg per annum if significant areas of vegetation were inundated and vigorously growing over the summer months. The inclusion of plant-mediated emissions at Greenfields Wetlands and Willunga Wetlands would have less impact on the annual emission estimates because the majority of the plants fringe the wetland channels and are seldom inundated.

Without having direct measurements of plant-mediated flux and robust estimates of species-specific plant cover and inundation depths, these calculations are speculative. However, it is evident that wetland size is a critical parameter in determining whole wetland emissions. Small increases or decreases in the areas assumed to have a given methane production or consumption rate can have large effects on the magnitude of the resultant emission estimates if the wetland is as large as Bool Lagoon. Greenfields Wetlands, which are relatively large for a constructed wetland, had the potential to release as much or more methane per annum than Bool Lagoon depending on the level of inundation assumed for Bool Lagoon. However, a wetland system as small as Willunga Wetlands does not have the capacity to release as much methane per annum as a natural wetland the size of Bool Lagoon, even though emissions from Willunga Wetlands were remarkably high on a per unit time and area basis compared with not only emissions from Bool Lagoon, but with most reports of methane emissions from freshwater systems (Sorrell and Boon, 1992; Kiene, 1991; Cao et al., 1996; Alford et al., 1997; Boeckx and Vancleemput, 1997; MacDonald et al., 1998; Verma et al., 1999).

6.5: Conclusions

1. The static chamber technique was suitably robust for measuring methane emissions and the associated variation provided that an adequate number of chambers were deployed or that the deployment periods were extended.

2. The coefficents of variance in the emission data ranged from 10-27% at the highest temporal and spatial resolution. Variation between simultaneous chamber estimates decreased when deployment time was increased from 2 hours to 24 hours.

3. Methane emissions from the different wetland sites did not occur in the site order predicted from the rates of methanogenesis reported in Chapter 5. The emissions were ranked on a seasonal basis as appears below:

   Summer:

WILLUNGA-INLET > (GREENFIELDS-INLET = WILLUNGA-OUTLET) > BOOL-HACKS >GREENFIELDS-OUTLET

Autumn:
(GREENFIELDS-INLET = WILLUNGA-INLET) > WILLUNGA-OUTLET > BOOL-HACKS > GREENFIELDS-OUTLET

Winter:
GREENFIELDS-INLET > WILLUNGA-INLET > WILLUNGA-OUTLET > BOOL-HACKS > GREENFIELDS-OUTLET

Spring:
WILLUNGA-INLET > WILLUNGA-OUTLET > GREENFIELDS-INLET > BOOL-HACKS > GREENFIELDS-OUTLET

The site order observed for spring most closely adheres to the predicted sequence although the higher than expected rates of methane emission from GREENFIELDS-INLET have advanced this site in the sequence to greater than BOOL-HACKS.

4. Emissions from these sites were among the highest reported. GREENFIELDS-OUTLET consistently had the lowest emissions and these were the most akin to those commonly reported for freshwater wetlands.

5. Methane emissions decreased with increasing flow path length at Willunga Wetlands and Greenfields Wetlands. Emissions were most strongly correlated with methane production rates (97%) and least strongly to sediment organic carbon content (63%).

6. Summer emissions were consistently higher than winter emissions at all of the wetland sites except GREENFIELDS-INLET where the inverse was true.

7. Inflow rate has a strong effect on methane emissions from Greenfields Wetlands. The unusual seasonal pattern in emissions from GREENFIELDS-INLET was caused by physical disturbance of the sediment by rapid inflows in winter rather than unseasonally high rates of methanogenesis.

8. Short periods of water column instability have the capacity to release significant quantities of methane if they occur when large amounts of methane are stored in the water column. For example, as much as 500 kg was released from WILLUNGA-INLET in two days at the end of summer 1996/1997 when the water column turned over after having been thermally stratified for several months.

