



# The fate of biologically produced methane in an anoxic fjord in British Columbia

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## NONTECHNICAL SUMMARY

Our climate is currently experiencing changes unlike those seen before in history. An increase of methane gas in the atmosphere has been shown to exacerbate these changes, much more so than an increase in carbon dioxide. Methanogens are microscopic organisms that produce methane, and live in oxygen poor environments. Methanotrophs are other microscopic organisms that consume methane, and can live in environments with or without oxygen. Both of these organisms can live and interact in an anoxic fjord; an environment containing areas with and without oxygen. Methane created by methanogens is consumed by methanotrophs, but it is not known if all the methane produced is consumed or if some reaches the surface and diffuses into the atmosphere. A perfect area to study this is Effingham Inlet, in British Columbia, Canada. Effingham Inlet contains two basins, one with oxygen throughout the basin and one that has both an oxic and anoxic zone. Six water samples were taken in both basins. Methane concentration and cell abundance were measured in each sample. Samples were also taken to determine if genes necessary for methanogenesis and methanotrophy were present. The presence of these genes indicates the presence of methanogens and methanotrophs, respectively. There was a strong increase in cell abundance in the anoxic zone of the upper basin, along with a relatively high methane concentration. The presence of methane in the anoxic zone along with an increase in cell abundance suggests the presence of methanogens. The absence of a high concentration of methane above the anoxic layer suggests that methanotrophs are present, and have consumed most if not all of the methane produced through methanogenesis.

## ABSTRACT

This study examined an anoxic fjord in British Columbia, Canada, to try to get a better understanding of what happens to biologically produced methane. Six samples were taken in two basins of the fjord, one with oxygen throughout the water column and one with both an oxic and anoxic layer. Methane concentrations were quantified using a gas chromatograph-flame ionization device, and microorganisms were quantified using fluorescence microscopy. DNA extraction and PCR amplification were performed on the key genes of both methanogenesis and methanotrophy, to determine if any methanogens and/or methanotrophs were present in each sample. Microbial abundance increased in the anoxic layer of the upper basin to approximately  $1.5 \times 10^6$  cells  $\text{mL}^{-1}$ , along with relatively high methane concentrations (0 ppm in the surface to ~50 ppm at depth). The presence of methane in the anoxic layer along with an increase in cells suggests the presence of methanogens. The absence of a high concentration of methane above the anoxic layer suggests that methanotrophs were present, and had consumed most if not all of the methane produced through methanogenesis.

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## INTRODUCTION

Our climate is currently experiencing changes never seen before in history. It is important to understand what contributes to these changes. Methane has been shown to be an

extremely powerful insulating gas, much more so than carbon dioxide (Schmidt and Schindell 2003). An increase in the amount of methane in the atmosphere would further increase climate change, so it is necessary to study what could produce or get rid of methane and whether the methane

produced could reach the atmosphere. One such way methane can be produced is biologically, through methanogenesis.

Methanogens are a diverse group of archaea that produce methane from compounds they use as a terminal electron acceptor, such as CO<sub>2</sub> (Pace 1997). Methanogens thrive in low oxygen environments, are very diverse, and evolved separately. Common to all methanogens regardless of species is the methyl-coenzyme M reductase (MCR), a gene that has been used before to quantify communities of methanogens (Thauer et al. 1998). Conversely, methane is biologically consumed through methanotrophy. Methanotrophs can be either bacteria or archaea, can live in environments with or without oxygen, and are very diverse as well (Hanson and Hanson 1996, Milucka et al. 2012). The particulate methane monooxygenase gene is common in almost all methanotrophs (Horz et al. 2001). The only methanotroph without this gene is *Methylocella*, an acidiphilic organism not found in marine environments (Dunfield et al. 2003). In environments containing areas with little or no oxygen both methanogenesis and methanotrophy can occur, with methanogens creating the methane consumed by methanotrophs. Though these two groups of organisms are known to interact with

each other, little is known about the details of their interactions in the marine environment (Adachi 2001). Marine anoxic zones are an ideal location to study these interactions because they contain environments with and without oxygen in the water column.

