Strong long-term interactive effects of warming and enhanced nitrogen and sulphur deposition on the abundance of active methanogens in a boreal oligotrophic mire

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SUMMARY

Peatlands play a key role in the carbon cycle by being a considerable source of atmospheric methane. Thus, an understanding of the microbial production of methane is important in relation to environmental changes of peatlands. We applied real-time PCR on the *mcr*A gene and transcript to investigate the peat methanogen community response to the combined effect of 18 years of simulated warming and deposition of nitrogen (N) and sulphur (S) at a boreal oligotrophic mire in Sweden. The long-term effects of the experimental treatments on the methanogens was highly dependent on interactions between the treatment factors. Enhanced N deposition amplified the effect of warming, resulting in a further increase of the abundance of active methanogens. The effect of the perturbations was modulated by the depth horizon, with the strongest effect at the water level, where the interaction between enhanced N and S deposition, and warming, resulted in an increase of active methanogens. These results indicate that increasing average temperatures and simultaneously higher N deposition rates will substantially increase the methanogenic activity in northern ombrotrophic peatlands. These findings strongly highlight the importance of accounting for any possible interactive perturbation effects when investigating the response of peat methanogens to environmental change.

KEY WORDS: field experiment, *mcr*A gene and transcript, nitrogen, real-time PCR, warming

INTRODUCTION

Boreal peatlands are net sinks of atmospheric carbon dioxide (CO_2). Their water-saturated conditions foster anaerobic decomposition of the peat organic matter, rendering peatlands important sources of atmospheric methane (CH_4) (Nilsson *et al.* 2001). The estimated annual emission rates of carbon as CH_4 from northern peatlands are 1–52 g m^{-2} yr^{-1} (Frolking *et al.* 2011, Abdalla *et al.* 2016). Methanogenesis in peatlands is mainly controlled by the water table position, peat temperature, plant functional type composition and nutrient content (Svensson & Sundh 1992, Granberg *et al.* 1997, Granberg *et al.* 2001, Ward *et al.* 2013).

Temperature strongly influences the abundance, activity and composition of methanogens, with higher abundance and methane production rates being related to increased temperature regimes among peatlands as well as *in situ* warming simulations (Turetsky *et al.* 2008, Yavitt *et al.* 2012, Blake *et al.* 2015, Martí *et al.* 2015). An increase in temperature also affects methanogenesis indirectly by driving e.g. permafrost thawing and changes in hydrological regimes, and in soil structure (Svensson *et al.* 1999, Andersen *et al.* 2013, McCalley *et al.* 2014). In turn, moisture regimes, S deposition and substrate amendment modulate the impact of temperature (Vile *et al.* 2003, Gauci *et al.* 2004, Turetsky *et al.* 2008, Liu *et al.* 2012, Peltionemi *et al.* 2016). Primary production and plant community composition determine the availability and degradability of organic matter which primarily regulates methanogenesis (Svensson & Sundh 1992, Bridgham *et al.* 2013). Increasing nitrogen availability results in a shift from *Sphagnum*-dominated peatlands towards vascular plants dominance (Wiedermann *et al.* 2007), which in turn enhances the availability of easily degradable substrates from root exudates and litter (Limpens *et al.* 2008, Eriksson *et al.* 2010b, Bragazza *et al.* 2012). Moreover, long-term increased deposition of nutrients (nitrogen, potassium and phosphorus) also results in peat subsidence and hence higher water table which lead to increased methane formation (Juutinen *et al.* 2018). Thus, the higher availability of

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easily degradable organic matter for the microorganisms in anoxic energy-constrained peat has a direct effect on the supply of substrate for the methanogens (Joabsson *et al.* 1999).

There is major concern about the effect of global change perturbations, such as increased temperature and anthropogenic atmospheric deposition of nitrogen and sulphur on peatland ecosystem function (Granberg *et al.* 2001, Gauci *et al.* 2004, Bridgham *et al.* 2013, IPCC 2013). Several studies have addressed the effect of global change drivers on soil microbial communities (Castro *et al.* 2010, Li *et al.* 2013, Andresen *et al.* 2014, Shen *et al.* 2014, Contosta *et al.* 2015, Garcia-Palacios *et al.* 2015). Yet, few assess the interactive effect of such perturbations. In addition, most studies are mainly based on relatively short-term timescales between one and five years, whilst in order to identify significant changes over relevant timescales it is necessary to carry out long-term (over a decade) experiments (e.g. Rinnan *et al.* 2007, Contosta *et al.* 2015).

The present study aimed to investigate individual and interactive effects of long-term (18 years) *in situ* simulation of warming, and enhanced nitrogen and sulphate deposition (Granberg *et al.* 2001) on the methanogen community population to gain a deeper understanding of peat CH_4 production. Previous studies of this experiment have shown that CH_4 production and emissions have increased in response to long-term enhanced N deposition, while they decreased in response to simulated warming (Eriksson *et al.* 2010a,b). Furthermore, the N amendment counteracted the effect of warming on the rate of methane emissions (Eriksson *et al.* 2010b). The rate of CH_4 production in laboratory incubations was reduced in samples with the S amendment, while no effect on the CH_4 emissions was observed from the S-amended plots. The enhanced N deposition has resulted in a shift from a *Sphagnum*-dominated towards vascular plant-dominated vegetation (Wiedermann *et al.* 2007) and in an increase of carbon incorporation rates, which in turn are counteracted in combination with enhanced S deposition (Olid *et al.* 2014). The peat geochemistry has been altered after long-term simulated N and warming, with an increased accumulation of Zn, Fe, P and Ca as a result of warming, and an increased accumulation of P and Ca after enhanced N deposition (Olid *et al.* 2017). Based on these previous observations it was expected that: 1) the abundance of the *mrc*A gene and its transcript would have increased with enhanced N deposition and decreased with simulated warming, 2) the simulations would display an interactive effect on the methanogens, reflected in either an amplification or attenuation of the *mcr*A gene and/or transcript abundances, and 3) an increase in *mcr*A gene and its transcript abundances would have taken place along with the shift from *Sphagnum*- towards vascular plant-dominated vegetation.

