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Influence of geochemistry and biology

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Abstract

Methylmercury (MeHg) is a neurotoxin mainly produced by microorganisms in suboxic and anoxic environments such as peatlands. Peatlands are an important source of MeHg in adjacent aquatic ecosystems, thus increasing the risk of human and wildlife exposure to this toxic compound. An improved understanding of factors limiting microbial net MeHg formation in peatlands could benefit the management and mitigation of this toxic compound particularly in peatland-rich landscapes.

In this thesis, a chronosequence trophic gradient of peatlands within the space of a few kilometers, all subjected to similar atmospheric deposition, underlying geology and climate patterns, was studied to determine the influence of biogeochemical factors on net MeHg formation in peat. Along the peatland chronosequence, higher net MeHg formation in peat soil of the younger peatlands was attributed to more nutrient rich conditions (Paper I). The same trend in net MeHg formation was observed in porewater, which was deemed more related to the shifts in the availability of electron acceptors for methylating microorganisms than to the abundance of electron donors (Paper II). The results of modeling the solubility of Hg(II) suggest that the net MeHg formation along the chronosequence could also be influenced by the supply of bioavailable Hg(II) to methylating organisms (Paper II).

The microbial community composition was significantly correlated to net MeHg formation along the chronosequence, with spatial patterns driven by environmental factors (Paper IV). Laboratory incubations with a combination of amended inhibitors/stimulators revealed the presence of different microbial processes in relation to the biogeochemistry. These differences are suggested to contribute to net MeHg formation along the chronosequence (Paper III). Quantitative gene expressions of specific microbial functional groups suggest that the role of SRB in net MeHg formation varied across the chronosequence, while methanogenic archaea were important for this across all the peatlands (Paper IV).

Keywords: methylmercury, mercury, peatlands, mercury methylation, microbial community, chronosequence

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Kvicksilvermetylering i boreala torvmark: Påverkan av geokemi och biologi

Sammanfattning

Metylkvicksilver (MeHg) är ett nervgift som huvudsakligen produceras av mikroorganismer i suboxiska och anoxiska miljöer, så som i torvmarker. Torvmarker är en viktig källa till MeHg i akvatiska ekosystem och kan medföra en ökad exponering av MeHg för djur och människor. En bättre förståelse av de faktorer som begränsar den mikrobiella netto-produktionen av MeHg i torvmarker skulle kunna leda till markanvändnings-strategier och effektiva åtgärder för att begränsa spridningen av denna giftiga förening i torvmarksrika skogslandskap.

I denna avhandling studerades en trofisk gradient av torvmarker som utgör en kronosekvens som sträcker sig över flera tusen år. Alla torvmarker ligger inom ett par kilometers avstånd från varandra, är utsatta för en liknande atmosfärisk deposition, och har jämförbar geologi och klimatmönster. Det övergripande syftet var att bestämma påverkan från olika biogeokemiska faktorer på netto-bildningen av MeHg. Längs kronosekvensen av torvmarker observerades högre netto-bildning av MeHg i de yngre mer näringsrika torvmarkerna (artikel I). Samma trend i netto-bildning av MeHg observerades i porvattnet och kunde då kopplas främst till förändringar i tillgängligheten av elektronacceptorer för Hg metylerande mikroorganismer, snarare än mängden elektrondonatorer (artikel II). Modellering av lösligheten och kemisk speciering av Hg (II) antyder att skillnaderna i netto-bildning av MeHg längs kronosekvensen också kan påverkas av förekomsten av biotillgängligt oorganiskt Hg för mikroorganismer som kan metylera Hg (artikel II).

Det mikrobiella samhällets sammansättning var signifikant korrelerat till netto-bildningen av MeHg i den studerade kronosekvensen, där rumsliga mönster påverkas av en rad miljöfaktorer (artikel IV). Kontrollerade inkubationsstudier i laboratoriemiljö bekräftade att skilda mikrobiella metabola processer låg till grund för produktion och omsättning av metylkvicksilver i kronosekvensens olika torvmarkerna, där skillnader i mikrobiella samhällen och kopplingen till biogeokemiska parametrar kan förklara skillnaden i netto-bildning av MeHg (artikel III). Fältobservationer av genuttryck från funktionella grupper av mikroorganismer tyder vidare på att betydelsen av svavel-reducerande bakterier för netto-bildningen av MeHg varierade längs kronosekvensen, samtidigt som metanogena arkéer också var viktig för netto-bildningen av MeHg i samtliga torvmarker (artikel IV).

Keywords: metylkvicksilver, kvicksilver, torvmark, kvicksilvermetylering, mikrobiella samhället, kronosekvens

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Dedication

To my beloved wife and son,
To my Mom and Dad, for always being there for me whenever I need you.

知之为知之，不知为不知，是知也 —— 孔子

If you know, to recognize that you know; If you don't know, to realize that you don't know. That is knowledge. Confucius

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. **Wang B.**, Nilsson M. B., Eklöf K., Hu H.*, Ehnvall B., Bravo A. G., Zhong S., Åkeblom S., Björn, E., Bertilsson S., Skyllberg U., Bishop K. (2020). Opposing spatial trends in methylmercury and total mercury along a peatland chronosequence trophic gradient. *Science of The Total Environment*. 718, 137306.

II. **Wang B.**, Zhong S.*, Bishop K., Nilsson M. B., Hu H., Eklöf K., Bravo A. G., Åkeblom S., Bertilsson S., Eklöf K., Skyllberg U. Net mercury methylation in peat: Biogeochemical influences along a peatland chronosequence (manuscript).

III. Hu H.*, **Wang B.**, Bravo A. G., Björn, E., Skyllberg U., Amouroux D., Tessier E., Zopfi J., Feng X., Bishop K., Nilsson M. B., Bertilsson S. (2020). Shifts in mercury methylation across a peatland chronosequence: From sulfate reduction to methanogenesis and syntrophy. *Journal of Hazardous Materials*. 387, 121967.

IV. **Wang B.**, Hu H.*, Bravo A. G., Buck M., Eklöf K., Björn, E., Skyllberg U., Nilsson M. B., Bishop K., Bertilsson S. Microbial mercury methylation along a peatland chronosequence: the role of mercury and non-mercury methylators (manuscript).

