The Boreal Journey of Methyl Mercury

From Forest Harvest to Black Alder Swamps

Rose-Marie Kronberg
Faculty of Forest Sciences
Department of Forest Ecology and Management
Umeå

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Abstract

The bioaccumulating neurotoxin methyl mercury (MeHg) is largely recognized as a problem in the boreal landscape. Both forest harvest sites and wetlands have been identified as MeHg sources. However, studies on logging effects have reported mixed results, and furthermore a black alder swamp has been recognized as a significant and consistent MeHg sink. This thesis aimed at clarifying the influence of these environments on the fate of MeHg in boreal landscapes.

The impact of forest harvest on MeHg production and export was studied in two types of landscapes, undulating and flatter terrain. Based on MeHg soil pools and fluxes of MeHg in streams, I conclude that MeHg was net produced in the soils after clear-cutting, with up to a 10-fold increase. Further, clear-cutting in undulating terrain approximately doubled the MeHg export to surface waters. The increase in load corresponded to 14% of the MeHg export from forested land in Sweden. When comparing to both forested and wetland areas, the increase in load was almost 8%. Moreover, laboratory incubation experiments using stable Hg isotopes were used to determine factors influencing methylation and demethylation rates. Experiments with soils from clear-cuts and reference sites suggested that the increase in methylation after clear-cutting was controlled by methanogens and sulfate reducing bacteria. In contrast, clear-cutting did not affect demethylation rates.

Furthermore, we wanted to know whether degradation or retention in the soil caused a black alder swamp to be a MeHg sink. A spatial analysis showed that MeHg was net degraded in the soil. In addition, based on snapshot budgets of nine additional swamps, net degradation of MeHg appeared as a common feature of alder swamps. Finally, incubation studies including sterile controls were performed to understand the processes affecting Hg methylation and MeHg demethylation in the black alder swamp. By targeting specific microbial guilds, we identified active microbial communities, showing that in particular methanogens were responsible for the biotic demethylation. Also, high rates of abiotic MeHg degradation were observed throughout the swamp.

Altogether, these findings are important for forestry practice recommendations and landscape planning. Forestry operations clearly increase the MeHg load while black alder swamps mitigate MeHg produced in upstream environments. Therefore, I propose that riparian buffer zones and restoration of black alder swamps situated downstream MeHg sources would decrease the MeHg load to downstream surface waters.

Keywords: mercury, methyl mercury, methylation, demethylation, boreal forest, soil, wetlands, forest harvest, water quality, restoration, *Alnus glutinosa*

Author's address: Rose-Marie Kronberg, SLU, Department of Forest Ecology and Management, SE-901 83, Umeå, Sweden
E-mail: rose-marie.kronberg@slu.se

In loving memory of Åke Kronberg

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Kronberg R-M, Drott A, Jiskra M, Wiederhold J G, Björn E, Skyllberg U. (2014a). Increased formation and export of methyl mercury after boreal forest harvest. *Under review in Nature Geoscience*.

II Kronberg R-M, Björn E, Skyllberg U. (2014b). The role of sulfate reducing bacteria and methanogens as potential methylators in soils of boreal forest harvest sites. *Manuscript*.

III Kronberg R-M*, Tjerngren I*, Drott A, Björn E, Skyllberg U. (2012). Net degradation of methyl mercury in alder swamps. *Environmental Science and Technology,* 46 (24), 13144-13151.

IV Kronberg R-M, Schaefer J K, Björn E, Skyllberg U. (2014c). Methanogens and abiotic processes drive MeHg degradation in a black alder swamp. *Manuscript*.

*Equal contributors

Abbreviations

16S	Component of the 30S small subunit of prokaryotic ribosomes
ANOVA	Analysis of variance
BES	Bromoethanesulfonic acid
cDNA	Complementary DNA
CH_2Cl_2	Dichloromethane
CH_4	Methane
CO_2	Carbon dioxide
$CuSO_4$	Copper sulfate
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
dsrB	Dissimilatory (bi)sulfite reductase
Fe(II)	Ferrous iron (reduced form)
Fe(III)	Ferric iron (oxidized form)
FeOOH	Iron oxide
GIS	Geographic information system
H_2SO_4	Sulfuric acid
Hg	Mercury
hgcA	Hg methylating corrinoid binding protein
Hg^{II}	Divalent mercury
Hg_{inorg}	Inorganic mercury
Hg_{TOT}	Total Hg
HS^-	Bisulfide
IRB	Iron reducing bacteria
KBr	Potassium bromide
k_d	Potential demethylation rate constant

k_m	Potential methylation rate constant
mcrA	Methyl coenzyme-M reductase
MeHg	Methyl mercury
merA	Mercuric reductase
merB	Organomercurial lyase
ML	Marine limit
MnO_2	Manganese(IV) oxide
MoO4	Molybdate
NaN_3	Sodium azide
NO_3	Nitrate
pH	$-\log[H_3O^+]$, measure of hydrogen concentration
qPCR	Quantitative (real-time) polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SO_4	Sulfate
SOC	Soil organic carbon
SRB	Sulfate reducing bacteria
STEB	Sodiumtetraethylborate
SUVA	Specific UV-absorbance

1 Introduction

1.1 Mercury in the Environment

Mercury (Hg) is naturally released to the atmosphere as the volatile elemental Hg (Hg^0) through e.g. weathering and volcano eruptions. In addition, Hg is released from fossil fuel combustion, mining, and industrial processes. These anthropogenic activities have increased the Hg load by a factor of about three since preindustrial times (Selin 2009). Hg^0 travels in the atmosphere for up to a year until it deposits, and accordingly Hg is present in both terrestrial and aquatic biomes. Another consequence is that also remote areas are polluted by Hg (Lindqvist & Rodhe 1985). Methyl mercury (MeHg) is the most common organic form of Hg and a potent neurotoxin. The major toxicological consequence of MeHg is that it readily passes the placental and blood-brain barriers with impairments on fetal development (Bridges & Zalups 2010). Additionally, MeHg efficiently bioaccumulates in the aquatic food web with fish consumption being the primary human exposure pathway (Mergler et al. 2007).

In Sweden, Hg levels in fish greatly exceed the European Union threshold value of 0.02 mg Hg kg^{-1} (EU 2008) in most of the hundred thousand lakes. Due to this situation there are Swedish recommendations of a limited consumption of certain fish species. This extensive Hg contamination of fish in Sweden is mainly attributed diffuse long-range Hg sources (Munthe et al. 2007). Although the Hg emissions have been reduced, a recent Swedish study reported increased pike Hg concentrations in 36% of the studied lakes (n=25) between 1994 and 2006 (Åkerblom et al. 2012). This increase was positively correlated to total organic carbon in the lake water. Further, increased hydrological export of organic matter carries with it inorganic divalent Hg (Hg^{II}) and MeHg from terrestrial to aquatic environments (Grigal 2003;

Cortizas & Biester 2007), which increases the potential for both methylation and biomagnification of Hg.