METHODS

Study site

The ongoing experiment at Degerö Stormyr (64° 11' N, 19° 33' E) was established in 1995 in a *Sphagnum*-dominated oligotrophic peat area of the mire. The experiment is a full factorial design including two levels of nitrogen (N): ambient (low level) at 2 kg ha^{-1} yr^{-1} and amendment (high level) with NH_4NO_3 to reach a deposition level of 30 kg ha^{-1} yr^{-1}; two levels of sulphur (S): ambient at 3 kg ha^{-1} yr^{-1} and amendment with Na_2SO_4 to a level of 20 kg ha^{-1} yr^{-1}; and two levels of warming (greenhouse, GH) treatment: ambient (low level) of GH, i.e. without a cover and high level GH with a cover (Granberg *et al.* 2001). The elevated levels of N and S correspond to the annual deposition amounts in southwest Sweden at the time of the start of the experiment. One third of the fertilisation is provided in May, then one sixth each month until September. The greenhouse plots are surrounded by transparent polycarbonate sheets and covered by a perforated plastic film, allowing precipitation to enter the plots and helping to minimise increases in humidity. The greenhouse covers are erected directly after snowmelt in late May and removed before snowfall. The photosynthetically active radiation is reduced by 10–15 % when compared to controlled conditions and up to 20–25 % in warm and moist conditions when condensed water is found on the plastic cover (Granberg *et al.* 2001). Each experimental combination is duplicated and distributed randomly (see Table A1 in the Appendix). The plot size is 2 m × 2 m and plots are separated by 1m buffer zones.

The dominant vascular plants are *Eriophorum vaginatum* L., *Andromeda polifolia* L. and *Vaccinium oxycoccos* L., and the dominant *Sphagnum* species are *S. balticum* (Russ) C. Jens. and *S. lindbergii* Schimp. The experimental site has a pH of ~ 4.5. The climate of the WMO (World Meteorological Organisation) reference period (1960–1991) is characterised by a mean annual total precipitation of 523 mm, a mean annual air temperature of 1.2 °C, a mean July air temperature of 14.7 °C and a mean January temperature of -12.4 °C. Average weather conditions during 2001–2012 were (Peichl *et al.* 2014): annual and growing season air temperatures 2.3 °C and 11 °C, respectively, annual and growing

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season precipitation 666 and 395 mm, respectively, and the growing season mean water table level 14 cm below peat surface.

Collection of peat samples

At each treatment plot (n = 16) one peat core (0–40 cm) was collected in August 2013, three weeks after the fertilisation in July. From each core, 5 cm^3 of peat was subsampled from five depths below the *Sphagnum* surface: 7–11 cm (A), 11–15 cm (B), 15–19 cm (C), 19–23 cm (D) and 23–27 cm (E). The samples were stored in 50 ml sterile tubes containing 2 ml of LifeGuard™ Soil Preservation Solution (MoBio Laboratories, Hameenlinna, Finland), and kept at room temperature for 24 hours before freezing at -20 °C.

Nucleic acid extraction and reverse-transcription PCR

Total RNA and DNA were co-extracted from 2 g of wet peat and recovered in 50 µl of buffer using the RNA PowerSoil® Total RNA Isolation Kit together with the RNA PowerSoil® DNA Elution Accessory Kit (MoBio Laboratories), according to the manufacturer's instructions. Concentrations of the RNA and DNA extracts were measured with the Quant-iT RNA HS assay and the Quant-iT dsDNA HS assay kits respectively, with the Qubit fluorometer (Invitrogen, Lidingö, Sweden). The ranges of RNA and DNA extraction yields were 2.5–120 ng μL^{-1} and 0.5–600 ng μL^{-1}, respectively. Two extractions from each sample were carried out and pooled. The pooled extractions of RNA were concentrated using the RNA Clean & Concentrator™-25 (Zymo Research, Täby, Sweden), in accordance with the manufacturer's instructions. The pooled extractions of DNA were concentrated using a vacuum concentrator (Vacufuge® 5301, Eppendorf, Horsholm, Denmark). DNA residues were removed from the concentrated RNA extracts by digestion using 2U TURBO DNAse (Ambion-Life Technologies, Stockholm, Sweden) for 1 h at 37 °C following the manufacturer's instructions. Reverse transcription was performed adding 2 µl of DNAse-treated RNA-extract to 17 µL reaction mixture containing 1X Expand Reverse Transcriptase Buffer (Roche Diagnostics, Mannheim, Germany), 10 mM Dithiothreitol solution (Roche diagnostics), 5 mM of dNTPs (New England BioLabs Inc., Glostrup, Denmark) and 250 nM of random hexamers (TAG Copenhagen A/S, Copenhagen, Denmark). After 2 min incubation at 42 °C in a DNA engine DYAD™ Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA), 1 µL of Expand Reverse Transcriptase (Roche Diagnostics) was added to the mixture and incubated at 42 °C for 40 min, followed by 30 min at 50 °C and 15 min at 72 °C. RNA template addition was in the range 8–300 ng.

Real-time PCR of *mcr*A genes and transcripts

The *mcr*A DNA and cDNA gene fragment copy numbers were quantified according to Martí *et al.* (2015) with slight modifications. Briefly, 4 µL of sample template was added to a 16 µL reaction mixture consisting of 1 SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 250 nM of each primer, 0.3 M betaine and 250 µg mL^{-1} of BSA. The detection limit was ten gene copies per reaction mixture, and the quantification limit was 100 gene copies per reaction mixture. Of the DNA samples, 10 % were below the detection limit, while another 8 % were detectable but not quantifiable. Of the cDNA samples, 6 % were below the detection limit. For the statistical analysis, 45 gene copies per reaction were used for the samples that were detectable but not quantifiable and 5 gene copies per reaction were used for the samples below the detection limit, as was done previously by Martí *et al.* (2015).