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The contribution of Baolin Wang to the papers included in this thesis was as follows:

I. The respondent contributed to formulating the scientific research objectives, conducted most of the lab work, and was the main person responsible for sampling, data analyses as well as writing.

II. The respondent contributed to formulating the scientific research objectives, was largely involved in the lab work, and was the main person responsible for sampling, data analyses, as well as writing.

III. The respondent conducted most of the lab work, was largely involved in formulating the scientific research objectives and approaches, data analyses and writing, was the main person responsible for sampling and sample preparation.

IV. The respondent conducted most of the lab work, was largely involved in formulating the scientific research objectives and approaches, was the main person responsible for sampling and sample preparation, data analyses and writing.

Abbreviations

16S rRNA	16S ribosomal RNA
ANOVA	Analyses of variance
BES	Podium 2-bromoethanesulphonate
C/N	Carbon/nitrogen ratio
Ca	Calcium
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
e.g.	*Exemple gratia* (for example)
Fe	Iron
Fe(II)	Ferrous iron (reduced form)
Fe(III)	Ferric iron (oxidized form)
FeRB	Iron-reducing bacteria
GWT	Ground water table
Hg	Mercury
Hg(0)	Elemental mercury
Hg(II)	Inorganic divalent mercury
HgS(s)	Metacinnabar
i.e.	*Id est* (that is)
K	Potassium

k_d	Demethylation rate constants
k_m	Methylation rate constants
m.a.s.l.	Meter above sea level
MeHg	Methylmercury
Mg	Magnesium
Mn	Manganese
Mo	Sodium molybdate (Na_2MoO_4)
MoBES	Sodium molybdate + sodium 2-bromoethanesulphonate
Na	Sodium
NMDS	Non-metric multidimensional scaling
NOM	Natural organic matter
PCA	Principle component analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PLS	Partial least square
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RSH	Thiol groups
S	Sulfur
S(−II)	Inorganic sulfide
S^0	Elemental sulfur
SO_4^{2-}	Sulfate ion
SRB	Sulfate-reducing bacteria
SUVA	Specific UV absorbance (at 254 nm)
THg	Total mercury
VPR	Vascular plant removal/removed

1. Introduction

Peatlands are globally important sources of methylmercury (MeHg) which is transferred to hydrologically connected streams and lakes where it can be bioaccumulated by fish (Bergman *et al.*, 2012; St. Louis *et al.*, 1996). This raises the risk of exposure to this potent neurotoxin. In Sweden, where peatlands are widespread, most of the more than 100 000 lakes have fish which are unsafe to eat because of high mercury (Hg) levels (Åkerblom *et al.*, 2014). The problem persists despite reduction of atmospheric Hg deposition in recent decades (Braaten *et al.*, 2019; Åkerblom *et al.*, 2012; Lindqvist *et al.*, 1991a; Lindqvist *et al.*, 1991b). It is thus urgent to obtain a better understanding of factors controlling Hg methylation in peatlands, especially since there is such a large amount of Hg already stored in those ecosystems (Osterwalder *et al.*, 2017).

1.1 Mercury

Mercury occurs naturally in the environment in three different forms: elemental mercury (Hg(0)), inorganic divalent mercury (Hg(II)) and organic methylmercury. All Hg species can pose health hazards depending on the dose and exposure pathway. Mercury is released to the atmosphere from various natural sources such as volcanic eruptions, weathering of rocks, geologic deposits of mercury as well as volatilization from the ocean (Nriagu & Becker, 2003). Human activities also release Hg to the atmosphere. These anthropogenic sources include fossil fuel combustion, waste incinerators as well as mining activities for gold and other metals (Pirrone *et al.*, 2010; Pacyna *et al.*, 2006). Mercury is termed a global pollutant because of its long-range atmospheric transport from local anthropogenic sources to remote regions (Lindberg *et al.*, 2007; Mason *et al.*, 1994; Lindqvist *et al.*, 1991b).

Since most Hg is deposited from the atmosphere in its inorganic forms (Schroeder & Munthe, 1998), *in situ* transformations of Hg must play a crucial role in the distribution and toxicity of Hg (Barkay & Poulain, 2007).

Methylmercury is the species of most concern compared with elemental and inorganic forms because of its high bioaccumulation in biota and biomagnification through food chains, risking the health of humans and wildlife (Diez, 2009; Mergler *et al.*, 2007). Human exposure to MeHg is mainly by consumption of fish and rice (Zhang *et al.*, 2010; Jiang *et al.*, 2006; Håkanson *et al.*, 1988) and primarily affects brain tissue such as nervous system, with symptoms ranging from mild numbness to blindness, and in severe cases, death (Clarkson, 2002; Clarkson, 1997; Harada, 1995). A concentration of 0.5 mg Hg/kg fish fresh weight is considered to be the threshold for safe fish consumption by the United Nations Environmental Programme (UNEP-Chemicals, 2002). In Sweden, where fish Hg concentrations commonly exceed this threshold, about 10% of children are born with Hg concentration above the Hg concentration limit of 0.58 μg/g Hg in hair, costing about 150 million € per year for Swedish society (Bellanger *et al.*, 2013). Therefore, despite the great strides taken to limit the use and spread of Hg globally, and by Sweden in particular, there remains a need to continue efforts to reduce exposure to Hg. Since peatlands are a source of MeHg to downstream aquatic ecosystems (Åkerblom *et al.*, 2020; Tjerngren *et al.*, 2012), and peatlands are a major component of the Swedish landscape, a better understanding of the factors influencing the formation of MeHg in peatlands may be able to help guide the efforts to further reduce Hg in the freshwater fish of Sweden and other countries with extensive peatlands.

1.2 Methylmercury formation and degradation

Net MeHg formation is determined by two competing processes in natural ecosystems, including Hg methylation (Jensen & JernelÖV, 1969) and demethylation (Spangler *et al.*, 1973). However, Hg(II) methylation has generally been regarded to be more important in determining net MeHg production than MeHg demethylation as methylation is largely driven by varying and high methylation rates with only a minor influence from demethylation reactions (Drott *et al.*, 2008; Hammerschmidt & Fitzgerald, 2006). This is because MeHg demethylation rates, even if they are significant

and often higher in absolute numbers, generally vary less among different environments, even though the seasonal variability may sometimes be high for demethylation (Kronberg *et al.*, 2012; Martin-Doimeadios *et al.*, 2003).

