"Vertical distribution of aerobic CH_4 consumption in cedar swamp soil: NH_4 implications."

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ABSTRACT

The vertical distribution of aerobic methane oxidation in cedar swamp soil was measured. In order to explore NH4 stimulation/inhibition patterns as a differential response of either methanotrophs type I or II, incubations were performed in soil layers from to the vertical profile. 16s rRNA clone library and quantitative (Real time-PCR) was used to relate components and size of populations with oxidation rates and response to NH_4 additions. Oxidation rates at high and low methane concentration incubations showed similar trends suggesting the dominance of methanotrophs Type II. Clone library analysis showed the presence of *Methylocystis* and *Methylosinus* related sequences (Type II), but also *Methylomonas* and *Methylomicrobium* related sequences (Type I). Quantitative PCR data and NH_4 inhibition patterns support the dominance of methanotrophs Type II along the vertical profile. Methanotrophs Type I were not able to be quantified by QPCR, however its presence in clone library and NH_4 methane oxidation stimulation in a layer with lower methanotrophs Type II population, may indicate its significant presence and Nitrogen dependent activity in a Nitrogen starved soil. Vertical changes of NH_4 inhibition patterns reflect vertical changes in the methanotrophic community composition within the soil vertical profile

INTRODUCTION

Methane (CH_4) is involved in a number of chemical and physical processes in the earth's atmosphere, including global warming. Atmospheric methane originates mainly from biogenic sources, such as rice paddies and natural wetlands. Wetlands represent one of the main sources of non anthropogenic methane emissions (~15-20% globally), being a place where methane cycling is actively performed (Le Mer, 2001). Cedar swamps at Woods Hole, belong to a wetlands system 100-mile wide strip located on the US Atlantic and Gulf coasts.

Consumption of atmospheric methane by soil is one of the important sinks in the global cycle of methane. This process is biologically mediated by microorganisms present in soil, whose regulating activity of atmospheric methane concentration is poorly understood (Le Mer 2001).

Methane-oxidizing bacteria (methanotrophs) are found in forest, agricultural, wetland soils among other environments. However the identity, physiology and activity is frequently unknown or poorly understood. Typical methanotrophs are bacteria of the phylum Proteobacteria, obligate methane oxidizers and aerobes. They occur in environments with stable gas exchange particularly in the interface between aerobic and anaerobic environments from which methane diffuses to the atmosphere.

There are two major groups of methanotrophs, Type I and Type II, whose phylogeny, assimilation pathways and structures differ considerably. Type I belongs to the Gamma Proteobacteria, can use NO_3 or NH_4 as nitrogen sources and a few strains have been detected to posses low nitrogenase activity; and some genera have been associated with low CH_4 affinity (meaning a requirement of

high levels of CH_4 for their metabolism) (Gucker J. et al 1991) . *Methylomonas, Methylomicrobium, Methylococcus, Methylobacter, Methylocaldum and Methylosphaera* are the genera belonging to the type I group. Type II, in the other hand, belongs to the Alpha Proteobacteria, can fix N_2 and also some strains are also able to use NO_3 or NH_4 as nitrogen source and have being associated to a high affinity capacity for CH_4 oxidation (e.i. can grow at low or high CH_4 concentrations). *Methylosinus, Methylocystis and Methylocapsa* are the genera belonging to Type II group.

The above highlighted differences in the physiology of each type of methanotrophs may leads towards a differential population structure, activity and response to environmental factors in soil ecosystems. In this sense, the influence of NH_4 concentration on CH_4 oxidation processes remains controversial and poorly understood. Some reports showed that NH_4 additions stimulate oxidation rates in the presence of high levels of CH_4 (Bender and Conrad, 1995; Boeckx et al 1996; Bodelier et al 200), while others showed inhibitions patterns when NH_4 fertilizers were applied (Hutsch, 1993). Visscher and Cleemput (2003) hypothesized that a nitrogen dependent or independent response by either type of methanotrophs can explain this inhibition/stimulation patterns.