Marine anoxic zones are formed when oxygen is continuously consumed through respiration in the water column. Primary production from photosynthesis produces oxygen to counteract respiration, but it can only occur down to the depth where light penetrates, whereas respiration of oxygen can occur anywhere oxygen is present. Anoxic conditions are formed when there is an area in the water column where respiration consumes all the oxygen and there is no photosynthesis or mixing of oxygenated water for a long period of time (Freeland et al 1997). In many of the world's oceans, including the subarctic eastern Pacific, anoxic regions are expanding (Whitney et al. 2007). There are a variety of places in which these zones can be created, but this study will focus on an anoxic fjord. A fjord is an area open to the ocean, but with a large sill extending from the seafloor fairly close to the surface (Fig. 1). In some fjords, flow of oxygenated water to the deep areas is restricted by the sill, causing anoxia in the deep water.

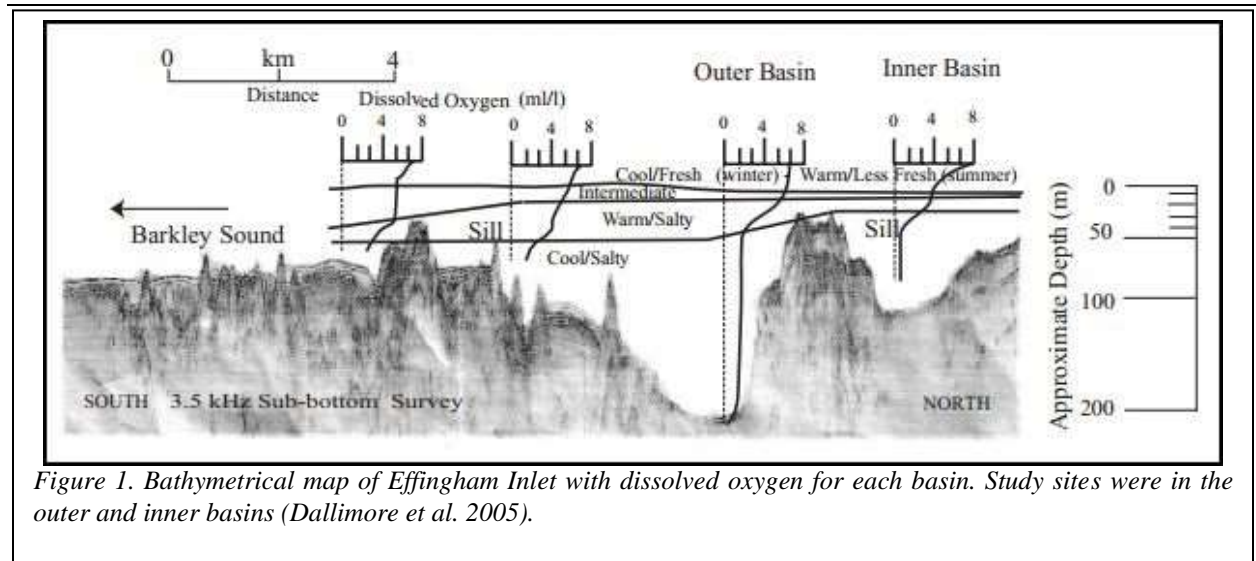


Figure 1. Bathymetrical map of Effingham Inlet with dissolved oxygen for each basin. Study sites were in the outer and inner basins (Dallimore et al. 2005).

Anoxia is a constant condition in fjords with nonexistent flow to the deep water. This allows for abundant and diverse communities of anaerobic bacteria and archaea to form over time in the anoxic zone. These bacteria and archaea are

exclusively chemolithoautotrophic, and include methanogens and methanotrophs (Diaz and Rosenberg 2008). As the community of methanogens develops they release methane into the water column, all of which could be consumed

by methanotrophs. However, if methanotrophs are limited by nutrients other than methane, consumed by predation, or a combination of the two, methane produced in anoxic zones could make its way to the surface and diffuse into the atmosphere. In Effingham Inlet, a fjord system on the west side of Vancouver Island in British Columbia, Canada, anoxia is an almost constant condition. Only during infrequent occurrences of flushing events when oxygen rich water is brought to the bottom of the fjord does the deep water column experience oxic conditions (Hay et al. 2009). During a flushing event, methane could be released en masse to the surface. There is a lack of knowledge about the methanogenic and methanotrophic communities in anoxic fjords. Due to the fact that the anoxic regions in the eastern subarctic Pacific are growing and the possibility that these regions could add methane to the atmosphere the interaction between these communities needs to be better understood, specifically whether methanotrophs are able to consume all the methane produced by methanogens (Zaikova et al. 2009, Whitney et al. 2007).