Methylation of Hg(II) requires the presence of suitable methyl donors, and both abiotic and biotic Hg(II) methylation naturally occur in the environment. Biotic Hg(II) methylation, which is proposed to be associated with the reductive acetyl-coenzyme A pathway and potentially linked to corrinoid-related proteins involved in this pathway (Choi *et al.*, 1994), accounts for most of environmental MeHg formation (Celo *et al.*, 2006), particularly under low oxygen conditions (Gilmour *et al.*, 2013; Hsu-Kim *et al.*, 2013; Hintelmann, 2010; Ullrich *et al.*, 2001). Most of the isolated environmental strains that have the ability to methylate mercury are in the class *Deltaproteobacteria* (Gilmour *et al.*, 2011; Ranchou-Peyruse *et al.*, 2009). Sulfate-reducing bacteria (SRB) have been identified as the main microbes capable of Hg(II) methylation (Gilmour *et al.*, 1992; Compeau & Bartha, 1985), but their presence is not a good indicator of MeHg production as not all SRB can methylate Hg (King *et al.*, 1999). Interestingly, SRB that use acetate as a carbon source can produce more MeHg than those that do not use acetate (King *et al.*, 2000), suggesting that MeHg formation among SRB is strain-dependent rather than species, genus or metabolic group dependent (Ranchou-Peyruse *et al.*, 2009).

In addition to SRB, iron-reducing bacteria (FeRB) (Yu *et al.*, 2012b; Fleming *et al.*, 2006; Kerin *et al.*, 2006) and methanogens (Hamelin *et al.*, 2011; Wood *et al.*, 1968) are also important contributors of MeHg formation. Similar to SRB, the capability of methylating Hg(II) is not ubiquitous among dissimilatory FeRB. *Geobacter* and *Desulfuromonas* (which are closely related to known SRB methylators within the *Deltaproteobacteria*) could produce MeHg when reducing Fe(III), nitrate, or fumarate. *Shewanella*, a subclass of *γ-Proteobacteria*, however, cannot produce MeHg (Kerin *et al.*, 2006). It has also been shown that methylotrophic *Methanosarcinaceae* and *Methanomethylovorans hollandica* could produce MeHg, with 1-4 % of the ^{201}Hg spike methylated (Gilmour *et al.*, 2013). Contrary to earlier assumptions that methanogens only played a minor role in MeHg formation (Ullrich *et al.*, 2001), more recent findings imply that methanogens may in fact be primary methylators under conditions where sulfate and iron reduction is not favored (Hamelin *et al.*, 2011).

It has long been demonstrated that SRB methylators may methylate Hg during fermentative growth and/or by syntrophic interactions with methanogens (Plugge *et al.*, 2011; Compeau & Bartha, 1985). The SRB may even contribute to MeHg formation when coexisting with FeRB populations, possibly by temporally and spatially separated processes (Yu *et al.*, 2012a). This suggests that Hg(II) methylation does not depend on a single methylating guild but on complex metabolic interactions among microorganisms, including prokaryotes, algae, fungi, and methanogens (Correia *et al.*, 2012). The recent discovery of a gene pair, *hgcAB*, which is necessary for microbial Hg methylation (Parks *et al.*, 2013), have since a few years created opportunities to assess the distribution of microbial Hg methylators in natural ecosystems and identify methylating taxa directly in complex environments. Not only has the range of known lineages of dominant methylators been broadened by identification of diverse *hgcAB* genes (Gilmour *et al.*, 2013), but also more methylators other than SRB, FeRB and methanogens, such as *Firmicutes* (Gilmour *et al.*, 2013; Parks *et al.*, 2013), have been discovered.

Microbial Hg methylation is limited by two classes of factors: One class relates to the activity of microorganisms involved in Hg(II) methylation. This involves factors such as availability of electron donors (e.g. organic matter) and availability of electron acceptors (e.g. SO_4^{2-} and Fe(III)) which also are sensitive to redox conditions (Ullrich *et al.*, 2001). The second class of factors concerns the bioavailability of Hg(II) to methylating microorganisms, which is controlled by factors such as inorganic sulfide (S(−II)) concentration, natural organic matter (NOM) functional groups, and pH (Benoit *et al.*, 2003). Because of the strong affinity of Hg(II) to reduced sulfur, the chemical speciation of Hg(II) is largely mediated by the relative proportions of S(−II) and thiol (RSH) groups (Skyllberg, 2008; Benoit *et al.*, 2003).

Similar to Hg methylation, both biotic and abiotic pathways of MeHg demethylation occur in nature. Photolytic decomposition is the dominant abiotic mechanism for demethylation in the environment, especially in surface water (Hammerschmidt & Fitzgerald, 2006; Seller *et al.*, 1996). Photolytic decomposition has been attributed to both direct and indirect processes. Direct processes involve the absorption of light by the MeHg molecule itself, potentially including energy transfer within a dissolved organic matter (DOM)−CH_3Hg complex, and breaking of the C−Hg bond

(Jeremiason *et al.*, 2015; Inoko, 1981). Indirect processes, however, involve secondary reactions with photochemically generated transient intermediates such as reactive oxygen species (Black *et al.*, 2012; Zhang & Hsu-Kim, 2010; Suda *et al.*, 1993).