The present report presents my attempt to address this possible differential response to NH4 by natural methanotrophic populations along the soil profile from Woods Hole Cedar Swamp. I address this by monitoring the vertical distribution of aerobic methane oxidation in WH Cedar swamp soil, experimentally test the influence of NH_4 additions on soil incubations, molecular phylogenetic detection of methanotrophs (16S rRNA) and quantification of specific populations by real time PCR.

MATERIAL AND METHODS.

Soil sampling.

Soil cores were obtained from the Woods Hole Cedar Swamp, Massachusetts. Soils surrounding the water bodies in the swamp were manually extracted with polystyrene corers of 20 cm diameter and 50 cm deep. In all the cases cores deeper than 12 cm were extracted, sealed in both extremes with rubber stopper, transported to the laboratory at environmental temperature and immediately sectioned in 2 cm layers for their correspondent analysis or incubation.

Soil analysis

Moisture content, nitrogen content, and methane content were immediately measure by duplicate in each correspondent 2 cm soil layer.
Moisture content was measured by dehydrating at least 15 gr of soil at 40 °C for 1 day. The percentage of moisture content was calculated on a dry soil basis.
Inorganic nitrogen content (NH_4/NO_3) was measured by "Soil kit" and " NH_4/NO_3 kit", where 25 g of soil were resuspended in 100 ml water, stirred for about 2 hours and then centrifuged for 10 min at 2000 rpm. The supernatant was used for inorganic nitrogen colorimetric detection.
Methane content was measured by triplicate in each layer by taking sub samples of 5 g of soil that were transferred into 55 ml serum bottles containing 20 ml NaOH (10 M) and sealed immediately with rubber stoppers. After vigorous mixing, methane was measured in the headspace by gas chromatography.

APPENDIX

S1:0-2 cm

S2:2-4 cm

S3:4-6 cm

S4:6-8 cm

S5: 8-10 cm

S6: 10-12 cm

Figure 1. Core and correspondent soil layers. Cedar Swamp Woods Hole Massachusetts.

Methane oxidation rates

All methane oxidation experiments were done in duplicate for each correspondent 2 cm layer from extracted soil. Of the soil samples taken form each core layer, about 30 g was transferred to 125 ml serum bottle, incubated at an initial concentration of ~100 ppm vol^{-1}, and sampled periodically for about 2 to3 hours until the concentration fell below 50 ppm vol^{-1} (low concentration kinetic experiment). The methane oxidation rate constant was determined by regressing the natural logarithm of the concentration against time. At least 5 data points were used for regression $r^2>0.90$ in almost all the cases. In the high concentration kinetic experiment the same bottle with soil was used but the methane initial concentration was raised to 2% and sampled for about 6 hours.

NH_4 additions and methane oxidation rates

The soils from the 125 ml serum bottles were split into two 10 g sub samples. One sub sample received 10 ml of demineralized water and the other part received 10 ml of a 180 mM aqueous $(NH_4)S0_4$ solution, sealed and incubated in 55 ml serum-bottles .As a result 4 treated soil samples were obtained for each correspondent core layer. These samples were incubated with ~200 ppm vol-1 for the low concentration kinetic experiments (see above), after the concentration decreased until 50 ppm vol-1 the bottles were opened aerated for 30 min and re -incubated with 2% initial methane for the high concentration experiments. Initial reaction rates were also calculated for these experiments, based on at least five data points. The variation coefficient (r2) exceeded 0.90 except in the cases in which the reaction rate was very low. Oxidation rates were consecutively measured in the same soil samples in a span of time no longer than 2 days.