In 2010, Flowers studied the amount of methane in the water column in three basins in Effingham Inlet (Fig. 1, Fig. 2). The data show a strong increase in methane with depth in the upper basin. This increase in methane suggests the presence of methanogens, while the lack of methane in shallower areas suggests the presence of methatrophs. Saanich Inlet is a seasonally anoxic fjord on the east side of Vancouver Island and was studied in depth by Zaikova et al. in 2009. The findings indicated an increase in microbial abundance with an increase in methane in the anoxic zone. These findings add to the possibility of the presence of methanogens in Effingham Inlet, due to the similarity of the two anoxic fjords and the fact that Effingham Inlet is a constantly anoxic fjord and has more potential to build an abundant and diverse chemolitho-autotrophic community.

The overall goal of this study is to see how methane is created and consumed throughout the water column in Effingham Inlet, and how important methanogens and methanotrophs are to this process. Based on previous studies I hypothesize that methanogenesis is the dominant process in the anoxic zone, while methanotrophy dominates in the oxic zone due to the high methane concentrations in the anoxic layers found by both Zaikova et al. and Flowers (Zaikova et al. 2009, Flowers 2010). The presence of key genes required for methanogenesis in the anoxic zone would indicate the production of methane deep in the fjord. The presence of key genes required for methanotrophy would indicate the consumption of methane. If there is no methane at the surface and genes required for methanotrophy are found, the methanotrophs have consumed all the methane created by the methanogens.

## METHODS

### Water Sample Collection

Samples were collected between 26 Jan and 3 Feb 2013, using a rosette from the R/V *Thomas G. Thompson* in Effingham Inlet. To collect samples from the water column, the rosette with a CTD, a dissolved oxygen meter, and 12 Niskin bottles was deployed down to the deepest

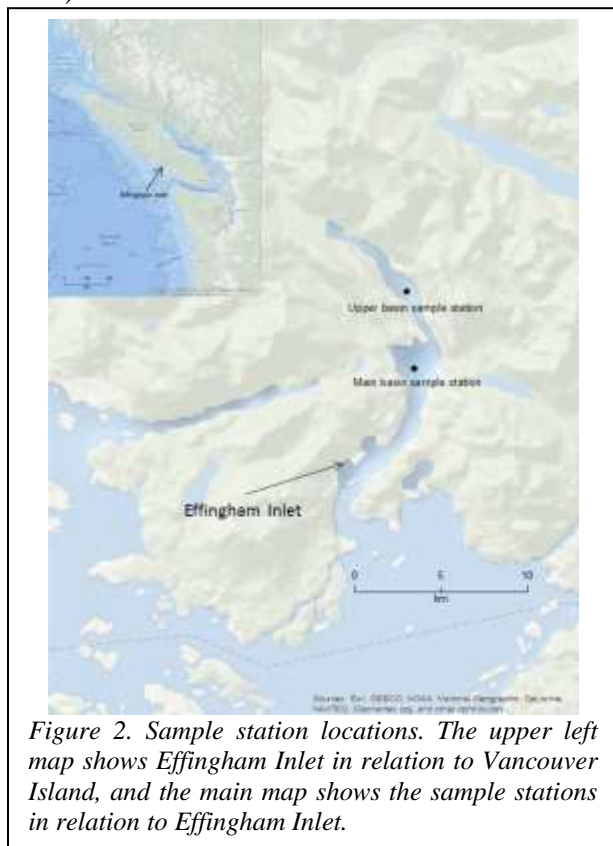


Figure 2. Sample station locations. The upper left map shows Effingham Inlet in relation to Vancouver Island, and the main map shows the sample stations in relation to Effingham Inlet.

part of the main and upper basins in Effingham Inlet (Fig. 1, Fig. 2). Latitude and longitude for the main and upper basin sampling stations are 49° 2.58' N 125° 9.17' W and 49° 4.27' N 125° 9.43' W, respectively. Six water samples were taken in each basin for a total of 12 samples. Each basin was sampled at 10, 20, and 30 meters, with three more samples taken at depth. The main basin was sampled at 100, 160, and 190 meters, and the inner basin was sampled at 60, 90, and 115 meters. This was done to try to get a good overall view of the environment in both the surface and deep water in the two basins.

