# THE FATE OF METHANE IN A NEW ZEALAND PULP AND PAPER MILL WASTEWATER TREATMENT SYSTEM

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# Abstract

The wastewater produced by Carter Holt Harvey's Kinleith pulp and paper mill in Tokoroa is currently treated by a series of aerobic ponds, designed to remove organic pollutants. The treatment system has experienced increases in Biological Oxygen Demand (BOD, a measure of organic pollution strength) at the outlet. The increases in BOD happened when no significant changes in wastewater influent flow and/or characteristics were recorded. The surge in BOD has caused the outlet discharge limit to be exceeded in the past. Based on previous studies, we believe that aerobic oxidation of methane ($CH_4$), by microorganisms called methanotrophs, can cause the generation of methanol (a compound that can cause an increase in BOD load in waste streams), under conditions that repress the further conversion of methanol into biomass and carbon dioxide ($CO_2$). The overall objectives of this project therefore were:

- To determine if the biosynthesis of $CH_4$ can occur in the treatment system
- To determine if the subsequent biological aerobic $CH_4$ oxidation to methanol can occur in the treatment system
- Whether enough methanol can be generated to affect the treatment system's performance in terms of BOD removal.

Evidence was found that confirmed $CH_4$ was formed in the pond sediments across the entire treatment system (with rates significantly higher near the inlet). $CH_4$ is therefore available in the treatment system for aerobic $CH_4$ oxidation.

The presence of aerobic conditions and the absence of dissolved $CH_4$ in the water column (part of the pond where wastewater flows above the sediment), showed that aerobic $CH_4$ oxidation can occur in the water column of the treatment system (except at the oxygen limited inlet). Laboratory testing also confirmed that aerobic $CH_4$ oxidation can occur and methanotrophs are present (albeit in small numbers) in the water column.

A model was used to determine if aerobic $CH_4$ oxidation to methanol can cause the BOD increase at the outlet. The model found that the rates ($CH_4$ production in the sediment and $CH_4$ oxidation in the water column) needed to cause the surge in BOD was significantly higher than the maximum rates calculated from laboratory tests of

samples collected from the treatment system. Aerobic $CH_4$ oxidation was therefore unlikely to cause the BOD increase at the outlet.

It is possible that the BOD increase is due to benthic feedback (anaerobic sludge layer becomes buoyant, suddenly releasing soluble compounds into the aerobic water column of a pond). The BOD increase experienced at the outlet followed similar characteristics associated with benthic feedback (event was random, occurred at a pond with an oxygen limited sludge layer and aerobic water column). If the cause of the BOD increase needs to be determined in the future, further investigation into benthic feedback is recommended.

To conclude, aerobic $CH_4$ oxidation to methanol can occur in the treatment system, but is unlikely to cause the BOD increase at the outlet of the treatment system.

The technical capabilities and knowledge developed during the project will likely benefit those in the pulp mill industry. Methods and techniques have been developed to investigate the generation and fate of $CH_4$ within a pulp mill aerated pond.

# Acknowledgements

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# 1 Introduction

The Kinleith pulp & paper mill is located on the outskirts of Tokoroa, New Zealand. The mill employs 570 people and covers an area of 60 hectares. Kinleith produces 270,000 tonnes of market pulp and 330,000 tonnes of paperboard (used for packaging) annually (CHH Pulp & Paper Ltd New Zealand, 2009).

The Kinleith wastewater treatment system consists of a clarifier, a non aerated holding basin and a series of five aerated ponds; the treated effluent is discharged into Lake Maraetai (Figure 1-1). The treatment system treats around 80,000 - 90,000 $m^3$ of wastewater every day (Maclean*, et al.*, 2007). Ninety percent of BOD (Biochemical Oxygen Demand (BOD) a measure of organic pollution strength) is removed, discharging approximately 1.85 tonnes BOD/day (t BOD/d) at the outlet (Slade, 2009).

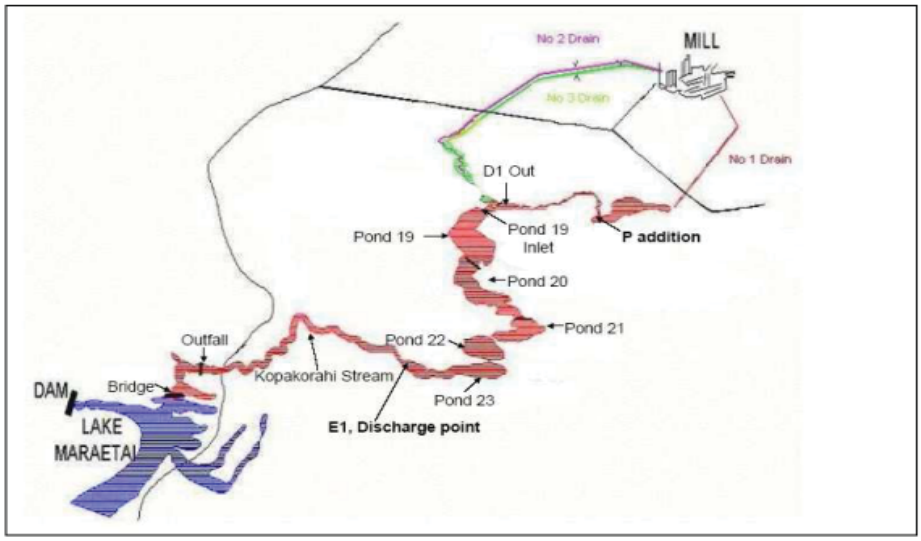


**Figure 1-1: Schematic diagram of Kinleith wastewater treatment system**

In 2006, a study by Slade showed increases in BOD at the outlet of the treatment system at times when there were no significant changes in the influent wastewater flow and/or characteristics, for a duration of three months (Figure 1-2). The increase in BOD load caused the BOD discharged from the treatment system to exceed the 2.5 t BOD/d discharge limit set by Environment Waikato. Changes in the operation of the treatment system during 2007 seemed to have decreased the duration of the unaccounted BOD increases at the outlet, however is still an ongoing problem (Slade, 2008, 2009).

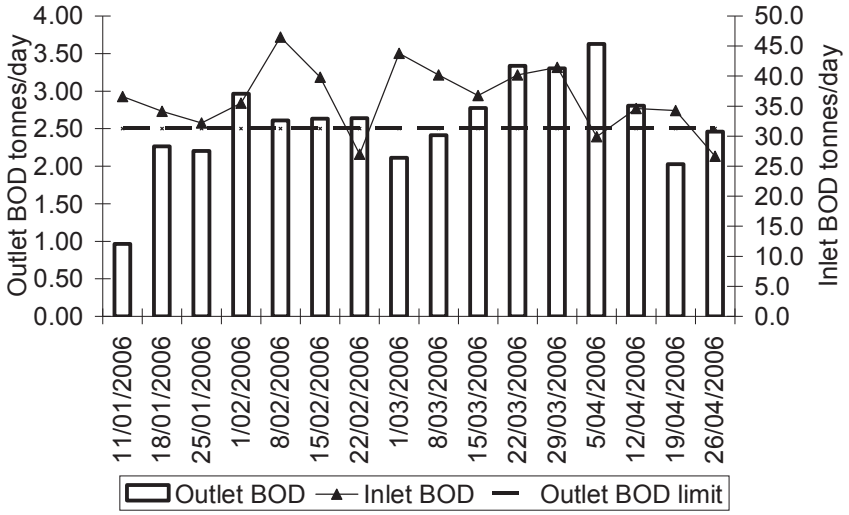


**Figure 1-2: Outlet BOD increases with no corresponding increases in Inlet BOD**

The results of previous studies on the treatment system have led to suggestions that the increase in BOD load could be due to methane ($CH_4$) generated in the sediment, under anaerobic conditions (Gielen*, et al.*, 2009; Slade, 2009; Thorn*, et al.*, 2009). The $CH_4$ generated can subsequently be aerobically oxidized to methanol by microorganisms called methanotrophs, and released into the treatment system, which may cause the increase in BOD load. Methanol was considered as the cause of the increase in BOD load instead of $CH_4$, due to the BOD assay method used during monitoring.

It should be noted that the BOD assay method used for monitoring wastewater treatment efficiency required site samples to be transferred to BOD bottles in the lab. This transfer would have most likely caused any $CH_4$ dissolved in the sample to diffuse out (based on tests done at Massey University). Therefore the initial amount of $CH_4$ in the BOD bottles was likely close to zero and the presence of dissolved $CH_4$ at the outlet of the treatment system was not considered as the cause of the increase in BOD load. Methanol, however, does not diffuse rapidly during sample transfer and would therefore contribute to the BOD measured.

The primary objective of the study was to determine if aerobic $CH_4$ oxidation to methanol could have caused the unaccounted BOD detected at the outlet of the treatment system. Therefore the following tests were done:

- Several parameters were monitored at different locations, to determine the potential for anaerobic $CH_4$ production and aerobic $CH_4$ oxidation in the treatment system.

- $CH_4$ gas emission and dissolved $CH_4$ concentration were monitored from different locations, in order to establish the $CH_4$ potential in the treatment system.

- The Biochemical Methane Potential (BMP: test for anaerobic $CH_4$ production potential) of sediment and water column (part of pond where wastewater flows above the sediment) samples collected at different locations in the treatment system was evaluated.

- Aerobic $CH_4$ oxidation assays using sediment and water column samples from different locations in the treatment system as inocula were conducted to find out if aerobic $CH_4$ oxidation can occur in the treatment system.

- The presence of methanotrophs in various samples was established using Fluorescent *in situ* hybridization (FISH).

# 2 Literature Review

The goal of this review was to examine:

- The biochemistry and microbiology of aerobic $CH_4$ oxidation
- The effect of different environmental parameters on aerobic $CH_4$ oxidation
- What is already known regarding $CH_4$ associated with pulp and paper mill wastewater
- The fate of $CH_4$ in aquatic environments, specifically lakes

The fate of $CH_4$ in lakes was reviewed, as lakes closely resemble the ponds in the treatment system (when compared to other aquatic environments). Lakes were reviewed because there was a lack of aerobic $CH_4$ oxidation data regarding aerated wastewater treatment ponds.

## *2.1 Biochemistry and Microbiology of Aerobic $CH_4$ Oxidation*

This section provides a brief explanation of methanogenesis, followed by an explanation of the aerobic $CH_4$ oxidation reaction pathway and finally the effect of different environmental parameters on aerobic $CH_4$ oxidation.

### 2.1.1 Methanogenesis

The main products of anaerobic degradation of organic compounds are: $CH_4$, carbon dioxide ($CO_2$) and biomass. Optimal conditions for anaerobic degradation requires the complete absence of oxygen ($O_2$), a pH of between 5 and 7 and a temperature range between 25 and 35 °C (Tchobanoglous*, et al.*, 2003). Anaerobic degradation occurs in two main steps. In the first step, called acidogenesis, microorganisms break down organic material into acids. In the second step, the acids are used as a carbon source by microorganisms called 'methanogens,' producing biomass and $CH_4$ (Tchobanoglous*, et al.*, 2003).

### 2.1.2 Aerobic $CH_4$ Oxidation

Methanotrophs are a subset of a bacterial group called methylotrophs; which only use 1-C compounds as a carbon and energy source (Bedard & Knowles, 1989; Hanson & Hanson, 1996). Methanotrophs use $CH_4$ as their sole carbon and energy source (Bedard & Knowles, 1989; Hanson & Hanson, 1996). They are commonly found in the aerobic interfaces of methanogenic environments (Knief & Dunfield, 2005). Such methanogenic environments include wetlands (Roden & Wetzel, 1996; Segers, 1998), rice paddies (Conrad & Rothfuss, 1991), lakes (Bastviken*, et al.*, 2004; Bastviken*, et al.*, 2008; Bastviken*, et al.*, 2002; Duc*, et al.*, 2010; Dzyuban, 2003, 2010) ponds and lagoons (Chabir*, et al.*, 2000; Koné*, et al.*, 2010).

### 2.1.3 Reaction Pathway

The aerobic $CH_4$ oxidation pathway involves multiple steps (Figure 2-1). Aerobic $CH_4$ oxidation to methanol is catalyzed by the enzyme methane monooxygenase (MMO) which exists in two forms; particulate MMO (pMMO) and soluble MMO (sMMO). MMO splits the bond between $O_2$, with one oxygen atom reduced to $H_2O$, the other oxygen atom incorporated to $CH_4$ to form methanol (Bedard & Knowles, 1989; Hanson & Hanson, 1996). Methanol is further degraded into formaldehyde (HCHO), which is assimilated into biomass or further oxidized to $CO_2$ via formic acid (HCOOH) (Bedard & Knowles, 1989; Hanson & Hanson, 1996). There are two pathways HCHO can be assimilated into biomass, the serine pathway and the RuMP pathway. In the serine pathway, HCHO reacts with glycine to form serine. In the RuMP pathway, HCHO combines with ribulose monophosphate (RuMP) to form Hexulose-6-phosphate (Hanson & Hanson, 1996). From this point on, different enzymes and products are involved. Methanotrophs are grouped according to the pathway they use to assimilate HCHO into biomass. Methanotrophs that use the serine pathway are classified as type I methanotrophs and methanotrophs that use the RuMP pathway are classified as type II methanotrophs.

Methanol extracellular accumulation does not normally occur during aerobic $CH_4$ oxidation by methanotrophs. Accumulation only occurs when specific inhibitors are present, which inhibit the enzyme methanol dehydrogenase (responsible for the

oxidation of methanol to formaldehyde). Inhibitors include cyclopropanol and sodium chloride. (Furuto*, et al.*, 1999; Lee S.G.*, et al.*, 2004; Takeguchi*, et al.*, 1997).

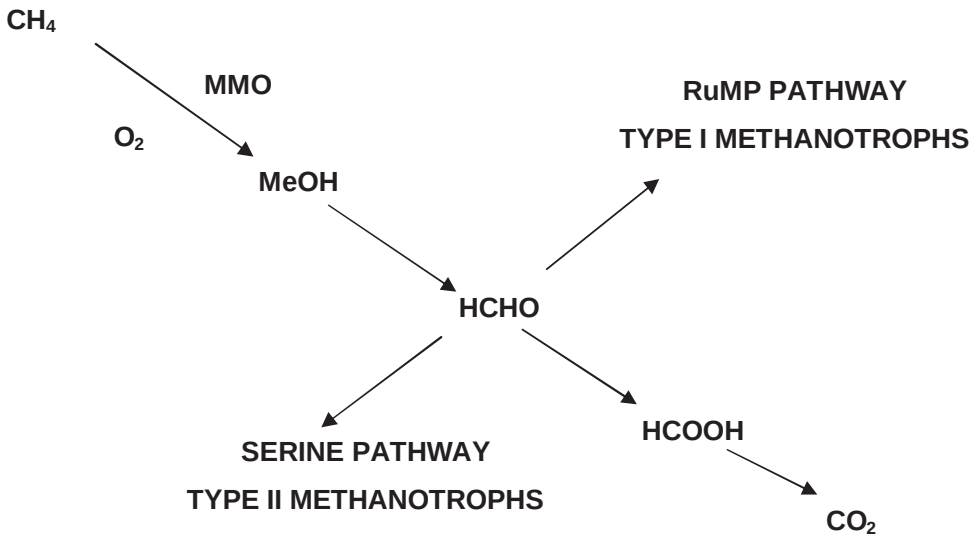


**Figure 2-1: Aerobic $CH_4$ oxidation reaction pathway**

Current research has focused on the first step of aerobic $CH_4$ oxidation, the conversion of $CH_4$ to methanol. This natural process has the potential to be developed into a cost effective process of producing methanol as a fuel source. Research has focused on determining the optimum conditions needed to produce the greatest amount of methanol (Furuto*, et al.*, 1999; Lee S.G.*, et al.*, 2004; Takeguchi*, et al.*, 1997; Xin*, et al.*, 2004). Research has also focused on providing a better understanding of the MMO enzymes, especially the pMMO which have been difficult to isolate (Balasubramanian*, et al.*, 2010; Hakemian & Rosenzweig, 2007; Himes*, et al.*, 2010; Lieberman & Rosenzweig, 2004; Murreil*, et al.*, 2000).

## *2.2 Parameters Affecting $CH_4$ Oxidation*

Environmental parameters such as temperature, pressure, pH and dissolved oxygen (DO) may affect the rate of aerobic $CH_4$ oxidation. Literature was therefore reviewed to find the effect of environmental parameter changes to aerobic $CH_4$ oxidation.

### 2.2.1 Temperature

Aerobic $CH_4$ oxidation occurs at a wide range of temperatures: Hanson & Hanson (1996) for example found that aerobic $CH_4$ oxidation in peat soils could occur between 0 to 35°C and Le Mer & Roger (2001) found that aerobic $CH_4$ oxidation in temperate forest soils occurred between 1 to 30°C. Temperature has also been shown to affect aerobic $CH_4$ oxidation rates, with maximum aerobic $CH_4$ oxidation rates consistently found to occur at temperatures between 25 to 35°C (Mor, et al., 2006; Nikiema, et al., 2007; Scheutz, et al., 2009). A drop below the optimum temperature range can cause a considerable decrease in aerobic $CH_4$ oxidation rates: For example, rates decreased by as much as 50% when temperature decreased from 30°C to 20°C (Nikiema, et al., 2007; Scheutz, et al., 2009). Therefore, aerobic $CH_4$ oxidation can occur within a wide range of temperatures and the rate of aerobic $CH_4$ oxidation is affected by temperature.

### 2.2.2 pH

pH does not seem to affect aerobic $CH_4$ oxidation. Hanson & Hanson (1996) found that the rate of aerobic $CH_4$ oxidation does not vary significantly, as the pH of peat soils varied from 3.5 to 8.0. Segers (1998) also found that the pH does not seem to affect aerobic $CH_4$ oxidation for different wetland samples. In lakes, aerobic $CH_4$ oxidation occurred between the pH values of 4.9 to 9.5 (Bastviken*, et al.*, 2008; Dzyuban, 2003, 2010).