DNA extraction, clone library, and quantitative PCR

Total DNA was extracted by duplicated from layer 2 (2-4 cm deep) and layer 5 (8-10 cm deep). Ultraclean soil DNA kit was used in the DNA extraction (MOBIO ®) from 1 gr of soil (see protocol in appendix)
Two clone libraries were generated using the TOPO A kit (Invitrogen ®) with amplification products generated with methanotrophs specific primers targeting the 16s rRNA gene and Super mix PCR solution ®. For methanotrophs Type I the Meth T1bR, and Meth T1 bF were used generating a 800bp product (Hanz-Peter et al, 2001) with the following program 95 C 30sec, 52 C 1 min and 72 C 1 min; Methanotrophs Type used the Am 445 F (Gulledge et al, 2001) and Hinby R (designed in this study with ARB) with the following conditions 95C 30 sec, 50 C 1 min and 72C 1 min . Clones were PCR amplified and sequenced. Phylogenetic analysis of sequences was performed using ARB.

The same primers and conditions were used for quantitative PCR, using the Biorad e-termocycler and SYBR green master mix.

RESULTS AND DISCUSSION

The cedar swamp soil profile presented a characteristic composition of saturated soils from wetlands. In general this is a slightly acidic soil (pH ~ 6.5), nutrient poor, and saturated with surrounding tannin-rich water. The site is dominated by Atlantic white cedar with the presence of sphagnum covering the cedar roots. The accumulation of organic matter in the superficial layers is a remarkable characteristic of this system. Specifically for the extracted core, its initial 6 cm were quite rich in organic matter (as noticed for the soil coloration and presence of vegetables' debris) and silt-dominated soils, turning into a sandy and very low in organic matter soil from 8 cm to deeper. (see figure 1)

Along the soil profile the moisture content, as well as the nitrogen content, decreased in each 2cm layers from the top to the bottom. The 0-2 cm layer presented the highest moisture and inorganic nitrogen content, indicating an area with high heterotrophic potential. Figure 2 shows a dramatic decrease of moisture and nitrogen content along the soil profile, suggesting the occurrence of very different environmental conditions and metabolic activities. Methane content presented in Figure 2, showed that the 2-4 cm layer had the highest methane content (~58 ppm vol/ g dws) followed by the 4-6 cm (~37 ppm vol/ g dws). Methane content decreased from the 2-4 cm layer to the bottom, which may be explained for the change in soil structure and organic matter content (whose degradation byproducts i.e. formate, acetate- are used for methanogens). In this case methanogenesis is improvable to happen in remarkable levels below 12 cm deep.

Methane oxidation has been shown to be a ubiquitous process in wetland soils (Bender and Conrad, 1994; Le Mer 2001). This is consistent with the measurements in the Woods Hole cedar swamp; the tested soils were able to oxidize methane at low concentrations (100 ppmv) and high concentrations (2%), and the magnitude of oxidation rates is close to those observed in undisturbed forest luvisoil (Bender and Conrad, 1994). Figure 3 shows the first- order reaction rate constants obtained from the low and high concentration kinetic experiments. The methane oxidation activity reached its highest value in the 2-4 cm layer in both cases low and high concentration, decreasing to no activity in the deepest layer of the profile. Low concentration incubations presented rates ranging from 0.4 to 0.15 uM/h/g DWsoil along the 0-2 cm to 8-10 cm layers, no methanotrophic but rather methanogenic activity was detected below these layers. Rates in high concentration incubations ranged from 0.018 to 0.035 uM/h/g DWsoil along 0-2 cm to 6-8 cm layers.

The decline of methanotrophic activity can be mainly related to the methane content in each soil level, however the nitrogen decline should also be considered in explaining the vertical methanotrophic profile. The natural methane concentration in the tested soils provided conditions for low affinity and high affinity methane oxidation (<12ppm and >40 ppm correspondently). Under > 40 ppm methane concentration conditions, both high affinity and low affinity methanotrophs can develop (Le Mer, 2001). Only slight differences were observed in our incubations experiments (Figure 3) suggesting that the profiles are enriched for a dominant population which present a similar response to either low or high methane concentrations; it can also be the case that our initial methane concentrations were not enough differentiated in order to detect different responses to methane levels.