1.2 Methyl Mercury Formation and Degradation

The pool of methyl mercury is the net of two processes: methylation of Hg^{II} and demethylation of MeHg. Since demethylation rates usually are lower and less variable (Lambertsson & Nilsson 2006), the net result of the two processes is most often a formation of MeHg.

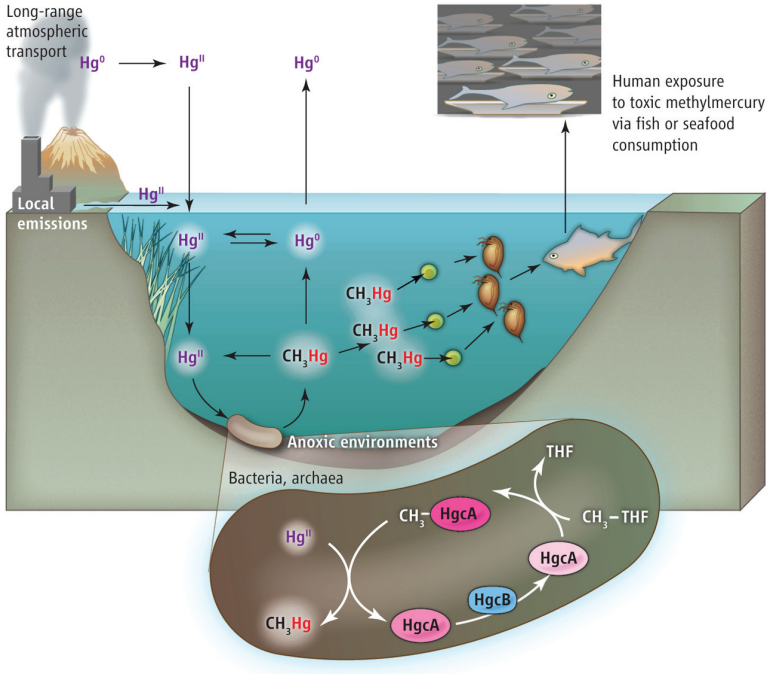

Figure 1. The mercury geochemical cycle. Hg is methylated to CH_3Hg (MeHg) in anoxic environments by microorganisms, one of which is illustrated in the insert. Different colors for the HgcA protein indicate different redox states of the corrinoid HgcA enzyme. HgcB is an electron donor required for corrinoid cofactor reduction. THF, tetrahydrofoalte. Reprinted from Poulain & Barkay (2013), with kind persmission from AAAS.

Methylation of Hg^{II} is a mainly biotic process (Jensen och Jernelöv 1969) carried out by a wide range of obligate and facultative anaerobic microorganisms. Recently a gene pair, *hgcAB*, was reported to be key for methylation (Parks et al. 2013), a finding which will be helpful in developing tools to study the formation of Hg and identifying novel methylation organisms

(Poulain & Barkay 2013). Figure 1 illustrates the Hg geochemical cycle, including the role of *hgcA* and *hgcB* in methylation by microorganisms. The microbial groups so far identified to methylate Hg are: 1) sulfate reducing bacteria (SRB) (Compeau & Bartha 1985), 2) iron reducing bacteria (IRB) (Fleming & Mack 2006; Kerin et al. 2006), 3) methanogens (Hamelin et al. 2011; Yu et al. 2013), and 4) firmicutes (Parks et al. 2013; Gilmour et al. 2013). Abiotic pathways of methylation have also been reported, e.g. by the action of humic substances (Nagase et al. 1982), but the importance of this mechanism compared to biotic methylation is arguable. Biotic methylation is controlled by several factors, such as electron donors, electron acceptors (e.g. sulfate (SO_4) and Fe(III)), temperature, and redox conditions (Ullrich et al. 2001). Another crucial factor is the bioavailability of Hg^{II} (Benoit et al. 2003) where dissolved organic matter (DOM), pH, redox, and sulfide concentrations all are influential by regulating the Hg^{II} speciation. Neutral, aqueous forms of Hg-sulfides, e.g. $Hg(SH)_2^0$ (Benoit et al. 1999; Drott et al. 2007), and low molecular mass organic thiols are suggested to increase the uptake of Hg for methylation in bacterial cells (Schaefer & Morel 2009).

For MeHg demethylation, both biotic and abiotic pathways are environmentally relevant. The most important abiotic pathway in freshwater environments is photodemethylation (Seller et al. 1996). Photodegradation takes place in open water bodies, where UV-irradiation induces a cleavage of the C-Hg bond. This process is affected by the presence of DOM, which promotes the process through radical formation and complexation, whereas light attenuation by DOM inhibits the process (Fernández-Gómez et al. 2013). A second abiotic demethylation pathway is MeHg reacting with hydrogen sulfides (Deacon 1978), and a third is degradation by selenoamino acids (Khan & Wang 2010; Asaduzzaman & Schreckenbach 2011), although the importance of this process in the environment is unknown. Biotic demethylation occurs through two known mechanisms, 1) the combined actions of the organomercurial lyase (MerB) and mercuric reductase (MerA) encoded on the Hg resistant *mer* operon (Schaefer et al. 2004) where MeHg is converted to Hg^0 and CH_4, and 2) an "oxidative" pathway where MeHg is released as Hg^{II} and either CO_2 and/or CH_4, allowing for potential recycling of Hg^{II} back to MeHg (Marvin-DiPasquale & Oremland 1998). Demethylation is just as methylation affected by several factors, such as microbial activity, organic matter content, and bioavailability of MeHg (Marvin-DiPasquale et al. 2000).

1.3 Impacts of Wetlands and Clear-cutting on Methyl Mercury

1.3.1 Clear-cuts

In the boreal zone, the dominating forest harvest technique is clear-cutting (Kreutzweiser et al. 2008), where all the trees in an area are felled and the soil is subsequently scarified before planting to enhance plant growth. Clear-cutting has a large impact on the soil, not only through scarification but also by the compaction due to the heavy machinery used. Additionally, the ground water table increases because of the loss of evapotranspiration from trees, and the soil temperature rises as an effect of increasing solar radiation reaching the ground.

Studies on the effect of clear-cutting on MeHg formation and export have shown varied results (Bishop et al. 2009). For example, Porvari et al. (2003) reported a large increase in stream MeHg concentrations in a Finnish catchment, while Sørensen et al. (2009) only observed a marginal increase concentrations in northern Sweden. Likewise, Eklöf et al. (2012) did not see any major effects after stump harvest, although they did see that forest harvest in many cases had an effect on MeHg concentrations. Further, a synoptic study of streams adjacent to clear-cut areas in Sweden reported increases in MeHg concentrations on sites above the marine limit (ML), but not below (Skyllberg et al. 2009). Also, there have been reports on increased Hg concentrations in biota of lakes in Canada impacted by clear-cutting (Garcia & Carignan 1999; Garcia & Carignan 2000; Desrosiers et al. 2006).