#### Methane analysis

Samples were taken from the Niskin bottles on the CTD on the Thompson with a syringe to prevent equilibration with the atmosphere, and immediately fixed with mercuric chloride to halt any biological consumption or production of methane. Samples were preserved in the freezer and brought back to the University of Washington. Methane was analyzed with a gas chromatograph flame ionization detector (GC-FID) in the Keil lab. The procedure for using the GC-FID followed the directions from the Chemical Analytical Laboratory, 1995. First, two standardized samples were injected into the GC-FID, to determine if it was working properly. After the standardization, samples were injected and results recorded.

#### DAPI counts

Samples for DAPI analysis were taken from Niskin bottles on the rosette, and immediately fixed with formaldehyde at a final concentration of 1% to halt any biological processes taking place. Samples were brought back to the lab on the *Thompson*, 10 mL were filtered through a 0.2 micron filter, and stored at -4°C. After filtration, approximately 300 µL of 4',6-diamidino-2-phenylindole (DAPI) solution, diluted to 300 nM in phosphate buffered saline, was added to each filter and placed on a coverslip. Cells were counted using fluorescence microscopy.

#### DNA extraction and PCR analysis

DNA extraction protocol followed the procedure done by Morris et al. 2012. Briefly, cells from 1 L of water were filtered onto Supor-200 0.2 mm filters. Filters were placed in 2 mL cryovials, treated with 1 mL of lysis buffer, flash frozen in liquid nitrogen, then stored at -20°C. Samples were later thawed on ice, sodium dodecyl sulfate was added to 1% and proteinase K was added at a concentration of 100 mg mL<sup>-1</sup>. The cell lysate was incubated at 37°C for 30 min, then at 55°C for 10 min. Community genomic DNA was extracted from 200 mL of cell lysate using a DNeasy Blood and Tissue kit (QIAGEN, Germantown, MD). PCR amplification followed DNA extraction. This procedure followed that done by Sharma et al. 2011. The presence or absence of methanogen DNA was determined using the forward primer 5'-GGTGGTGTMGGA TTCACACARTAAYGCWACAGC -3' and the reverse primer 5'-TTCATTGCRTAGTTWGGRT AGTT-3' (Steinberg and Regan 2009). The presence or absence of methanotroph DNA was determined using the forward primer 5'-TTCTGGGGNTGGACNTAYTTYCC-3' and the reverse primer 5'-CCNGARTAYATHMGNATG GTNGA-3' (Kolb et al. 2003).

#### RESULTS

In the main basin of Effingham Inlet, there was almost no methane sampled. The only sample with methane was at 10 m depth, with a very small concentration of 2.16 ppm. In the upper basin of Effingham, there was much more methane. Methane was sampled at a concentration of 53.1 ppm at 115 m, 43.3 ppm at 90 m, and 4.48 ppm at 30 m. Cell abundance in the main basin was high in the surface samples with a concentration of 7x10<sup>5</sup> cells mL<sup>-1</sup>, and decreased with depth to 5x10<sup>4</sup> cells mL<sup>-1</sup> at 190 m. In the upper basin, cell abundance decreased from 10<sup>6</sup> cells mL<sup>-1</sup> at 10 m to 8x10<sup>5</sup> cells mL<sup>-1</sup> at 30 m, increased to 1.2x10<sup>6</sup> cells mL<sup>-1</sup> at 60 and 90 m, and increased with an increase in methane concentration to 1.5x10<sup>6</sup> cells mL<sup>-1</sup> at 115 m (Fig. 3).