Microorganisms related to Type II methanotrophs have been shown to be dominant population under low and/or high methane conditions in non-agricultural soils (Bull et al 2000). Concordantly, Type II methanotrophs have been suggested as the probably dominant in rice fields because they are the only type producing soluble methane-mono-oxigenase, which avoid the accumulation of NO_2

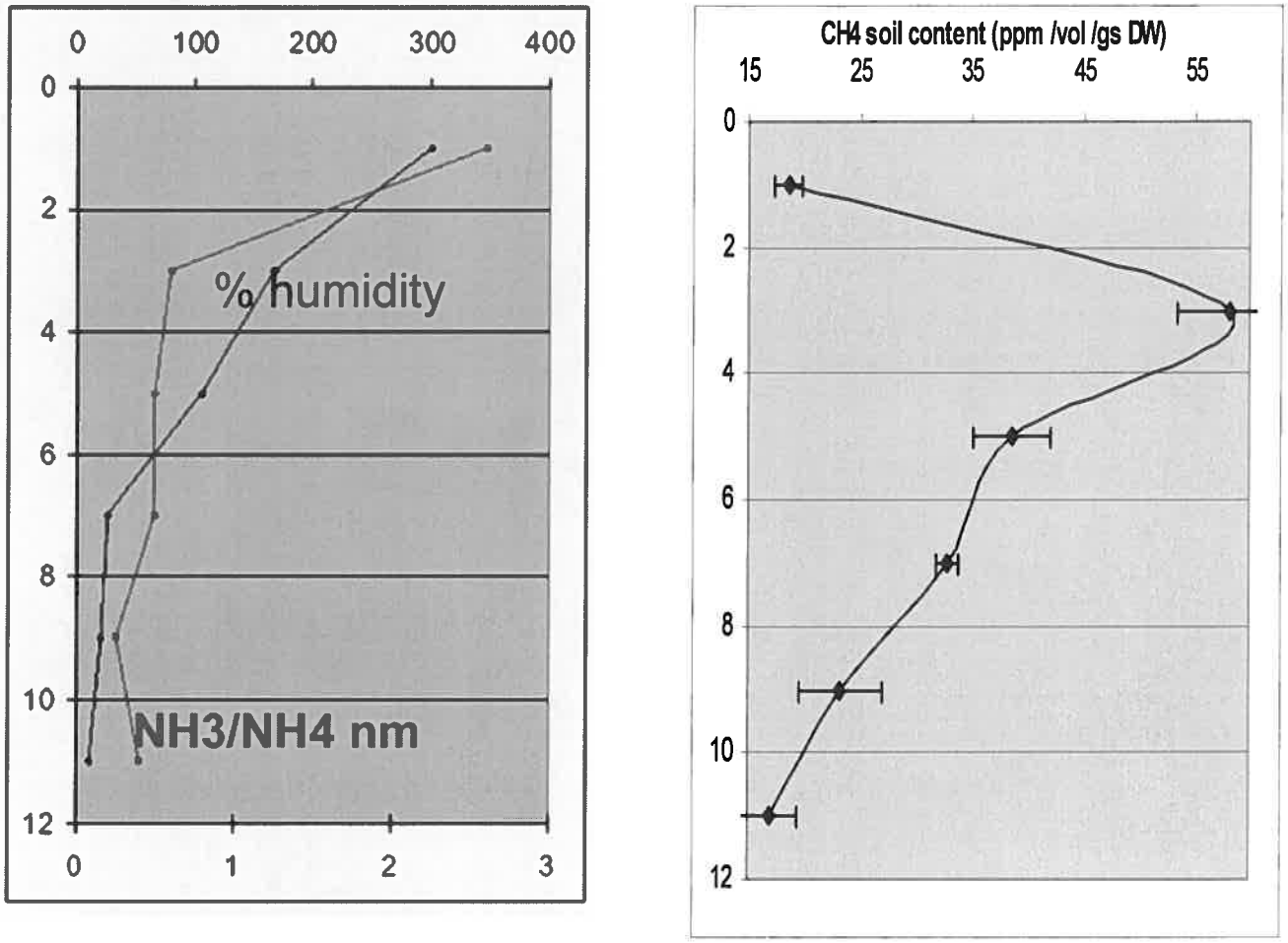
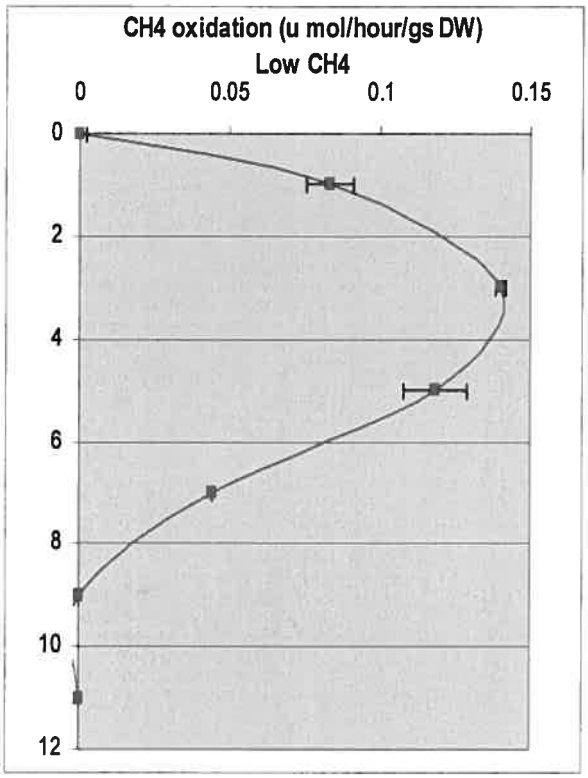
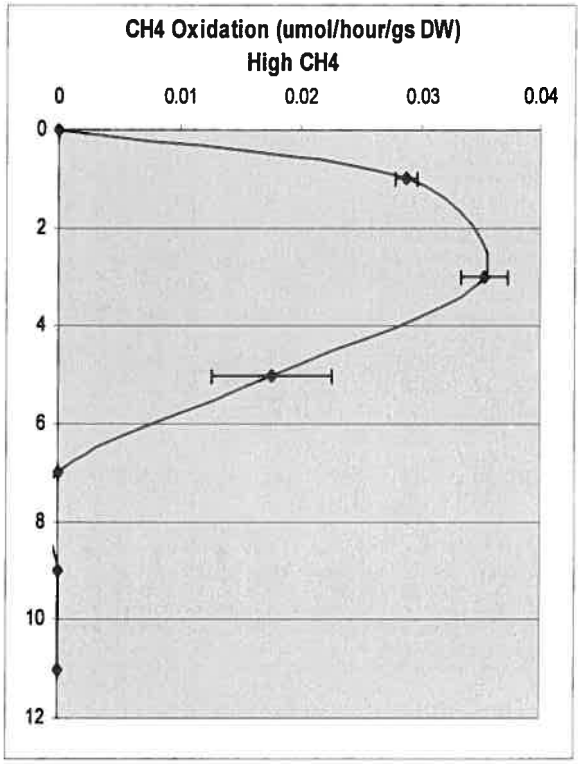