1.3.2 Wetlands

There are many definitions of wetlands, usually including criteria of the water table close to or at the soil surface during long enough time of the year to support hydrophilic vegetation (Gunnarsson & Löfroth 2009). Futhermore, there are many types of wetlands, and relevant for this thesis are peatlands and forest swamps. We refer to peatlands as peat-accumulating wetlands, such as bogs and fens dominated by *Sphagnum* spp. Forest swamps are defined as wetlands not accumulating peat, with mineral soil, a tree cover and relatively nutrient rich understory vegetation.

Wetlands are largely anaerobic environments, but can also have seasonal fluctuations with the oxic/anoxic interface moving during the year, making them dynamic redox environments. This feature stimulates methylation and most wetlands are thus sources of MeHg (Tjerngren et al. 2012, St Louis et al. 1994) and very important environments in the Hg cycling despite their relatively small area (4-6%) of the total terrestrial biome (Mitsch & Gosselink 1993). Also, due to their ecosystem services, as improving water quality and supporting high biodiversity (Mitsch & Gosselink 2000), many wetlands are

now restored after previously being drained for agricultural or silvicultural purposes (Zedler & Kercher 2005). The restoration of wetlands poses a dilemma when also considering the mercury issue: restoring ecosystem services while increasing the MeHg load to downstream environments?

1.4 Objectives

- Are some types of landscapes more prone to increases in MeHg load after clear-cutting?
- Is the increase in load mostly due to mobilization of already existing MeHg, or is MeHg net formation greater after forest harvest?
- Which factors influences the export of MeHg after clear-cutting?
- Which factors are key for the increase in MeHg formation in soils of clear-cut areas?
- What makes a black alder swamp (Edshult) a MeHg sink, soil retention or degradation?
- Are black alder swamps generally MeHg sinks?
- Which processes control the degradation of MeHg in alder swamps?

2 Materials and Methods

2.1 Study Sites

2.1.1 Clear-cuts and References

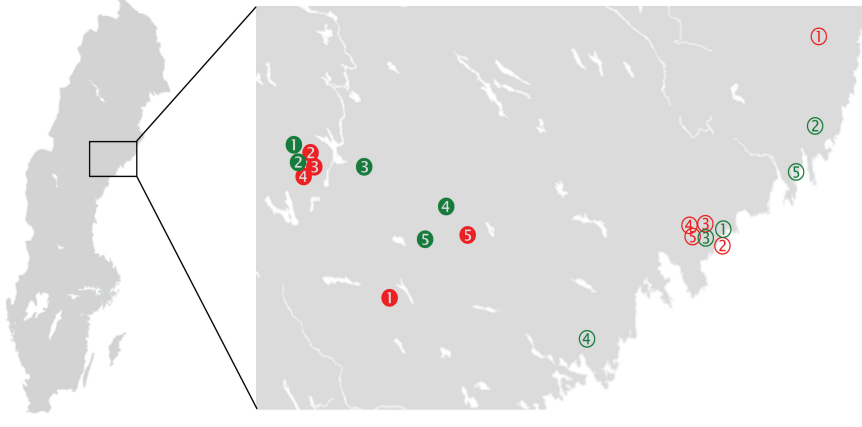

Figure 2. Map of Sweden illustrating the locations of clear-cuts (red) and references (green). Above ML is represented by filled circles and below ML is represented by un-filled circles.

For paper I, ten reference sites and ten clear-cut sites were selected with the assistance of Holmen Skog AB, serving as land host. Five clear-cuts and five references were situated above and below the ML of the ancient Baltic Sea, respectively (Figure 2). Above ML was defined as >300 m m.a.s.l. and below ML as <200 m.a.s.l. The location of sites above and below the ML was used as a method to get two contrasting sets of sites. Above the ML the terrain is more undulating, dominated by uplands with steeper slopes. Below ML the percentage of wetlands and formerly drained peatlands is higher and in some

areas the geochemistry can differ significantly from above ML, due to that the land below ML has been a sea floor. The tree layer of all sites, before clear-cutting, consisted of >70% Norwegian spruce (*Picea abies*), and the ground vegetation was dominated by billberries (*Vaccinium myrtillus*). The reference sites were dominated by >80 years old Norway spruce. All clear-cuts were harvested in 2009 followed by soil scarification. A first-order stream drained each site and the watersheds at the sampling point in each stream were defined. The watersheds of the clear-cut sites had between 30-79% of clear-cut area. In paper I, stream water data from all sites were used to calculate flow-weighted concentrations and catchment exports. Soil samples were taken at one occasion from a subset of sites for determination of MeHg and Hg_{inorg} pools. For paper IV, two references and two clear-cuts were selected for the determination of methylation and demethylation rate constants in soils. More details are found in paper I and II.

2.1.2 Wetlands

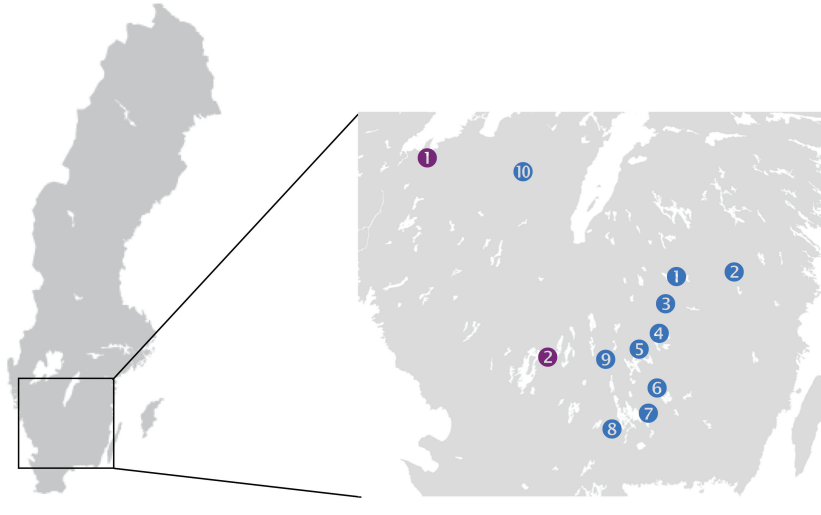

Figure 3. Map of Sweden showing the locations of the wetland sites. Purple represents *Sphagnum* peatlands: **1** Långedalen, and **2** Ystebo. Blue represents forest swamps. All sites were dominated by black alder (*Alnus glutinosa*), except Åryd that was dominated by downy birch (*Betula pubescens*). **1** Edshult, **2** Kvillehult, **3** Löneberg, **4** Speltorpet, **5** Kolsboda, **6** Åryd, **7** Nybygget, **8** Steglehylt, **9** Klasentorp, and **10** Trestena.