Data analyses

The factorial experiment consists of three treatment factors at two levels (2^3-design) with field duplicates for each treatment. Thus, the statistical evaluation is based on $n = 8$ for the main factors N, S and GH, $n = 4$ for the 2-way interaction factors (N×S, N×GH and S×GH) and $n = 2$ for the 3-way interaction (N×S×GH); see Table A1 for the treatment effects evaluation matrix.

After ten years of treatment some of the plots exhibited different distances from the surface to the mean water table level (Eriksson *et al.* 2010b). To account for this gradual change, the sampled depths were classified into three depth horizons: above the annual mean water table level (AWT), a layer around the annual mean water table level (WT), and below the annual mean water table level (BWT). These depth horizons were set according to their positions relative to the average seasonal water table level within each plot as given by (Eriksson *et al.* 2010a) (Table A2).

Statistical analyses were performed in R version 3.1.2 (R Core Team 2014), mainly with the ecology package *vegan* (Oksanen *et al.* 2013). The effect of the treatments on the abundances of the *mcr*A gene and transcript, and on the transcript/gene quotient (response variables, Y) was assessed by multiple linear regression (MLR): $log_{10}Y = \beta_0 + \beta_1 N + \beta_2 S + \beta_3 GH + \beta_4 (NxS) + \beta_5 (NxGH) + \beta_6 (SxGH) + \beta_7 (NxSxGH) + \varepsilon$. Although there is a difference in

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sampling time between the present study (analysing the abundance of methanogens by real-time PCR) and previous studies reporting on CH_4 production rates and the plant distribution changes (Wiedermann *et al.* 2007, Eriksson *et al.* 2010a,b), Pearson's correlations between the molecular dataset and the CH_4 and plant datasets were made within specific depth horizons. This is motivated by the fact that, at the time for those observations, the experimental effects are considered to have reached a stage where initial progressing effects have passed and stabilised conditions have been reached. This is strongly corroborated by establishment of the plant distribution patterns which have been retained since then. The correlation was applied to analyse the degree of association between the abundance of *mcr*A and the cover percentage of the dominant vascular plants species (*E. vaginatum*, *A. polifolia* and *V. oxycoccos*) as well as the dominant *Sphagnum* species (*S. balticum* and *S. lindbergii*). Pearson's correlation was also applied to analyse the relationship between the abundance of *mcr*A and the potential CH_4 production (average rate for the incubation duplicates in units of µg d^{-1} g^{-1}, dry weight basis). A significance level of 0.05 was used for all analyses.

RESULTS

Abundance of *mcr*A gene and transcript among treatments

The average *mcr*A gene abundances of the peat profiles differed significantly in response to increased warming (GH) and enhanced N and S depositions (Figure 1A). On average, 1.5×10^5, 2.3×10^5 and 2.4×10^5 *mcr*A gene copies per gram of soil were detected for the high levels of simulated N deposition, S deposition and warming, respectively. For the corresponding ambient levels, the averages were 2.4×10^5 (N), 1.6×10^5 (S) and 1.5×10^5 (GH) *mcr*A gene copies per gram of soil. Thus, the abundance of the *mcr*A gene decreased significantly in the enhanced N treatment and showed significant