9. Rates of methane emission at Bool Lagoon were strongly affected by the depth of inundation and the presence of aquatic vegetation. Emissions decreased with

decreasing water depth and exposed but waterlogged sediments appeared to consume methane.

10. Wetland size is the single most important factor in determining annual methane emissions on the whole wetland scale. Emissions from Willunga Wetlands on a per unit area and time basis far exceeded those from Greenfields Wetlands and Bool Lagoon but Willunga Wetlands had the lowest annual emission on a whole wetland basis due to its relatively small size. Bool Lagoon, on the other hand, had the capacity to release almost 200 hundred times more methane than Willunga Wetlands on a whole wetland basis primarily because the inundated area of Bool Lagoon can be up to 800 times larger than that of Willunga Wetlands.

Chapter 7

Chapter 7: Final Discussion

7.1 Methane dynamics in the three wetland systems

It is evident that Willunga Wetlands, Greenfields Wetlands and Bool Lagoon differed with respect to their relative rates of methane production, oxidation and emission. These rate differences were supported by the different physico-chemical processes operating at the three wetlands. A schematic model of seasonal methane emissions and trends in key wetland parameters appears in Figure 7.1.

The constant inflow of secondary treated sewage into Willunga Wetlands provided a highly stable and productive environment for methanogens and methanotrophs, particularly over the summer months when the water column was persistently thermally stratified. Water levels were relatively static, ranging from 3.1m in winter to 2.9m in summer. Oxygen was rapidly depleted from the overlying water and thus the oxic: anoxic interface occurred within the water column of Willunga Wetlands at between 1 and 1.5m depth depending on site and season, being more shallow at WILLUNGA-INLET and during the summer months.

The complimentary conditions of a stable, anoxic hypolimnion and high organic loading (DOC > 25 mg L^{-1}) supported extremely high rates of methanogenesis in the hypolimnion and floccuent sediment (> 350 nmol mL^{-1} h^{-1}) of WILLUNGA-INLET. It is also likely that fermenter and methanogenic organisms were washed into Willunga Wetlands in the inflowing sewage. Rates of this magnitude are more akin to those reported for sewage biodigestors (Salminen and Rintala, 1999) than for natural wetlands (Boon and Sorrell, 1991; Bachoon and Jones, 1992; Boon and Mitchell, 1995; Casper, 1996). Methanogenesis occurred in both the anoxic water layers and sediment. It was dependent on season, being higher in summer than winter and was limited by the availability of suitable organic substrates. At WILLUNGA-INLET, the methanogenic carbon supply was primarily limited by the rate of fermentation but at WILLUNGA-OUTLET organic carbon availability per se limited methanogenesis along with the rate of fermentation. There was some evidence for competitive interactions between methanogens and sulphate-reducing bacteria in WILLUNGA-INLET (at limiting sulphate concentrations) but the process of anaerobic methane oxidation may have confounded these results (see Chapter 5) and it may be that the methanogens and sulphate-reducers were not in direct competition which was the case in the sewage bioreactors studied by Ueki and Ueki (1990).

A gradient from aerobic to anaerobic methanotrophy occurred with increasing depth in WILLUNGA-INLET, concurrent with decreasing oxygen concentrations and increasing methane concentrations. Aerobic methanotrophy consumed up to 8 nmol CH_4 mL^{-1} h^{-1} in the surface waters in summer and anaerobic methanotrophy consumed up to 140 nmol CH_4 mL^{-1} h^{-1} in the benthos. Nevertheless, net methanogenesis was observed at a rate of 106 mmol CH_4 m^{-2} h^{-1} in February 1997. This is unusual for a natural freshwater wetland where the potential for methanotrophy usually exceeds that for methanogenesis (Moore and Knowles, 1987; Moore and Knowles, 1990: Boon and Mitchell, 1995; Krumholz et al., 1995; Nedwell and Watson, 1995; Boon and Lee, 1997) and this observation strengthens the conclusion that the WILLUNGA-INLET system behaves more like a sewage bioreactor than a freshwater wetland as described by Zinder (1993). Rates of aerobic methanotrophy may have been depressed by the presence of ammonium ions in the sewage, as has been reported in upland soils and rice paddies (Bedard and Knowles, 1989; Steudler et al., 1989; Bronson and Mosier, 1994; Crill et al., 1994; Schnell and King, 1994). If so, this would have resulted in increased rates of net methane production at Willunga Wetlands. It is likely that the inhibitory effect of ammonium was minimal because a) the pH at Willunga Wetlands became strongly alkali during the daytime when oxygen levels were at their highest (and thus when aerobic methanotrophy would be most favoured) and b) the ammonium inhibition studies cited above occurred in upland and relatively oxic paddy soils where aerobic methanotrophy would have been dominated by type I methanotrophs. At Willunga Wetlands the methane to oxygen ratio was more likely to favour the dominance of type II methanotrophs and the effect of ammonium in these conditions is largely unstudied.