### 2.2.3 DO and $CH_4$ Concentrations

Aerobic $CH_4$ oxidation commonly occurs at the chemocline of stratified lakes (Dzyuban, 2010; Hanson & Hanson, 1996; Harrits & Hanson, 1980; Schubert*, et al.*, 2010). The chemocline is the layer of water between the hypolimnion (anaerobic water layer) and the epilimnion (where DO concentration is comparably higher than the chemocline) (Figure 2-2). The chemocline provides methanotrophs with a favourable environment, as a steady supply of $O_2$ and a plentiful supply of dissolved $CH_4$ from the anaerobic water layer (as aerobic $CH_4$ oxidation cannot occur in the anaerobic conditions) are available. However, some studies have suggested that DO concentration may not significantly affect aerobic $CH_4$ oxidation. High $CH_4$ oxidation rates occurred in lakes where DO concentration was between 6 -12 mg/L (Utsumi*, et al.*, 1998), while another study found that optimum aerobic $CH_4$ oxidation rates occurred at DO concentrations of between 0.1 to 1.0 mg/L (Rudd*, et al.*, 1976). Soni *et al.* (1998) tested how pressure affected aerobic $CH_4$ oxidation. The results showed that increasing the headspace pressure using a $CH_4/O_2$ gas mixture increased the growth rate of the methanotrophs. This was due to an increase in dissolved $CH_4$ and DO concentration. However after a certain pressure (20psig in this case), growth was inhibited. Inhibition did not happen when an inert gas (nitrogen) was used to increase pressure. Therefore, high concentrations of $CH_4$ and $O_2$ may actually inhibit the growth of methanotrophs.

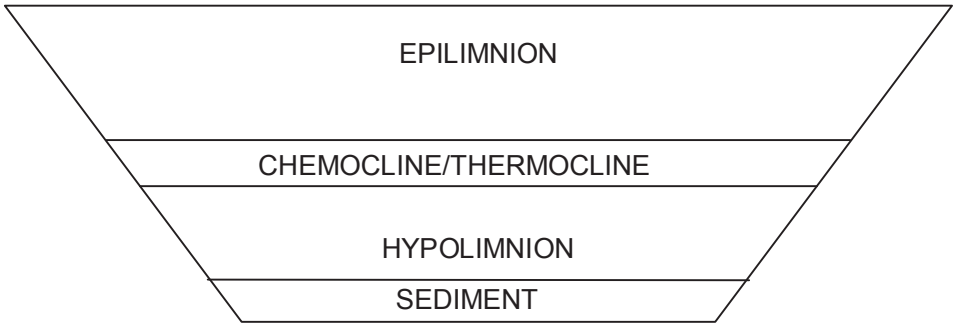


**Figure 2-2: Different water column layers in a lake**

### 2.2.4 Inhibitory Compounds

Hanson & Hanson (1996) found that ammonia inhibited aerobic $CH_4$ oxidation (at concentrations of 4 - 10 mM) and could be due to ammonia inhibiting the MMO enzymes. Copper ions have also been found to inhibit the enzyme sMMO and pMMOs

at high concentrations (Hanson & Hanson, 1996; Himes*, et al.*, 2010; Murreil*, et al.*, 2000). Bussman (2005) found that algae, calcium carbonate and calcium phosphate inhibited aerobic $CH_4$ oxidation. There is also evidence that light can inhibit aerobic $CH_4$ oxidation in lakes (Murase*, et al.*, 2005).

## 2.3 $CH_4$ and Pulp and Paper Mill Aerated Ponds

Numerous studies have been done on the anaerobic degradability of paper mill wastewaters. These studies have shown that wastewater from pulp and paper mills are suitable for anaerobic degradation, as they contain easily degradable organic compounds and are generally non toxic to anaerobes (Lee*, et al.*, 1989; Rintala & Puhakka, 1994; Sierra-Alvarez*, et al.*, 1990). However, effluents from bleaching and debarking have been shown to inhibit anaerobic degradation. A study has been done on the microbial population of a Canadian Kraft Pulp mill's sediment from an aerated pond (Liss & Allen, 1992). The study found that 85% of bacteria present were anaerobic. $CH_4$ gas emissions from Kraft pulp and paper mill wastewater treatment plants have also been investigated. The study found that $CH_4$ gas emissions appeared to be related to anaerobic biological activity and sludge accumulation (Crawford, 2008). The $CH_4$ gas emissions were highly variable, ranging from 0.02 - 32 grams/second (g/s) for anaerobic treatments, 1.9 - 5.8 g/s for aerated ponds, and 0.2 g/s for activated sludge.

## 2.4 Fate of $CH_4$ in Lakes

Literature regarding $CH_4$ oxidation in aquatic environments mostly focused on lakes and deep marine environments. Marine environments have been known to exhibit anaerobic $CH_4$ oxidation and lakes commonly exhibit aerobic $CH_4$ oxidation (Dale*, et al.*, 2008; Hanson & Hanson, 1996; Thieben*, et al.*, 2006). Lakes are the closest system to resemble aerated ponds. This literature review therefore focuses on the fate of $CH_4$ in lakes.

### 2.4.1 Sediment

#### *2.4.1.1 Sediment $CH_4$ Production*

Methanogenesis occurs when conditions at the sediment are anaerobic and where there is a good supply of biodegradable material (Bastviken*, et al.*, 2008; Huttunen*, et al.*, 2001; Lojen*, et al.*, 1999). For example, Dzyuban (2003) found that lakes with an aerated water column above the sediment produced less $CH_4$ (15 - 210 mL $CH_4/m^2d$) than lakes with an anaerobic water column above the sediment (120 - 5000 mL $CH_4/m^2d$).

Sediments can be grouped into two categories: littoral sediments (shallow sediments close to the shore) and profundal sediments (deep sediments where there is no light penetration). Littoral sediments are more prone to being disturbed by wind or wave action (Bastviken*, et al.*, 2008; Bussmann, 2005; Murase*, et al.*, 2005), increasing the possibility of sediment resuspension, causing a sudden release of $CH_4$.

Changes in water column temperature seem to have no effect on anaerobic $CH_4$ production for profundal sediments (Dzyuban, 2003), while changes in water column temperatures can affect anaerobic $CH_4$ production for littoral sediments (Bastviken*, et al.*, 2008; Bryant, 1979; Duc*, et al.*, 2010).

Other conditions that affect sediment $CH_4$ production are the organic carbon load and the organic carbon utilization rate in the sediment. Murase *et al.*(2005) found that littoral sediments produced more $CH_4$ than profundal sediments, while the opposite was found by a study by Dzyuban (2003). In Murase's study, the organic carbon sedimentation rate caused the difference. Organic carbon settled quicker for the shallower littoral sediments compared to the profundal sediments. Therefore more organic carbon was available for consumption in the littoral sediments. In Dzyuban's case, the rate of anaerobic degradation at the profundal sediment (2200 mg carbon/$dm^3$d) was much faster than at the littoral sediment (760 mg carbon/$dm^3$d); therefore organic material was degraded to $CH_4$ faster in the profundal sediment. The same relationship between organic carbon and sediment $CH_4$ production was found in a study done by Yang (1998), on lake and river sediments in Taiwan. $CH_4$ production

rates were lower for sediments with lower organic carbon content (0.24 - 1.06 mg/L) compared to sediments with higher organic carbon content (11.75 - 54.5 mg/L).

#### *2.4.1.2 Sediment Aerobic $CH_4$ Oxidation*

$CH_4$ is commonly oxidized at the part of the sediment where it is in contact with the aerobic water column, commonly referred to as the sediment-water column interface (Juutinen*, et al.*, 2008; Remsen*, et al.*, 1989). Aerobic $CH_4$ oxidation in the sediment can be substantial, with up to 16 - 90% of $CH_4$ produced also oxidized in the sediment (Bussmann, 2005; Dzyuban, 2003; Murase*, et al.*, 2005).

The rate of aerobic $CH_4$ oxidation in the sediment seems to be correlated to the rate of $CH_4$ production in the sediment. Hence, faster rates of anaerobic $CH_4$ production have been correlated with faster rates of aerobic $CH_4$ oxidation (Duc*, et al.*, 2010). Aerobic $CH_4$ oxidation has also been known to deplete $O_2$ levels at the sediment-water column interface (Bussmann, 2005; Huttunen*, et al.*, 2006; Juutinen*, et al.*, 2008).

### 2.4.2 Water Column

A general pattern found in lakes was that dissolved $CH_4$ concentrations are greatest in the hypolimnion (Figure 2-2), and lowest at the thermocline (Figure 2-2) (Bastviken*, et al.*, 2004; Bastviken*, et al.*, 2008; Bastviken*, et al.*, 2002; Juutinen*, et al.*, 2008; Utsumi*, et al.*, 1998). This pattern is largely due to aerobic $CH_4$ oxidation, as $CH_4$ oxidation rates depend on $CH_4$ and $O_2$ concentrations. $CH_4$ oxidation rates peak under conditions where $CH_4$ concentration is high and there is adequate $O_2$ supply, which typically occur at the thermocline (Bastviken*, et al.*, 2008; Dzyuban, 2003; Murase*, et al.*, 2005; Schubert*, et al.*, 2010). Dissolved $CH_4$ concentration is higher at the epilimnion (Figure 2-2) compared to the thermocline, as the epilimnion contains dissolved $CH_4$ which has diffused from the littoral sediments.

### 2.4.3 Emission to Atmosphere

Littoral sediments provide the majority of $CH_4$ gas emissions to the atmosphere, the majority emitted as bubbles (25 - 98%) (Bastviken*, et al.*, 2004; Bastviken*, et al.*, 2008; Juutinen*, et al.*, 2008; Keller & Stallard, 1994). $CH_4$ gas bubble emission rates are lower for sites with water columns greater than seven metres (10 - 200 mg $CH_4/m^2d$) compared to sites with water columns less than two metres (300 - 2000 g $CH_4/m^2d$) (Keller & Stallard, 1994). $CH_4$ gas bubble emissions have been found to be highly variable. In the study done by Schubert *et al.* (2010), $CH_4$ gas bubble emission ranged from 0.2 - 7 mg $CH_4/m^2d$. Reasons given for the variability were: the turbulence from wind stirring the littoral sediment (Keller & Stallard, 1994) and bubbles from sediments rising in a shallow water column have to overcome less hydrostatic pressure, before the bubbles are released to the atmosphere (Bastviken*, et al.*, 2004).

The rate of $CH_4$ gas emission through diffusion is generally lower than bubble emissions and is largely due to two reasons. The first reason, dissolved $CH_4$ from profundal sediments are generally consumed before reaching the surface of the lake. The second reason, $CH_4$ produced by littoral sediments generally do not diffuse into the water column, as the conditions suit $CH_4$ gas bubble emission (Bastviken*, et al.*, 2004; Bastviken*, et al.*, 2008).

## *2.5 Literature Review Summary*

There is considerable evidence that $CH_4$ can be formed in the anaerobic zone of various aquatic environments and subsequently aerobically degraded by methanotrophs in the aerobic zone. Both $CH_4$ formation and consumption are affected by temperature but can occur within a broad temperature range. However, we know very little regarding the occurrence, and most importantly, the quantitative significance of these mechanisms during pulp and paper wastewater treatment. With the objective of the thesis to determine the potential source of BOD formation during pulp and paper wastewater treatment, it was hypothesised that $CH_4$ formation and subsequent oxidation could generate enough methanol to cause the unaccounted BOD increase at the outlet of the treatment system. However, methanol accumulation is rarely observed (this compound is normally rapidly oxidized by methanotrophs) but can occur under

specific conditions. As the focus of the thesis was whether methanol production can cause the increase in BOD load at the outlet, the metabolism of methanol was assumed to be inhibited in the thesis.

# 3 Methodology

## *3.1 Sample Collection and Preparation*

### 3.1.1 Sites

Five sites were chosen for sampling:

- P19 In: located near the start of Pond 19
- P19 Middle: an unused dredge, approximately halfway between the start and end of Pond 19
- P19 Out: at the outlet of Pond 19
- P22 Out: a site near the outlet of Pond 22
- P23 Out: at the outlet of Pond 23

All sampling sites (Figure 3-1) were located near the banks of ponds for their easy accessibility (except for P19 In) and therefore were not close to the aerators. The sampling sites were also sufficiently spread out to provide a good snapshot of the treatment system.

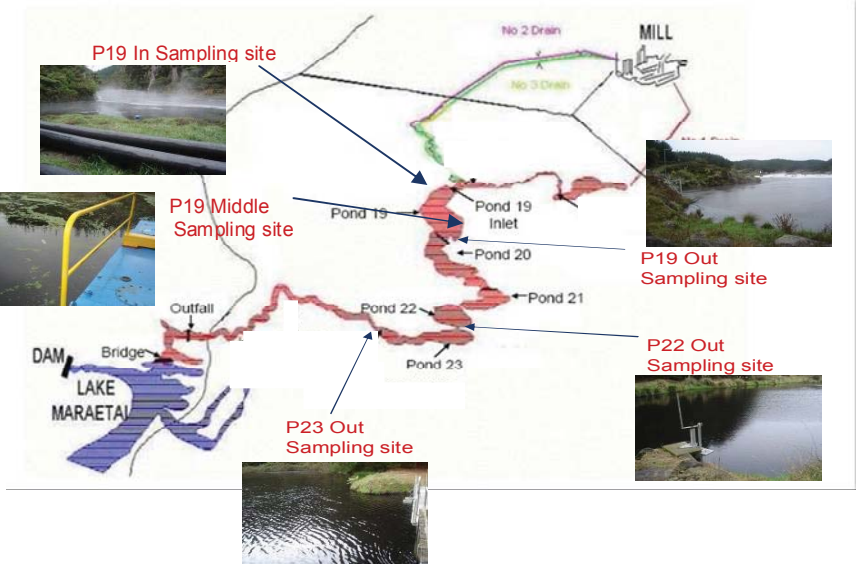


**Figure 3-1: Sampling site locations**

### 3.1.2 $CH_4$ Gas Emission

$CH_4$ gas either bubbles or diffuses out of the water column into the atmosphere. To quantify each of these contributions, two different surface gas collectors were used at each location (Figure 3-2). One collector was equipped with a baffle, to divert rising bubbles and only collect the diffuse fraction. The other collector was used to sample the total $CH_4$ gas emitted (from bubbles and diffusion). The difference in $CH_4$ emission rates between the two collectors is considered equal to the $CH_4$ bubble gas emission rate. The surface gas collectors were installed with half of the collector in the water, for an hour, at the five sampling sites. Gas (25 mL) was removed from the surface gas collectors using a syringe and immediately injected into a 60 mL serum bottle. Triplicates were collected for each site, for both gas collectors, therefore a total of six serum bottles were used for each site.

Prior to sampling, the serum bottles were flushed with nitrogen ($N_2$) or helium, sealed with a rubber seal, and 35 mL of the inert gas was removed from the bottle.

**Figure 3-2: Surface gas collectors**

NB: Gas collector for bubbles and diffusion (left picture) and diffusion only (right picture)

### 3.1.3 Dissolved $CH_4$

Triplicate samples of dissolved $CH_4$ were collected at each of the five sampling sites at a depth of one metre. The pond water sampled (50 mL) was immediately injected into a sealed 120 mL serum bottle previously flushed with air. The bottle also contained

1 mL of 5% sodium azide ($NaN_3$) solution as growth inhibitor (to prevent $CH_4$ formation or consumption prior to analysis). An air volume of 80 mL was removed from the bottle before adding the pond water sample, to prevent pressure from increasing during sampling.

The serum bottles were taken back to the laboratory and shaken for 24 hours at 30°C to ensure the sample had reached equilibrium. The gas composition of the bottles' headspace was then determined by TOGA analysis. With the $CH_4$ headspace gas composition known, the dissolved $CH_4$ concentration in the sample was calculated using Henry's law, as shown in Appendix 8.1.2.

### 3.1.4 Methanol and Volatile Fatty Acids (VFAs)

Water column samples (60 mL) one metre below the water surface were collected at all sampling sites. The samples were then frozen on return to the laboratory.

A Scion in-house method was used to prepare the samples for analysis (shown in the Appendix 8.4.2). This method involved pH correction with formic acid and use of Butan-1-ol as an internal standard. Turbid samples were pre-filtered through a polytetrafluoroethylene (PTFE) syringe filter (0.45μm).

### 3.1.5 Biochemical $CH_4$ Potential (BMP) Assay

Triplicate sediment and water column samples were collected at all sampling sites. The samples were kept in a chilly bin filled with ice slurry after collection on site, then stored at 4°C back in the laboratory. Sediment samples were collected at the top of the sediment layer and water column samples were collected at a depth of one metre.

Samples were prepared according to standards 'ASTM E2170 - 01(2008) Standard Test Method for Determining Anaerobic Biodegradation Potential of Organic Chemicals Under Methanogenic Conditions' (ASTM International, 2008).

### 3.1.6 Aerobic $CH_4$ Oxidation Assay

Sediment samples were collected from the P19 Middle and P23 Out sampling sites and water column samples were collected from the P19 In and P19 Middle sampling sites. Sediment samples were collected at the top of the sediment layer and water column samples were collected at a depth of one metre. At the laboratory, 60 mL of sample was transferred into a 160 mL serum bottle and the serum bottle was sealed. The bottle's headspace was then flushed with a $O_2$:$N_2$ gas mixture (20.7:76.8 vol:vol). After flushing, 3 mL of gas was removed from the headspace and replaced with 3 mL pure $CH_4$ producing a 3% $CH_4$ in air gas mix for the headspace. The bottle was then incubated for an hour at 35 °C under mixing. After one hour, the bottle was flushed again with the 3% $CH_4$ in air gas mix. The sample was then incubated until further analysis was required. The purpose of the one hour incubation between flushes was to equilibrate the gases at the incubation temperature. The purpose of the second flush was to remove gases released from the water phase (e.g. $CO_2$ re-equilibrating).

The water column samples were also tested for methanol to determine if methanol was produced and released from the bacterial cell.

Two controls and two blanks were used. The controls and blanks are described below:

- ***$N_2$ and $O_2$ control:*** Contained 60 mL of sediment or water column sample with the headspace flushed with the $O_2$:$N_2$ gas mixture (20.7:76.8 vol:vol). This control determined the background oxidative potential of other substrates which are present in the sample.

- ***Sodium azide ($NaN_3$) control:*** Contained 57.5 mL of sediment or water column sample, 2.5 mL of 5% $NaN_3$ with the headspace flushed with 3% $CH_4$ in air mix. This control was used to quantify potential $CH_4$ abiotic losses.

- ***Sodium azide ($NaN_3$) and water ($H_2O$) blank:*** Contained 57.5 mL of $H_2O$ and 2.5 mL of 5% $NaN_3$ with the headspace flushed with 3% $CH_4$ in air mix. This control was used to determine if $NaN_3$ had any effect on the gas concentrations in the headspace.

- ***Water ($H_2O$) blank:*** Contained 60 mL of $H_2O$ with the headspace flushed with 3% $CH_4$ in air mix. This control was used to show if there was any absorption of $CH_4$ or any significant leaks from the serum bottles.

Triplicate site, control and blank tests were prepared for each day aerobic $CH_4$ oxidation was tested.

### 3.1.7 Fluorescent *In Situ* Hybridization (FISH)

FISH is a technique used to enumerate bacteria from a mixed community. It is rapid and relatively simple, the main advantage being that organisms do not need to be cultured before observation. FISH works by targeting specific DNA sequences in chromosomes using fluorescent probes. Three probes were used for FISH analysis. The EUB mix probe was used to identify all bacteria present in the sample. The two other probes were used to specifically identify certain type I and type II methanotrophs in the sample.