Fluxes and transformation processes of Hg^{II} and MeHg were studied in all wetlands at one occasion in either 2009 or 2010. Edshult was sampled during 2006 to 2012 with focus on soil samples for incubation studies to quantify and

learn about methylation and demethylation processes. Wetland sites were selected with the assistance of the Swedish Forest Agency (Skogsstyrelsen), who provided GIS material of identified black alder (*Alnus glutinosa*) environments. A brief description of the sites follows below, and more details are found in paper III.

The site Edshult is located in southern Sweden, about 50 km from the Baltic Sea coast (Figure 3) and has an area of 0.042 km^2. The inlet stream drains a total area of 0.48 km^2, of which 17.4 % is a pine bog, 6.2 % a clear-cut area, and 76.4 % consists of drained peat soils forested with Norway spruce (*Picea abies*). These upstream environments generate humic substances, resulting in inlet stream water rich in dissolved organic carbon (DOC). Edshult is dominated by black alder in the upper half of the swamp while in the lower half the tree cover is sparse and consisting mostly of downy birch (*Betula pubescens*). The understory vegetation in this part is dominated by a broad-leaved sedge, wood club-rush (*Scirpus sylvaticus*).

A *Sphagnum* peatland, Långedalen, was studied alongside Edshult as a contrasting site, representing the more common bog and fen type wetlands. Långedalen is a valley-bottom wetland located at the table mountain Hunneberg (70 m.a.s.l.), approximately 40 km from the west coast of Sweden (Figure 3). This site has two defined parts: the upstream section is a ombrotrophic bog and the downstream part develops from a poor into intermediate fen (A and B, respectively, Figure 5). The bog is dominated by *Sphagnum* spp., and the fen by both *Sphagnum* and *Carex* spp. species. In addition the fen has a sparse deciduous tree cover.

Figure 4. From left to right: pictures from sites Klasentorp, Kolsboda, Löneberg, and a black alder trunk at site Edshult.

An additional ten forest swamps and one *Sphagnum* peatland were sampled at one occasion for paper III: Speltorpet and Trestena in 2009 and the remaining sites in 2010. All forest swamps were dominated by black alder except for Åryd, which was dominated by downy birch. All sites had clearly defined inlet and outlet streams with DOC rich waters entering the sites.

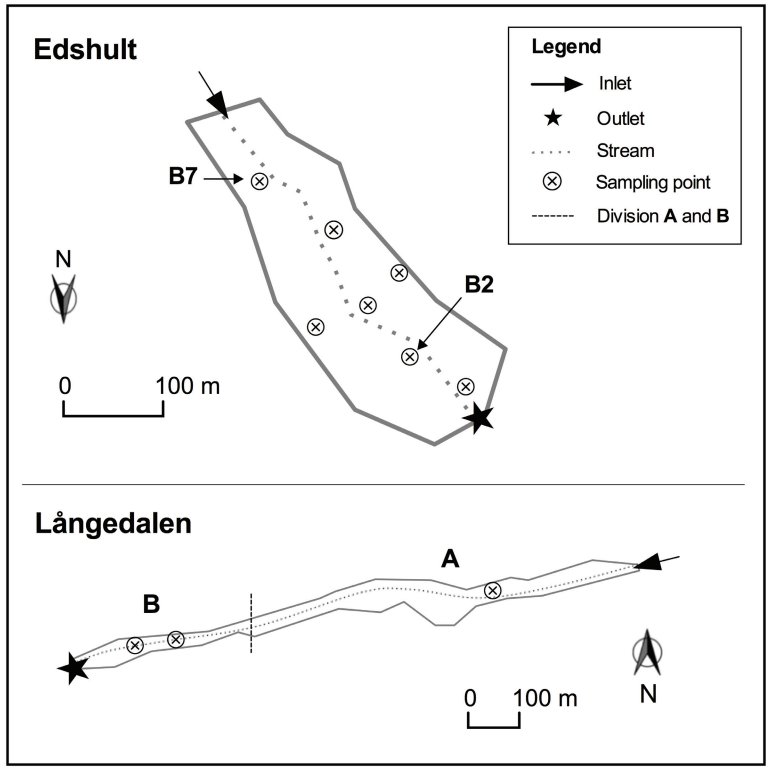

Figure 5. Illustration of sites Edshult and Långedalen with stream, inlet and outlet, and sampling locations indicated.

2.2 Sampling and Chemical Analyzes

Stream waters were sampled to evaluate the elements and compounds related to the Hg biogeochemical cycle, while soil samples were collected mainly to assess Hg and MeHg pools, and methylation and demethylation processes. Based on the finding that Edshult was a consistent MeHg sink, snapshot budgets were also determined based on one sampling occasion to evaluate whether black alder swamps in general act as MeHg sinks.

In the clear-cuts and reference sites, soil sampling was done in late June and early July of 2011 for determination of total Hg (Hg_{TOT}), MeHg, and other relevant elements. In August 2012, soils were collected for incubation studies as described below. Stream waters from clear-cuts and references were sampled at eight occasions during the ice-free season for one year, in total ~160 samples. Stream water samples were taken from all wetlands in May 2010, except sites Trestena and Speltorpet that were sampled in May 2009. In 2010, soil was sampled from all sites at the same occasion as the stream sampling and Edshult was sampled every year from 2006 – 2012. Soil samples were taken with minimal exposure to oxygen and the cores were stored, handled, and incubated in N_2 atmosphere using a glovebox.

All stream water samples and porewater samples were analyzed for elements and compounds relevant for Hg biogeochemistry, e.g. SO_4, inorganic sulfides (mainly HS^-), Fe(II)/(III), DOC, and specific UV absorbance (SUVA). For MeHg determination in soil, an in-house aqueous stable isotope enriched tracer (Snell et al. 2000) was added to the sample prior to extraction. Extraction was done by $KBr/CuSO_4/H_2SO_4/CH_2Cl_2$ and in the last step MeHg was back-extracted to ultrapure water, and samples were frozen until analysis. For stream water MeHg determination, an enriched stable isotope tracer was added as internal standard. The samples were refrigerated and left to equilibrate for 24 h before they were frozen until analysis. After thawing, the MeHg was ethylated with sodiumtetraethylborate (STEB) and purged and trapped onto Tenax® traps (Lambertsson & Björn 2004) before thermal desorption and analysis on GC-ICPMS (Larsson & Frech 2003). Stream water samples for Hg_{TOT} determination were sent to ITM, Stockholm University, for analysis according to the US EPA method 1631 (EPA 2002). Soil Hg_{TOT} was analyzed through thermal decomposition atomic absorption spectrometry on a Leco AMA 254-analyzer.