Figure 2. Moisture content, nitrogen content, and methane content.





High affinity: 100 ppm/ml CH4

Low affinity: 2% CH4

Figure 3. Vertical distribution of methane oxidation rates.

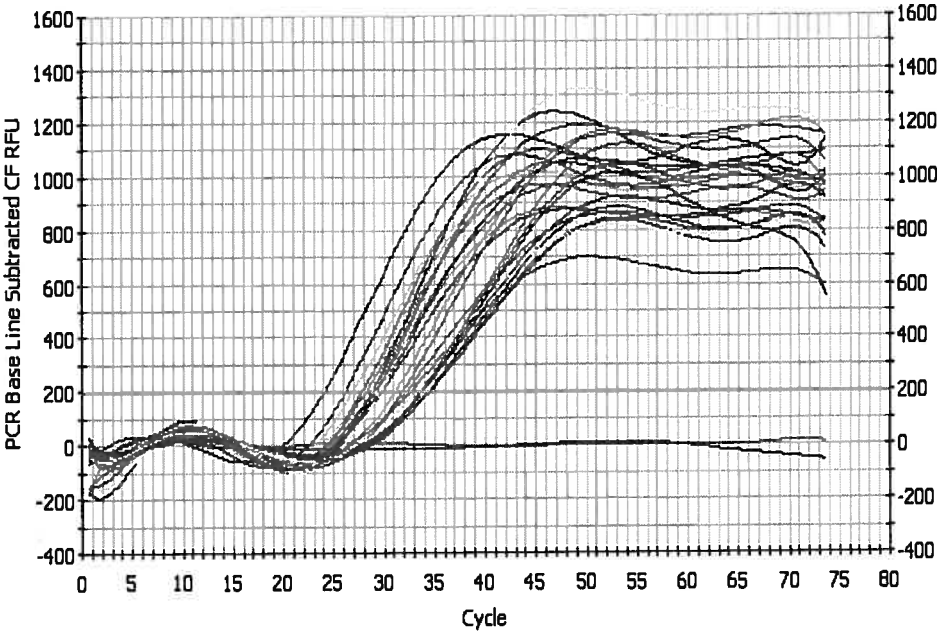
toxic to methanotrophs and they also posses resistant spores more efficient than type I (King G, 1994; Le Mer et al, 1996) Considering these facts and the similar response to either high or low methane concentrations, it is reasonable to consider the occurrence of dominant populations of methanotrophs II along the soil layers in our profile. However even though Type II may be the dominant, because of the natural high methane levels, populations of Type I can still develop and play a role in this soil system. In fact, the clone library analysis revealed the presence of both groups of methanotrophs. Of 86 clones sequenced, 30 of them clustered with the methanotrophs Type I (gamma Proteobacteria) being associated to *Methylomonas sp – Methylomonas methanica* (23 clones) and *Methylomicrobium* (17 clones). While 50 clones clustered with methanotrophs Type II (alpha Proteobacteria) all of them either closely related to *Methylosinus or Methylocystis*. Clone library analysis tend to support the abundance of methanotrophs Type II, but since each clone library was done with different primers it can only provide information about the presence of both types of microorganisms.

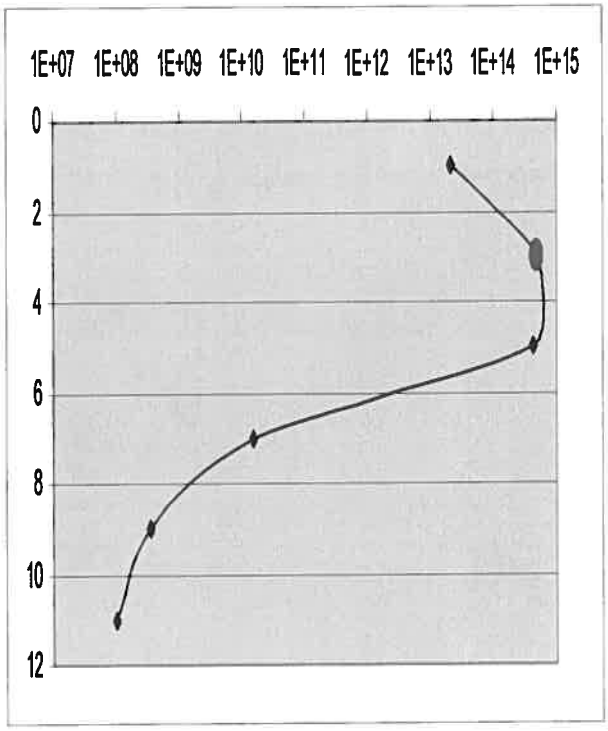
Quantitative (real time) PCR determination, complimentary support the suggestion that methanotrophs Type II- related are the dominant population in the soil profile (Figure 4). Even though I did not perform an absolute population number quantification, the estimation of specific initial DNA (nMol) present in each sample provided with enough reliability to compare abundance of the same group of organisms along the soil layers. The estimated numbers of methanotrophs type II clearly corresponded to the oxidation rates along the soil profile. Despite of the fact that we tried to do a similar quantification of Methanotrophs I populations, our real time PCR did not work as good as for the methanotrophs Type II. This difference in amplification may be due to PCR conditions, type of primers used (Type I were degenerated primers while type II were not), and presence of humic acids in the extracted DNA. Quantification of Methanotrophs Type will be attempted back in my lab. Primers targeting the pmoA, mmoX, and 16S rRNA have been developed for real time applications; it is suggested to use this set of primer for more refined population size estimations.