Water column samples were collected at one metre depth at the P19 In and P19 Middle sites. Samples from the P19 In and P19 Middle water column $CH_4$ oxidation assays were also collected for FISH analysis. The samples were prepared for FISH analysis according to a Scion in-house method shown in Appendix 8.4.3.

## *3.2 Analytical Procedures*

### 3.2.1 Gas Composition

The concentration of $CH_4$ in the headspace of the gas emission, dissolved $CH_4$ and $CH_4$ oxidation samples were analyzed using the Titrimetic off gas analysis (TOGA) system. The TOGA system was originally developed for detailed analysis of aerobic wastewater treatment systems, but is capable of detecting organic compounds associated with anaerobic fermentation such as hydrogen, $CO_2$ and $CH_4$. The TOGA system also has the capability of real time analysis of dissolved gas concentrations through a membrane inlet mass spectrometry (MIMS) probe. A headspace gas sample

of 25 mL was injected into a vessel containing a MIMS probe. The mass spectrometer used by the TOGA system was a Hiden Analytical HPR20 (United Kingdom) with a $CH_4$ detection limit of 100 ppmv.

During BMP assays, the headspace gas composition in the flasks were analyzed by gas chromatography (GC) using a GC 8700 basic gas chromatograph produced by Carle Instruments Inc, with a thermal conductivity detector and helium as the carrier gas. Gas (250 µL) from the headspace was injected into the GC. A Scion in house method was used for the injection procedure required for this GC which is shown in the Appendix 8.4.1.

### 3.2.2 Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS)

The concentrations of TSS and VSS in BMP and aerobic $CH_4$ oxidation assays were determined by following the standard methods (APHA, 1998) sections 2540D and 2540E.

### 3.2.3 Methanol and VFAs

Methanol and VFA samples were analyzed using an Agilent Technologies 7890A capillary gas chromatography with flame ionisation detection (GC-FID). The column used was a 30 m Supelco 23327 column (0.5 µM ID) ramped from 40ºC to 180ºC. The full method is shown in the Appendix 8.4.2.

### 3.2.4 Temperature and DO Measurement

The temperature and DO concentration at the sampling sites were measured using a John Morris Scientific YSI55 DO/temperature meter. During the dissolved $CH_4$ profile, temperature, DO, pH and conductivity were measured using a Hydrolab MS5 meter.

### 3.2.5 FISH

FISH samples were analyzed using an Olympus BX61 at 600x magnification epifluorescence microscope and photographs of the samples were taken using an Olympus Colourview digital camera under epifluorescence.

### 3.2.6 Data Analysis

Data was analyzed using Microsoft Excel and Minitab for $CH_4$ gas emission, dissolved $CH_4$ concentration, methanol, VFA and TSS/VSS results. BMP assays were analyzed using a computer graphing program called 'Graph Pad Prism' developed by Graph Pad Software Inc, USA.

# 4 Results & Discussion

## *4.1 Wastewater Characteristics*

This section describes the wastewater characteristics recorded from monitoring temperature, DO concentration, methanol and VFA concentrations from five different locations located throughout the treatment system (Figure 3-1). Temperature and DO concentrations for the sediment and water column of the five sampling sites were monitored weekly, during the months of May 2010 to March 2011.

Boxplots were used to show some of the data in the next couple of sections. An example of a boxplot with the symbol definitions are shown below (Figure 4-1).

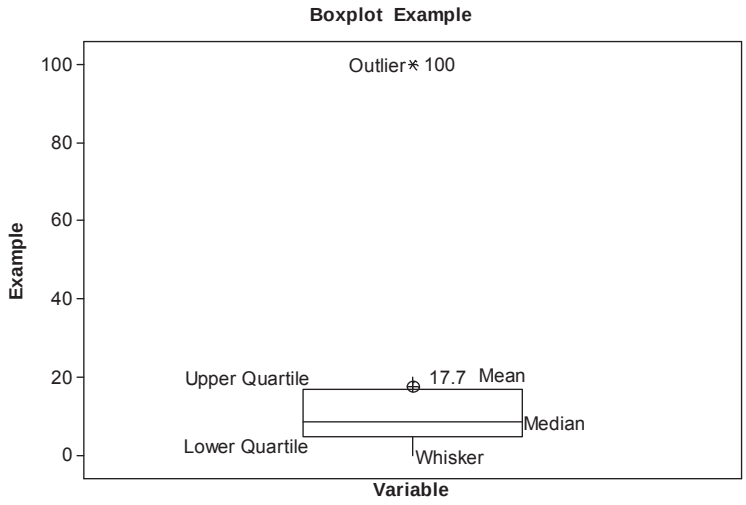


**Figure 4-1: Boxplot example**

- Outlier: The most extreme observation in the data set
- Upper Quartile: The top 25% of the data set
- Mean: The average value in the data set
- Median: The value that separates the upper half and lower half of the data set
- Lower Quartile: The lowest 25% of the data set
- Whisker: Indicates the minimum and maximum values of a data set if there are no outliers

### 4.1.1 DO Concentration

In the water column, conditions characteristic of oxygen limitation were recorded at the inlet (e.g. 0.19 mg/L at P19 In), the rest of the treatment system being characterized by DO concentration above the 2 mg/L threshold recommended for aerobic growth in industrial microbiology (Figure 4-2). The DO threshold for aerobic conditions in complex environments is not clear, because the core of large particles found in well aerated water can still be subject to anaerobic conditions. For simplification, we describe water column exhibiting high DO concentrations (> 2 mg/L) as being 'aerobic' in the following discussion. The DO concentrations for the sampling sites located at the first pond (P19 Middle and P19 Out) were lower than the concentrations recorded for the sites near the outlet of the treatment system (0.5 mg/L at P19 Middle and 0.58 mg/L at P19 Out compared to 2.08 mg/L at P22 Out and 2.67 mg/L at P23 Out, Figure 4-2). The DO concentration at P19 Middle and P19 Out increased significantly during the mill shutdowns (to approximately 5 mg/L), which are shown as outliers (Figure 4-2). The increase in DO is most likely due to a decrease in organic matter entering the treatment system, which reduced the rate of $O_2$ consumption by aerobic microorganisms. This in turn caused DO concentration to increase in the water column.

The low DO concentrations detected in the sediment layer, for all five sampling sites, showed the occurrence of severe oxygen limitation, a condition necessary (but not in itself sufficient) for methanogenesis (Tchobanoglous, et al., 2003). The low DO concentration is due to the consumption of $O_2$ in the water column. This consumption prevented $O_2$ diffusing in the sediment layer. The sediment at Pond 19 were more $O_2$ limited (average DO concentration was under 0.10 mg/L for all three sampling sites at Pond 19), than the sediments located near the outlet of the treatment system (average DO concentration was 0.21 mg/L for the P23 Out sampling site, Figure 4-2). DO concentrations in the sediments were fairly constant, with P23 Out the only site showing some variability.

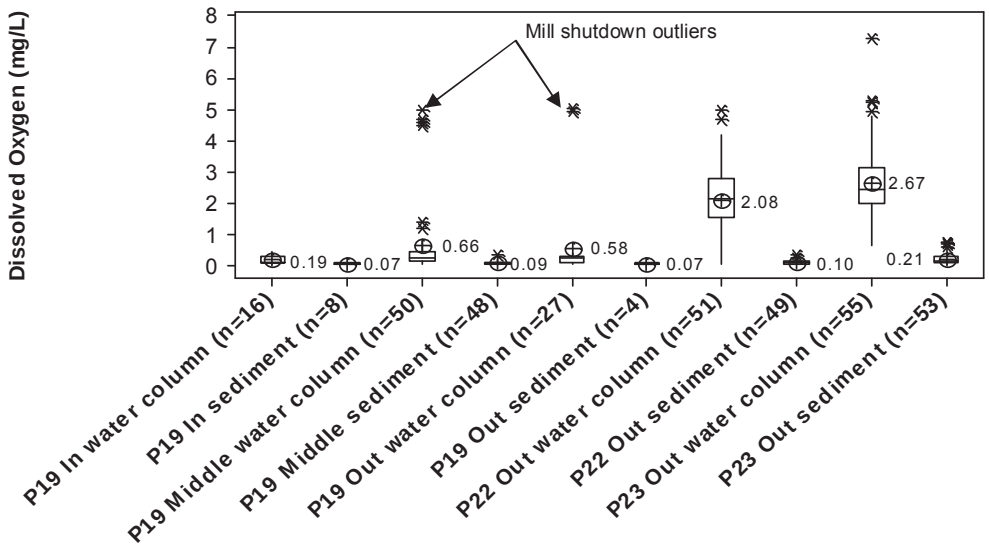


**Figure 4-2: DO concentration for the water column and sediment of the five sampling sites**

The severe oxygen limitation consistently recorded in the sediment can provide the environment for methanogenesis to occur (Tchobanoglous, et al., 2003). The DO concentrations recorded at the water column are within the literature values in which aerobic $CH_4$ oxidation has been known to occur (Rudd, et al., 1976; Utsumi, et al., 1998). Therefore, the DO concentrations recorded at the sediment and in the water column of the five sampling sites suggest that anaerobic $CH_4$ production in the sediment and aerobic $CH_4$ oxidation in the water column (with the exception of the treatment inlet) is possible.

### 4.1.2 Temperature

Sediment and water column temperatures decreased down the treatment system. There was also very little difference in temperature between the water column and the sediment for the sites monitored (Figure 4-3). The average sediment temperature for the P19 Out site was higher than the average temperature for the P19 Middle site, which is located upstream from P19 Out. The difference in temperature between the water column (28.1°C) and sediment (32.4°C) was also high for the P19 Out site (Figure 4-3). This anomaly was likely artificial because only four temperature recordings were made at the sediment of P19 Out.

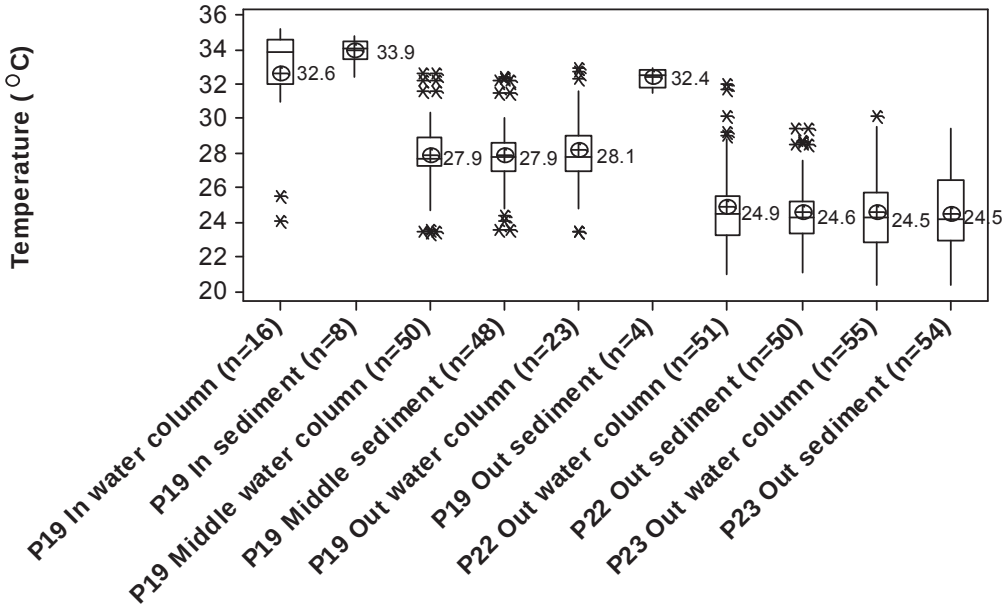


**Figure 4-3: Sediment and water column temperature of the five sampling sites**

The water column and sediment temperatures of the five sampling sites were fairly stable, increasing the possibility of anaerobic $CH_4$ production occurring at the sediment.

The effect of seasonal temporal variations on $CH_4$ gas emissions and dissolved $CH_4$ concentration for the five sampling sites were also investigated (Appendix 8.2) but no correlation was found.

### 4.1.3 Methanol and Volatile Fatty Acids (VFA) Concentration

Methanol (43.20 ± 9.8 mg/L) and acetic acid (29.00 ± 14.6 mg/L) were detected at the treatment inlet (P19 In). The methanol detected most likely originated from the paper making effluent (Maclean*, et al.*, 2007), as methanol is a pollutant known to be found in the mill wastewater.

## 4.2 $CH_4$ Production in the Treatment System

This section describes where $CH_4$ was produced in the treatment system, and its magnitude. $CH_4$ production in the treatment system was quantified by:

- Monitoring the $CH_4$ gas emissions from the pond surface
- Monitoring the dissolved $CH_4$ concentrations in the water column
- Running biochemical $CH_4$ potential (BMP) assays on samples collected from the five sampling sites

### 4.2.1 $CH_4$ Gas Emission from Pond Surface to Atmosphere

$CH_4$ gas emissions were recorded at all five sampling sites and significant differences were detected depending on location. $CH_4$ gas was emitted more frequently at the start of the treatment system (P19 In and P19 Middle sites). $CH_4$ gas emission rates were also higher at the treatment inlet, on average 3.25 and 3.40 g $CH_4/m^2d$ for P19 In and P19 Middle sites respectively (Figure 4-4). The majority of $CH_4$ gas was emitted as bubbles, which is in agreement with the literature on $CH_4$ gas emission from lakes (Bastviken*, et al.*, 2004; Bastviken*, et al.*, 2008; Keller & Stallard, 1994; Schubert*, et al.*, 2010). Emission through $CH_4$ diffusion was only consistently detected at the P19 In sampling site.

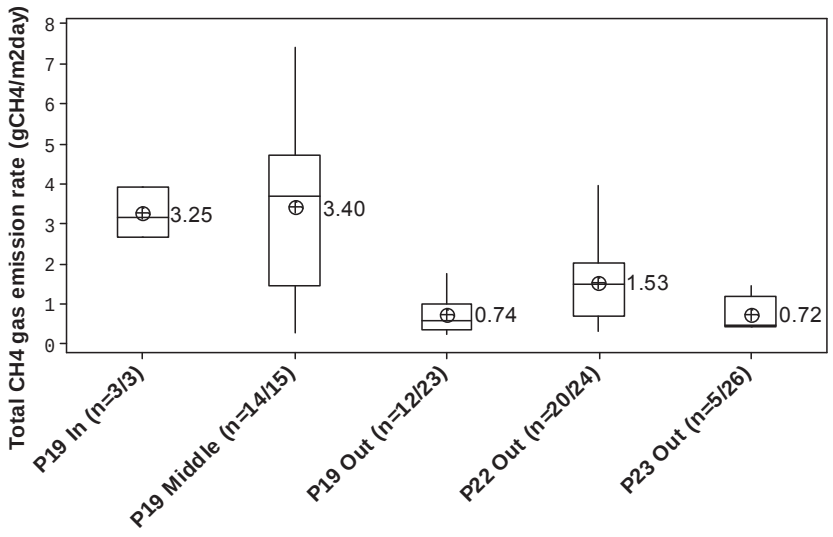


**Figure 4-4: Boxplot of total $CH_4$ gas emission rates for the five sampling sites**

NB: n=5/26 means that $CH_4$ gas was detected on only 5 of the 26 sampling days at that site. The boxplot values are based on the days when $CH_4$ gas was detected.

The variability in $CH_4$ gas emission rates detected in the treatment system corroborates with findings from past studies on lakes (Bastviken*, et al.*, 2004; Keller & Stallard, 1994; Schubert*, et al.*, 2010) and pulp mill wastewater treatment plants (Crawford, 2008). The magnitude of the $CH_4$ gas emission rates detected in the treatment system are comparable to the rates found from a lake in Panama, are slightly below the average rates found from swine lagoons in North Carolina and are well below the average rates found from an uncovered municipal landfill in Austria (Table 4-1).

**Table 4-1: Comparison of $CH_4$ gas emission rates observed from the treatment system to literature values**

| Pond | $CH_4$ gas emission rate (g $CH_4/m^2d$) |
|---|---|
| Pond 19 Inlet | 2.630 - 3.920 |
| Rest of Pond 19 | 0.240 - 7.430 |
| Pond 22 | 0.290 - 3.970 |
| Pond 23 | 0.400 - 1.440 |
| Gatun Lake, Panama (Keller & Stallard, 1994) | 0.010 - 2.000 |
| Swine lagoons (Sharpe, et al., 2002) | 2.000 - 11.50 |
| Uncovered landfill (Scheutz, et al., 2009) | 27.30 - 725.2 |

### 4.2.2 Dissolved $CH_4$ Concentration in the Water Column of the Treatment System

The highest dissolved $CH_4$ concentrations were detected at the start of the treatment system (1 - 2.5 mg/L at P19 In). Dissolved $CH_4$ concentration then gradually decreased until no more $CH_4$ was detected at the middle of Pond 19 (Figure 4-5). The incremental decrease in dissolved $CH_4$ concentration was most likely due to the availability of DO to methanotrophs. DO is largely provided by the aerators installed at Pond 19. No aerators were installed at the inlet (shown on Figure 4-5 as P19 In), which meant conditions were oxygen limited, which favoured the production of $CH_4$ and possibly inhibited aerobic $CH_4$ oxidation. Hence why dissolved $CH_4$ concentration at the inlet was fairly constant. Dissolved $CH_4$ concentration started to decrease after the inlet, which is also where the first aerator was installed.

There were also no significant changes in dissolved $CH_4$ concentration with depth (one to three metres) for the water column of Pond 19 (Figure 4-5).

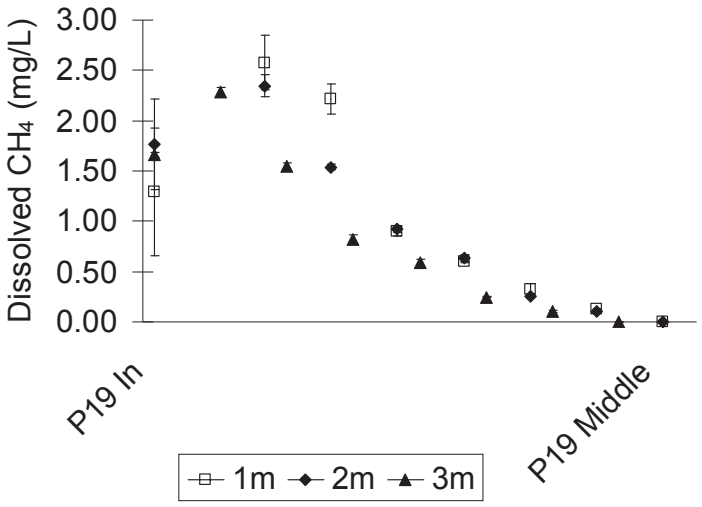


**Figure 4-5: Dissolved $CH_4$ profile of Pond 19**

The same pattern was also found for the five sampling sites. Dissolved $CH_4$ was only detected at significant concentrations at the start of the treatment system. Dissolved $CH_4$ concentration was highest at the start of Pond 19 (P19 In), with only low concentrations being detected for the rest of Pond 19 (P19 Middle and P19 Out), (Figure 4-6). Dissolved $CH_4$ was never detected at the sampling sites near the outlet of the treatment system (P22 Out and P23 Out).