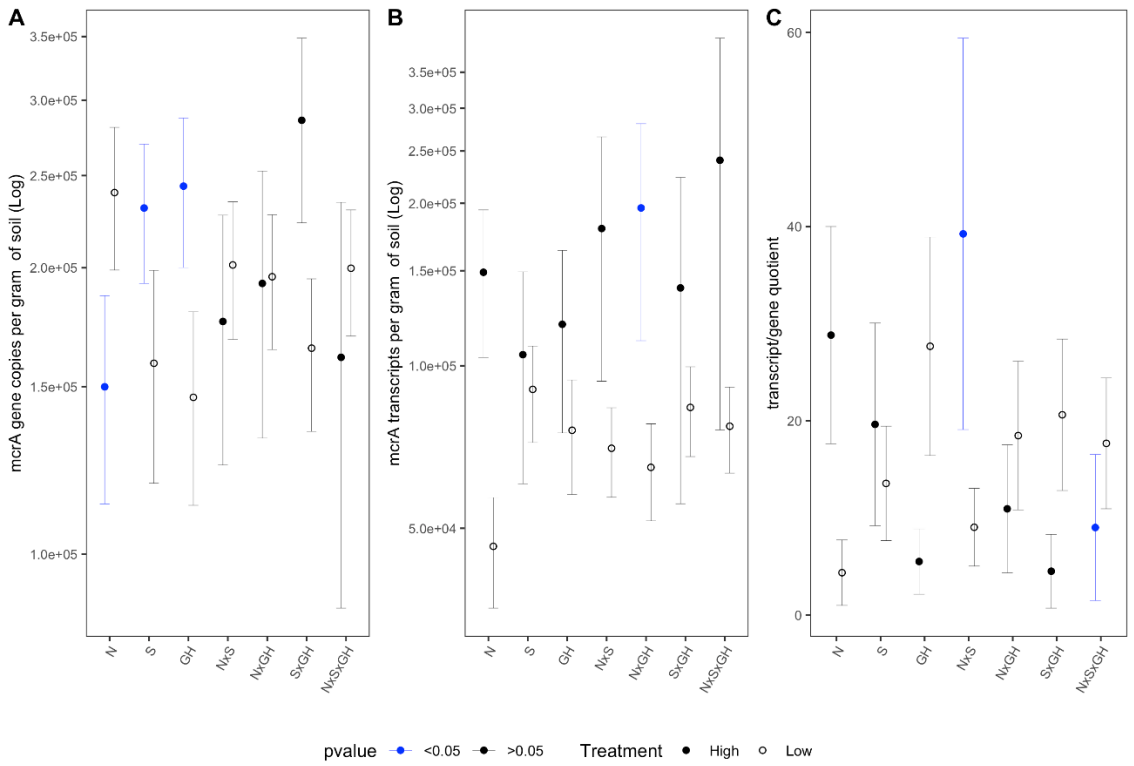

Figure 1. Overall results per treatment effect for (A) abundance of *mcr*A gene copies, (B) abundance of *mcr*A transcripts, and (C) transcript/gene quotient (note the logarithmic scales on vertical axes). Error bars represent one standard error. The significance estimates are based on the MLR model ($log_{10}Y = \beta_0 + \beta_1 N + \beta_2 S + \beta_3 GH + \beta_4(N \times S) + \beta_5(N \times GH) + \beta_6(S \times GH) + \beta_7(N \times S \times GH) + \varepsilon$). 'Low' refers to the low (ambient) treatment level and 'High' to treatments involving N (30 kg ha^{-1} yr^{-1}) or S (20 kg ha^{-1} yr^{-1}) amendment or warming (GH), singly and in combination. Number of replicates: for main effects, $n = 40$; for two-way interaction effects, $n = 20$; and for three-way interaction effects, $n = 10$. For further details see Table A1 (Appendix).

increases in the S-amended plots and the GH treatments. Although not statistically significant, interaction effects including high N levels (N, N×S, N×GH, N×S×GH) indicated lower gene abundances than the ambient levels (Figure 1A). The combination of high S and GH (S×GH) showed the highest average abundance, at 2.8×10^5 *mcr*A gene copies per gram of soil compared to 1.7×10^5 *mcr*A gene copies per gram of soil for the interaction of the ambient levels.

The abundance of the *mcr*A transcript was significantly affected by the interaction of high-N and warming (Figure 1B). This had a synergistic effect, amplifying the effect of the respective individual main factors *per se* (i.e. low-N and low-GH). With high levels of the treatment factors we detected more *mcr*A transcripts per gram of peat, ranging from 1.1×10^5 in high-S to 2.4×10^5 in high-N×S×GH, than with ambient levels of these factors for which the range was 4.6×10^4 in low-N to 9.0×10^4 in low-S.

The distribution patterns of the transcript/gene quotient differed from those of transcript abundance (Figure 1C). The quotients differed significantly in response to the high levels of the N×S and N×S×GH factors. N×S had a synergistic effect, amplifying the individual effect of low-N and low-S, while including the interaction with warming counteracted the effect (Figure 1C).

Vertical distribution of methanogens in the peat

As expected, the abundances of *mcr*A genes and transcripts were mainly higher in the intermediate depth layers than at the upper and lower peat depths (Figure 2). The depth distributions of *mcrA* gene abundance were similar for the simulated