The NH4 addition experiment was directed to test the response of possibly naturally occurring enrichments of either methanotrophs population type in a Nitrogen dependent or independent fashion. The initial hypothesis considered that methanotrophs Type I can become dominant in a Nitrogen dependent phase, meaning that they can become dominant in presence of fixed and available nitrogen. While the methanotrophs Type II, can be de dominant in Nitrogen independent phase, relying on their capacity to fix Nitrogen, not necessarily being stimulated when Nitrogen is added to the system as could be the case of Type I.

The low methane concentration plus NH_4 experiment showed some stimulation in the methane oxidation rate only in the first layer (0-2 cm) while in the rest no stimulation but rather a light inhibition occurred (Figure 5). Methanotrophs type II are associated with high affinity for methane consumption and a N independent response, almost all the layers seemed to reflect this pattern by not responding to this NH_4 addition in low CH_4 concentrations, supporting the initial hypothesis. However, the stimulation observed in the 0-2 cm layer could initially disagree with the proposed Nitrogen independent response. Some *Methylosinus* and *Methylocystis* strains have been found able to use NO_3 and NH_4 as nitrogen source, and since the 0-2 cm layer is the one naturally possessing the highest NO_3/NH_4 content (~5 times higher than the rest of layers), it is hypothesized that NO_3/NH_4 utilizing methanotrophs Type II could have been enriched at this soil level and are the main responsible for the response to NH_4 addition.