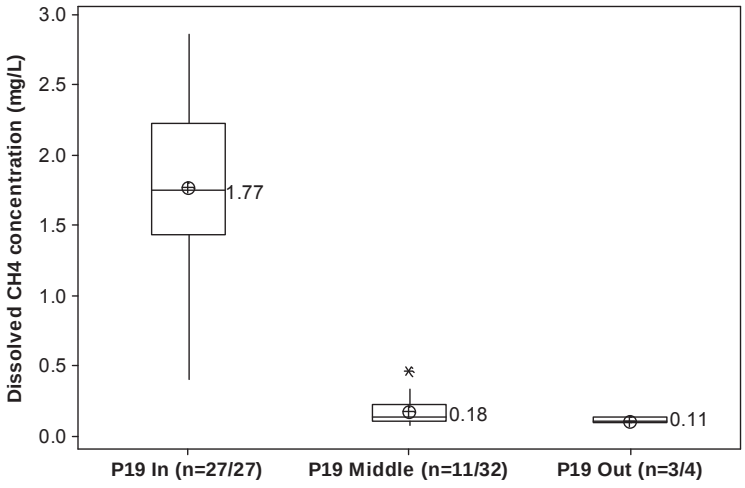


**Figure 4-6: Boxplot of dissolved $CH_4$ concentration at the P19 In, P19 Middle, P19 Out sampling sites**

NB: n=11/32 means the number of days dissolved $CH_4$ was detected / number of days the site was sampled. The boxplot values are based on the days dissolved $CH_4$ was detected.

### 4.2.3 Biochemical $CH_4$ Potential (BMP) Assays

To quantitatively compare the $CH_4$ production rates across the treatment system, the 60 day $CH_4$ production yield, the ultimate $CH_4$ potential and the $CH_4$ production rates were calculated from the BMP assay results.

The 60 day $CH_4$ production yield for the sediment samples (Figure 4-7) showed similar production at four of the five sampling sites (200-280 mL $CH_4$/gVSS). The sediment sample from the treatment inlet (P19 In) produced the highest amount of $CH_4$ (450 mL $CH_4$/gVSS).

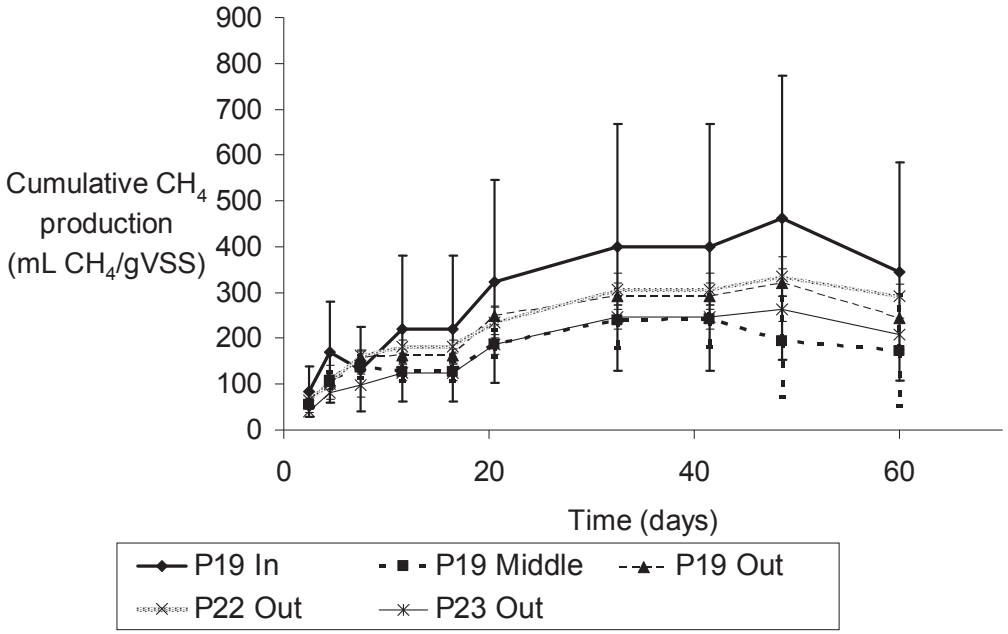


**Figure 4-7: Cumulative $CH_4$ production for sediment samples**

The ultimate $CH_4$ potential (the maximum amount of $CH_4$ that can be biologically produced from a given sample) and the $CH_4$ production rate were calculated using the software 'Graph Pad Prism,' by fitting a one phase association graph to the BMP data. The fitted one phase association graph approximated the value where $CH_4$ production reached a plateau, which represents the ultimate $CH_4$ potential of the sediment sample. The $CH_4$ production rate was based on the slope of this graph, before $CH_4$ production

reached a plateau. The sediment sample collected from the P19 Middle site exhibited the fastest $CH_4$ production rate (0.072 mL $CH_4$/d) and the sediment from the treatment inlet (P19 In), had the highest $CH_4$ potential (419.4 mL $CH_4$/gVSS) (Table 4-2).

These results suggest that a more active methanogen population is present in the P19 middle sediment and a higher amount of biodegradable substrate is available at the treatment inlet.

**Table 4-2: $CH_4$ production rate and ultimate $CH_4$ potential for the sediments collected from the five sampling sites**

| Sample | $CH_4$ production rate (mL $CH_4$/d) | Ultimate $CH_4$ potential (mL $CH_4$/gVSS) |
|---|---|---|
| P19 In | 0.062 | 419.4 |
| P19 Middle | 0.072 | 212.6 |
| P19 Out | 0.069 | 297.3 |
| P22 Out | 0.058 | 334.9 |
| P23 Out | 0.058 | 256.2 |

BMP assays were also done for samples from the water column of the five sampling sites. However, an error occurred during the analysis of total organic carbon (TOC) concentration for the water column samples, affecting the calculation of $CH_4$ production values. Anaerobic $CH_4$ production however is unlikely to occur at the water column, as aerobic conditions are present for the majority of the treatment system. The only section of the treatment system where anaerobic $CH_4$ production can occur is at the treatment inlet, where the water column is oxygen-limited.

## *4.3 Evidence of Aerobic $CH_4$ Oxidation in the Treatment System*

The previous sections have shown that:

i. Anaerobic $CH_4$ production can occur in the sediment
ii. Aerobic conditions exist in the water column, making aerobic $CH_4$ oxidation possible in the treatment system

This section evaluates the potential of aerobic $CH_4$ oxidation occurring in the sediment and water column of the treatment system, through the use of aerobic $CH_4$ oxidation assays and the detection of methanotrophs using FISH.

### 4.3.1 Aerobic $CH_4$ Oxidation Potential Assay

Water column samples from the P19 In and P19 Middle sampling sites were tested for their potential to aerobically oxidize $CH_4$. These two sites were selected, as $CH_4$ gas emissions were frequently detected and dissolved $CH_4$ was consistently detected at the P19 In site.

Sediment samples were also tested for their potential to aerobically oxidize $CH_4$, as the sediment-water column interface (oxygen limited sediment and aerobic water column) exists in the treatment system. Aerobic $CH_4$ oxidation has been known to occur at the sediment-water column interface of lakes (Bussmann, 2005; Dzyuban, 2003; Juutinen*, et al.*, 2008; Murase*, et al.*, 2005; Remsen*, et al.*, 1989). Sediment samples from the P19 Middle and P23 Out sites were tested, to determine if there are significant differences in aerobic $CH_4$ oxidation potential between the inlet and outlet of the treatment system.

After sample collection, samples were taken back to the laboratory and tested under controlled conditions as specified in section 3.1.6. Control and blank samples were also used and their results are shown in Appendix 8.3.

#### *4.3.1.1 Water Column Samples*

For the flasks containing the P19 middle sample, $CH_4$ and $O_2$ concentration decreased simultaneously as $CO_2$ concentration increased (Figure 4-8). This suggests that aerobic $CH_4$ oxidation occurred in the sample. For the flasks containing the P19 In sample, $O_2$ consumption and $CO_2$ production preceded $CH_4$ consumption (Figure 4-8). This suggests that other compounds were aerobically degraded in the sample before $CH_4$ was oxidized. A comparison of the two tests showed that $CH_4$ concentration decreased faster for the P19 Middle sample than the P19 In sample, as $CH_4$ was consumed within 99 hours for the P19 Middle sample and 267 hours for the P19 In sample. A more active methanotroph population may be present at the P19 middle site.

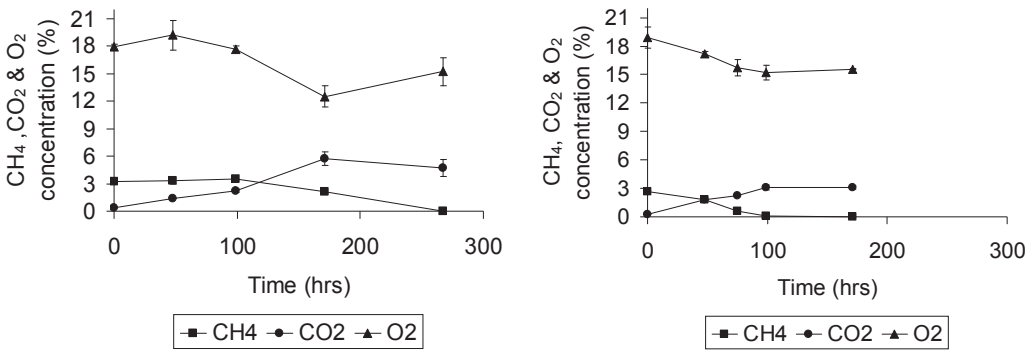


**Figure 4-8: Gas concentrations for P19 In (left) and P19 Middle (right) water column samples during the aerobic $CH_4$ oxidation assay**

The highest $CH_4$ consumption rate (357.5 µmol $CH_4$/gVSShr) occurred for the P19 Middle sample, after 99 hours of incubation (Figure 4-9). The highest $O_2$ consumption rate (905.2 µmol $O_2$/gVSShr) and $CO_2$ production rate (722.6 µmol $CO_2$/gVSShr) occurred for the P19 In sample after 171 hours of incubation (Figure 4-9). The presence of methanol and acetic acid at the P19 In sampling site may explain why the highest $O_2$ consumption and $CO_2$ production rates occurred for the flasks inoculated with the P19 In sample. The aerobic degradation of methanol and acetic acid would increase the overall rates of $O_2$ consumption and $CO_2$ production in the sample.

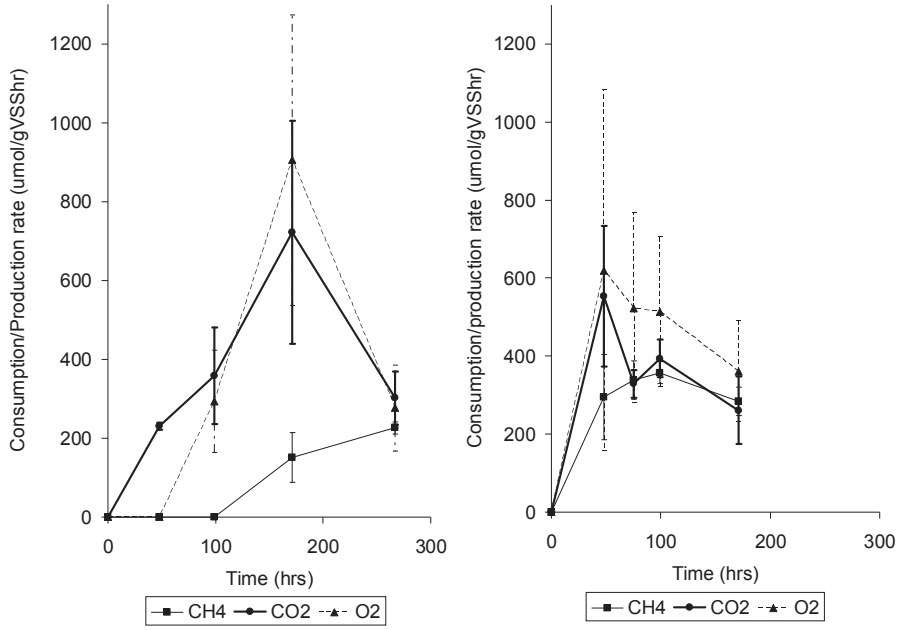


**Figure 4-9: Production/consumption rates for P19 In (left) and P19 Middle (right) water column samples during the aerobic $CH_4$ oxidation assay**

Methanol concentration was monitored during the assays of both samples (P19 In and P19 Middle). Methanol was only detected for the P19 In sample and would most likely have been present when the samples were collected, and not formed during the test. In fact, methanol concentration decreased during the test, most likely due to aerobic degradation by other microorganisms. Therefore evidence of extracellular methanol accumulation was not seen during the test.

In conclusion, the results of the aerobic $CH_4$ oxidation assays showed that aerobic $CH_4$ oxidation can potentially occur in the water column of the treatment system. The assays also suggest that the aerobic degradation of other carbon sources takes place before aerobic $CH_4$ oxidation by methanotrophs.

#### *4.3.1.2 Sediment Samples*

$O_2$ consumption and $CO_2$ production occurred simultaneously with $CH_4$ consumption in the flasks inoculated with sediment samples (P19 Middle and P23 Out) (Figure 4-10). This suggests that aerobic $CH_4$ oxidation occurred for these samples. However, $O_2$ concentration decreased and $CO_2$ concentration increased when there was no corresponding decrease in $CH_4$ concentration for both samples, suggesting other substrates were being aerobically degraded (Figure 4-10). $CH_4$ concentration decreased faster in the flasks containing the P19 Middle sample (60 hours) than the flasks containing the P23 Out sample (134 hours).

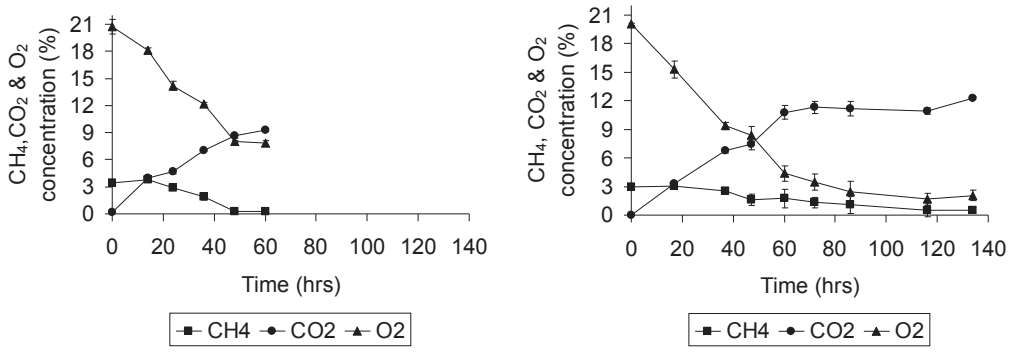


**Figure 4-10: Gas concentrations for P19 Middle (left) and P23 Out (right) sediment samples during the aerobic $CH_4$ oxidation assay**

The highest $CH_4$ consumption rate (3.67 µmol $CH_4$/gVSShr), was recorded for the P19 Middle sample and occurred after 48 hours of incubation (Figure 4-11). The highest

$CH_4$ consumption rate recorded for the P23 Out sample was approximately a third (1.19 µmol $CH_4$/gVSShr) of the highest $CH_4$ consumption rate recorded for the P19 Middle sample (Figure 4-11). A smaller, less active methanotroph population could be the reason why $CH_4$ consumption was slower in the P23 Out sample than in the P19 Middle sample.

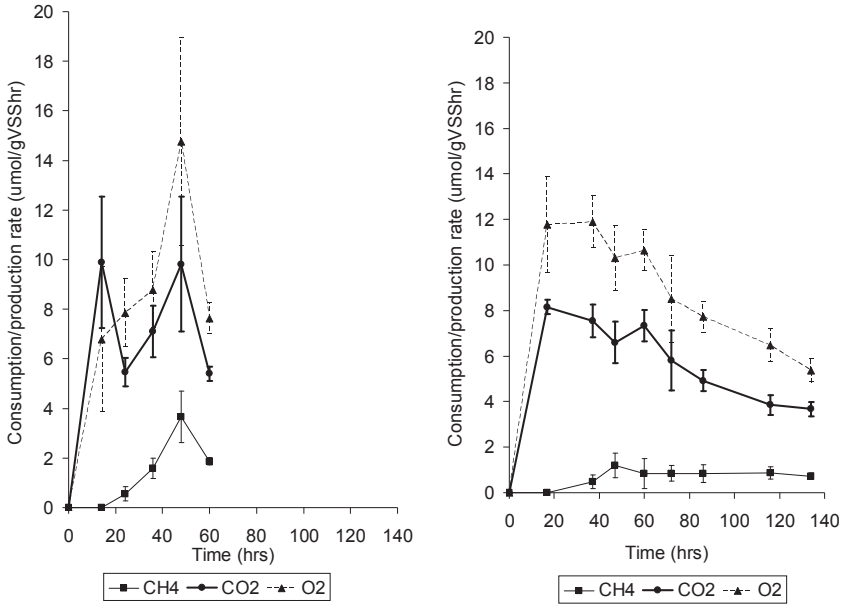


**Figure 4-11: Production/consumption rates for P19 Middle (left) and P23 Out (right) sediment samples during the aerobic $CH_4$ oxidation assay**

In conclusion, the aerobic $CH_4$ oxidation assays showed that aerobic $CH_4$ oxidation can potentially occur in the sediment-water column interface of the treatment system. The assays also showed that the potential for aerobic $CH_4$ oxidation is greater near the inlet than the outlet of the treatment system and the aerobic degradation of other carbon sources may compete with aerobic $CH_4$ oxidation by methanotrophs.

### 4.3.2 FISH Analysis

Three probes were used for FISH analysis. The EUB mix probe was used to identify all the bacteria present in the sample. The other two probes were used to identify certain type I and type II methanotrophs in the sample.

FISH analysis was carried out on water column samples collected from the P19 In and P19 Middle sites.

Methanotrophs were detected for the water column sample collected from the P19 In site (Figure 4-12). The left picture shows all the bacteria present in the sample (EUB mix probe); the middle picture shows type I methanotrophs (red fluorescence) present in the sample; and the right picture is a combination of the left and middle picture, which shows that type I methanotrophs only make up a small part of the total bacterial population. Methanotrophs were not detected in the water column samples collected from the P19 Middle site and type II methanotrophs were not detected in any of the samples.