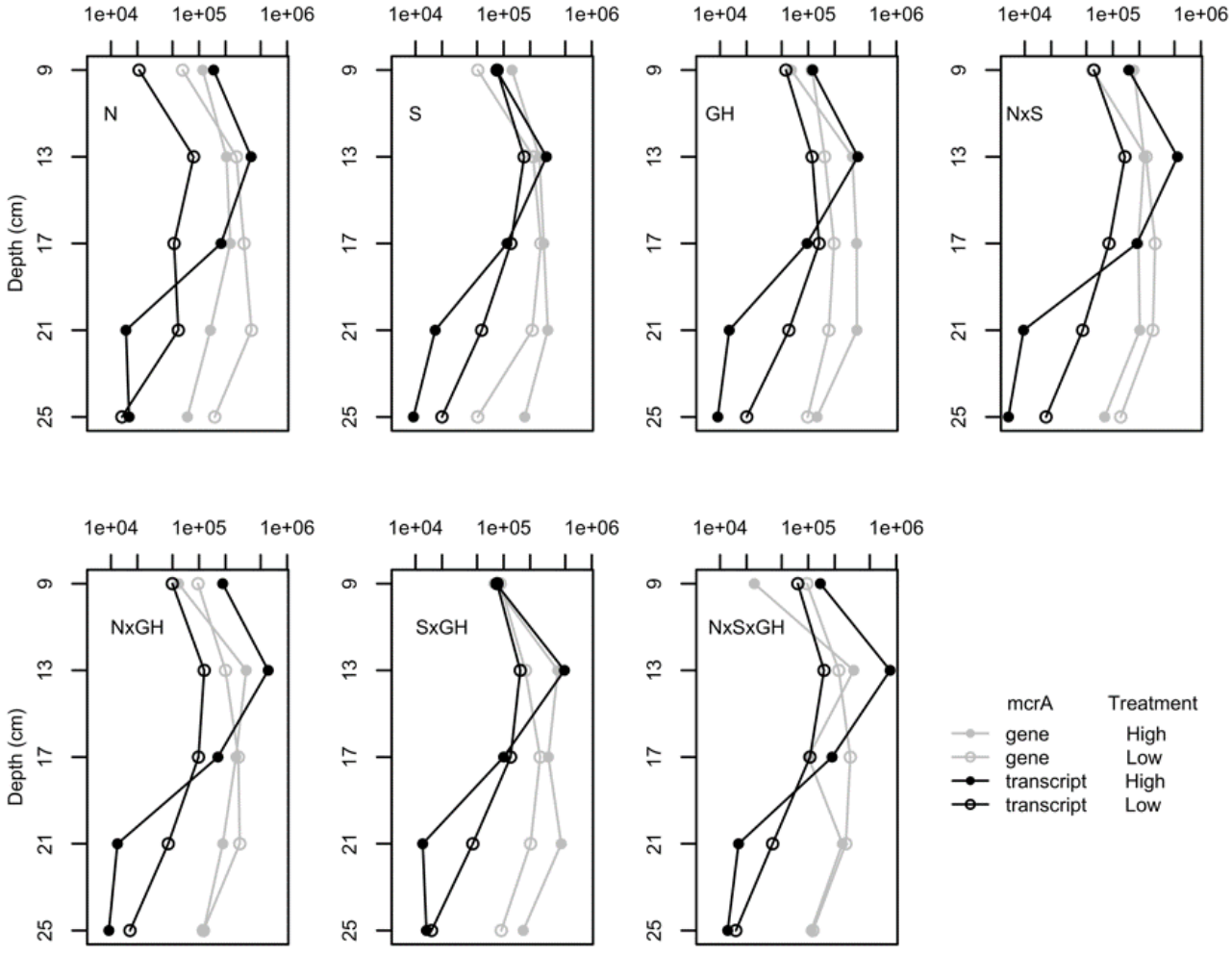

Figure 2. Depth profiles, per treatment effect, of the abundance of *mcr*A gene copies and transcripts per gram of soil (note the logarithmic scales on horizontal axes). 'Low' refers to the low (ambient) treatment level and 'High' to treatments involving N (30 kg ha^{-1} yr^{-1}) or S (20 kg ha^{-1} yr^{-1}) amendment or warming (GH), singly and in combination. Number of replicates: for main effects, $n = 8$; for two-way interaction effects, $n = 4$; and for three-way interaction effects, $n = 2$.

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perturbations and the ambient levels, i.e. with highest abundance between 11 and 23 cm depth, except for the interactions with high-GH where a lower gene abundance was found at 15–19 cm depth. The depth distributions of transcripts were clearly affected by the simulated N and S depositions, and by GH (high levels of N, S and GH) as well as their interactions. This was reflected by a higher abundance of transcripts at 11–15 cm relative to the upper sample, i.e. mostly covering the WT horizon (Table A2), followed by a considerably lower abundance between 15 and 27 cm (Figure 2).

Responses of methanogens to the treatments, within specific depth horizons

To assess the responses of the methanogens to the individual and interactive effects of the perturbations, the coefficients from stepwise MLR were analysed separately for each of the three depth horizons (AWT, WT and BWT). These analyses revealed few significant responses to the simulated perturbations, reflected in either an increase or a decrease in the abundance of genes or transcripts relative to ambient levels (Figure 3). The majority of significant responses were associated with interactions of the treatment factors.

The effect of the treatments on *mcr*A gene abundance was most pronounced in the WT horizon, which showed an increase of genes as a result of simulated N or S depositions with simultaneous warming (N×GH and S×GH). In contrast, the 3-way interaction (N×S×GH) resulted in a decrease of gene abundance (Figure 3A). At the AWT horizon the gene abundance increased in response to the combination of enhanced N and S deposition (N×S), while at the BWT horizon simulated N deposition resulted in a decrease and the high-S×GH interaction resulted in an increase of *mcr*A genes.

Similarly, the magnitude of the significant response of *mcr*A transcript abundance to the treatment factors was most pronounced at the WT horizon, where the abundance of transcripts increased as a result of the 3-way interaction (N×S×GH; Figure 3B). At the AWT horizon the abundance of transcripts decreased in response to the3-way interaction but showed an increase with the

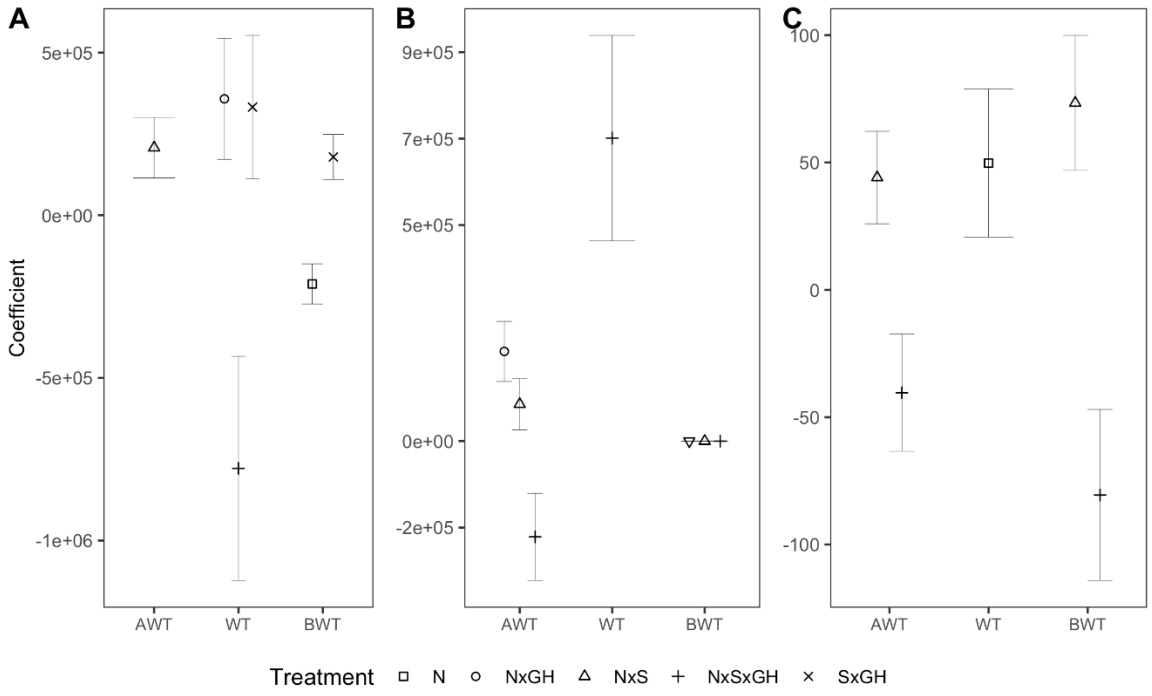

Figure 3. Multiple linear regression coefficients for (A) abundance of *mcr*A gene copies, (B) abundance of *mcr*A transcripts, and (C) transcript/gene quotient; in the peat profile above the mean annual water table level (AWT), around the mean annual water table level (WT) and below the mean annual water table level (BWT). Each treatment response is represented by the coefficient reflecting the change in *mcr*A abundance (relative to ambient conditions) in response to the 'High' treatments involving N (30 kg ha^{-1} yr^{-1}) or S (20 kg ha^{-1} yr^{-1}) amendment or warming (GH), singly and in combination. Error bars represent one standard error. For number of replicates, see Table A1 in the Appendix. The results are based on the MLR model ($Y = \beta_0 + \beta_1 N + \beta_2 S + \beta_3 GH + \beta_4(N\times S) + \beta_5(N\times GH) + \beta_6(S\times GH) + \beta_7(N\times S\times GH) + \varepsilon$). Only the significant coefficients are shown.

simulated N×GH and N×S interactions. At the BWT horizon the abundance of transcripts decreased slightly in response to GH and high-N×S but increased slightly in response to the N×S×GH combination (Figure 3B).