Figure 6. From left to right: soil sampling at site Edshult in May 2010 and October 2011, and at site Steglehylte in May 2010.

2.3 Incubation Studies

Incubation studies were performed on the collected soil cores to determine the potential methylation and demethylation rate constants, k_m and k_d (Lambertsson & Lundberg 2001). These incubations were performed both with and without addition of a variety of electron acceptors, electron donors, and inhibitors of specific microbial groups (see papers II, III and IV for details).

After homogenization, approximately 10 g of soil was weighed in 50 mL falcon tubes, denoted T0. Enriched stable isotope tracers of Hg_{inorg} and MeHg, e.g. $^{201}Hg(NO_3)_2$ and $Me^{204}Hg$, used for determination of k_m and k_d were added along with potential amendments. The sample was thoroughly mixed and subsequently half of the soil was transferred to a second tube denoted T48. This tube was incubated in the dark at room temperature for 48 h in the glovebox, after which it was frozen. The T0 tube were frozen within maximum 15 minutes of isotope and amendment addition and was used as a control of the processes at 0 hour. In 2011 and 2012, soil from the 48 h incubations were taken out for phylogenetic analyses (described below).

As electron donors, a mix of ethanol, acetate, pyruvate, lactate and butyrate was added, and as electron acceptors nitrate (NO_3), SO_4, amorphous manganese and iron oxides (MnO_2 and FeOOH) were used. As inhibitors of specific microbial groups, we added molybdate (MoO_4) to inhibit sulfate reducers and bromoethanesulfonic acid (BES) to inhibit methanogens. In 2012, we also sterilized samples through either autoclaving or by azide (NaN_3) addition to investigate possible abiotic processes. Samples incubated for 0 and 48 hours were then analyzed for MeHg as described above.

After signal deconvolution (Qvarnström & Frech 2002), the rate constants k_m and k_d were calculated by a first-order kinetic model, assuming negligible demethylation of $Me^{201}Hg$ and methylation of ^{204}Hg during the incubation (Hintelmann et al. 2000). Potential Hg_{inrog} methylation rate constants (k_m, d^{-1}) and potential MeHg demethylation rate constants (k_d, d^{-1}) were calculated using equations 1 and 2.

$$k_m = ([Me^{201}Hg]_{T48} - [Me^{201}Hg]_{T0}) / [^{201}Hg(NO_3)_{2tracer}] \quad (1)$$

$$k_d = -1 \times \ln([Me^{204}Hg_{tracer}]-([Me^{204}Hg_{T0}]-[Me^{204}Hg_{T48}]))- \ln([Me^{204}Hg_{tracer}]) \quad (2)$$

2.4 Phylogenetic Analyzes

Phylogenetic analyses were done to investigate the microbial communities present and active in the soils in relation to the methylation and demethylation

incubation studies. During 2010 to 2012, native and T0 samples were taken in the field during sampling and after homogenization in the glovebox. Subsamples were also taken from all T48 incubation samples. About 1 g of soil was added to 3 mL LifeGuard solution (MoBio, Carlsbad, CA), thoroughly shaken, and shipped to the lab where stored in at -80°C until analysis.

At the lab, genomic DNA and total RNA were extracted and separated, and cDNA was generated. Genomic DNA and cDNA were then subjected to PCR amplification, cloning and sequencing to investigate the diversity of genes and transcripts targeting the methyl coenzyme-M reductase (*mcrA*), dissimilatory (bi)sulfite reductase (*dsrB*), and the Hg methylating corrinoid binding protein (*hgcA*). Only high quality sequences were kept for further study. *Geobacter*-specific 16S rRNA abundance was determined through qPCR amplification according to the method described in Cummings et al. (2003).

2.5 Flow Weighted Stream Data and Export Budgets

Water fluxes of streams were calculated from water height measures collected hourly using data loggers. The water height was recalculated to water flux using water flow measurements from salt dilution (Moore 2005) at minimum four occasions covering both low and high flows. The water flux was divided into eight periods over the year, each representing one sampling, with the sampling occasion in the center of the period. For the first and last period, the sample is instead at the start respectively at the end of the period. The annual average water flux weighted concentrations were calculated by multiplying the concentration of each specific parameter with the accumulated water flux for of the eight periods, summing the products and dividing by the total annual water flux.

To obtain exports, we determined the watersheds at the logger position of each site by GIS hydrology tools and visual inspection during a rain event. At clear-cut sites, the clear-cut and standing forest areas were quantified. Areas of wetlands and previously drained peatlands were determined, as well as elevation differences and topography roughness. Details are found in paper I.

2.5.1 Uncertainties in Water Flux Determinations

To calculate the water flux, linear, exponential and/or power functions were used. The uncertainties in the calculated fluxes are mainly due to the model performance at high flow rates. To incorporate this uncertainty in the results, fluxes were calculated as an average from several models simulating the water flux beyond the largest flux we captured in our measurements. We estimate that the uncertainty is ±15%. At two sites, (CC4 BML and REF3 BML) we

were not able to determine the watershed areas, and those sites were therefore excluded from the export calculations. For details, see paper I.

2.5.2 Scaling Up to a National Perspective

To explore the relevance of our MeHg flux weighted stream data, we estimated the implications of our results on a national level. Because DOC is a parameter commonly measured in streams, and is by far the dominant transporting agent for MeHg, we based this calculation on our measured MeHg to DOC mass ratio in combination with previously reported DOC concentrations in northern coniferous forests (Schelker et al. 2012; Laudon et al. 2013). We calculated the mass of annual MeHg export as a consequence of clear-cutting in Sweden as compared to the annual MeHg export from both the Swedish forest without clear-cuts, as well from wetlands. Based on earlier studies (Skyllberg et al. 2009; Bishop et al. 2009) we assumed the effect would last five years. Details are found in paper I.

2.6 Statistics

In paper III, MeHg concentrations at Edshult were evaluated by dividing the site into an upstream part (-40 to 250 m) and a downstream part (270 to 400 m). Soil MeHg concentrations were normalized both to the mean within each year and soil organic carbon (SOC). Data were tested for normal distribution and equal variance using Shapiro-Wilkinson test, and a one-way ANOVA was used to test the difference between the two populations, using the software PASW stastistics 18 (SPSS, Inc, USA). Further, Minitab 16 (Minitab Inc., Saltsjöbaden) was used for linear regressions to analyze the relationship between %MeHg, k_m, and k_d with distance from stream inlet. Linear regression was used to test the relation between %MeHg and k_m/k_d as well. The data sets were tested for normal distribution and equal variance prior to analysis.