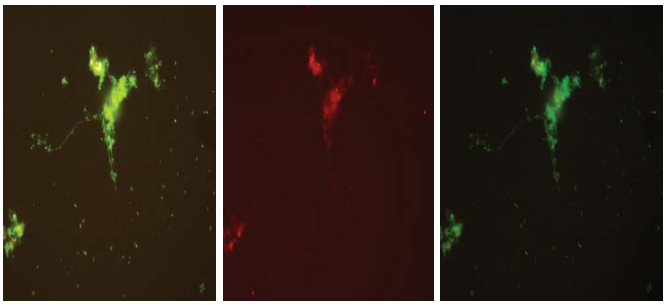

**Figure 4-12: EUB mix probing (left), probing for Type I methanotrophs (centre), Probes combined (right) during FISH analysis of P19 In sample**

The FISH results provided further evidence that aerobic $CH_4$ oxidation can potentially occur in the water column of the treatment system. The results also showed that methanotrophs may only be a small part of the total aerobic microorganism population present in the treatment system.

## *4.4 Fate of $CH_4$ in the Treatment System*

An overview diagram summarising the monitoring results is shown below (Figure 4-13). Based on the monitoring and experimental results, the fate of $CH_4$ in the treatment system would most likely follow the steps below:

- $CH_4$ is produced in the sediment and released as bubbles, based on the results of BMP assays of sediment samples and $CH_4$ gas emission monitoring.

- As $CH_4$ bubbles rise up the water column, some of the $CH_4$ in the bubble diffuses into the water column, and the remaining $CH_4$ is emitted to the

atmosphere as bubbles. A fraction of the $CH_4$ that has diffused into the water column can be oxidized by methanotrophs; the rest will diffuse to the atmosphere or be discharged with the effluent. This explanation is based on the results obtained from DO concentration monitoring, aerobic $CH_4$ oxidation assays and FISH analysis of the water column samples.

- Aerobic $CH_4$ oxidation can occur at the sediment-water column interface based on the aerobic $CH_4$ oxidation assay results of the sediment samples.

- Aerobic $CH_4$ oxidation can lead to methanol release in the water column, if the enzyme methanol dehydrogenase is inhibited.

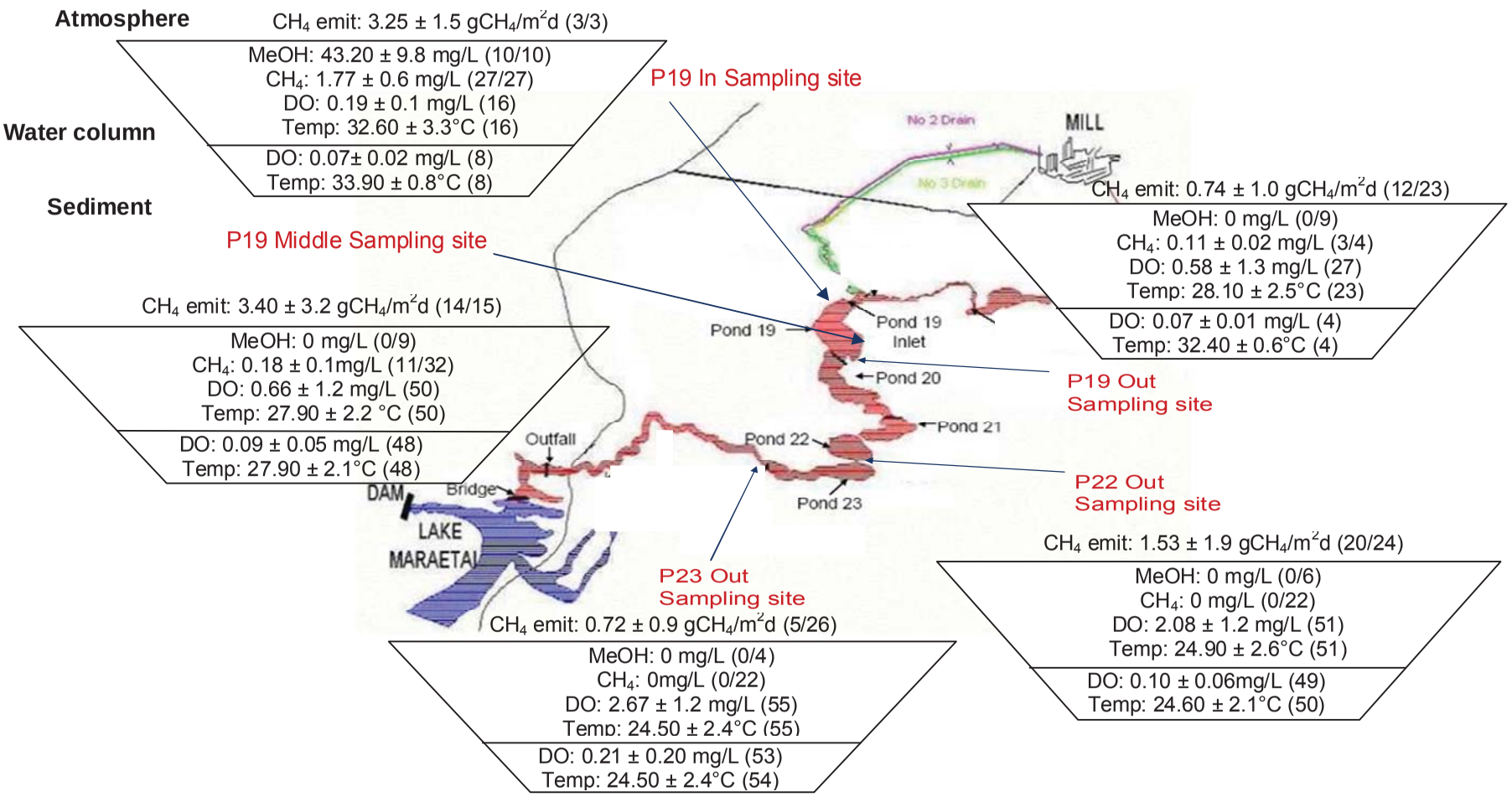


**Figure 4-13: Overview diagram of the monitoring results from the treatment system**

NB: n = 11/32 means the number of days $CH_4$ gas, dissolved $CH_4$ or methanol was detected / number of days the site was sampled. The average values are based on the days when $CH_4$ gas/dissolved $CH_4$/methanol was detected

## *4.5 Possibility of Aerobic $CH_4$ Oxidation increasing BOD Load*

A simple model was constructed to model the fate of $CH_4$ at the outlet of the treatment system. The model was used to determine if the aerobic oxidation of $CH_4$ into methanol could be quantitatively significant and cause the unaccounted BOD increase at the outlet of the treatment system.

### 4.5.1 Objectives

There have been instances in the past when BOD at the outlet of the treatment system has exceeded the 2.50 t BOD/d discharge limit enforced by Environment Waikato. The surge in BOD happens even when there were no significant changes in wastewater flow and/or wastewater characteristics entering the inlet. Aerobic $CH_4$ oxidation to methanol may cause the unaccounted BOD increase. A simple model was constructed based on the diagram shown below (Figure 4-14). The highest BOD value recorded at the outlet was 3.63 t BOD/d during a study done by Slade (2006). This study first identified the BOD discharge problem at the outlet. The model predicted the rates of sediment $CH_4$ production, dissolved $CH_4$ consumption and $CH_4$ gas emission that are needed to cause an unaccounted BOD increase of 0.10 and 1.13 t BOD/d. This equates to 2.60 and 3.63 t BOD/d discharging from the outlet.

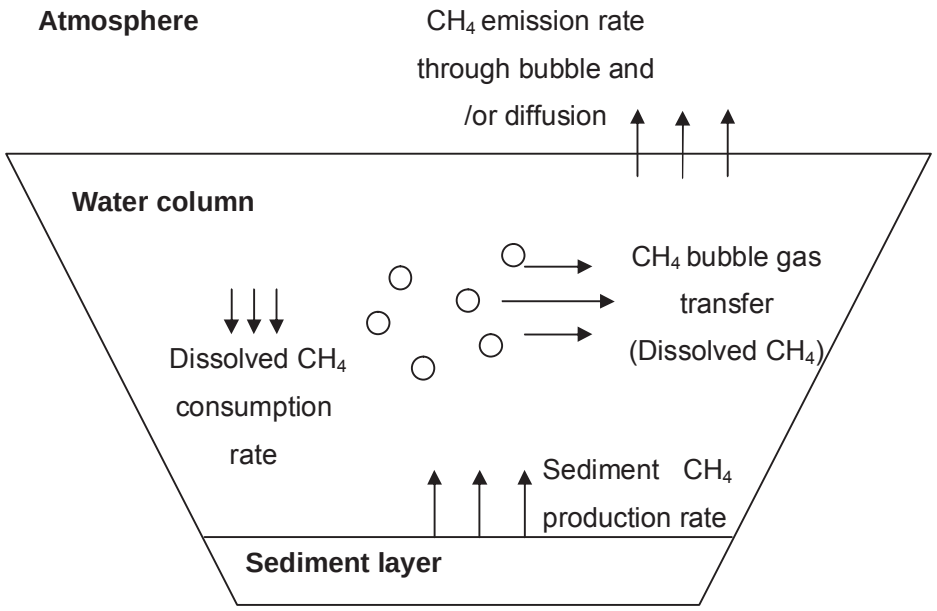


**Figure 4-14: Process diagram used for the model showing the likely fate of $CH_4$ in the treatment system**

### 4.5.2 Assumptions

The assumptions used in the model are based on the monitoring and lab test results and from previous studies done on the treatment system. Other assumptions were used to provide the most favourable conditions for $CH_4$ production, transfer and biological conversion.

- $CH_4$ is converted into methanol with a high experimental yield of 0.38 - 0.61 g MeOH/g $CH_4$. This is a conservative assumption, as methanol is normally only released extracellularly if the enzyme methanol dehydrogenase is inhibited.

- $CH_4$ is produced as bubbles at the sediment. The direct diffusion (without gas transfer) of $CH_4$ from the sediment to the water column is neglected. The impact of this assumption is discussed later.

- $CH_4$ gas bubbles are small and spherical. This assumption maximises $CH_4$ transfer to the water column because small spherical bubbles have higher mass transfer than large bubbles. A high mass transfer between a $CH_4$ gas bubble and the water column favours aerobic $CH_4$ oxidation (as more dissolved $CH_4$ is available).

- The majority of $CH_4$ is produced within the top 0.2 m of the sediment layer. A previous study on the same treatment system by Gielen *et al.* (2009) found that $CH_4$ concentrations were highest at the top 0.2 m of the sediment and the total thickness of the sediment layer ranged between 0.75 - 0.84 m. The sediment core samples from the study were also collected from an area in the treatment system that has not been dredged before.

- The water column is two metres deep. The average depth for the pond closest to the outlet (Pond 23) is between two to three metres. Therefore if the sediment layer is approximately one metre (Gielen*, et al.*, 2009) the water column will be between one to two metres.

- The water column is non turbulent. This assumption simplifies the model as modelling the turbulence produced by other variables such as wind would have been too complex.

- The unaccounted BOD increase is caused by methanol formation during aerobic $CH_4$ oxidation.

- Dissolved $CH_4$ concentration is negligible.

- $CH_4$ production is homogenous across the sediment of Pond 23, and all the $CH_4$/methanol production occurs in Pond 23 (no carry over from the other ponds).

- $CH_4$ consumption at the sediment-water column interface was not included in the model because BOD samples from the outlet were taken from the water column. Therefore any unaccounted BOD increase recorded at the outlet must have occurred at the water column.

### 4.5.3 Explanation of Model Rate Calculations

The following sections aim to determine at what rates $CH_4$ production, transfer and consumption can cause the unaccounted BOD increase. For this purpose:

1. The lowest BOD increase necessary to breach the discharge limit at the outlet was estimated to be 0.10 t BOD/d. The model assumed that $CH_4$ production was uniform in Pond 23 (pond closest to the outlet). Therefore the BOD increase can be expressed as a surface yield, by dividing the BOD increase (0.1 t BOD/d) by the area of Pond 23 (approximately 8000 $m^2$) which equates to 12.5 g BOD/$m^2$d. The calculation of the sediment $CH_4$ production and dissolved $CH_4$ consumption rate required to cause the BOD increase are explained below (points 2 to 4).

2. A theoretical methanol BOD value of 0.75 g/g (this value was assumed to be half of the methanol theoretical oxygen demand value of 1.5 g/g) was used for the methanol productivity necessary to achieve the unaccounted BOD increase.

3. The dissolved $CH_4$ consumption rate needed to cause the unaccounted BOD increase was calculated using an experimental $CH_4$ to methanol biological yield of 0.38 - 0.61 g MeOH/g $CH_4$ (Furuto*, et al.*, 1999; Lee S.G.*, et al.*, 2004; Takeguchi*, et al.*, 1997). An example of the calculation can be found in the Appendix 8.1.4.4.

4. The sediment $CH_4$ production rate was estimated based on the dissolved $CH_4$ consumption rate and the rate a $CH_4$ gas bubble transfers to the water column. This is discussed below:

The behaviour of a $CH_4$ gas bubble's ascent through a non turbulent water column first had to be modelled before the sediment $CH_4$ production rate could be calculated. During a bubble's ascent, $CH_4$ is transferred to the water column. The percentage of $CH_4$ transferred to the water column depends on a variety of parameters, such as the water column depth, $CH_4$ concentration in the bubble, the bubble diameter, the terminal velocity of a rising bubble ($V_t$), and the mass transfer coefficient ($K_L$). For simplicity and according to the pond characteristics, the bubbles were assumed to be pure $CH_4$ and the water column depth is set at two metres. The $V_t$ and $K_L$ values were obtained from

a study by Ahmed (2002), on the gas transfer of small spherical bubbles in natural and industrial systems. According to the literature, $CH_4$ gas bubble diameters ranged from 0.13 to 2 cm in lakes (Boudreau*, et al.*, 2001; Martens & Val Klump, 1980; McGinnis*, et al.*, 2006; Ostrovsky, 2009). Ahmed's study only considered bubble diameters from 0.01 to 0.2 cm, therefore the analysis was restricted to this range. An example calculation of the amount of $CH_4$ that will be transferred from a gas bubble to the water column can be found in the Appendix 8.1.4.3.

Table 4-3 shows that the maximum amount of $CH_4$ that can be transferred from a gas bubble to the water column was 70% (bubble size of 0.10 cm). Table 4-3 also shows that the variation in transfer is quite large (3-70%) due to the variation in $K_L$ values.

To calculate the sediment $CH_4$ production rate, the dissolved $CH_4$ consumption rate calculated to cause the unaccounted BOD increase was divided by the maximum percentage transfer (70%).

**Table 4-3: Bubble to water column $CH_4$ transfer according to bubble diameter**

| Bubble diameter (cm) | $K_L$ (cm/s) | $V_t$ (cm/s) | Percentage transfer from $CH_4$ gas bubble to water column (%) |
|---|---|---|---|
| 0.10 | 0.005 - 0.031 | 12 | 15 - 70 |
| 0.15 | 0.005 - 0.045 | 18 | 6 - 50 |
| 0.20 | 0.004 - 0.050 | 22 | 3 - 37 |

The amount of $CH_4$ emitted to the atmosphere was assumed to be the difference between the sediment $CH_4$ production rate and the dissolved $CH_4$ consumption rate. Since the model assumed that 70% of $CH_4$ produced in the sediment was dissolved and consumed in the water column, 30% of the $CH_4$ produced from the sediment must therefore be emitted to the atmosphere.

### 4.5.4 Explanation of Experimental Rate Calculations Used to Compare with the Model

The rates of $CH_4$ production, consumption and emission predicted by the model were compared with experimental rates obtained from laboratory testing and site monitoring.

The experimental sediment $CH_4$ production rate was calculated from the results obtained from the sediment BMP tests. An example calculation can be found in Appendix 8.1.4.1. The rates obtained from the BMP assays cannot be directly compared to rates achieved under continuous treatment (which occurs at the treatment system). However, the BMP tests were conducted under conditions favourable for anaerobic $CH_4$ production (e.g. constant temperature of 35°C, no oxygen, inoculum collected from an active anaerobic digester). The rate calculated from these tests can therefore be regarded as the maximum sediment $CH_4$ production rate possible in the treatment system sediment.

The experimental dissolved $CH_4$ consumption rate was calculated from the results obtained from the aerobic $CH_4$ oxidation water column assays. An example calculation can be found in Appendix 8.1.4.2. The aerobic $CH_4$ oxidation samples were conducted under conditions favourable for aerobic $CH_4$ oxidation (e.g. constant temperature of 35°C, high $CH_4$ concentration in air mix and continuous mixing). The rate calculated can therefore be regarded as the maximum dissolved $CH_4$ consumption rates possible in the water column of the treatment system.

The experimental $CH_4$ gas emission rates were the rates observed during monitoring of the five sample sites.

### 4.5.5 Model Results

The $CH_4$ production, consumption and emission rates predicted by the model were significantly higher than the rates calculated from laboratory tests and monitoring results of the five sampling sites (Table 4-4). For example, the maximum $CH_4$ production rate calculated from the sediment BMP test results (3.66 - 4.61 g $CH_4/m^2d$) would need to increase by approximately 9 fold (39.14 g $CH_4/m^2d$) and 96 fold (442.80 g $CH_4/m^2d$) to cause the lowest and highest unaccounted BOD increase respectively (Table 4-4). It is therefore very unlikely that enough $CH_4$ can be produced by the sediment to cause the lowest unaccounted BOD increase at the outlet.

The maximum dissolved $CH_4$ consumption rates calculated from the aerobic $CH_4$ oxidation assay water column results (6.15 - 14.59 g $CH_4/m^2d$), would need to increase by at least 2 fold (27.43 g $CH_4/m^2d$) and 21 fold (309.96 g $CH_4/m^2d$) to cause the lowest and highest unaccounted BOD increase respectively (Table 4-4). The samples used to calculate the maximum dissolved $CH_4$ consumption rates were collected from the pond near the inlet of the treatment system (Pond 19). The dissolved $CH_4$ consumption rates at the outlet would be expected to be of similar or lower value than the maximum dissolved $CH_4$ consumption rates calculated, as the active methanotroph population is expected to be smaller near the outlet (Thorn*, et al.*, 2009). The dissolved $CH_4$ consumption rate required to cause the lowest unaccounted BOD increase is therefore unlikely to occur at the outlet.