Atmospheric emissions from WILLUNGA-INLET were very high (mean > 8 mmol m^{-2} h^{-1}; Figure 7.1) compared to those reported for other freshwater systems (Kiene, 1991; Boon and Sorrell, 1995; Cao et al., 1996), even highly eutrophic ones (Verma et al., 1999). Rates of methane production, oxidation and emission all decreased with increasing flow path length, as did the organic carbon and methane concentrations in the water and sediment. This indicates that water flowing through Willunga Wetlands decreased in methanogenic action and was therefore improved in overall quality. However, rates of methane emission from WILLUNGA-INLET were high compared to those from Bool Lagoon, suggesting that the treatment was not complete and that the outflow from Willunga Wetlands would still demand significant quantities of oxygen from any receiving water body. Therefore the treatment provided by this wetland was inadequate.

In comparison with the highly stable inflow regime at Willunga Wetlands, inflows to Greenfields Wetlands were variable, ranging from zero to over 1000 L s^{-1}. Stormwater inflows peaked in winter/spring, were negligible in summer except for occasional storms, and were intermediate but variable during autumn. The organic carbon content of the sediments at Greenfields Wetlands was very similar to that in the sediments of Willunga Wetlands but the organic content of the stormwater entering Greenfields Wetlands (DOC < 7 mg L^{-1}) was four times lower than that of the sewage entering Willunga Wetlands (DOC > 25 mg L^{-1}). In addition, the water column at Greenfields Wetlands was too shallow to support persistent thermal stratification during summer thus oxygen was available at the sediment surface, rendering it only moderately reducing (-50 to -250 mV). Overall, the methanogenic habitat provided at Greenfields Wetlands was less favourable than that provided at Willunga Wetlands. This was evident by the methanogens only being detected in the sediment environment at Greenfields Wetlands.

Methanogenesis at Greenfields Wetlands was more rapid in summer than winter. It was strongly limited by organic carbon availability in both seasons as well the rate of fermentation. These effects became more apparent with increased flow path length. The control rate of methanogenesis at GREENFIELDS-INLET (80 nmol mL^{-1} h^{-1}) in summer was 13 times higher than that at GREENFIELDS-OUTLET (6 nmol mL^{-1} h^{-1}). The methanogens at Greenfields Wetlands were also competing with nitrate-reducing and sulphate-reducing bacteria for these limiting substrate supplies, although it is uncertain to what degree anaerobic methanotrophy may have confounded these results. The decrease in rates of methane production along the flow path was more rapid at Greenfields Wetlands that at Willunga Wetlands, suggesting that methanogenesis in the Greenfields Wetlands complex was more strongly limited by organic carbon supply. The low rates of methanogenesis at GREENFIELDS-OUTLET also suggest that the outflow from Greenfields Wetlands was very low in methanogenic action and was therefore unlikely to adversely affect oxygen regimes in receiving water bodies.