The statistical analyses for paper I, II, and IV were done using the software GraphPad Prism (GraphPad Software Inc., USA). For the wetland incubation studies (paper IV), significant differences between the control and the treatments were tested through ANOVA followed by Tukey's multiple comparisons test, 4 and 8 comparisons in total for 2011 and 2012, respectively. For the incubation studies of clear-cuts and references (paper II), ANOVA followed by Tukey's multiple comparisons test was used as well, resulting in 4 comparisons per sample. All tests were carries out using a 0.05 significance level.

3 Results and Discussion

3.1 The Impact of Clear-cutting on Methyl Mercury Net Formation and Export

Previous studies have shown effects of clear-cutting on MeHg export or concentrations in streams, although most of these studies only include one or two sites making it difficult to generalize the results. Our aim with this study was to have a sufficient number of sites in two contrasting environments, allowing us to show a significant difference between sites, provided there would be one. We had an indication from a synoptic study that sites above the ML are more sensitive to clear-cutting in this respect (Skyllberg et al. 2009). Therefore, in paper I, the streams of 20 sites were studied during one year and soils were sampled at one occasion for Hg_{TOT} and MeHg surface soil pools. In paper II, soil samples were collected for incubation studies to determine potential methylation and demethylation rate constants in relation to various amendments.

3.1.1 MeHg Net Formation in Soils

Figure 7 illustrates the spatial distribution of %MeHg and soil wetness at both references and clear-cuts. The clear-cuts had significantly higher absolute concentrations and soil pools of MeHg as well as %MeHg of total Hg than reference sites, especially at intermediate distance from the stream. We hypothesize that this increase is due to a formation of new discharge areas after clear-cutting. The higher %MeHg appears to follow the increase in soil wetness, not surprising given that methylation of Hg is an anaerobic process. In contrast to the MeHg soil pool, the Hg_{TOT} soil pool did not differ between references and clear-cuts. Thus, these results infer that there is a great increase in MeHg net formation in soils after clear-cutting. More details are found in paper I.

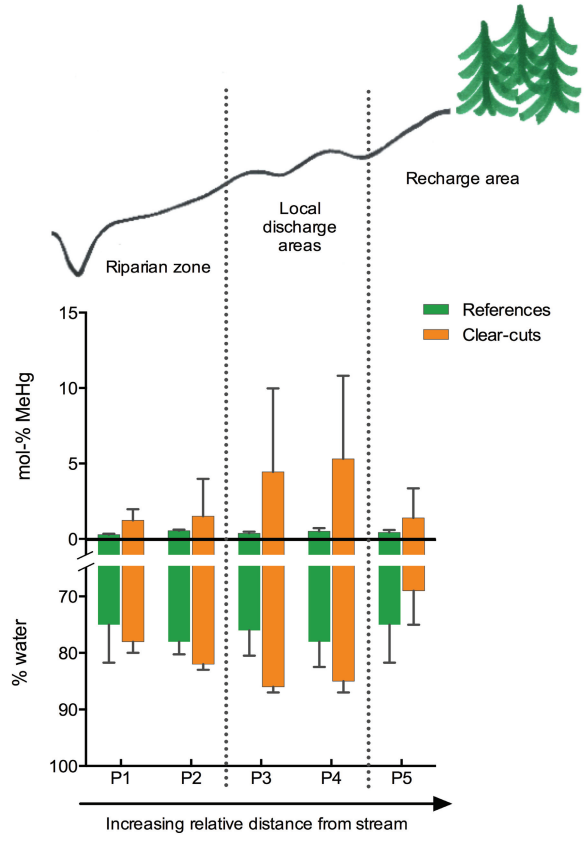

Figure 7. Spatial distribution of %MeHg (of Hg_{tot}) and %wet mass. Data are averages of four references and four clear-cuts and samples taken at five locations (P1 to P5) at each site. Error bars designate standard deviation, (n=4).

3.1.2 MeHg Stream Concentrations and Export

The 20 sites, 10 clear-cuts and 10 references, were distributed equally above and below the ML to cover two contrasting environments in the boreal landscape. Above the ML the landscape is more undulating, as reflected by the topography roughness (Table 1), whereas below the ML the land is flatter and wetlands and drained peat soils are more common. Above the ML, MeHg concentrations in references were less variable and lower than the stream concentrations at the clear-cut sites (0.30±0.092 and 0.54±0.32 ng L^{-1}, respectively). Interestingly, we observed no difference in MeHg concentrations between reference (0.68±0.24) and clear-cut sites (0.44±0.13) below ML. This

lack of difference was probably due to already high steam concentrations in the reference sites compared to above ML. The MeHg watershed export follows the same pattern as the stream concentrations (Table 1). Below ML the sites are impacted by wetlands to a much higher degree, explaining the already high MeHg stream concentrations at reference sites. When the percentage of clear-cut area in the harvested watersheds was taken into consideration, the effect above ML became even more pronounced. Below ML this calculation made no difference for the result. To conlcude, in areas above the ML with undulating terrain and few wetlands the MeHg load approximately doubles after clear-cutting.

Table 1. *Average ± standard deviation for MeHg concentration and export from catchments (not considering % clear-cut area), % peat soil (wetland + draind peat soils) of the watershed area, and the topography roughness (average difference in altitude between the highest and lowest point in the watershed normalized to total watershed area).*

Sites	MeHg	MeHg export	Peat soil area	Topography roughness
	ng L^{-1}	mg ha^{-1}	%	m ha^{-1}
CCs above ML	0.54 ±0.32	1.6 ±0.44	0 ±0	2.6 ±0.9
REFs above ML	0.30 ±0.092	0.65 ±0.10	4.0 ±5.8	3.2 ±1.6
CCs below ML	0.44 ±0.13	1.9 ±0.08	8.8 ±6.8	0.3 ±0.3
REFs below ML	0.68 ±0.24	2.5 ±0.43	15 ±12	0.6 ±0.5

By applying our results to a national scale, we estimated the impact of clear-cutting in Sweden. The MeHg to DOC ratios measured at our sites and known DOC concentrations from a range of northern coniferous forests (Schelker et al. 2012; Laudon et al. 2013) were used in the calculations. We also classified the forested area of Sweden into categories representing the different types of landscapes in our study, and we assumed a five year duration of the increase in MeHg concentrations. The outcome of these calculations was that the practice of clear-cutting increases the MeHg load to streams with 14% compared to managed forests without clear-cutting. When adding the MeHg export from wetlands, the increase in MeHg load by clear-cutting is 7-8% of the total export. For more details, see paper I.

3.1.3 Incubation Studies Determining Methylation and Demethylation

Figure 8 shows the effect of adding BES, MoO_4, SO_4, and Fe to three soils from the clear-cut site CC2. Results from the other sampled clear-cut site were similar, and details for that site and the two references are found in paper II.