The highest $CH_4$ gas emission rate observed during monitoring was 7.43 g $CH_4/m^2d$, which is approximately half (11.74 - 15.73 g $CH_4/m^2d$) the $CH_4$ gas emission rate predicted by the model, which would cause the lowest unaccounted BOD increase. The highest observed $CH_4$ gas emission rate was actually observed at the sampling site located near the treatment inlet. $CH_4$ gas emission rates near the outlet of the treatment system ranged between 0.40 - 1.44 g $CH_4/m^2d$, providing further evidence that aerobic $CH_4$ oxidation is unlikely to cause even the lowest unaccounted BOD increase at the outlet.

**Table 4-4: Comparison of rates predicted by the model to the experimental and observed rates from testing and monitoring**

| | (g $CH_4/m^2d$) | | |
|---|---|---|---|
| | Sediment $CH_4$ production rate | Dissolved $CH_4$ consumption rate | $CH_4$ gas emission rate |
| Predicted for unaccounted BOD increase of 0.10 t/d | 39.14 - 63.74 | 27.43 - 44.62 | 11.74 - 15.73 |
| Predicted for unaccounted BOD increase of 1.13 t/d | 442.8 - 720.3 | 309.1 - 504.2 | 132.8 - 216.1 |
| Experimental & observed from testing and monitoring | 3.660 - 4.610 | 5.600 - 16.49 | 0.240 - 7.430 |

Although the model supports that aerobic $CH_4$ oxidation was unlikely to cause the unaccounted BOD increase at the outlet, one could argue that the accumulation of $CH_4$ in the sediment and its sudden release into the water column may have caused such an increase. However, if this event did occur, the majority of the $CH_4$ gas bubbles released would be expected to reach the atmosphere, due to the shallow water column (< 2 m) near the outlet. Furthermore, even if the majority of the $CH_4$ from the bubbles did transfer into the water column, the sudden increase in dissolved $CH_4$ could not be all consumed by the methanotrophs, due to the small population expected at the outlet (Thorn*, et al.*, 2009).

Another possibility could be that a very large amount of $CH_4$ diffused directly from the sediment into the water column without bubble formation. However, for this mechanism to produce enough $CH_4$, sediment $CH_4$ production had to be 9 - 96 fold higher than the rates monitored during BMP. This seems highly improbable given the BMP rate represent a near-ideal productivity.

Carry over of $CH_4$ and/or methanol from ponds upstream of Pond 23 (the pond closest to the outlet) is another possibility. This is unlikely however, as methanol and dissolved $CH_4$ were only detected near the inlet of the treatment system. Also, methanol and dissolved $CH_4$ would have been degraded before reaching the outlet, due to the 6 day hydraulic retention time between the inlet and outlet of the treatment system (Maclean*, et al.*, 2007). Therefore the BOD increase must be caused by a mechanism that happens near the outlet of the treatment system.

### 4.5.6 Uncertainty Analysis

This section examines whether changes in some assumption values in the model, could change the model's conclusion (aerobic $CH_4$ oxidation cannot cause the unaccounted BOD increase at the outlet). Assumptions regarding active sediment thickness and bubble diameter were examined.

Changes in active sediment thickness would affect the calculation of the experimental $CH_4$ production rates, which was based on sediment BMP test results. In the model, $CH_4$ production was assumed to be restricted to the top 0.2 m of the sediment layer (Gielen*, et al.*, 2009). Gielen *et al.* (2009) also found that that the total thickness of the sediment layer ranged between 0.75 - 0.84 m, at sites where no prior dredging had occurred. The total sediment thickness at the outlet would therefore be expected to be similar or smaller than what was found in Gielen's study (approximately one metre), as the majority of the particulate organic matter entering the treatment system should settle in Pond 19 (Pond 19 has a hydraulic retention time of five days, compared to one day for the rest of the treatment system, (Maclean*, et al.*, 2007). When the active sediment thickness of one metre was used, the resulting sediment $CH_4$ production rates (18.34 – 23.03 g $CH_4/m^2d$, (Table 4-5), were still not within the range of rates needed to cause the lowest unaccounted BOD increase (39.14 - 63.74 g $CH_4/m^2d$).

**Table 4-5: The effect of changes to active sediment thickness to the sediment $CH_4$ production rate**

| Active sediment thickness (m) | sediment $CH_4$ production rate (g $CH_4/m^2d$) |
|---|---|
| 0.2 | 3.660 - 4.610 |
| 0.6 | 11.01 - 13.82 |
| 1.0 | 18.34 - 23.03 |

However, if the active sediment layer was one metre thick and if all the $CH_4$ produced in the sediment was transferred to the water column and consumed, the dissolved $CH_4$ consumption rate (18.34 - 23.03 g $CH_4/m^2d$), would be close to the rate predicted by the model (27.43 - 44.62 g $CH_4/m^2d$), to produce the lowest unaccounted BOD increase. However, complete transfer is unlikely to occur. Complete transfer would require the $CH_4$ gas bubble diameter to be less than 0.10 cm, lower than the diameter range (0.13 to 2 cm) found in lakes (Boudreau*, et al.*, 2001; Martens & Val Klump, 1980; McGinnis*, et al.*, 2006; Ostrovsky, 2009).

## 4.6 Alternative Causes to the Increased BOD Load at the Outlet

Based on the model results, aerobic $CH_4$ oxidation to methanol is unlikely to cause the unaccounted BOD increase at the outlet of the treatment system. Another mechanism or event must have occurred near the outlet to cause the increase in BOD. This section explores the other possibilities.

Other waste streams (such as agricultural runoff) could have been received by the final pond, increasing the BOD load. However this situation is unlikely, as the land that surrounds the treatment system is almost exclusively used for forestry. Furthermore, if other organic waste streams were present, it would only make up a small amount of the total wastewater flowing through the ponds, as the pulp and paper mill discharges approximately 90 ML/day.

The mechanism called 'benthic feedback' could have occurred near the outlet causing the increase in BOD. Benthic feedback is the process in which the anaerobic sludge layer becomes buoyant, suddenly releasing soluble compounds (e.g. ammonia, VFAs, inorganic nutrients) into the aerobic water column of a pond. The soluble compounds may be subsequently oxidized, producing extra oxygen demand (Shilton, 2005). Benthic feedback occurs randomly in ponds, hence the lack of literature. Benthic feedback is a viable mechanism which could explain the increases in BOD at the outlet. The BOD increases were fairly random at the outlet, as the increases could not be correlated with DO, temperature and the BOD at the inlet of the treatment system. Furthermore, the conditions for benthic feedback (oxygen limited sludge layer and aerobic water column) do exist at the outlet.

Further research is needed to investigate the possibility of whether benthic feedback can release enough soluble compounds to the water column to produce an increase in BOD.

# 5 Conclusions

The project's objective was to determine if the unaccounted BOD increase experienced at the outlet of the treatment system was due to aerobic $CH_4$ oxidation to methanol in the water column. To achieve this goal, evidence to support biosynthesis of $CH_4$ and the subsequent biological aerobic oxidation of $CH_4$ to methanol needed to be found in the treatment system.

$CH_4$ gas was emitted throughout the treatment system, mostly through bubbles and at higher rates near the inlet. Therefore $CH_4$ must be produced in the treatment system. The results of BMP assays for sediment samples confirmed that $CH_4$ is produced in the treatment system.

The presence of aerobic conditions, combined with the lack of dissolved $CH_4$ detected in the water column (except at the oxygen limited inlet) showed that aerobic $CH_4$ oxidation most likely occurs in the water column of the treatment system. Laboratory testing provided further evidence that aerobic $CH_4$ oxidation can occur in the water column. For example, aerobic $CH_4$ oxidation assays detected simultaneous decreases in $CH_4$ and $O_2$ concentration with an increase in $CO_2$ concentration, and methanotrophs were detected using FISH analysis.

Other significant results were found during laboratory testing. The aerobic $CH_4$ oxidation assays showed that aerobic $CH_4$ oxidation can also occur at the sediment-water column interface in the treatment system, and the oxidation of other carbon compounds can compete with aerobic $CH_4$ oxidation. FISH analysis suggested that methanotrophs only make up a small fraction of the total bacterial population in the water column.

The monitoring and laboratory test results showed that the mechanisms needed to cause an increase in BOD load through aerobic $CH_4$ oxidation exists in the treatment system. The simple model however, showed that aerobic $CH_4$ oxidation was unlikely to cause the unaccounted BOD increase at the outlet of the treatment system. The rates predicted by the model (sediment $CH_4$ production and dissolved $CH_4$ consumption), that would cause the lowest unaccounted BOD increase at the outlet, were significantly higher than the rates calculated from the results of laboratory tests. Some of the model assumptions were examined, to determine if the model's conclusion was affected. The

changes had no effect on the conclusion and reaffirmed the assumption that the increase in BOD must be caused by a mechanism or event that occurs near the outlet.

A possible mechanism which could have caused the BOD increase at the outlet is benthic feedback. The BOD increase at the outlet followed similar characteristics associated with benthic feedback (randomness of the event, occurs at a pond with an anaerobic sludge layer and aerobic water column). If the cause of the BOD increase needs to be determined, further investigation into benthic feedback is recommended.

To summarise, the biosynthesis of $CH_4$ and subsequent biological oxidation of $CH_4$ can occur in the treatment system, but not at rates which can cause the unaccounted BOD increase at the outlet. Therefore aerobic $CH_4$ oxidation is unlikely to cause the unaccounted BOD increase at the outlet.

# 6 Future Work

## *6.1 Benthic Feedback Monitoring*

Benthic feedback may be difficult to monitor on site, due to the random nature of this mechanism. Benthic feedback could however be induced to occur under controlled laboratory conditions. Changing conditions such as temperature, DO and mixing speed on sediment and water column samples, until benthic feedback occurs, is a possibility. The experimental conditions that induced benthic feedback can then be compared to the conditions monitored when an unaccounted BOD increase at the outlet occurred. The similarity or lack of parity between the experimental and monitored conditions can potentially validate whether benthic feedback is possible at the pond, and cause the BOD increase.

## *6.2 $CH_4$ Gas Emission Monitoring*

The capability to monitor $CH_4$ gas emissions was developed during the course of the project. This capability may prove useful for the mill in the near future, as green house gas (GHG) emissions will need to be monitored under the Emissions Trading Scheme (ETS) proposed by the New Zealand Government. The $CH_4$ gas emission costs of the treatment system were calculated, using the $CH_4$ gas emission rates recorded during the course of the project.

To calculate the cost, $CH_4$ gas was assumed to have a global warming potential 21 times higher than $CO_2$ (European Commission, 2001) and the cost of one tonne of $CO_2$ was set at $12.50 (Ministry for the Environment, 2011a). The results are shown in Table 6-1.

The annual $CH_4$ gas emissions from the treatment system could cost as low as $6,800 or as high as $49,800 (Table 6-1). The majority of $CH_4$ gas emissions occurred at the first pond of the treatment system (Pond 19). Also, the emission rates were highly variable for the rest of Pond 19, which is the reason why there is a large variation in the estimated annual cost of $CH_4$ gas emissions.

**Table 6-1: Estimated $CH_4$ gas emission cost for the treatment system**

| Pond | $CH_4$ gas emission range (g $CH_4/m^2d$) | Pond Area ($m^2$) | $CH_4$ emission (t $CH_4$/yr) | $CO_2$ equivalent emission (t $CO_2$/yr) | Cost ($) *rounded to nearest hundred |
|---|---|---|---|---|---|
| Pond 19 Inlet | 2.63 - 3.92 | 19200 | 18.43 - 27.47 | 387.1 - 577.0 | 4,900 - 7,300 |
| Rest of Pond 19 | 0.24 - 7.43 | 52800 | 4.63 - 143.2 | 97.13 - 3,007 | 1,300 - 37,600 |
| Pond 22 | 0.29 - 3.97 | 9900 | 1.05 - 14.35 | 22.01 - 301.3 | 300 - 3,800 |
| Pond 23 | 0.40 - 1.44 | 7969 | 1.16 - 4.22 | 24.43 - 88.57 | 300 - 1,100 |
| **TOTAL** | | 89869 | 25.27 - 189.2 | 530.7 - 3,974 | 6,800 - 49,800 |

The cost of $CH_4$ gas emissions from the treatment system were compared to the $CO_2$ emission cost of running the aerators located at Pond 19. The total installed aeration capacity at Pond 19 is 550 kW (Maclean*, et al.*, 2007) and a kW hr of electricity was assumed to produce 0.16 kg of $CO_2$ (Ministry for the Environment, 2011b). The aerators were unlikely to run at full capacity for 24 hours a day, so to provide a more conservative estimate, the aerators were assumed to run at 70% capacity, 18 hours a day. The results are shown on Table 6-2.

**Table 6-2: Comparison of GHG emission costs for the P19 aerators and $CH_4$ gas emissions for the treatment system**

| | kW hr/yr | $CO_2$ equivalent emissions ($tCO_2$/yr) | Cost ($) |
|---|---|---|---|
| P19 aerators | 2,529,450 | 404.7 | 5,059 |
| $CH_4$ gas emissions | NA | 530.7 - 3,974 | 6,800 - 49,800 |
| | | **Total Cost ($)** | 11,859 - 54,859 |

Both Table 6-1 and Table 6-2 show that the majority of GHG emission costs occur at Pond 19. Therefore future reductions in GHG emission costs must focus on reducing $CH_4$ gas emissions and aerator use at Pond 19. However, reducing aerator use may actually increase $CH_4$ gas emissions from Pond 19 as DO concentrations may decrease, which may inhibit methanotroph growth and/or increase $CH_4$ production. Therefore, more novel solutions may be required, such as the feasibility of using anaerobic processes to treat the concentrated wastewater streams at the mill before entering the treatment system. This would reduce the BOD load entering the treatment system, reduce aerator use and have the potential of converting the $CH_4$ produced in the process to other forms of energy.

The feasibility of using anaerobic processes on concentrated wastewater streams depends on the make up of the wastewater. Most wastewater streams from kraft pulp and paper mills can be anaerobically treated, especially wastewaters from mechanical pulping, secondary fibre pulping and condensates from chemical and semi chemical pulping (Pokhrel & Viraraghavan, 2004; Rintala & Puhakka, 1994). However, wastewater streams which contain resin acids from bleaching operations are not suitable for anaerobic treatment (Ali & Sreekrishnan, 2001; Pokhrel & Viraraghavan, 2004; Rintala & Puhakka, 1994). The pulping/drying and the hot water circuit/recovery wastewater streams from the mill may be suitable for anaerobic treatment, however anaerobic treatment at the mill must also be able to handle the variability in wastewater flows, due to the mill shutdowns and spills that occasionally happen in the mill.

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# Appendices

## *8.1 Calculations*

### 8.1.1 $CH_4$ Gas Emission Rate Calculations

The headspace of the $CH_4$ gas emission samples were analyzed using the TOGA. The analysis provided a parts per million (ppm, calculated using a calibration curve) $CH_4$ value for each sample. The ppm value was converted into a rate value with units of g $CH_4/m^2d$, using the following calculations:

- Account for the dilution effect that occurs in the serum bottle (serum bottles were used to collect the samples). Before collection, the 60 mL serum bottle is flushed with an inert gas and 35 mL of gas is removed. Gas from the surface gas collectors (25 mL) is then injected into the serum bottle. The sample gas would be diluted by the inert gas still remaining in the bottle. The number of moles (n) in the sample was calculated using the ideal gas law. The following n values needed to be calculated.

    - n overall = n 60mL – n 35mL + n 25mL
    - n 60mL = total n in 60mL serum bottle
    - n 35mL = total n in 35 mL removed from serum bottle
    - n 25mL = 25 mL sample gas added to serum bottle

For n 35mL the pressure needed to be multiplied by:

$$\frac{total\ volume\ serum\ bottle}{total\ volume\ serum\ bottle + volumeremoved} = \frac{60}{60+35}$$

- $= 0.63$

Using the following values:

Pressure (P) = 1 atm

Sample volume (V) = sample volume (mL)/1000 (to convert into litres)

Gas constant (R) = 0.082 L atm/mol K

Temperature (T) = 298 K

- $n\ 60mL = \frac{1\ x\ (60/1000)}{0.082\ x\ 298} = 2.46x10^{-3}\ mol$
- $n\ 35mL = \frac{(0.63\ x\ 1)\ x\ (35/1000)}{0.082\ x\ 298} = 9.02x10^{-4}\ mol$
- $n\ 25mL = \frac{1\ x\ (25/1000)}{0.082\ x\ 298} = 1.02x10^{-3}\ mol$
- $n\ overall = 2.46x10^{-3} - 9.02x10^{-4} + 1.02x10^{-3} = 2.58x10^{-3}\ mol$

To calculate the dilution effect, the following equation was used:

- $Dilution\ effect = \frac{n\ overall}{n\ 25ml} = \frac{2.58x10^{-3}\ mol}{1.02x10^{-3}\ mol} = 2.53$

The ppm value taking into account the dilution effect can now be calculated (real ppm)

- $real\ \ ppm = ppm\ x\ dilution\ effect$

- The moles of $CH_4$ gas in the surface gas collector headspace ($nCH_4$) was then calculated:
  - $nCH_4 = real\ \ ppm\ x\ 1x10^{-6}\ \ x\ mols\ of\ \ ideal\ gas\ in\ headspace$

Where:

$$mols\ of\ \ ideal\ gas\ in\ headspace = \left( \frac{total\ volume\ of\ \ gas\ collector}{volume\ of\ 1\ mol\ ideal\ gas\ at\ 25°C} \right) x\ 0.5$$

- $= \left( \frac{30L}{24L} \right) x\ 0.5$

$$= 0.625\ mol$$

Multiplied by 0.5 as only half the gas collector was submerged in the water column

- $nCH_4$ is then converted into a rate term with units of g $CH_4/m^2d$

$$CH_4 \ gas\ emission\ rate = \left( \frac{nCH_4 \ \times \ molecular\ weight\ of\ CH_4}{Area\ of\ surface\ gas\ collector} \right) \times 24\ hours$$

o

$$= \left( \frac{mol\ x\ 16\ g/mol}{0.2m^2} \right) x24\ hours$$

$$= \ g\ CH_4/m^2 d$$

$CH_4$ gas emission samples were collected after an hour for each site, which is the reason why the equation is multiplied by 24 hours.
Table 8-1 shows an example of the calculation.