Methanotrophy was an exclusively aerobic process at Greenfields Wetlands. Methanotrophic activity was detected in the water column and floc, being highest active in the floc of GREENFIELDS-INLET (-490 nmol mL^{-1} h^{-1}) and lowest in the surface waters of GREENFIELDS-OUTLET (-0.15 nmol mL^{-1} h^{-1}). Potential rates of methanotrophy exceeded those for methanogenesis, which is apparently typical for natural freshwater systems (Moore and Knowles, 1987; Moore and Knowles, 1990: Boon and Mitchell, 1995; Krumholz et al., 1995; Nedwell and Watson, 1995; Boon and Lee, 1997). Nevertheless, emissions from GREENFIELDS-INLET were greater than or equal to those from either Bool Lagoon or Ryan's Billabong (Muller et al., 1994; Boon and Sorrell, 1995)

and are among the highest reported for any freshwater system (Cao et al., 1996; Kiene, 1991; Verma et al., 1999).

Methane emissions from Greenfields Wetlands were so strongly affected by the rate of inflow that seasonal rates of methane emission were uncoupled from seasonal rates of methanogenesis. In winter, inflow rates and emissions were at their annual peak even though rates of methanogenesis and methanotrophy were at their lowest annual levels. In summer, when flow rates were low and rates of both methanogenesis and methanotrophy were at their highest, methane emissions were lower than in winter. It is hypothesised that rapid inflows during winter scoured the sediment and released accumulated methane primarily through enhanced rates of methane ebullition. In summer, the water column was stratified and emissions were lower than in winter due to slower transport times between the sediment and the atmosphere and greater relative rates of methanotrophy (see Chapter 6).

The water regime at Bool Lagoon was more variable than that in the other two systems. None of the basins filled to capacity at any time during the study period. Each summer the water table dropped, exposing the sediment over most of the system. The complex became so dry in late summer 1995/1996 that the site BOOL-HACKS had to be abandoned because it was no longer inundated and therefore no longer represented a suitable site to compare with the permanently inundated sites at Willunga Wetlands and Greenfields Wetlands.

The water column, like that at Greenfields Wetlands, was too shallow to support persistent thermal stratification over summer and thus the oxic:anoxic interface occurred in the sediments. The sediment organic carbon content at Bool Lagoon was higher than that at Greenfields Wetlands but the sediments were, if anything, less reducing ($^{+}50$ to $^{-}280$ mV). The net result was that the methanogens were restricted to the sediment environment and the net rates of methanogenesis were very similar to those at GREENFIELDS-INLET. Methanogenesis at Bool Lagoon was not limited by organic carbon per se, as had been the case at Greenfields Wetlands, but rather it was limited by the rate of fermentation. The addition of fermenters enhanced rates of methanogenesis independently of substrate additions, suggesting that the organic carbon supplies in the sediment were adequate for the extant methanogenic population but too refractory to allow for rapid and sustained supply of suitable methanogenic precursors. Methanogenesis at Bool Lagoon also appeared to be limited by competition with nitrate reducers, but again the magnitude of anaerobic methanotrophy was unknown and may have confounded the results.

Potential rates of methanotrophy (-388 nmol mL^{-1} h^{-1}) exceeded those for methanogenesis (51 nmol mL^{-1} h^{-1}) in the benthos at BOOL-HACKS by an order of magnitude, a similar ratio to that observed at GREENFIELDS-INLET and in other freshwater systems (Moore and Knowles, 1987; Moore and Knowles, 1990: Boon and Mitchell, 1995; Krumholz et al., 1995; Nedwell and Watson, 1995; Boon and Lee, 1997). Methane emissions from BOOL-HACKS (>2 mmol m^{-2} h^{-1}) were similar to those from GREENFIELDS-INLET in summer but were much lower in winter due to seasonal coupling of methanogenesis and methane emissions at Bool Lagoon.