Location P1 is in the riparian zone (~0.5 m from the stream), and P3 and P4 are located at an intermediate distance from the stream (20 - 40 m) in an area of newly created discharge areas. The potential methylation rate constants display a quite similar pattern for all three soils. Additions of BES and MoO_4 results in an almost 50% decrease in methylation in all cases but one (MoO_4, CC2 P3). Furthermore, the amendment of sulfate roughly doubles the methylation rate constants in P3 and P4. The k_d is stimulated by most amendments, and most interestingly by both BES and MoO_4. Sulfate additions increase demethylation rates in all cases, suggesting a large potential role for SRB when not sulfate limited. Aother possibility is that SRB at the time of incubation are living in syntrophy with methanogens (Pak & Bartha 1998; Hamelin et al. 2011), which could cause a lower methylation rate (in the control) and would explain the increase in k_d by both BES and MoO_4.

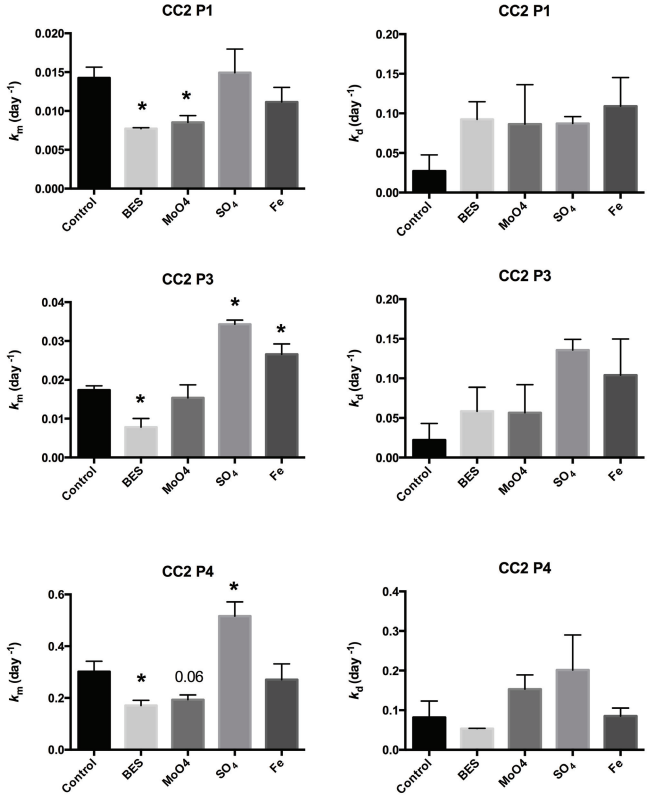

Figure 8. The effect of specific metabolic inhibitors (BES and MoO_4) and electron acceptors (SO_4 and FeOOH) on potential methylation and demethylation rate constants, k_m and k_d, respectively, for three locations with increasing distance from the stream (see figure 7) at site CC2. *indicates significant differences ($p<0.05$) as compared to the control treatment.

At the time of incubation though, the demethylation was not contributing significantly to the MeHg yield, implied by the strong correlation between %MeHg and k_m for all sites (see paper II). In fact, there was no difference in demethylation rates between harvested sites and references. Accordingly, this strongly suggests that methylation is the most important process in the net formation of MeHg, and from the effect of inhibitors, that methanogens and SRB are the most important microbial communities controlling methylation in these environments.

3.2 Net Degradation of Methyl Mercury in Black Alder Swamps

In a previous mass balance input-output study (Tjerngren et al. 2012), a black alder swamp was found to be a consistent MeHg sink over four years. In the light of this discovery, more studies were undertaken at this particular site: Edshult.

3.2.1 Black Alder Swamps as MeHg Sinks

For paper III, the soils of site Edshult was sampled and studied during five years, 2006 - 2010. At each occasion, Hg_{TOT}, MeHg, k_m and k_d was determined. Although there was a large inter-annual variation in Hg_{TOT} and MeHg concentrations, %MeHg (of Hg_{TOT}) always decreased from stream inlet to stream outlet while Hg_{TOT} varied without showing any trends. A one-way ANOVA showed that the downstream sampling points (270 – 400 m) had significantly lower MeHg concentrations than the upstream ones (0.66 as compare to 1.34 ng MeHg g^{-1} SOC^{-1} annual $average^{-1}$, p=0.0006). Hence, the soil concentrations and pools of MeHg decreased following the flow direction of the stream through the swamp.

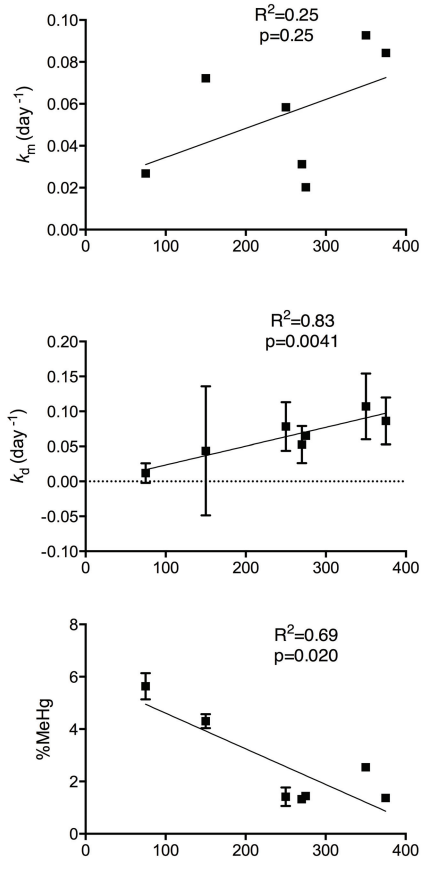

Figure 9. Potential methylation and demethylation rate constants and %MeHg with increasing distance from stream inlet, Edshult 2008.

The most extensive soil sampling was done in 2008, and linear regressions of k_m, k_d and %MeHg plotted against increasing distance from stream inlet is shown in Figure 9. The pattern supports the result from the ANOVA of the MeHg concentrations. %MeHg increases with distance from inlet, while k_d clearly increases and k_m varies greatly but has no trend. These trends were similar during all five years of studies. Altogether, these results clearly illustrate the link between soil processes and MeHg yields. In this case that net degradation of MeHg was the reason for Edshult being a net MeHg sink during four years (Tjerngren et al. 2012).

To evaluate the generality of black alder swamps as MeHg sinks, snapshot budgets for additional nine black alder swamps (Figure 3) were determined, based on one sampling occasion. All of the swamps were either sinks or had steady state budgets for MeHg. As contrasting sites, the two *Sphagnum*

peatlands were sampled, and they were both shown to be significant sources of MeHg. These results indicate that net degradation of MeHg is a common feature of black alder swamps. More details are found in paper III.

3.2.2 Methylation and Demethylation Studies

During 2010 to 2012, incubation studies at Edshult were performed with the addition of various electron acceptors, electron donors, and inhibitors of specific microbial groups. In 2012 a sterile control was included to evaluate the abiotic versus the biotic demethylation.