**Table 8-1: Example of $CH_4$ gas emission rate calculation**

| ppm $CH_4$ | Real ppm $CH_4$ | moles of $CH_4$ gas in headspace | Rate of $CH_4$ gas emission (g $CH_4/m^2d$) |
|---|---|---|---|
| 1057.25 | 2662.15 | $1.66 \times 10^{-3}$ | 3.19 |
| 681.33 | 1715.58 | $1.07 \times 10^{-3}$ | 2.06 |

### 8.1.2 Dissolved $CH_4$ Concentration Calculations

The headspace of the dissolved $CH_4$ samples were also analyzed using the TOGA. The ppm values obtained from the samples was converted into mg/L. Table 8-2 shows an example calculation. The following calculations were undertaken to convert from ppm to mg/L for the samples:

- To calculate the total moles of gas in the headspace, for the 120 mL serum bottle after sampling (n after sampling), the moles of gas before sampling (n initial) and the moles of gas removed from the serum bottle (n removed) was calculated using the following values:

P = 1 atm
V = sample volume (mL)/1000 (to convert into litres)
R = 0.082 L atm/mol K
T = 298 K

$$n\ initial = \frac{P\ x\ total\ volume\ serum\ bottle}{RT}$$

- $= \frac{1\ atm\ x\ 0.12L}{0.082L\ atm/molK\ x\ 298K}$

$$= 4.90x10^{-3}\ mol$$

$$n\ removed = \frac{P\ after\ 80ml\ removed\ x\ volume\ of\ gas\ removed\ before\ sampling}{RT}$$

- $= \frac{\left(\frac{0.12}{0.12+0.08}\right) atm\ x\ 0.08L}{0.082L\ atm/molK\ x\ 298K}$

$$= 1.96x10^{-3} mol$$

$$n\ after\ sampling = n\ initial - n\ removed$$

- $= 4.90\ x\ 10^{-3} mol - 1.96\ x\ 10^{-3} mol$

$$= 2.94\ x\ 10^{-3}\ mol$$

- Calculate mol $CH_4$

$$= \frac{ppm\ CH_4}{1x10^6}$$

- 

$$= mol\ CH_4$$

- Calculate mol $CH_4$ in headspace

$$mol\ CH_4\ in\ headspace = mol\ CH_4\ x\ n\ after\ sampling$$

o $$= mol\ CH_4\ x\ 2.94\ x 10^{-3}\ mol$$

- Convert to mg $CH_4$ in headspace

$$mg\ CH_4\ in\ headspace = mol\ CH_4\ in\ headspace\ x\ molecular\ weight\ CH_4\ x 1000$$

o $$= mol\ CH_4\ in\ headspace\ x 16\ g/mol\ x 1000$$

- The partial pressure of $CH_4$ ($PCH_4$) in the headspace was calculated. To calculate $PCH_4$, pressure of the headspace after sampling (P after sampling) was also calculated.

$$P\ after\ sampling = \frac{n\ after\ sampling\ x\ RT}{volume\ of\ serum\ bottle - volume\ of\ gas\ removed\ before\ sampling}$$

o $$= \frac{2.94\ x 10^{-3} mol\ x\ 0.082\ L\ atm/molK\ x\ 298K}{0.12L - 0.08L}$$

$$= 1.03\ atm$$

$$P\ CH_4 = P\ after\ sampling\ x\ mol\ CH_4$$

o $$= 1.03\ atm\ x\ mol\ CH_4$$

- Concentration of $CH_4$ in the liquid phase $CH_{4\ (liq)}$ can now be calculated using Henry's law constant ($k_H$) for $CH_4$ at 25°C.

$$CH_{4\ (liq)} = \frac{PCH_4}{K_H}$$

  - $$= \frac{atm}{Latm/mg}$$

  $$= mg/L$$

- The total amount of $CH_4$ in the sample (both headspace and liquid) can now be calculated

$$Amount\ of\ CH_4\ in\ sample = mg\ of\ CH_4\ in\ headspace \quad x \left(CH_{4(liq)}\ x\ volume\ of\ liquid\ sample\right)$$

  - $$= mg\ x\ (mg/L\ x\ 0.05L)$$

  $$= mg$$

- The dissolved $CH_4$ concentration of the sample can now be calculated

$$Dissolved\ CH_4 = \frac{amount\ of\ CH_4\ in\ sample}{volume\ of\ liquid\ sample}$$

  - $$= mg/L$$

**Table 8-2: Example of dissolved $CH_4$ concentration calculation**

| ppm $CH_4$ | mol $CH_4$ | mol $CH_4$ in head space | mg $CH_4$ in head space | $PCH_4$ (atm) | $CH_4$ in liquid phase (mg/L) | Amount of $CH_4$ in sample (mg) | Dissolved $CH_4$ (mg/L) |
|---|---|---|---|---|---|---|---|
| 2632 | $2.63 \times 10^{-3}$ | $7.75 \times 10^{-6}$ | 0.12 | $2.71 \times 10^{-3}$ | $5.89 \times 10^{-2}$ | 0.13 | 2.54 |
| 2606 | $2.61 \times 10^{-3}$ | $7.67 \times 10^{-6}$ | 0.12 | $2.68 \times 10^{-3}$ | $5.83 \times 10^{-2}$ | 0.13 | 2.51 |
| 2076 | $2.08 \times 10^{-3}$ | $6.11 \times 10^{-6}$ | 0.10 | $2.14 \times 10^{-3}$ | $4.64 \times 10^{-2}$ | 0.10 | 2.00 |

### 8.1.3 Production/Consumption Rates for $CH_4$ Oxidation Assays

The TOGA was also used to analyze the aerobic $CH_4$ oxidation assay samples. The ppm values were converted to µmol/gVSShr. The following steps were used to convert from ppm to µmol/gVSShr and Table 8-3 shows an example calculation.

- Convert ppm gas in the headspace of the serum bottle to µmol. This required the calculation of the volume of 1 mol of ideal gas in the headspace.

$$volume\ of\ 1\,mol\ of\ \ ideal\ gas = \left(\frac{nRT}{P}\right) x\,1000$$

o

$$= \left(\frac{1\,mol\ x\,8.314\,\frac{m^3 Pa}{Kmol}\ x\,308K}{101300\ Pa}\right) x\,1000$$

$$= 25.29\ L/mol$$

Multiplied by 1000 to convert units from $m^3$ to L

$$\mu mol\ \ gas\ in\ headspace = ppm\ \ x\,\frac{headspace\ \ volume}{volume\ of\ 1\,mol\ ideal\ \ gas}$$

o

$$= ppm\ x\,\frac{0.10\ L}{25.29\ L/mol}$$

$$= \mu mol$$

- Calculate the µmol difference. This is the difference in µmol between the sample at the start of the test and the sample after a specific time.

- Calculate production/consumption rate in µmol/g VSShr

$$\text{Production/consumption rate} = \frac{\mu mol\ difference}{sample\ VSS\ x\ CH_4\ consumption\ time\ x\ sample\ volume}$$

o

$$= \frac{\mu mol}{gVSS/L\ x\ hrs\ x\ 0.060L}$$

$$= \mu mol/gVSS\ hr$$

**Table 8-3: Example of consumption/production rate calculation**

| $CH_4$ consumption time (hrs) | ppm $CH_4$ | µmol $CH_4$ gas in headspace | VSS (g/l) | µmol $CH_4$ gas difference | µmol $CH_4$/gVSS hr |
|---|---|---|---|---|---|
| 0 | $2.96 \times 10^4$ | 117.2 | 16.53 | | |
| 17 | $3.06 \times 10^4$ | 121.1 | 15.62 | -3.95 | -0.25 |
| 37 | $2.53 \times 10^4$ | 99.9 | 15.94 | 17.20 | 0.49 |
| 47 | $1.62 \times 10^4$ | 64.1 | 15.86 | 53.06 | 1.19 |
| 60 | $1.74 \times 10^4$ | 68.7 | 16.13 | 48.49 | 0.84 |

## 8.1.4 Process Model Calculations

### *8.1.4.1 Maximum Sediment $CH_4$ Production Rate*

The maximum sediment $CH_4$ production rate calculated from the results of the BMP assays had units of mL $CH_4$/d. The rate needed to be converted to g $CH_4/m^2d$. The following calculations were undertaken to convert from units of mL $CH_4$/d to g $CH_4/m^2d$ and a sample calculation is shown on Table 8-4:

- Convert mL $CH_4$/d to mol $CH_4$/d using the ideal gas law:

$$mol\ CH_4/d = \frac{P\ x\ mL\ CH_4/d}{RT}$$

o

$$= \frac{1\ atm\ x\ (mL\ CH_4/d\ x\ 1000)}{0.082\ atm/molK\ x\ 308K}$$

$$= mol\ CH_4/d$$

- Convert mol $CH_4$/d to g $CH_4$/d using the equation n=m/Mr:

$$g\ CH_4/d = \frac{mol\ CH_4/d}{Mr}$$

o

$$= \frac{mol\ CH_4/d}{16\ g/mol}$$

$$= g\ CH_4/d$$

- Convert from g $CH_4$/d to g $CH_4$ /$m^3$d by dividing by 2 x $10^{-6}$ $m^3$ (as 2 mL of sediment sample was used in the BMP assays).

- Convert from g $CH_4$ /$m^3$d to g $CH_4$/$m^2$d by multiplying by 0.2 m (as the active sediment thickness was assumed to be 0.2 m).

**Table 8-4: Example of maximum sediment $CH_4$ production rate calculation**

| mL /$CH_4$d | mol $CH_4$/d | g $CH_4$/d | g $CH_4$/$m^3$d | g $CH_4$/$m^2$d |
|---|---|---|---|---|
| 0.073 | 2.88 x $10^{-6}$ | 4.61 x $10^{-5}$ | 23.03 | 4.61 |

#### *8.1.4.2 Maximum $CH_4$ Sediment & Water Column Consumption Rate*

The $CH_4$ consumption rates calculated from the aerobic $CH_4$ oxidation assay results were converted from units of µmol $CH_4$/gVSShr to g $CH_4$/$m^2$d. An example calculation is shown on Table 8-5. The following calculations were undertaken:

- Convert units from µmol $CH_4$/gVSShr to µg $CH_4$/gVSShr by multiplying by the molecular weight of $CH_4$ (16 g/mol).

- Convert from µg $CH_4$/gVSShr to µg $CH_4$/Lhr by multiplying by the grams of VSS per L of sample (g/L).

- Convert from µg $CH_4$/Lhr to g $CH_4$/$m^3$d using unit conversions

- Convert g $CH_4$/$m^3$d to g $CH_4$/$m^2$d by multiplying by the active sediment layer depth for sediments (0.2 m) and the water column depth for the water column (2 m).

**Table 8-5: Example of $CH_4$ consumption rate calculation**

| Sample | µmol $CH_4$/ gVSShr | VSS (g/l) | ug $CH_4$/ gVSShr | µg $CH_4$/ Lhr | g $CH_4$/ $m^3$d | g $CH_4$/ $m^2$d |
|---|---|---|---|---|---|---|
| P19 Middle Sediment | 3.66 | 11.84 | 58.64 | 694.28 | 16.66 | 3.33 |

#### *8.1.4.3 Bubble Transfer to Water Column*

The following equation was used to predict the gas transfer from a gas bubble to the water column:

$$\frac{dCH_4}{dt} = k_L\, a\, (C_s - C_t)$$

Where:

$\frac{dCH_4}{dt}$ = the rate $CH_4$ is transferred from a bubble to the water column (mg/s)

$K_L$ = mass transfer coefficient (m/s)

a = area of a bubble ($m^2$)

$C_s$ = Equilibrium concentration at the gas/water interface ($mg/m^3$) calculated using Henry's law ($p= k_H C$)

$C_t$ = Bulk aqueous phase concentration ($mg/m^3$)

This equation was integrated to calculate the amount of $CH_4$ that can be transferred from a bubble at a specific depth, as a bubble rises up the water column. The velocity of a rising bubble (terminal velocity, $V_t$) was used to calculate the time a bubble takes to rise to a certain height, which changes for different sized bubbles.

After the bubble has transferred $CH_4$ to the water column at a specific depth, the bubble's volume would have decreased. The new bubble volume was calculated using the ideal gas law. The number of moles (n) used in calculating the new bubble volume was the difference in the total number of moles the $CH_4$ gas bubble had at the previous

depth and the number of moles of $CH_4$ transferred to the water column. The $CH_4$ transferred to the water column at each specific depth was then summed. The total $CH_4$ transferred to the water column was then divided by the amount of $CH_4$ in the bubble at the start before the ascension of the bubble, thereby calculating the total percentage of $CH_4$ transferred from the bubble to the water column. Table 8-6 shows a sample calculation of $CH_4$ gas bubble transfer to the water column for a specific bubble diameter. For this example:

- Henry's law coefficient ($k_H$) = 0.051 L atm/mg (Ahmed & Semmens, 2002)
- Bubble diameter = 0.50 mm
- Mass transfer coefficient ($K_L$) = 5.00 x $10^{-5}$ m/s (Ahmed & Semmens, 2002)
- Terminal velocity (Vt) = 0.06 m/s (Ahmed & Semmens, 2002)

**Table 8-6: Example of $CH_4$ gas bubble transfer to the water column calculation**

| Metres from sediment (m) | Bubble diameter (mm) | Bubble radius (m) | Bubble volume ($m^3$) | Bubble volume (mL) | Bubble area ($m^2$) | $pCH_4$ (atm) | Cs ($mg/m^3$) | bubble rise time (s) | $CH_4$ transferred to water column (mg) | n $CH_4$ transferred to water column (mol) | n $CH_4$ bubble (mol) | new bubble volume (mL) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.00 | 0.50 | $2.50x10^{-4}$ | $6.54x10^{-11}$ | $6.54x10^{-5}$ | $7.85x10^{-7}$ | 1.20 | $2.35x10^{4}$ | 0.00 | | | $3.18x10^{-9}$ | |
| | | | | | | | | | $3.08x10^{-6}$ | $1.92x10^{-10}$ | | $6.25x10^{-5}$ |
| 0.20 | 0.49 | $2.46x10^{-4}$ | $6.25x10^{-11}$ | $6.25x10^{-5}$ | $7.62x10^{-7}$ | 1.18 | $2.31Ex10^{4}$ | 3.33 | | | $2.99x10^{-9}$ | |
| | | | | | | | | | $2.94x10^{-6}$ | $1.84x10^{-10}$ | | $5.87x10^{-5}$ |
| 0.40 | 0.48 | $2.41x10^{-4}$ | $5.87x10^{-11}$ | $5.87x10^{-5}$ | $7.30x10^{-7}$ | 1.16 | $2.27x10^{4}$ | 3.33 | | | $2.76x10^{-9}$ | |
| | | | | | | | | | $2.77x10^{-6}$ | $1.73x10^{-10}$ | | $5.50x10^{-5}$ |
| 0.60 | 0.47 | $2.36x10^{-4}$ | $5.50x10^{-11}$ | $5.50x10^{-5}$ | $6.99x10^{-7}$ | 1.14 | $2.24x10^{4}$ | 3.33 | | | $2.54x10^{-9}$ | |
| | | | | | | | | | $2.61x10^{-6}$ | $1.63x10^{-10}$ | | $5.15x10^{-5}$ |
| 0.80 | 0.46 | $2.31x10^{-4}$ | $5.15x10^{-11}$ | $5.15x10^{-5}$ | $6.69x10^{-7}$ | 1.12 | $2.20x10^{4}$ | 3.33 | | | $2.34x10^{-9}$ | |
| | | | | | | | | | $2.45x10^{-6}$ | $1.53x10^{-10}$ | | $4.81x10^{-5}$ |
| 1.00 | 0.45 | $2.26\ x10^{-4}$ | $4.81x10^{-11}$ | $4.81x10^{-5}$ | $6.40x10^{-7}$ | 1.10 | $2.16x10^{4}$ | 3.33 | | | $2.14x10^{-9}$ | |
| | | | | | | | | | $2.30x10^{-6}$ | $1.44x10^{-10}$ | | $4.49x10^{-5}$ |
| 1.20 | 0.44 | $2.20\ x10^{-4}$ | $4.49x10^{-11}$ | $4.49x10^{-5}$ | $6.11x10^{-7}$ | 1.08 | $2.12x10^{4}$ | 3.33 | | | $1.96x10^{-9}$ | |
| | | | | | | | | | $2.16x10^{-6}$ | $1.35x10^{-10}$ | | $4.18x10^{-5}$ |
| 1.40 | 0.43 | $2.15\ x10^{-4}$ | $4.18x10^{-11}$ | $4.18x10^{-5}$ | $5.83x10^{-7}$ | 1.06 | $2.08x10^{4}$ | 3.33 | | | $1.80x10^{-9}$ | |
| | | | | | | | | | $2.02x10^{-6}$ | $1.26x10^{-10}$ | | $3.89x10^{-5}$ |
| 1.60 | 0.42 | $2.10\ x10^{-4}$ | $3.89x10^{-11}$ | $3.89x10^{-5}$ | $5.55x10^{-7}$ | 1.04 | $2.04x10^{4}$ | 3.33 | | | $1.64x10^{-9}$ | |
| | | | | | | | | | $1.89x10^{-6}$ | $1.18x10^{-10}$ | | $3.61x10^{-5}$ |
| 1.80 | 0.41 | $2.05x10^{-4}$ | $3.61x10^{-11}$ | $3.61x10^{-5}$ | $5.28x10^{-7}$ | 1.02 | $2.00x10^{4}$ | 3.33 | | | $1.49x10^{-9}$ | |
| | | | | | | | | | $1.76x10^{-6}$ | $1.10x10^{-10}$ | | $3.34x10^{-5}$ |
| 2.00 | 0.40 | $2.00x10^{-4}$ | $3.34x10^{-11}$ | $3.34x10^{-5}$ | $5.02x10^{-7}$ | 1.00 | $1.96x10^{4}$ | 3.33 | | | $1.35x10^{-9}$ | |
| | | | | | | | | | $1.64x10^{-6}$ | $1.02x10^{-10}$ | | $3.09x10^{-5}$ |
| | | | | | | | | **Sum $CH_4$(mg)** | $2.56x10^{-5}$ | | | |

#### *8.1.4.4 Unaccounted BOD Increase Calculations*

The dissolved $CH_4$ consumption rate required to cause the unaccounted BOD increase at the outlet was calculated. The following equation was used:

$$Dissolved\ CH_4\ consumption\ rate = \frac{\left(\frac{1}{Methanol\ BOD\ equivalent}\right) x\ unaccounted\ BOD\ increase}{\frac{methanol}{CH_4}\ yield\ x\ Area\ of\ \ pond\ at\ the\ outlet}$$

$$Dissolved\ CH_4\ consumption\ rate = \frac{\left(\frac{1}{0.75 g/g}\right) x\ unaccounted\ BOD\ increase}{0.38 - 0.61\frac{gmethanol}{gCH_4}\ yield\ x\ 7968.5\ m^2}$$

$$= g\ CH_4 / m^2 d$$

Unaccounted BOD increase values of $1.00 \times 10^5$ and $1.13 \times 10^6$ g BOD/d were used. These values represent the lowest and highest BOD loads which would cause the BOD discharged at the outlet of the treatment system to exceed the discharge limit (2.50 t BOD/d). Table 8-7 is an example of a dissolved $CH_4$ consumption rate calculation, which would produce an unaccounted BOD increase of $1.13 \times 10^6$ g BOD/d at the outlet.