The overarching hypothesis erected in Section 1.6 that *'the wetland which provides conditions most favourable for methanogenesis will support the highest rates of methane production, oxidation and emission'* was generally supported by the data presented in this thesis. Willunga Wetlands provided the most favourable conditions for methanogenesis and had the highest potential rates of methanogenesis and the highest atmospheric emissions (in summer). Greenfields Wetlands provided a less favourable environment for methanogens, particularly at the outlet in winter, and this was reflected in lower rates of methanogenesis and methane emissions. The underlying assumption to this hypothesis that rates of aerobic methane oxidation and methane emission will be coupled to rates of methanogenesis was also generally supported although several important deviations were detected, such as the seasonal uncoupling of methane production and emission at Greenfields Wetlands and the apparent dominance of anaerobic methanotrophy at Willunga Wetlands.

These studies suggest that significant differences occur in methane dynamics across natural and constructed wetlands and between different constructed wetland systems. Methane emissions at a given wetland site at a given time cannot necessarily be predicted from knowledge of key wetland parameters (such as sediment organic carbon content) which are known to be strong predictors of methane emissions from natural wetlands (Conrad, 1996; Segers, 1997; Vandernat and Middelburg, 1998). Instead, influent organic carbon content was a strong predictor and other factors such as inflow velocity or water column thermodynamics had very strong but temporary impacts. The factors listed below have been identified as the key controllers of methane dynamics in wetlands constructed for wastewater treatment:

- influent organic carbon content
- influent organic carbon quality
- distance from the inlet
- inflow velocity and variability
- thermal structure of the water column
- depth to the oxic:anoxic interface
- rate of fermentation
- rate of methanogenesis
- availability of alternative electron acceptors
- basin design
- wetland surface area; and
- flow path length.

7.2: Implications for the Australian Inventory

Rates of methane emission detected from the constructed wetland sites were variable but were generally high compared to other freshwater systems in temperate, south-eastern Australia (Muller et al., 1994; Boon and Sorrell, 1995) and abroad (Cao et al., 1996; Kiene, 1991; Verma et al., 1999). Specific rates of methane emission from WILLUNGA-INLET in summer were the highest (mean 8.5 mmol m^{-2} h^{-1}) and emissions from GREENFIELDS-OUTLET in winter were the lowest (mean 0.1 mmol m^{-2} h^{-1}) compared with intermediate rates from GREENFIELDS-INLET and Bool Lagoon.

However, it was found that the size of the wetland system was a more important variable than the specific rate of methane emission with respect to whole wetland emissions estimates and thus national inventories. For example, the expansive Bool Lagoon system (2500 ha) would emit 1,326,600 kg of methane in a year if it were assumed that the system filled to capacity in winter and mean summer emissions were 2.1 mmol m^{-2} h^{-1}. In contrast, the relatively small Willunga Wetlands system would emit only 7,110 kg per year assuming an area of 3 ha even if summer methane emissions exceeded 8 mmol m^{-2} h^{-1} for part of the system. Greenfields Wetlands at 40 ha was intermediate in size and in annual emission (67,400 kg). However, the annual budget for Bool Lagoon could be reduced to 37,700 kg (approximately half of that from Greenfields Wetlands) if the system were assumed to have experienced a dry winter and only 10 ha were inundated in summer (see Chapter 6 for details). Therefore accurate estimation of the inundated area or wetland size is a critical factor in determining the contribution a wetland or group of wetlands makes to a national inventory.

Knowledge of methane dynamics in Australian wetlands was, until this current thesis, limited to five wetland systems in south-eastern Australia; Ryan's Billabongs 1 and 5, Ryan's Bog, Raftery's Swamp, Kiewa Billabong and Bool Lagoon (Boon and Sorrell, 1991; Sorrell and Boon , 1992; Muller et al., 1994; Boon and Sorrell, 1995; Ganf et al., 1998). The data presented here for Willunga Wetlands and Greenfields Wetlands make a significant contribution by providing directly comparable information on methane dynamics for constructed wetlands in the same geographical location as that obtained for natural systems. Such comparisons show that wetlands constructed for wastewater treatment have the capacity to emit more methane on a per unit time and area basis than natural wetlands, primarily because of their enhanced rates of organic carbon input and methanogenesis. This observation further suggests that emissions from natural wetlands in south-eastern Australia will increase if these systems were to become more eutrophic or

if productivity, and therefore autochthonous carbon supplies, were to increase in response to the Greenhouse Effect.