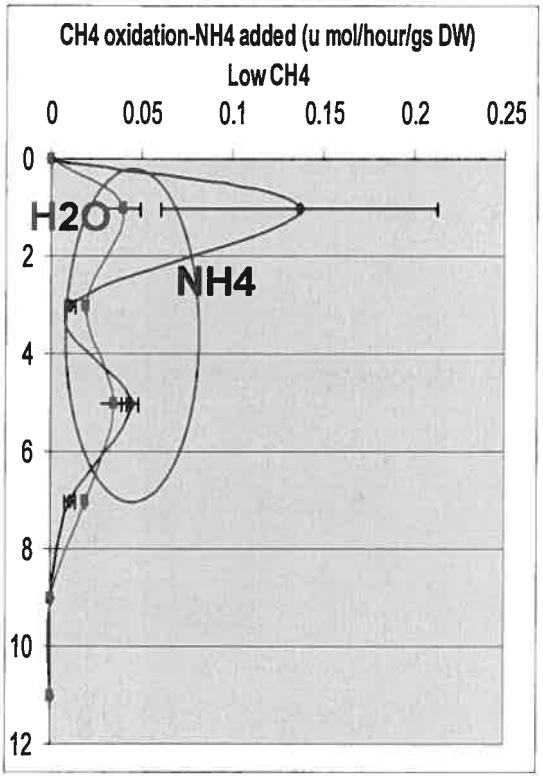
600 bp

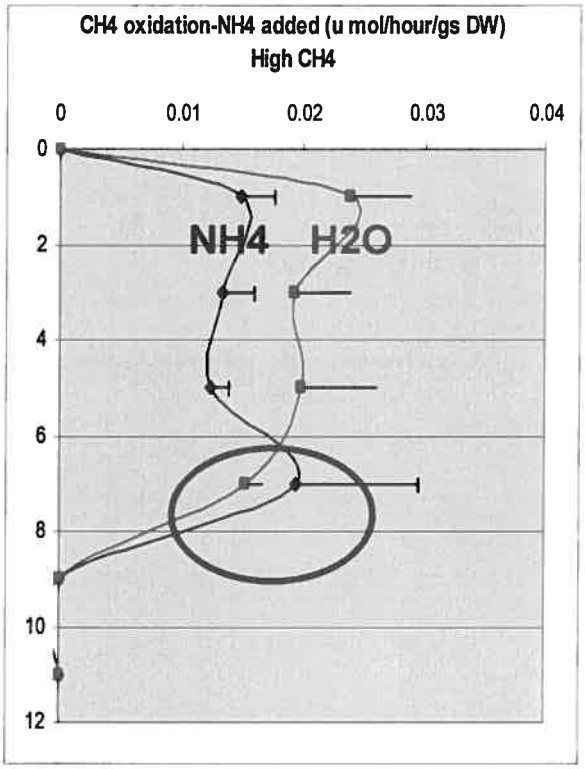




ngr ul-1

Figure 4. Quantitaive (real time) PCR of methanotrophs Type II. 16S rRNA primers. Comparison of specific ngr DNA present along soil profile.





High affinity: 200 ppm/ml CH4 **Low affinity: 3% CH4**

Figure 5. Effects of NH4 additions on methane oxidation rates along soil profile.

In the case of high methane concentration incubation (Figure 5), a marked inhibition was observed in the layers from 0-6 cm, while in the layer 6-8 cm methane oxidation was stimulated. The results showing NH_4 related inhibition of methane oxidation in the presence of high methane concentration is in agree with several reports (De Visscher et al. 1999, 2003; Bodelier et al 200); particularly in the report of De Vischer and Van cleemput 2003, it is indicated that land fill methanotrophs II-dominated soils presented this inhibition response. Methane oxidation inhibition as a consequence of increased NH_4 levels, have bee reported to be a competitive inhibition, where the inhibition takes place due to NO_2 built up produced by NH4 oxidation (Dunfield and Knowles, 1995).

The observed NH4 methane-oxidation inhibition pattern is in agree with the proposed hypothesis of methanotrophs Type II Nitrogen independent response. The highest inhibition was observed in the layer with highest methanotrophs Type II populations according to the data from quantitative PCR. Regarding the stimulation observed in the layer of 6-8cm, it can be speculated that a potential stimulation of Nitrogen limited methanotrophs Type I may be responding to the NH4 addition. This layer is depleted in Nitrogen, with a methane content close to the requirement for low affinity methane oxidation and the methanotrophs Type II is low or close to their lowest values in our Q PCR quantification. Methanotrophs Type I may be playing a role in this layer; however the lack of a reliable quantification of this group suggests being cautious about the interpretation of its possible role in this particular site.

In summary, this study was able to show how the occurrence of a vertical distribution of methane oxidation rates that are closely related to soil physical and biological characteristics. Oxidation rates under low or high methane concentrations, quantitative PCR and clone library approach indicate that Methanotrophs Type II are dominant at least in the 0-6 cm layer. Ammonium inhibitions patterns reflect differenced between microbial communities in soil where vertical changes of NH_4 inhibition patterns reflect vertical changes in the methanotrophic community within the soil vertical profile. It seems that as a long trend in this cedar swamp soils nitrogen starvation allows the take over of Nitrogen fixing methanotrophs usually related to Type II. Evidence of methanotrophs Type I presence was given by clone library analysis, however no activity rates or relative population sizes could be clearly associated to them being required more studies about this subject.

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