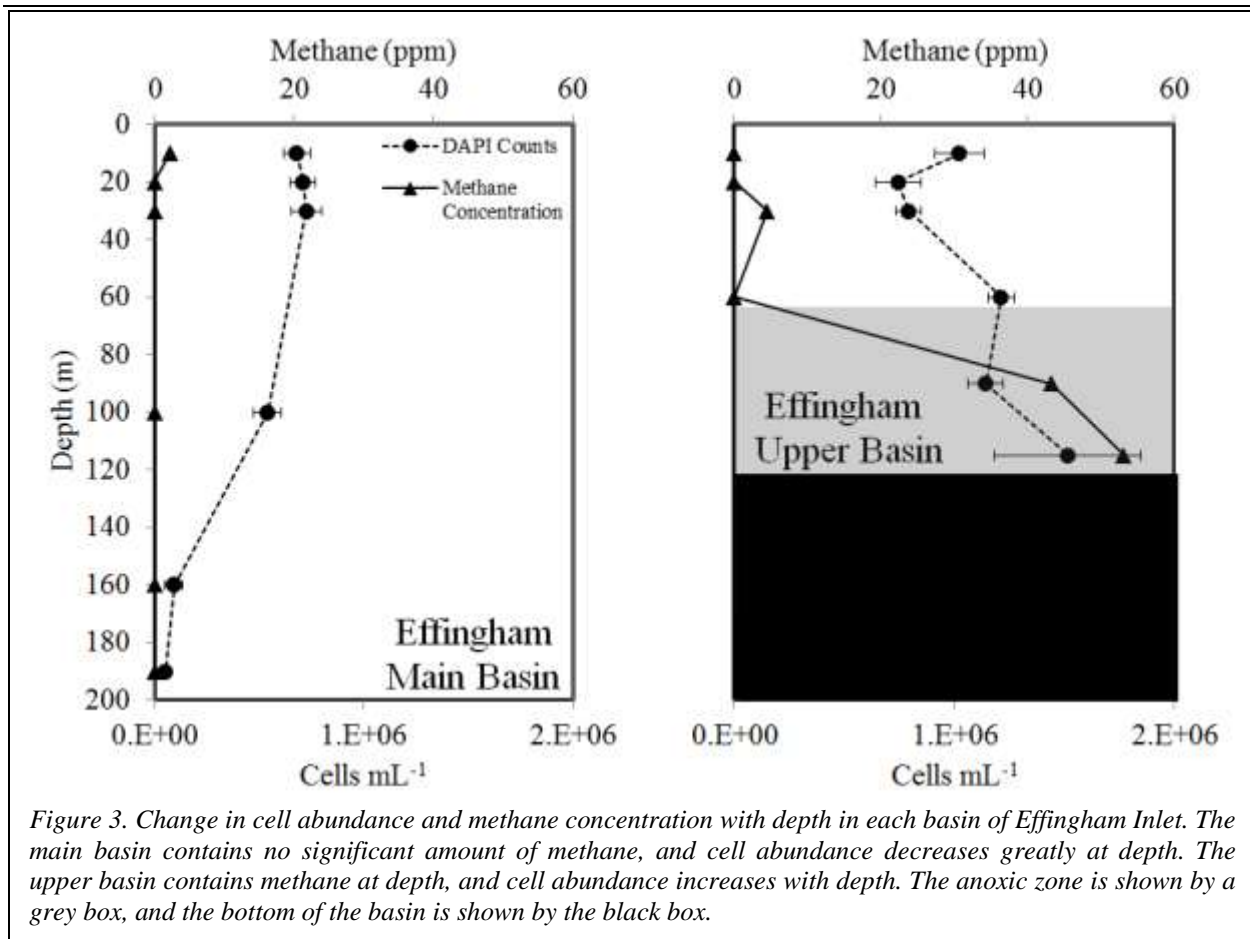


Figure 3. Change in cell abundance and methane concentration with depth in each basin of Effingham Inlet. The main basin contains no significant amount of methane, and cell abundance decreases greatly at depth. The upper basin contains methane at depth, and cell abundance increases with depth. The anoxic zone is shown by a grey box, and the bottom of the basin is shown by the black box.