However, there is a lack of data on methane emissions from tropical, desert and alpine regions as well as from different types of aquatic systems such as peatlands, salt lakes, saline marshes, regulated rivers and estuaries in Australia upon which to test these hypotheses. This lack of base data will limit our understanding of future trends in methane emissions and our abilities to manage them.

No comprehensive national methane budget has been conducted for Australia thus far. One study by CSIRO Division of Atmospheric Research (Galbally et al., 1992) attempted to estimate methane emissions from Australian wetlands as part of a wider study to determine the influence of the biosphere and the impact of different land-uses on greenhouse gas processes in Australia. This paper showed that anthropogenic methane emissions (3.3 Mt y^{-1}) were significantly greater than natural emissions (-0.7 Mt y^{-1}), a conclusion largely based on the ability of terrestrial systems to consume large quantities of methane. Neither the categories for anthropogenic or natural emissions contained wetlands constructed for wastewater treatment, sewage treatment facilities, irrigation storage facilities, regulated rivers or impounded reservoirs (although rice paddies were included). These omissions are notable because significant emissions are likely to occur from these types of biological systems in Australia, particularly over the warm summer months.

The emission estimates presented by Galbally et al. (1992) were based almost exclusively on data from northern hemisphere systems and assumed rates of 5, 10 and 40 g CH_4 m^{-2} y^{-1} for methane emissions from littoral wetlands, inland wetlands and rice paddies, respectively. They omitted the wetlands of central Australia on the basis that knowledge of the dynamics in surface area was too poor to be useful. However, given that summer temperatures exceed 40°C and wetland areas can exceed 5.7 million ha in central Australia (Spiers and Finlayson, 1999), it is likely that when these systems are inundated they release significant quantities of methane. These systems should be further studied and adequately represented in national inventories.

Galbally et al. (1992) estimate that Australian wetlands emit 0.2 Mt CH_4 y^{-1}. This estimate is an order of magnitude less than that estimated by Matthews and Fung (1987). Galbally et al. (1992) considered this disparity to be due to overestimation by Matthews and Fung (1987) of central Australian wetland areas and thus emissions. The accuracy of that statement is difficult to assess given that Galbally et al. (1992) omitted central Australian

wetlands entirely. But it is apparent that the generation of a *bona fide* estimate of methane emissions from Australian wetlands is limited by:

1) a lack of information about wetland types, water depths and surface areas, particularly for constructed and ephemeral wetlands; and
2) a lack of methane emission estimates from a range of wetland systems across the different climates of Australia.

Therefore, at this time, the magnitude of methane emissions from Australian wetlands is effectively unknown and a comprehensive assessment of the impacts that the data on methane dynamics in constructed wetlands contained in this thesis have on the national inventory would be premature.

7.3: Future Research and Management Directions

The studies undertaken for this thesis provide valuable data on factors controlling methane dynamics in constructed wetlands from which suggestions for future research and management directions can be drawn.

Methanogenesis was found to be limited by substrate availability at all of the wetland sites even WILLUNGA-INLET which was fed with effluent containing more than 25 mg L^{-1} DOC. At the more organic sites, such as WILLUNGA-INLET and BOOL-HACKS, methanogenesis was limited by the rate of fermentation. Whilst at the less organic sites, such as GREENFIELDS-INLET, methanogenesis was limited by the availability of organic carbon per se. These limitations were defined by observing increases in the rate of methanogenesis when organic carbon and/or fermentative bacteria were added to incubating samples. These experiments suggest that increasing the number of fermentative bacteria will lead to an increase in the rate of decomposition and thus the rate of methanogenesis. However, the interactions between different groups of anaerobes that are capable of producing and consuming methane are highly complex thus it cannot be unequivocally concluded that the rate of methanogenesis increased. It may have been that the rate of anaerobic methanotrophy decreased.