The extent of response of the transcript/gene quotient to the treatments increased with peat depth, showing a higher response to the N×S interaction and a lower response to the N×S×GH interactions at the AWT and BWT horizons, respectively (Figure 3C). At the WT horizon the transcript/gene quotient increased in response to simulated N deposition.

Methanogens in relation to composition of the plant community

At the AWT horizon, the abundance of the *mcr*A transcript correlated positively with the abundances of *E. vaginatum* ($r = 0.52$, $p = 0.0075$) and *A. polifolia* ($r = 0.66$, $p = 0.0011$). At the WT horizon, the abundance of the *mcr*A transcript correlated positively with the abundances of *E. vaginatum* ($r = 0.71$, $p = 0.0005$) and *V. oxycoccus* ($r = 0.50$, $p = 0.0361$). The transcript/gene quotient and the abundances of *E. vaginatum* and *V. oxycoccus* were positively correlated ($r = 0.58$, $p = 0.01$ and $r = 0.47$, $p = 0.04$ respectively). At the BWT horizon, significant weak positive correlations were found between the *mcr*A gene abundance and the total *Sphagnum* spp. cover ($r = 0.36$, $p = 0.0202$), and weak negative correlations with *A. polifolia* ($r = -0.31$, $p = 0.0475$) and *V. oxycoccus* ($r = -0.31$, $p = 0.0483$). Although not statistically significant, there was a tendency towards a negative correlation between the abundance of the *mcr*A transcript and *Sphagnum* cover at all three depth horizons.

Correlation between methanogens and CH_4 production

There were significant correlations between the abundances of the *mcr*A gene and its transcript and the CH_4 production rates reported by Eriksson *et al.* (2010a) (Table 1). At the AWT horizon there were strong correlations between *mcr*A gene abundance and CH_4 production rates for the treatments that included simulated warming, i.e. GH, N×GH, S×GH and N×S×GH. At the WT horizon the *mcr*A gene abundance and CH_4 production rates strongly correlated in the N×S treatment. At the BWT horizon, *mcr*A transcript abundance and CH_4 production rates were strongly correlated to all of the treatment factors. Correlations between the transcript/gene quotient and CH_4 production were also found at BWT, for the main N and S factors and their interaction (Table 1).

Table 1. Pearson correlation coefficients between CH_4 production rate and *mcr*A gene abundance (Gene), *mcr*A transcript abundance (Transcript) and transcript/gene quotient (Quotient) per horizon in the peat profile. Horizons: AWT = above the annual mean water table level; WT = layer around the annual mean water table level; BWT = below the annual mean water table level. All: includes AWT, WT and BWT. Significance levels are indicated as **: $p \leq 0.001$; *: $p \leq 0.05$; ns: non-significant.

Treatment level	Effect	Gene	Horizon	Transcript	Horizon	Quotient	Horizon
High level (perturbation)	N	ns	All	0.08 **	BWT	0.70 **	BWT
	S	-0.42 *	BWT	0.78 **	BWT	0.66 **	BWT
	GH	0.88 **	AWT	0.70 **	BWT	ns	All
	N×S	0.90 *	WT	0.82 **	BWT	0.71 *	BWT
	N×GH	0.92 *	AWT	0.86 **	BWT	ns	All
	S×GH	0.96 **	AWT	0.77 **	BWT	ns	All
	N×S×GH	0.99 *	AWT	0.85 *	BWT	ns	All
Low level (ambient)	N	ns	All	0.39 *	BWT	ns	All
	S	ns	All	0.43 *	BWT	0.74 *	BWT
	GH	ns	All	0.61 **	BWT	0.63 *	BWT
	N×S	ns	All	0.44 **	BWT	ns	All
	N×GH	ns	All	0.61 **	BWT	0.59 **	BWT
	S×GH	ns	All	0.62 **	BWT	0.60 **	BWT
	N×S×GH	ns	All	0.62 **	BWT	0.58 **	BWT

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DISCUSSION

Abundance of *mcrA* gene and its transcript

The effect of both treatment and depth horizon on *mcr*A was consistently more pronounced at the transcriptional level than at the gene level. This is in line with previous studies which found that variation in *mcr*A gene abundance, indicative of the amount of methanogens, does not necessarily correspond to the variation in CH_4 production (Roling 2007, Freitag & Prosser 2009). Based on laboratory incubations of peat under a temperature gradient (4, 15, 25 and 30 °C) it has been suggested that the transcript/gene quotient is potentially a better indicator of *in situ* methanogenic activity than the transcript abundance in environmental samples (Freitag & Prosser 2009). However, in the present study the abundance of the *mcr*A transcript was clearly a better indicator of methanogenic activity than the transcript/gene quotient. Moreover, there was no correlation between the *mcr*A gene and its transcript. For the different perturbations, strong correlations between the abundance of *mcr*A transcript in the present study and the maximal CH_4 production reported by Eriksson *et al.* (2010a) were found mainly in the anoxic zone, i.e. below the water table. Given the temporal and spatial discrepancies, i.e. a 7-year interval between samplings at different sampling points within the treatment plots, this shows that the relative stabilisation reported for the plant distributions is valid also for the active methanogens. Furthermore, the vertical distributions of the abundance of transcripts were much more distinct and revealed stronger correlation with vegetation patterns than those of the transcript/gene quotient. Altogether, these circumstances strengthen the view that, in peatland ecosystems, the abundance of transcripts (normalised to the mass of soil) better reflects potential methane production activity than does the transcript/gene quotient.