## DISCUSSION

The results from this study are similar to others done in the same area, with some key differences. Flowers et al. sampled the methane concentration in both the main and upper basins of Effingham Inlet. Her results showed a similar profile of methane in the upper basin, with methane increasing at depth. However, there was a significant increase in methane near the bottom of the main basin in 2010, which was not seen in 2013. The lack of methane in the main basin suggests a relatively recent deep mixing event. Mixing events are known to be common in the main basin but do not occur often in the upper basin, which is supported by the data from the upper basin. A strong increase in methane at depth was seen in both 2010 and 2013, suggesting a lower frequency of mixing events (Fig. 3). This notion is supported by the higher concentrations of methane in the upper basin of Effingham

compared to the main basin and the seasonally anoxic zone of Saanich Inlet, suggesting the upper basin has had more time to build up methane (Zaikova et al. 2009).

Results of the DAPI counts reflected what was expected. Cell abundance is high in the surface in both basins. In the main basin, there was no anoxic zone and cell abundance decreased significantly with depth. In the upper basin, cell abundance was high in the anoxic zone where methane was present, suggesting a diverse and abundant chemolithoautotrophic community. These results are similar to those obtained by Zaikova et al. from Saanich Inlet in 2009, which also showed an increase in methane and high cell abundance at depth in the anoxic zone. While the results are similar, there are some important differences. Both cell abundance and methane concentration are much higher in the anoxic zone in the upper basin of Effingham Inlet than in Saanich Inlet. Since the upper basin of Effingham

Inlet is almost permanently anoxic and has had a much longer amount of time to build up a large chemolithoautotrophic community, methane concentrations have been able to increase over long periods of time (Fig. 3). In contrast, Saanich Inlet is only seasonally anoxic meaning the chemolithoautotrophic community has to rebuild after each flushing event, leading to a smaller community.

Because the PCR analysis did not yield any results, there is no way of definitively knowing whether methanogens or methanotrophs were present anywhere sampled. However, the results of this study compared with the results obtained by Zaikova et al. suggest the presence of both methanogens and methanotrophs in the water column of the upper basin of Effingham. In Saanich Inlet, bacterial diversity was well examined and showed the presence of *Methylococcales*, a known methanotroph. Unfortunately, archaeal diversity was not well examined, and only showed the presence or absence of Marine Groups 1 and 2. The presence of methanogenesis in Saanich Inlet can be inferred from the high concentration of methane in the anoxic zone where methanogens thrive. The similarity between the two basins suggests the presence of both methanogens and methanotrophs in the upper basin of Effingham Inlet. The high concentration of methane in the deep anoxic water of the upper basin also suggests the presence of methanogens. The lack of methane outside of the anoxic zone and the presence of methanotrophs in Saanich Inlet suggest that methanotrophs are present in the upper basin.

In both Saanich and Effingham Inlet, methane is not seen in the surface waters, suggesting that all biologically produced methane from methanogens is consumed through methanotrophy before reaching the surface and diffusing into the atmosphere. While it does not reach the surface during anoxic periods, methane could be released into the atmosphere during a flushing event when the entire basin is mixed. Mixing is seasonal in Saanich Inlet, frequent in the main basin of Effingham Inlet and infrequent in the upper basin. Since it has been seen at depth in all of these areas, it is not unlikely that methane is

released to the atmosphere during a mixing event. Because a release of methane during a mixing event has not been specifically seen in any anoxic fjord, seasonal or otherwise, it cannot be assumed that this process undeniably takes place.

Since methane in the atmosphere exacerbates climate change and anoxic regions in the subarctic Pacific are growing, it is important to fully understand the fate of methane produced in anoxic fjords during flushing events to determine if anoxic fjords could contribute to climate change. An ideal way to study this would be to measure methane and oxygen concentrations and quantify the community of methanogens and methanotrophs in a known seasonally anoxic fjord before, during, and after a flushing event. This data would show whether or not methane produced is consumed during a flushing event, or released into the atmosphere.

## CONCLUSIONS

An increase in methane in the anoxic layer of Effingham Inlet's upper basin was accompanied by high cell abundance in the same anoxic layer. The similarity of these data compared with data obtained from Saanich Inlet indicates the presence of methanogens and methanotrophs in the upper basin. The lack of methane in the oxic layer suggests methane is consumed through methanotrophy before it has a chance to reach the surface and diffuse into the atmosphere. More studies are necessary to determine if high concentrations of methane in anoxic layers of some fjords could reach the surface during mixing events and be a source of methane to the atmosphere.

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