To further understand anaerobic methanotrophy, intensive research effort needs to be directed towards understanding the interactions between different anaerobic bacterial functional groups and towards identifying the full suite of microbes present. In particular, the capacity for different organisms to produce and/or consume hydrogen needs to be investigated as this is a critical process in syntrophic methanogenesis (and thus anaerobic methanotrophy) and these processes have been found to be more widespread in nature

than previously thought. The role of fermenters in controlling the switch between methanogenesis and reverse methanogenesis also needs to be investigated because the mode of fermentation may dictate the relative rates of methane production and consumption in anaerobic, freshwater sediments. Homoacetogenesis is also poorly understood as a potential factor controlling net methane production or as an alternative electron sink. The use of quantitative PCR techniques to enumerate different populations of methanogens and methanotrophs would be highly profitable.

The Willunga Wetlands site may provide an unique environment for addressing these future research objectives. The high organic loading, coupled with thermal stability of the water column over summer, provides a stable gradient from oxic to anoxic conditions. The system operates similarly to a sewage reactor with respect to methane dynamics but is open to the environment and thus uncontrolled migration of bacterial species. The open nature of the site may prove valuable with respect to identifying novel species with the capacity to produce or consume methane. Molecular techniques such as 16srRNA probes would be ideal because they do not require the culturing of cells in order to identify species or functional groups.

Experiments conducted at Willunga Wetlands on methane dynamics have, thus far, been hampered by using changes in dissolved methane concentration over time as the only measure of both methanotrophic and methanogenic activities. This technique confounds the results in anaerobic conditions where both processes may be operating. It is imperative that future investigations utilise a combination of molecular techniques, including:

- ion-sensitive sensors for core and *in situ* measurements;
- *in situ* incubation techniques with isotopic tracers and benthic chambers; and
- molecular genetic methods such as polymerase chain reaction and mRNA to measure microbial activity.

A profitable line of research may be to characterise methane dynamics at intervals down the water column during summer stratification, autumnal turnover and winter mixing. It would be predicted that during the summer stratification period a graduation from aerobic communities (methane-consuming) to anaerobic communities (net methane-producing) would occur. When the water column turns over in autumn, the microbial distribution would be disturbed and many of the organisms would be subjected to sub-optimal oxygen conditions due to water column mixing. Mixing is expected to persist over winter until a

new gradient is formed in the next summer stratification period when the microbial gradient can reform.

These experiments could be designed to answer the following questions:

- does a gradient from aerobic methanotrophic communities to anaerobic methanogenic communities form during summer stratification?
- is this gradient lost during water column mixing?
- if so, does exposure to sub-optimal oxygen concentrations adversely affect the activities of methanotrophs and/or methanogens in warm autumn months?
- is this effect dependent on the water temperature at the time of mixing and thus the seasonal level of cellular activity?
- is methane produced in the ostensibly oxic water layers during stratification?
- is methane consumed in the ostensibly anoxic water layers?
- what is the fate of accumulated methane during autumnal turnover?

These types of investigations could also be performed at Greenfields Wetlands and Bool Lagoon in order to determine whether anaerobic methanotrophy occurs in these sediments that have a permanently oxic interface with the water column and thus are only moderately reducing. Further understanding the extent of anaerobic methanotrophy and the factors controlling it may be the most profitable line for mitigating methane emissions from natural and constructed wetlands because anaerobic methanotrophs can access methane at the point of production unlike aerobic methanotrophy, which is relatively dependent on transport from the anaerobic methanogenic zone (even if this were only on the scale of micrometres). These investigations could be expanded to develop full methane budgets for the three wetlands and may lead on to predicting the effects on methane dynamics of processes such as aquatic plant growth and senescence and burrowing by aquatic fauna.