Vertical distribution of methanogens

There were active methanogens throughout the sampled part of the depth profile (from 7 to 27 cm below the surface), covering both the oxic and the anoxic horizons. It is well established that potential methane production in the surface layers is not inhibited by dry and oxic periods (Fetzer *et al.* 1993, Öquist & Sundh 1998). In the present study the maximum abundance of the *mcr*A transcripts generally occurred within 11–15 cm of the mire surface for all treatments. This depth interval is associated with the average position of the water table during the growing season (14 cm below the moss surface), which is identified as a major regulator of methane emissions to the atmosphere (Granberg *et al.* 1997). Previous studies of the depth distribution of methane formation and oxidation in a set of boreal mires found that the highest methanogenic activity occurred mainly 5–10 cm below the growing season average water table level, and methanotrophic activity mainly around the average water level (Sundh *et al.* 1994, 1995). Fluctuation of the water table around its average level was shown to regulate methane formation and oxidation (Granberg *et al.* 1997). Our observations of the vertical distribution of active methanogens using real-time PCR confirms this pattern for the methanogens. Furthermore, vertical profiles of methanogen diversity together with CH_4 production measurements have previously shown that diversity is related to depth and that the maximum CH_4 production occurs immediately below the water table (Galand *et al.* 2002, Cadillo-Quiroz *et al.* 2006, Hoj *et al.* 2006, Ganzert *et al.* 2007).

Under ambient conditions the abundances of the *mcr*A gene and its transcript had similar depth distributions, indicating that in the natural situation the methanogenic activity is proportional to the abundance of methanogens. This is supported by the similar depth distribution pattern of the transcript/gene quotient for these conditions. In contrast, when facing a perturbation, even after 18 years since its onset, the depth distribution of the transcript/gene quotient did not correspond to the occurrence of methanogen biomass (*mcr*A gene), reflecting an effect of the perturbation on their activity (*mcr*A transcript). This implies that the environmental conditions for growth of methanogens in the peat profile are harsh. Thus, even when they experience enhanced energy input from the anaerobic degradation of elevated levels of substrate e.g. from roots introduced due to the N-supplement in our study, their growth rate seems to be very low. This conforms with the generally low growth efficiency of anaerobes. Furthermore, hydrogenotrophic methanogenesis is reported to be the dominant methanogenic pathway in ombrotrophic bogs (Horn *et al.* 2003, Galand *et al.* 2005, Martí *et al.* 2015). This is confirmed by the studies of the $^{13}CH_4$-signals observed in methane emitted from this mire type (e.g. Whiticar *et al.* 1986). The role of the hydrogenotrophs (e.g. sulphate reducers and methanogens) is to maintain a low partial pressure of hydrogen in order to allow for as complete a degradation of organic matter as possible in an anaerobic environment (Conrad 1999, Heimann *et al.* 2010). This in turn means low energy supply for the

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growth of methanogens in peat bogs. Thus, even when there is a higher availability of easily degradable organic matter in the peat as result of an introduction of roots due to a higher N regime, the growth response may not be enough to be discriminated from the control plots without N addition. However, given that our understanding of the physiology of the unknown hydrogenotrophic methanogens in acid peat environments, especially substrate affinity kinetics in relation to thermodynamic conditions and competition by other electron acceptors (see review by Heimann *et al.* 2010) is little developed so far, there is a need for further studies to underpin this view.

Methanogens and plant community composition

The Degerö Stormyr mire is highly oligotrophic, and increased N availability has affected the plant community composition by shifting it from *Sphagnum* to vascular plant dominance (Wiedermann *et al.* 2007). This, in turn, has resulted in a larger root biomass and most likely also an increase in root exudation (Limpens *et al.* 2008). Consequently, more easily degradable organic matter will be available, which might ultimately increase methanogenesis. This is supported by the positive correlation found between the *mcr*A gene expression and the vascular plants, as well as the increase in *mcr*A gene expression observed with enhanced N deposition. It is reasonable to assume that the root distribution has shifted towards shallower depths in response to the N amendment, resulting in a lower root exudation in the anoxic zone. Granberg *et al.* (2001) observed a decrease in methane emissions after short-term simulated N deposition, which was argued to be due to a likely reallocation of the vascular plants roots to a shallower horizon. Indeed, Olid *et al.* (2017) reported an increase in root abundance 10–15 cm below the peat surface in these plots after twelve years of treatment. Moreover, a decline in *Sphagnum* may imply a reduction of leachates such as uronic acid and polyphenols, which are strong inhibitors of microbial decomposition (Clymo & Hayward 1982, Stalheim *et al.* 2009, Hájek *et al.* 2010) and thus also indirectly promote microbial activity.

Responses of the methanogens to the treatments

The effect of long-term enhanced N on the active methanogens was modulated by warming and enhanced S deposition. Thus, the combined N×S×GH interaction showed the strongest effect. An increase in temperature would enhance the overall microbial activity involved in the anaerobic degradation pathway and the amounts of organic matter degraded would be likely to increase as a result of the shift in plant composition. The sulphate added may act in several ways, of which one would be to compete for the methanogenic substrates.

As a result of the simulated warming, the temperature measured at 18 cm below the surface had increased by 2 °C (Granberg *et al.* 2001) and the sedge cover had expanded. Although these changes may support methanogenesis, a reduction in CH_4 production rates was observed by Eriksson *et al.* (2010a). This was confirmed by the lower extent of *mcr*A gene expression observed with simulated warming. This may be explained by the fact that the peat temperature increase in response to the greenhouse treatment covers the whole oxic zone (with higher levels above 18 depth) and therefore probably promotes aerobic decomposition. Consequently, the organic matter left available and eventually reaching the anoxic zone will be of lower degradability, leading to lower methane formation.