Microbial demethylation is thought to be the primary pathway for biotic degradation of MeHg in the environment (Robinson & Tuovinen, 1984). Numerous microbes known as methylators are also capable of degrading MeHg (Bridou *et al.*, 2011; Baldi *et al.*, 1993; Oremland *et al.*, 1991). There are two commonly accepted mechanisms of microbial MeHg degradation. One is a reductive pathway, which is mediated by the *mer*-operon system, resulting in the cleavage of the C−Hg bond by the organomercurial lyase enzyme, yielding Hg(II), followed by the reduction of Hg(II) to Hg(0) by the mercuric reductase enzyme, with methane as the sole gaseous carbon product (Barkay *et al.*, 2003; Robinson & Tuovinen, 1984); The other is an oxidative pathway, in which MeHg is decomposed to Hg(II), with carbon dioxide and small amounts of methane as the gaseous metabolic byproducts (Oremland *et al.*, 1991). Reductive MeHg degradation appears to be prevalent particularly in aerobic surface water *(*Matilainen & Verta, 1995; Spangler *et al.*, 1973). Methanogens and SRB, and probably other anaerobic microorganisms, may be involved in oxidative demethylation in anoxic environments, with methanogens dominating degradation at *in-situ* MeHg concentrations (Marvin-DiPasquale & Oremland, 1998). Similar to microbial Hg(II) methylation, microbial demethylation of MeHg is affected by the activity of microorganisms involved in demethylation and the availability of MeHg to those microorganisms, which are largely driven by environmental factors (Schaefer *et al.*, 2004; Marvin-DiPasquale *et al.*, 2000).

Both biotic and abiotic demethylation processes play important roles in global mercury cycling. The Hg(0) resulting from reductive demethylation can evade from water, soil, and sediments. The Hg(II) produced by oxidative demethylation can be remethylated, bound by sulfur species, or volatilized as dimethylmercury.

1.3 Boreal peatlands

Peatlands are peat-accumulating wetlands that support nutrient-poor vegetation. These are found in extensive areas of Scandinavia, Russia,

Canada, and Alaska. The relatively low level of nutrients is related to the source of water in peatlands, which often comes primarily from precipitation that has a low concentration of nutrients. Peatlands can also receive water draining from surrounding uplands or regional groundwater systems. Both of these sources may contain more nutrients than precipitation.

The vegetation in peatlands includes sedges, mosses, and shrubs. The mix of vegetation types is a sensitive indicator of trophic status and a strong driver of wetland functioning (Selvendiran *et al.*, 2009). In general, nutrient-poor bogs dominated by non-vascular plants such as *Sphagnum* mosses, tend to inhibit terminal processes like methanogenesis while supporting acetogenesis and acetate accumulation. Richer fens with a dominance of vascular plants, especially sedges which can produce easily-degraded and high-quality carbon substrates via root exudation (Conrad, 1996), support increased methanogenesis and less acetate accumulation.

Peatlands have seasonally varied water table regimes that influence the spatial and temporal distribution of oxic, suboxic and anoxic conditions in the peatland. These seasonal fluctuations create redox oscillations in the vicinity of the groundwater table that are likely to favor microbial processes, such as reduction of sulfate and ferric iron, both of which are implicated in Hg methylation (Bravo *et al.*, 2018; Bergman *et al.*, 2012), as well as organic matter degradation that offers electrons to Hg methylating microbes, including syntrophs (Yu *et al.*, 2018; Bae *et al.*, 2014). In a global perspective, peatlands are a major source of MeHg to downstream high-latitude aquatic ecosystems (Tjerngren *et al.*, 2012; Mitchell *et al.*, 2008; St. Louis *et al.*, 1996), which entail the risk of harm to humans and wildlife that consume fish that have bioaccumulated MeHg from these waters (Munthe *et al.*, 2007; Ratcliffe *et al.*, 1996; Driscoll *et al.*, 1994).

Along the Baltic Sea's Bothnian Bay in northern Fennoscandia, post-glacial land uplift has created a chronosequence of peatlands within the space of a few kilometers, all subjected to similar atmospheric deposition, underlying geology and climate patterns. As peatlands age, newly formed peat at the top of the soil profiles in these peatlands is progressively isolated from the underlying mineral substrates. The delivery of weathering products (e.g. dissolved minerals) to these peatlands from the surrounding watersheds is also likely to decrease over time. These factors create a natural biogeochemical gradient across the chronosequence (Tuittila *et al.*, 2013)

that can facilitate studies of long-term biogeochemical influences and related vegetation changes on the net formation of MeHg.

1.4 Aim and objectives

The principal aim of this thesis was to investigate the production of MeHg along the biogeochemical gradient created by a peatland chronosequence in order to better determine the factors influencing net MeHg formation in boreal peatlands. This is intended to build the evidence-base for guiding strategies to limit exposure of people and wildlife to Hg in northern landscapes with a large component of peatlands.

The thesis focused on three types of geochemical influence: 1) electron donors like high-quality root carbon exudates from vascular plants, 2) electron acceptors such as sulfate and ferric iron, and 3) the bioavailability of Hg(II). Recent breakthroughs in genomics were also used to define the microbial communities involved in the formation of MeHg. While the chronosequence creates a natural gradient in a number of relevant biogeochemical factors, vascular plant removal was used to reduce root exudates and thus complement the natural variation of relevant factors across the peatland chronosequence. The effects were measured in solid peat and porewater chemistry, as well as in the microbial communities. Laboratory incubations provided further insights into the processes involved in MeHg formation by experimentally manipulating the activity of different microbial metabolic pathways. The outcome of these studies were used to speculate on strategies for management of peatland-rich landscapes to limit Hg methylation.

The specific objectives were:

- To determine the influence of biogeochemical factors, including the presence of vascular plants, on net MeHg formation in peat along the geochemical gradient created by the chronosequence (Paper I and Paper II).
- To investigate whether the solubility and chemical speciation of Hg(II) controls net MeHg formation along the chronosequence (Paper II).

- To experimentally identify microbial metabolic processes driving net MeHg formation in relation to trophic status along the peatland chronosequence (Paper III).
- To investigate the microbial community composition and the abundance of specific microbial functional groups in relation to net MeHg formation in peat soil along the peatland chronosequence (Paper IV).

2. Materials and methods

2.1 Study sites

Fifteen open peatlands were selected from a vegetation survey of 70 peatlands along a peatland chronosequence that was created by post-glacial land uplift. Ages span from 0 to > 4000 years within < 10 km from the sea in northern Sweden along the Gulf of Bothnia. The fifteen peatlands were then divided into three age classes, young (< 10 m.a.s.l., < 1000 years, n = 5), intermediate (10 − 20 m.a.s.l., 1000 − 2000 years, n = 5) and old (20 − 40 m.a.s.l., > 2000 years, n = 5) (Figure 1). The three peatland age classes were different with respect to elevation, acidity (pore water pH), and vegetation composition (Table 1). More about the vegetation can be found in Paper I.