Modelling of methane emissions from constructed wetlands in order to predict the effect of processes such as Global Warming or the amount of methane a proposed constructed wetland will produce, is currently hampered by a lack of knowledge on the effects of basin design, flow rate, short-term enhanced methane emissions (e.g. winter emissions from Greenfields Wetlands, Chapter 6) and the presence of aquatic vegetation. It is necessary that we understand these processes in order to manage methane emissions from wetlands effectively. For example, during a stormy period in February 1997, when the water column at WILLUNGA-INLET underwent one of the first turnover events for the year,

500 kg of methane was apparently 'lost' from the water column over twelve days (see Chapter 6 for details). Was this methane lost to the atmosphere or was the rate of methanotrophy temporarily enhanced to levels high enough to consume the upwelling methane prior to release? Results from this experiment could be compared with those of Utsumi et al., (1998) for dimictic Lake Nojiri, Japan.

At GREENFIELDS-INLET, winter emissions were greater than those in summer, unusual for a freshwater wetland. This was attributed to high velocity, intermittent stormwater inflows scouring the sediment surface and releasing trapped methane. How important are these episodes to the annual methane budget for Greenfields Wetlands? Or for the regional budget? These types of questions cannot be answered by interrogating existing data sets or by routine sampling protocols and remain fundamental questions in our understanding of wetlands methane dynamics.

Fundamental too, is an understanding of how methane dynamics change as a constructed wetland matures from having predominantly mineral sediments to predominantly organic sediments. Will there be a point where methane emissions are too high to be acceptable? Will rates of methanogenesis change as the organic carbon pool changes from one dominated by allochthonous carbon to one dominated by autochthonous carbon? Can these data be used to predict whether a proposed constructed wetland is capable of processing the expected carbon loads adequately and rountinely? Are downstream and connected aquifer systems at risk of high oxygen demands from poorly treated water leaving the constructed wetland system?

In conclusion, the data presented in this thesis have raised many research questions but have also clearly identified the following management guidelines for minimising methane emissions from wetlands constructed to treat wastewater:

- Divert high flows from entering the treatment wetland. High flows may lead to enhanced short-term emissions (e.g. GREENFIELDS-INLET) and are likely to undermine the treatment capacity of the wetland by disturbing aquatic plant beds and sediments.
- Maintain oxic water column. The lowest methane emissions came from wetlands where the oxic:anoxic interface occurred in the sediment rather than in the water column.
- Install pre-treatment ponds and field trial the use of fermenters. Rapid initial decomposition of the incoming pollutants may reduce the flow path

length required for adequate treatment and thus the amount of land needed.

- Encourage methanotrophy in dry wetlands and terrestrial systems, particularly those adjacent to or downwind of methanogenic wetlands. Methane sources and sinks need to be managed on a landscape scale. It is likely that agricultural improvement of rural land has decreased the capacity of terrestrial soils to consume methane thus wise management of constructed wetlands should aim to increase the regional methanotrophic capacity as well as mitigating methane emissions from the new point source.
- Routinely monitor oxygen concentrations at the discharge point of constructed wetlands to increase understanding of different management strategies and to protect downstream and underground water resources from high oxygen demands.
- Develop a register of constructed wetlands including critical design and water quality parameters. Such a register would enable a national inventory of constructed wetlands and estimations of their methane emissions to be generated.
- Control wetland construction. Development approval processes for wetlands need to include an assessment of the potential environmental impacts of the development including greenhouse gas emissions and strategies for mitigating these emissions.
- Develop a Code of Practice. Management protocols that are used in industries with a developed understanding of their methane emissions, e.g. wetland rice growers, dairy farming and municipal sewage treatment, should be reviewed and developed into a Code of Practice for constructed wetland design and management to optimise wetland performance and minimise greenhouse gas emissions. The treatment capacity of a constructed wetland, once commissioned, should be routinely assessed and a series of triggers developed to alert managers as to when to intervene.

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