**Table 8-7: Example of dissolved $CH_4$ consumption rate calculation**

| Methanol/$CH_4$ yield (g/g) | Dissolved $CH_4$ consumption rate (g $CH_4/m^2d$) |
|---|---|
| 0.38 | 504.21 |
| 0.61 | 309.96 |

## 8.2 Effect of Seasonal Changes to $CH_4$ Gas Emission and Dissolved $CH_4$ Concentration

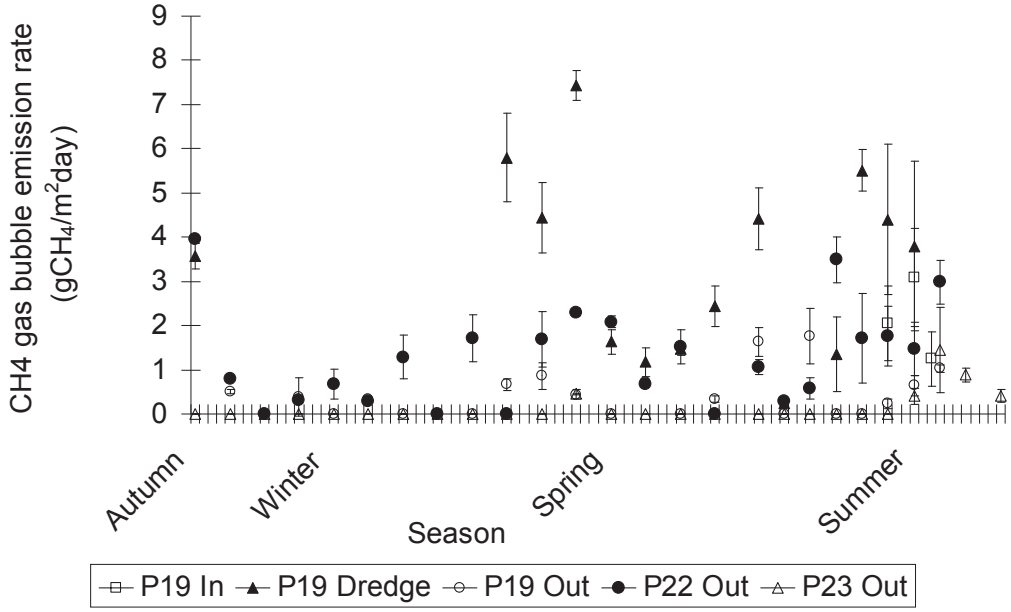


**Figure 8-1: $CH_4$ gas emission rates for the five sampling sites during different seasons**

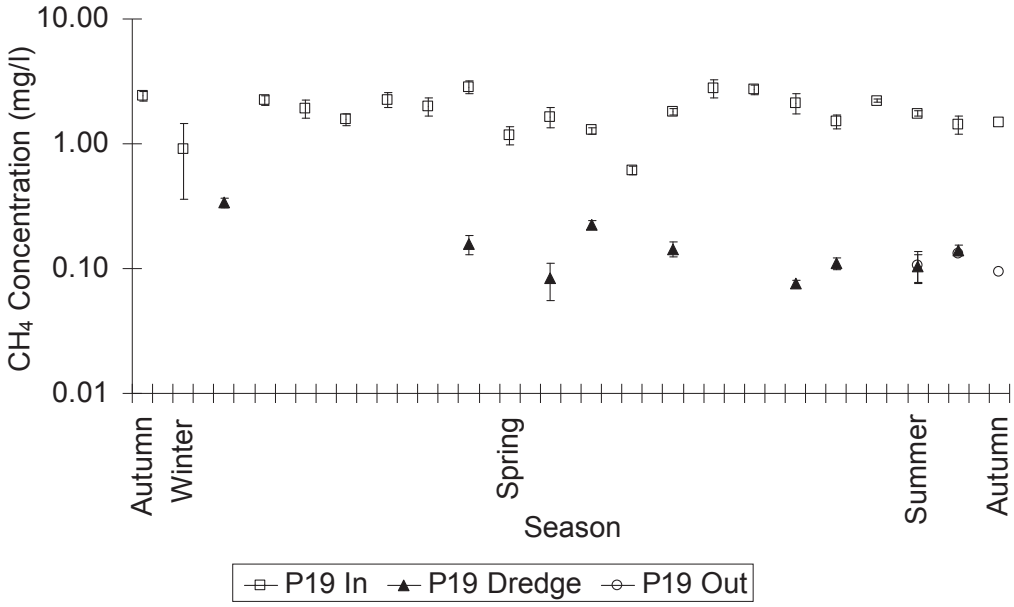


**Figure 8-2: Dissolved $CH_4$ concentration for the sampling sites where dissolved $CH_4$ was detected during different seasons**

## *8.3 Potential $CH_4$ Oxidation Assay Controls*

### 8.3.1 Water Column Controls

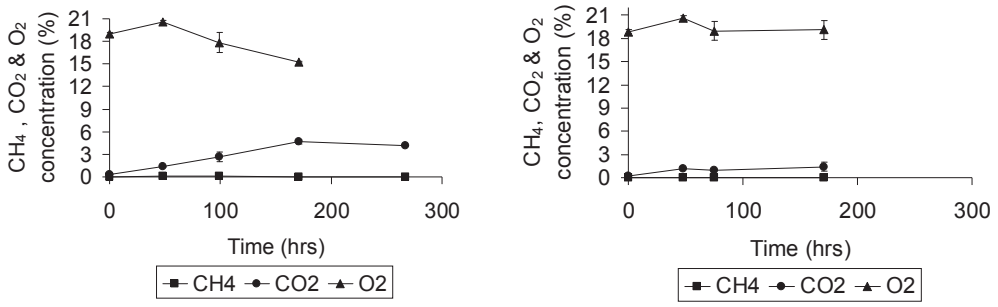


**Figure 8-3: Gas concentrations during aerobic $CH_4$ oxidation assay for P19 In (left) and P19 Middle (right) $N_2$+$O_2$ control**

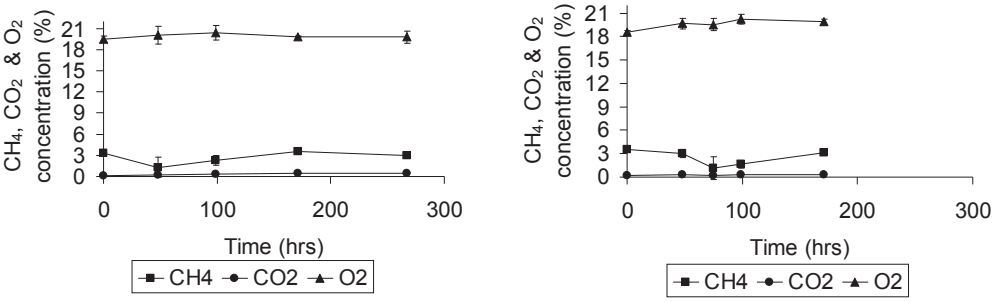


**Figure 8-4: Gas concentrations during aerobic $CH_4$ oxidation assay for P19 In (left) and P19 Middle (right) $NaN_3$ control**

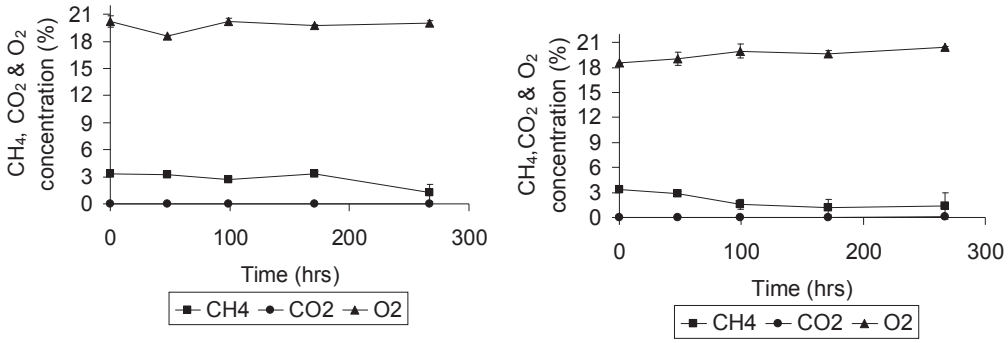


**Figure 8-5: Gas concentrations during aerobic $CH_4$ oxidation assay for P19 In and P19 Middle blanks. $NaN_3$+$H_2O$ blank (left) and $H_2O$ Blank (right)**

### 8.3.2 Sediment Controls

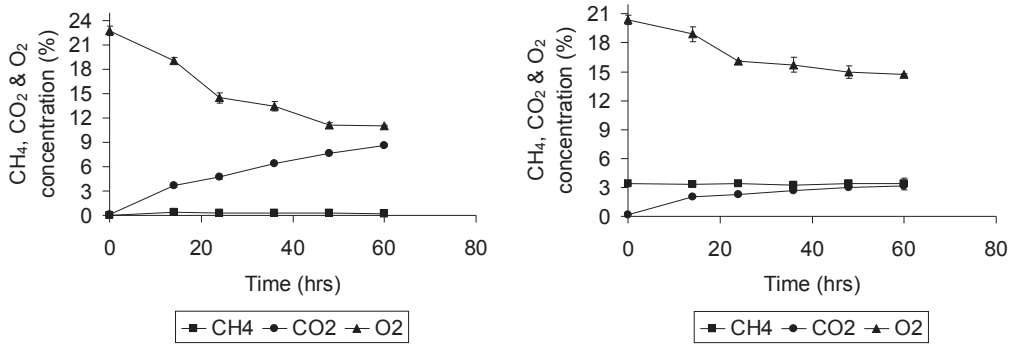


**Figure 8-6: Gas concentrations during aerobic $CH_4$ oxidation assay for P19 Middle sediment $N_2$ + $O_2$ control (left) and $NaN_3$ control (right)**

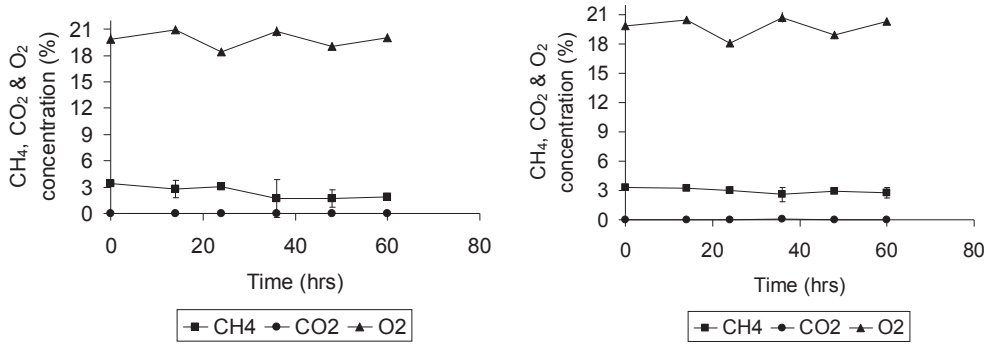


**Figure 8-7: Gas concentrations during aerobic $CH_4$ oxidation assay for P19 Middle sediment $NaN_3$ & water blank (left) and $H_2O$ blank (right)**

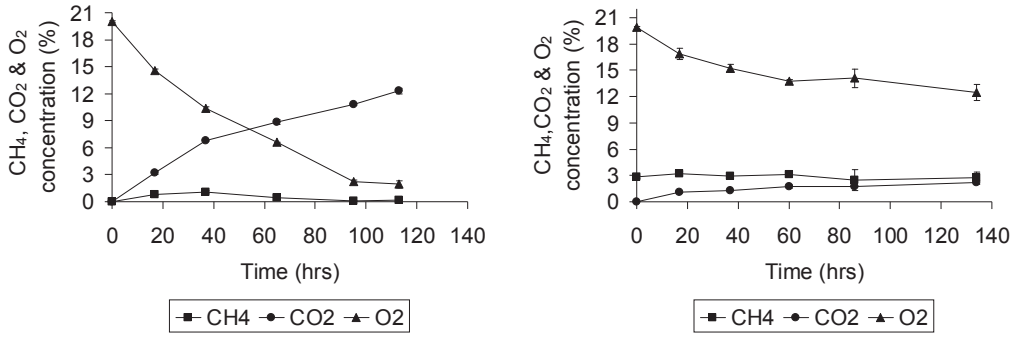


**Figure 8-8: Gas concentrations during aerobic $CH_4$ oxidation assay for P23 Out sediment $N_2$ + $O_2$ control (left) and $NaN_3$ control (right)**

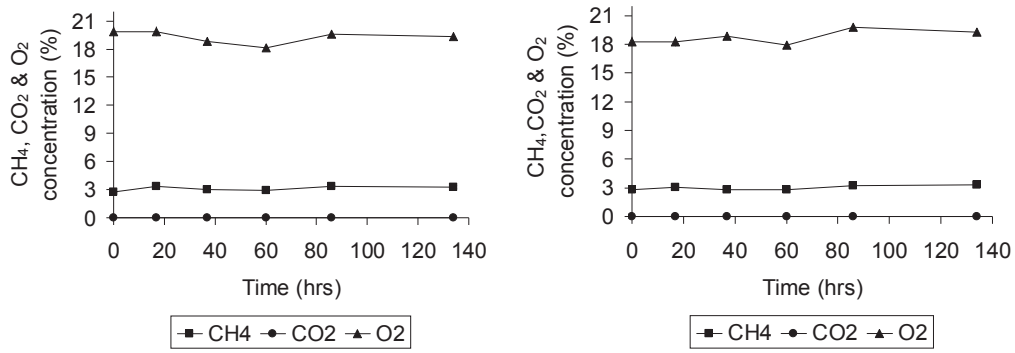


**Figure 8-9: Gas concentrations during aerobic $CH_4$ oxidation assay for P23 Out $NaN_3$ + $H_2O$ blank**

## *8.4 Methods*

### 8.4.1 BMP Measurements

The first thing to do is measure the pressure of the serum bottle. This is to establish gas production and whether samples' gas composition needs to be qualified using the GC. If the pressure measurement is above 30 then the samples need to be quantified using the GC.

A 250 µL sample is taken from the serum bottle and injected into the GC. The serum bottle is then bled and taken back to the incubator.

### 8.4.2 Methanol & VFA Method

**Principle:** Gas chromatography (GC) can be used to detect volatile fatty acids (VFA), in their acid form, after separation in a polar column. By starting at a low column temperature, alcohols can also be detected in the sample solution using a flame ionisation detector (FID).

**Apparatus:**

- Fused silica capillary column, Supelco 23327: 200°C: 30m x 530µm x 0.5µm
- GC equipped with an FID detector
- GC sample vials and caps

**Safety issues:**

The iso-butyric, butyric and valeric acids are putrid smelling compounds. Take all practicable care to reduce that amount of odour released into your immediate environment when weighing out these compounds.

**Method:**

1. Dilute samples as required. Add 1 ml of diluted sample to each vial or dilute directly into the vial. For example: to get a 20 fold dilution add 50uL sample to 950uL MilliQ water in a 2 mL screw top GC vial. Use auto pipettes for all small volumes.
2. Always analyse samples in duplicate.
3. If samples are dirty (i.e. turbid) then filter the sample through a 0.45μm nylon syringe filter before dilution.
4. Add 80 μL of formic acid (3% solution) to the GC vial.
5. Add 40 μL of internal standard (1000 ppm Butan-1-ol solution) to the GC vial
6. Cap and ensure well mixed.
7. Prepare standard solutions containing 1 mL of standard, 80 μL of formic acid (3% solution) and 40 μL of internal standard. Place the standards after 3 blanks at the start of your run. Analyse each standard level in duplicate.
8. Prepare blank solutions containing 1 mL of MilliQ, 80 μL of formic acid (3% solution) and 40 μL of internal standard. Place the blanks at the start of your run to settle the baseline prior to sample analysis. Also use blank between samples to limit acetic acid carry over.
9. Occasionally run a 40ppm VFA standard to check the calibration and retention times ($t_R$).
10. Run the samples on the GC.
11. Calculate concentrations of volatiles using standard curves, which have the response plotted against the concentration of the standards. The response is determined by dividing the integration area of each VFA standard by the integration area of the internal standard.

**Chromatography conditions:**

| | |
|---|---|
| Injector temperature | 170°C |
| Liner | Gooseneck with glass wool |
| Injection technique | Purge splitless |
| Purge on time | 1 minute |
| Oven temperature profile | Initial temperature 40°C (2 min)<br>Ramp 10°C/min to 180°C (2 min) |
| Run length | 21min (28min total - includes cooling to 40°C) |
| Detector | FID (190°C) |
| Head pressure | 3.3862psi |
| Carrier gas (Helium) | 5 mL/min |
| Hydrogen | 35 mL/min |
| Air | 350 mL/min |
| Nitrogen | 25 mL/min |

### 8.4.3 FISH Method

**Sample fixation**

Add 1 mL of sample to 3mL PFA and refrigerate for 1-3hrs.

Centrifuge and remove fixative (discard in PFA waste bottle). Resuspend in 3 mL of 1 x PBS and centrifuge. Discard supernatant.

Resuspend cells in 1 mL of 1 x PBS. Add 1mL of ethanol (96%-100%) and mix.

Cells are now fixed, store in the freezer.

**Dehydration**

Apply 10μL of pure culture or 5μL of activated sludge to a well and air dry.

Dehydrate in an ethanol series – 50%, 80% and 98% for 3min each.

Air dry, can store in the dark in the freezer.

**Hybridisation**

Prepare hybridisation buffer in a 2 mL tube:

| | | |
|---|---|---|
| 360μL | 5M NaCl | |
| 40μL | 1M TrisHCl | |
| xμL | Formamide | (700μL for 35%) |
| xμL | Sterile $H_20$ | (898μL for 35%) |
| 2μL | 10% SDS | (add last in the lid) |

Add 8μL to each well.

Add 0.5μL of each probe (from a stock solution of 50ng/μL) to the desired wells and mix gently.

Pour the remaining hyb buffer into a 50 mL falcon tube with tissue paper inside. Place slide carefully in tube and incubate at 46°C for 1-2hrs.

**Washing**

Prepare wash buffer in a 50 mL falcon and place in water bath at 48°C:

| | | |
|---|---|---|
| xμL | 5M NaCl | |
| xμL | 0.5M EDTA | |
| 1000μL | TrisHCl | |
| 43.8 mL | Sterile $H_2O$ | |
| 50μL | 10% SDS | (add last) |

Pipette a small amount of wash buffer over the slide (collect waste – formamide), then place slide gently in wash buffer at 48°C for 10-15mins. Discard hyb tube.

Remove slide, wash gently but quickly with sterile $H_2O$ and dry quickly by shaking it. Wash buffer tube can be reused as a hyb tube next time.

When slide is completely dry, view or store in a fresh tube in the freezer.