2.2 Sampling and chemical analyses

Four sampling campaigns were carried out in June (the beginning of growing season) and August (growing season) of 2016 and 2017. In June 2016, five 70 × 210 cm plots were established at least 5 m away from each other along a line across the center of each of the fifteen peatlands. Then each of the five 7 × 210 cm plots was divided into three 70 × 70 cm subplots: one subplot with all vascular plants removed (VPR) and one control subplot as well as one buffer subplot between them (Figure 1).

Peat cores were extracted immediately below the average growing season ground water table (GWT) from each control and VPR subplot within each peatland using a custom-made knife. These peat soil samples were used for analyses of chemical elements/compounds as well as phylogenetic studies on the microbial community composition. These data were then used to evaluate

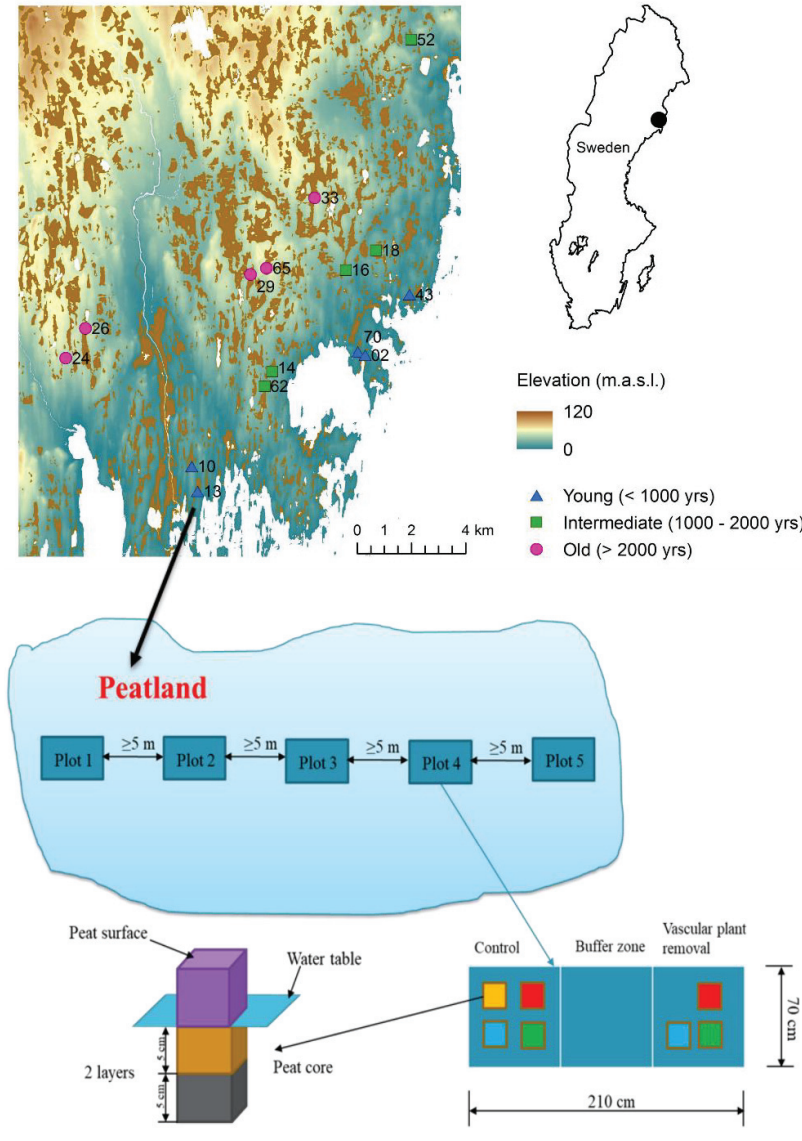

Figure 1. The experimental plot setup and peatland sampling sites located along the northeast coast of Sweden where post-glacial uplift has created a relationship between elevation above sea level and the age of the peatland. The color of the symbol marking each sampling site indicates the age class (blue triangles = young, green boxes = intermediate, and pink dots = old) assigned to the peatland in this study. The numbering relates to an initial vegetation inventory of some seventy peatlands along this chronosequence. (Figure adapted from Paper I)

Table 1. Characteristics of the study peatlands along the chronosequence. (Adapted from Paper I)

Peatland	Elevation (m.a.s.l)	Peat depth (cm)	Age (year)	N	E	Veg. Class[a]	pH[b]
02	0.72	70	72	63°51'3.90"	20°42'54.12"	6	5.0 ± 0.2
70	1.49	46	149	63°51'8.86"	20°42'35.14"	5	4.0 ± 0.2
43	3.43	70	341	63°52'11.67"	20°45'8.07"	3	4.0 ± 0.1
13	3.53	154	352	63°48'38.55"	20°34'51.54"	6	4.5 ± 0.1
10	5.07	140	503	63°49'9.09"	20°34'41.77"	6	4.1 ± 0.3
Y[c]	2.8 ± 1.6	98 ± 45	283 ± 154				4.3 ± 0.5
52	12.60	114	1221	63°57'16.73"	20°46'15.09"	4	4.7 ± 0.2
14	13.89	244	1341	63°50'54.39"	20°38'39.93"	3	4.5 ± 0.1
18	14.54	66	1401	63°53'8.15"	20°43'48.29"	4	3.6 ± 0.1
16	14.56	76	1402	63°52'47.97"	20°42'22.49"	2	3.9 ± 0.3
62	15.57	106	1495	63°50'37.38"	20°38'16.74"	1	3.8 ± 0.2
I[d]	14.2 ± 1.0	121 ± 72	1372 ± 90				4.1 ± 0.5
29	27.53	96	2547	63°52'52.61"	20°38'5.52"	1	3.7 ± 0.2
26	29.19	246	2686	63°52'5.08"	20°30'28.78"	1	3.7 ± 0.2
33	30.54	210	2799	63°54'17.68"	20°41'16.43"	2	3.9 ± 0.2
24	31.46	140	2874	63°51'31.51"	20°29'29.14"	1	3.8 ± 0.2
65	34.82	130	3146	63°52'58.48"	20°38'50.03"	1	3.8 ± 0.2
O[e]	30.7 ± 2.4	164 ± 61	2810 ± 200				3.8 ± 0.2

a Vegetation classes, from an initial survey of seventy peatlands along the chronosequence (data unpublished), with increasing number representing vegetation that requires more nutrients to thrive and spread.

b Mean pH ± SD of the four sampling occasions in 2016 and 2017.

c, d, e Rows represent average parameter values ± SD for young, intermediate and old peatland classes, respectively.