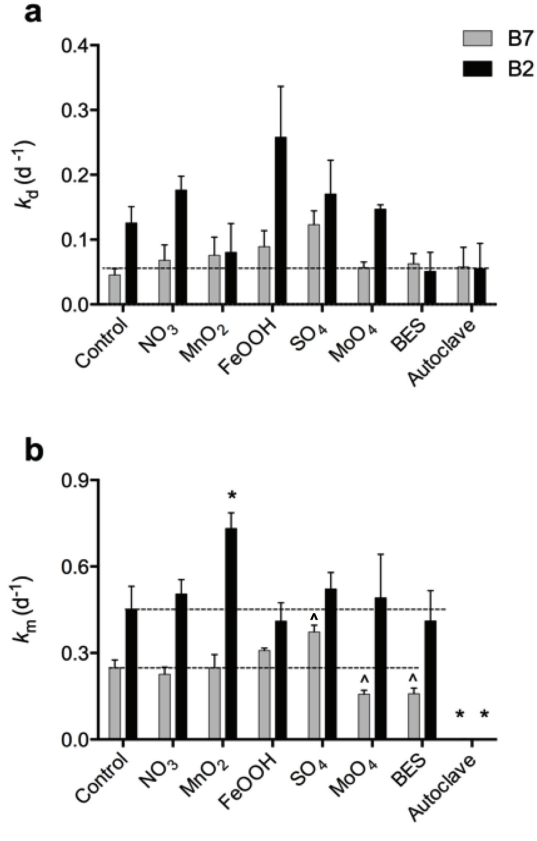

Figure 10. The effect of electron acceptors (NO_3, MnO_2, FeOOH, SO_4), two specific inhibitors (MoO_4, BES), and sterilization (Autoclave) on potential demethylation and methylation rate constants, **a** and **b**, respectively, as compared to control treatment. In **a**, the dashed line indicates the height of the Autoclave bars, while in **b**, the dashed lines indicate the height of the control treatment bar for B7 and B2 respectively. Asterisk (*) indicates significant difference ($p<0.05$), and caret (^) marginally significant difference ($p<0.08$), as compared to control. Soil sampled at two locations in site Edshult, August 2012.

The result was a display of high and consistent abiotic k_d (0.5 d^{-1}) in both parts of the swamp (Figure 10a). In the upstream part (B7), the demethylation was mainly abiotic but showed potential for stimulation of biotic demethylation. However, in the downstream part (B2) there was a biotic demethylation signal of the same magnitude adding to the abiotic signal. Accordingly, the combined affect of both abiotic and biotic demethylation in B2 explains the high net degradation and thus low %MeHg in this part of the swamp, relative to B7. Moreover, the addition of BES resulted in the same k_d as in the abiotic control, and the inhibition of SRBs (by MoO_4) stimulated demethylation. Thus, the microbial community mainly responsible for the biotic demethylation in B2 appear to be methanogens. Further, SO_4 and FeOOH additions stimulated demethylation, indicating that SRB and IRB could be degrading MeHg under certain conditions. The phylogenetic analyzes supports this hypothesis with *mcrA* transcripts being the most abundant, compared to *dsrB* and *Geobacter* rRNA (Table 2). Additionally, all *mcrA* transcripts clustered within *Methanomicrobia*, a group containing known Hg methylators (Yu et al. 2013). In conclusion, demethylation at Edshult is suggested to be due to a combination of abiotic pathways and the activity of mainly methanogens.

Table 2. *Results of phylogenetic analyzes from Edshult soils during 2011 and 2012.*

			Geobacter	**Transcripts**	
Year	**Site**	**Incubation**	rRNA	*dsrB*	*mcrA*
2011	B7	T0	+	-	nd
	B7	T48, Control	++	+/-	+
	B7	T48, +SO4	+++	-	nd
	B7	T48, +SO4/+C	+++	-	nd
	B7	T48, +C	+++	-	nd
2012	B7	T0	nd	-	-
	B7	T48, Control	nd	+/-	++
	B7	T48, +SO4	nd	++	++
	B7	T48, +Fe	nd	+	++
2012	B2	T0	nd	-	+/-
	B2	T48, Control	nd	-	-
	B2	T48, +SO4	nd	-	++
	B2	T48, +Fe	nd	-	++

- = below detection
+/- = very faint band, near detection limit
+ = low abundance, ++ = medium abundance, +++ = high abundance
nd = not determined

Considering the effect of autoclaving, methylation is purely biotic at both sampling locations. In the upper part, B7, methylation was stimulated by SO_4 and inhibited by BES and MoO_4, respectively inhibiting methanogenesis and sulfate reduction. Correspondingly, *mcrA* and *dsrB* transcripts were abundant in August 2012 at B7. Together, this indicates that the methylation potential in B7 is associated with the activity of methanogens and SRB. One possibility is that methanogens and SRB live syntrophically at Edshult, a common strategy in sulfate poor environments (Pak & Bartha 1998; Hamelin et al. 2011). The portion (~1/3) of the methylation potential unaccounted for could be attributed IRB, in this case quite likely due to their acitvity (Table 2) in the soils. The attempt to stimulate and inhibit methylation at B2 gave no clear result. The only significant difference, as compared to the control, was the addition of MnO_2, resulting in an increase in k_m, probably due to reduction of Mn by IRB (Lovley & Giovannoni 1993). Altogether, methanogens, SRB and IRB are suggested to play roughly equal roles in the methylation potential of the swamp. See paper IV for more details.

4 Conclusions and Future Perspectives

- Clear-cutting of coniferous forstes increases the net formation of MeHg in soils. The increase in net methylation is likely due to incraesed microbial activity, as stimulated by an improved availability of high quality organic matter and nutrients after clear-cutting. Furthermore, an increased groundwater table contributes to a higher anaerobic microbial activity, altogether generating more MeHg.
- Clearcutting in undulating terrain roughly doubles the MeHg load to surface waters.
- The increase in load after clear-cutting corresponds to 14% of the MeHg export from forested land in Sweden, and when comparing to wetlands about the same number. When comparing to forested and wetlands together the contribution from five years of clear-cutting effect makes up 7-8% of the total MeHg export.
- Microbial communities controlling the increase in methylation after forest harvest are mainly methanogens and SRB
- Black alder swamps act as sinks and net degrade MeHg
- In the black alder swamp Edshult, the net degradation is due to biotic demethylation by primarily methanogens in addition to a high abiotic demethylation signal
- Methylation rates are also high in Edshult, where methanogens together with IRB and SRB are important contributors
- Because many alder swamps are situated downstream majors sources of MeHg, such as forest clear-cuts and peatlands, they help to protect lakes and other surface waters from receiving high loads of MeHg. This role of alder swamps may be enhanced by actively restoring these environments.

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