In conclusion, by considering the *mcr*A transcript as a proxy for methanogenic activity, our results indicate that methane production in northern peatlands might increase with warming in combination with higher rates of atmospheric N deposition, as it results in a higher abundance of active methanogens. While the effect of N on the methanogens may be mainly indirect via an induced shift in vegetation, the effect of warming may be mainly direct, promoting microbial degradation through an increase in peat temperature. Moreover, our results suggest that the methanogenic activity will react to higher rates of atmospheric S deposition only if it occurs in combination with warming and increased N deposition. Thus, our results highlight a need to investigate the response of methanogens, as well as the overall microbial community in peatlands, to simultaneously applied multiple environmental stressors since their interactions counteract or amplify the effects of the individual stressors *per se*.

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AUTHOR CONTRIBUTIONS

MM, MBN and BHS conceived the idea and MM led the investigation; MBN designed the experiment and maintains the long-term experiment; MM, MBN and BHS performed the fieldwork; MM conducted the laboratory analyses, analysed the data, drafted the manuscript and led the writing. All co-authors contributed to writing the manuscript according to their expertise in: peatland functions and statistical design (MBN), statistical analyses (AD), molecular biology (PE), and anaerobic microbiology (BHS).

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Appendix

Table A1. Evaluation matrix for the treatments effect. Horizons in the peat profile: AWT = above the annual mean water table level; WT = layer around the annual mean water table level; BWT = below the annual mean water table level. 'Low' and 'High' refer to the treatment levels, involving the following application rates: for sulphur (S), Low (ambient) = 3 kg ha^{-1} yr^{-1}, High = 20 kg ha^{-1} yr^{-1}; for nitrogen (N), Low (ambient) = 2 kg ha^{-1} yr^{-1}, High = 30 kg ha^{-1} yr^{-1}; and for warming (GH), Low = ambient, High = with a transparent cover (see Methods for further details).

Plot number	Field treatment	Number of replicates			Main effect						2-way interaction effect						3-way interaction effect	
					N		S		GH		N×S		N×GH		S×GH		N×S×GH	
		AWT	WT	BWT	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
11	control	1	1	3	x		x		x		x		x		x		x	
19	control	1	1	3	x		x		x		x		x		x		x	
4	GH	1	1	3	x		x			x	x							
16	GH	1	1	3	x		x			x	x							
12	N	1	2	2		x	x		x						x			
18	N	1	2	2		x	x		x						x			
9	N×GH	1	2	2		x	x			x				x				
17	N×GH	1	1	3		x	x			x				x				
8	N×S	2	2	1		x		x	x			x						
14	N×S	1	1	3		x		x	x			x						
2	N×S×GH	2	1	2		x		x		x		x		x		x		x
13	N×S×GH	1	1	3		x		x		x		x		x		x		x
3	S	1	1	3	x			x	x				x					
7	S	1	1	3	x			x	x				x					
5	S×GH	1	1	3	x			x		x						x		
10	S×GH	1	1	3	x			x		x						x		
Statistical replicates	Factorial design				8	8	8	8	8	8	4	4	4	4	4	4	2	2
	AWT				8	10	8	10	9	9	4	6	4	5	4	5	2	3
	WT				8	12	11	9	11	9	4	5	4	5	6	4	2	2
	BWT				24	18	21	21	20	22	12	9	12	10	10	11	6	5
	All depths × Factorial design				40	40	40	40	40	40	20	20	20	20	20	20	10	10

Table A2. Depth normalisation based on plot-specific average values as measured in the years 2004–2006 (Eriksson *et al*. 2010a). MTW05 = mean water table level in 2005 at each plot. Difference = the MTW05 of each plot subtracted from the overall MTW05 average (8.8 cm). Adjusted water level = Difference added to 14 cm (corresponding to the 2004–2006 mean annual water table level). A–E: sampled depths classified to horizon in the peat profile: AWT = above the annual mean water table level, WT = around the annual mean water table level, BWT = below the annual mean water table level.

Treatment	Plot	MTW05 (cm)	Difference (cm)	Adjusted water level	A (7–11 cm)	B (11–15cm)	C (15–19cm)	D (19–23cm)	E (23–27cm)
GH	4	10.0	-1.2	12.8	AWT	WT	BWT	BWT	BWT
GH	16	10.8	-2.0	12.0	AWT	WT	BWT	BWT	BWT
N	12	7.7	1.1	15.1	AWT	WT	WT	BWT	BWT
N	18	7.9	0.8	14.8	AWT	WT	WT	BWT	BWT
N×GH	9	7.1	1.7	15.7	AWT	WT	WT	BWT	BWT
N×GH	17	10.0	-1.2	12.8	AWT	WT	BWT	BWT	BWT
N×S	8	3.6	5.2	19.2	AWT	AWT	WT	WT	BWT
N×S	14	9.7	-0.9	13.1	AWT	WT	BWT	BWT	BWT
N×S×GH	2	5.7	3.1	17.1	AWT	AWT	WT	BWT	BWT
N×S×GH	13	10.4	-1.7	12.3	AWT	WT	BWT	BWT	BWT
S	3	9.3	-0.5	13.5	AWT	WT	BWT	BWT	BWT
S	7	8.7	0.1	14.1	AWT	WT	BWT	BWT	BWT
S×GH	5	12.0	-3.3	10.7	AWT	WT	BWT	BWT	BWT
S×GH	10	10.1	-1.3	12.7	AWT	WT	BWT	BWT	BWT
Zero Treatment	11	9.1	-0.4	13.7	AWT	WT	BWT	BWT	BWT
Zero Treatment	19	14.8	-6.0	8.0	AWT	WT	BWT	BWT	BWT