Hg biogeochemistry and trophic status along the peatland chronosequence as well as the effects of VPR on net MeHg formation. Peat soils were taken from the control plots during all four campaigns (June 2016, August 2016, June 2017 and August 2017) and from the VPR plots on all but the first of those campaigns, since no VPR effect was expected in June 2016 at the time of the first vascular plant removal. Details on soil sampling and soil sample preparations can be found in Papers I and IV.

Porewaters were sampled from both control and VPR subplots in June and August 2017 at the 10 cm peat profile where peat soils were collected. Elements/compounds in the porewater samples were analyzed to evaluate the

Hg biogeochemistry as well as the effects of vascular plant removal along the peatland chronosequence. Detailed protocols of clean, oxygen-free porewater sampling and sample preparation can be found in Paper II.

2.3 Incubation studies

Peat soils and porewaters for incubation experiments were sampled in August 2016 on spots near control subplots from the three peatlands (i.e. site 02, 16 and 65; Figure 1). Peat soil and porewater were homogenized to make peat slurries (approximately 95% (wt/wt) water content) for incubations to determine the potential methylation and demethylation rate constants, k_m and k_d, as well as contributions of different microbial metabolism pathways to Hg(II) methylation. Briefly, approximately 30 mL slurries contained in 120 mL serum bottles were pre-incubated in the dark at 18 °C for 14 days in order to remove residual oxygen. This was followed by addition of different combinations of electron acceptors, electron donors, and inhibitors of specific microbial groups, as well as enriched stable isotope tracers of Hg(II) and MeHg. Two types of control incubations were included, sterile controls with autoclaved (121 °C for 30 min) peat soil and reference controls with original peat soil but without any addition of inhibitors or other treatment. For each treatment, triplicate "T0" samples were collected within 5 min after Hg tracer amendment. Part of each slurry was frozen at − 80 °C for the determination of k_m and k_d. The rest of the slurry was centrifuged at 3100 × g and 4 °C for 10 min to collect the pore water for specific chemical analyses. After Hg tracer amendment, triplicate slurries for each treatment were incubated for 24 h in a N_2-filled glovebox, then a similar sampling protocol as for T0 samples was applied to collect pore water and solid phases for chemical analyses. The relative contributions of different microbial metabolisms to Hg(II) methylation was calculated by the differences of k_m between the treatments of specific microbial inhibitors and the control using equations (1), (2) and (3).

$$k_{m\_}\text{Mo} = k_{m\_}\text{control} - k_{m\_}\text{SR} - k_{m\_}\text{Syn} \quad (1)$$

$$k_{m\_}\text{BES} = k_{m\_}\text{control} - k_{m\_}\text{Meth} - k_{m\_}\text{Syn} \quad (2)$$

$$k_{m\_}\text{MoBES} = k_{m\_}\text{control} - k_{m\_}\text{SR} - k_{m\_}\text{Meth} - k_{m\_}\text{Syn} \quad (3)$$

$k_{m_}$Mo, $k_{m_}$BES, $k_{m_}$MoBES refer to the k_m in the treatment of Mo, BES and MoBES, respectively; $k_{m_}$control refers to the k_m in the control incubations; $k_{m_}$SR, $k_{m_}$Meth, $k_{m_}$Syn refer to the k_m contributed by sulfate

reduction, methanogenesis, and syntrophic metabolism between methanogens and SRB fermenters, respectively. More details can be found in Paper III.

2.4 Modeling for solubility and speciation of Hg(II)

The modeling of chemical speciation of Hg(II) in the peatland soils and porewaters along this peatland chronosequence was based on the method of Liem-Nguyen *et al.* (2017). Briefly, thermodynamic calculations were made in the Microsoft Excel program using an iterative procedure where either $Hg(NOM{-}RS)_2$ (ads) or metacinnabar (HgS(s)) determined the free concentration of Hg(II). Inputs to this modeling were the total soil concentrations of Hg(II) and MeHg, S, organic C, concentrations of NOM associated RSH (including adsorbed and aqueous phases: NOM−RSH(ads) and NOM−RSH(aq) respectively) and aqueous phase concentrations of DOC, Cl^-, HS^-, Fe(II) as well as pH. The polysulfide species HgS_nHS^- and $Hg(S_n)_2^{2-}$ were also considered in the thermodynamic calculations. The model fit (merit-of-fit) was evaluated from the modeled and observed distribution of Hg(II) and MeHg between solid/adsorbed and aqueous phases, calculated as merit-of-fit = $\Sigma(data_{modeled}\text{-}data_{measured})^2/\Sigma data_{measured}^2$ ($data_{modeled}$ = modeled data; $data_{measured}$ = measured data). More details can be found in Paper II.

2.5 Microbial communities

Sequence-based analyses of microbial community composition were done to investigate the composition and abundance of microbial communities in peat soil in relation to Hg methylation along the chronosequence. Peat soils for DNA and RNA extractions were randomly sampled from different spots of the two layers (0-5 and 5-10 cm) of the 10 cm peat cores taken from both the control and VPR subplots on all four sampling occasions during 2016 and 2017. The peat samples were immediately frozen and kept in liquid nitrogen during transport and stored at − 80 °C until further processing and analysis.

In the lab, genomic DNA and total RNA were extracted from peat soil, and the purified RNA was then used for synthesizing complementary DNA (cDNA). The amplicons of 16S rRNA gene were generated by a two-step PCR amplification with DNA as templates and then sequenced by Illumina

MiSeq platform to investigate the microbial community composition in relation to Hg methylation along the chronosequence. The cDNA was subject to quantitative PCR (qPCR) to determine the abundance of specific microbial functional groups that were metabolically active. This was done by targeting relevant marker genes. More details can be found in Paper IV.

2.6 Statistics

All data were checked for their normality and homogeneity prior to statistical analysis using the Shapiro-Wilk and Levene tests, respectively. Log transformation was applied when the original data were not normally distributed. For data that were non-normally distributed with/without homogeneous variance even after log-transformation, a non-parametric Kruskal-Wallis test was conducted and followed by a pairwise Wilcoxon rank sum test if there was any significant difference. For these tests, the 0.05 level of significance was used in all four papers.

In Paper I and II, significant differences in geochemical parameters as well as concentrations of Hg species in both peat soil and porewater between experimental factors, such the three peatland age classes (young, intermediate and old peatlands), sampling depth (two layers), month of sampling (June and August) and treatment (control and VPR), were tested using Analyses of Variance (ANOVA), followed by Tukey's multiple comparison test. All these tests were conducted using R (Version 3.6.0 or 3.6.3, https://www.r-project.org/). In Paper I, Principle Component Analysis (PCA) and Partial Least Square (PLS) analysis were conducted in the SIMCA software package (Version 14, Umetrics Umeå, Sweden), using only data from the two sampling occasions in 2016. In Paper III, IBM SPSS Statistics 24 (SPSS Inc., USA) was used for statistical analyses. In Paper IV, all the statistical analyses were based on the dataset after the rarefaction of sequences and performed in R (Version 3.6.3) using specific packages. Non-metric multidimensional scaling (NMDS) analyses with Bray-Curtis distance were used to characterize microbial community variation along the peatland chronosequence. Permutational multivariate analysis of variance (PERMANOVA) was used to test the differences in environmental and taxonomic composition among categories. A Mantel test was conducted to examine the relationship between bacterial similarities and overall environmental variables.

3. Results

3.1 Peatland trophic gradient

The three age classes along the chronosequence exhibited a distinct gradient of trophic status and hydrogeochemistry that was evident from vegetation composition and several other geochemical features of peat soil (Figure 2) and porewater (Figure 3). The older peatlands, with higher elevations above sea level, were more acidic and nutrient poor (oligotrophic) than the younger peatlands. This was reflected by decreasing vascular plant cover and concentrations of major minerogenic elements (e.g. Fe, Ca, Mn, Mg, K, Na) but increasing total concentrations of carbon in superficial peat soil (Figure 2), as well as decreasing pH and major ion concentrations (e.g. Fe(III), SO_4^{2-}, Mn, Mg, K, Na) but increasing C/N in porewater with peatland age (Figure 3). Furthermore, microbial community composition also showed a clear spatial pattern along this nutrient gradient, with young and intermediate peatlands having higher microbial diversity and abundance compared to old peatlands (Table 2). For more details, see Papers I, II and IV.

Table 2. Microbial community diversity indices across the three peatland age classes. Superscript letters along with values indicate significant differences ($p < 0.05$) among the three age classes, values with the same letter do not differ significantly. (Table adapted from Paper IV)

Peatland	n	OTU number	Chao1	Shannon	Pielou
Young	40	236 ± 33^{a}	347 ± 60^{a}	4.6 ± 0.3^{a}	0.85 ± 0.03^{a}
Intermediate	40	222 ± 28^{a}	317 ± 48^{a}	4.5 ± 0.3^{a}	0.84 ± 0.03^{a}
Old	40	194 ± 24^{b}	279 ± 38^{b}	4.3 ± 0.4^{b}	0.82 ± 0.05^{b}

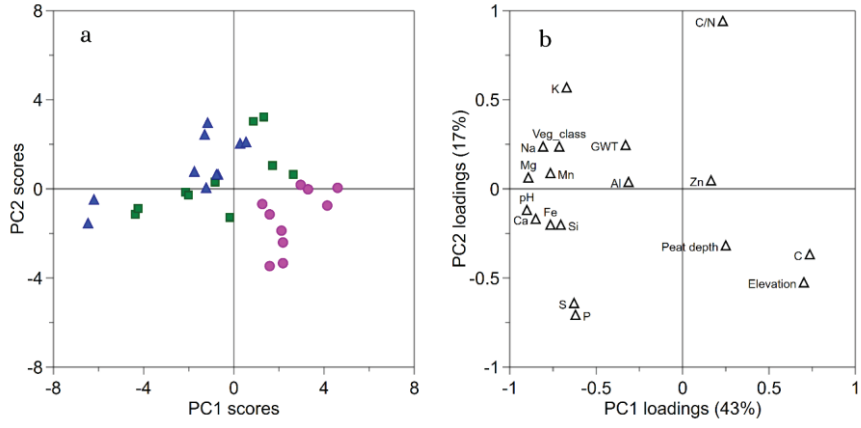

Figure 2. PCA of the explanatory variables (biogeochemistry and vegetation) measured on two sampling occasions during 2016 along a chronosequence of fifteen peatlands. (a) Scores for the three age classes of peatlands, young (blue triangles), intermediate (green boxes) and old (pink dots); (b) Variables strongly contributing to separate the old and young peatland classes have high or low loading respectively for PC1, reflecting the gradient of nutrients and related factors created by peatland aging. (Figure adapted from Paper I)

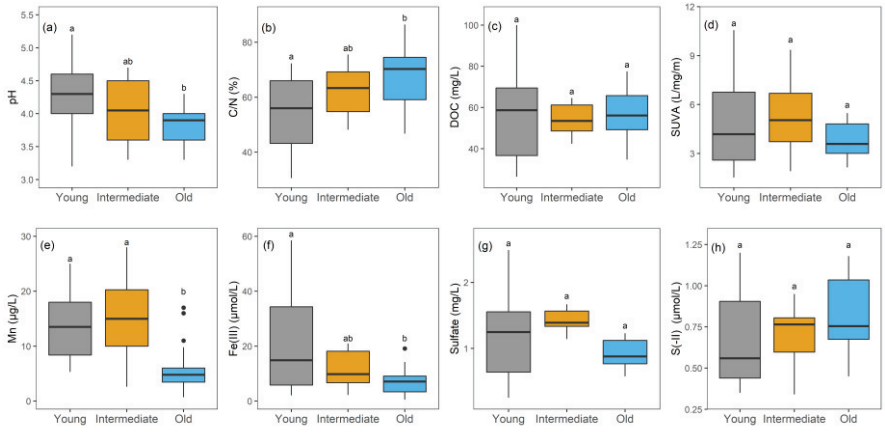

Figure 3. Porewater chemistry in peatlands of the three age classes along the chronosequence. Since removing the vascular plants had no significant effect (cf. Paper II, Figure S5), the values from plots with and without the vascular plant removal treatment are grouped together from the two sampling occasions in 2017. The letters above the boxes indicate significant differences ($p < 0.05$) between the three peatland age classes. Boxes with the same letters indicate no significant difference. (Figure adapted from Paper II)

3.2 Vascular plant removal

Our initial hypothesis was that the vascular plant removal would reduce input of high quality carbon sources (i.e. root exudates) for methylating microorganisms in the rhizosphere and subsequent microbial MeHg net formation. However, there were no differences in net formation of MeHg between the control plots and those where the vascular plants had been removed, in terms of either MeHg or %MeHg in solid peat or porewater in young, intermediate or older peatlands (Figure 4). The vascular plant removal also did not create any detectable changes in major biogeochemical parameters in either peat soil (e.g. C/N, element concentrations) or porewater (e.g. pH, DOC, ion concentrations, or low molecular weight organic substances such as acetate). Porewater samples collected from the VPR subplots were therefore treated as replicates of control samples for subsequent data analyses in Paper II. Moreover, the patterns in microbial community composition in VPR subplots were similar to those in control subplots along the peatland chronosequence (Figure 5).

3.3 Net MeHg formation along the chronosequence

In Paper I, the distribution of Hg species in the solid peat along this peatland chronosequence was investigated. In the 10 cm soil layer just below the average annual growing season water table, concentrations of MeHg and %MeHg (of total Hg) were higher in younger, more mesotrophic peatlands than in older, more oligotrophic peatlands. In contrast, total mercury (THg) concentrations were higher in the older peatlands (Figure 6). Similar to the trend in solid peat, concentrations of MeHg and %MeHg in porewater were also higher in the younger peatlands than in the older peatlands along this peatland chronosequence (Figure 4).

3.4 Chronosequence influences on net methylation

Along this peatland chronosequence, net MeHg formation was associated with peatland trophic status, with higher net MeHg formation in younger peatlands where there was higher nutrient availability. This association was evident from the significant correlations between net MeHg formation and geochemical parameters in both peat soil and porewater as well as microbial community composition along the chronosequence.

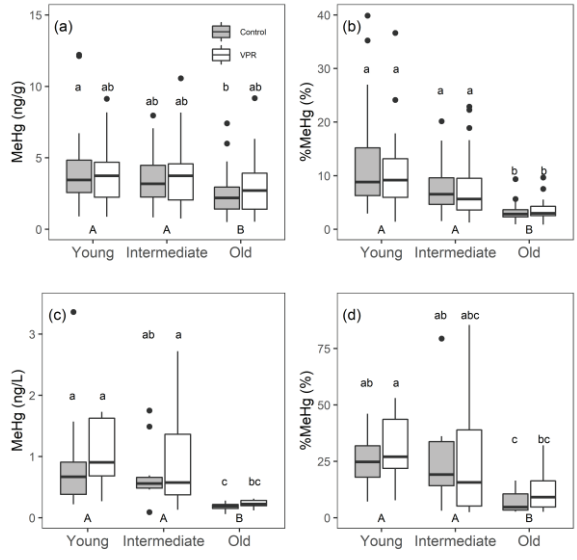

Figure 4. Effects of vascular plant removal (VPR) on net MeHg formation in peatlands of the three age classes along the chronosequence. (a) Concentrations of MeHg and (b) %MeHg in peat soils of both 0-5 and 5-10 cm layers from the three sampling occasions in 2016 and 2017. While (c) concentrations of MeHg and (d) %MeHg in porewaters of 0-10 cm layers from the two sampling occasions in 2017. The lowercase letters above the boxes indicate significant differences ($p < 0.05$) between control and vascular plant removal plots. The uppercase letters below the boxes indicate significant differences between the three peatland age classes. The same letters indicate no significant difference. n = 180 for plots (a) and (b), n = 60 for plots (c) and (d).

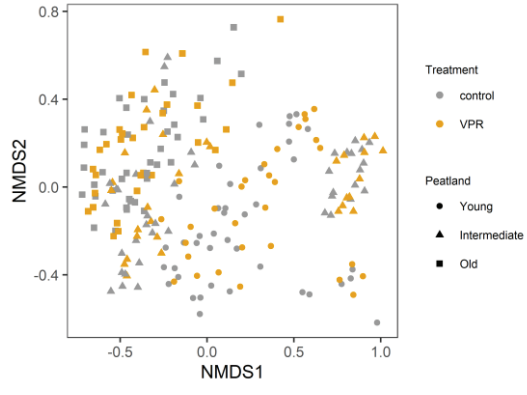

Figure 5. Effects of vascular plant removal (VPR) on microbial community composition in peatlands of the three age classes along the chronosequence.

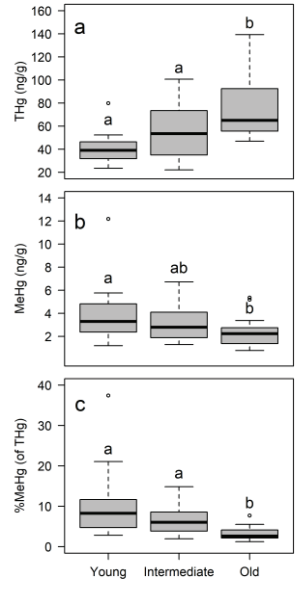

Figure 6. THg (a), MeHg (b) and %MeHg (c) of the 10 cm peat layer immediately beneath the average annual growing season water table along a chronosequence of fifteen peatlands divided into three age classes. The samples were collected on four occasions (June and August, 2016-2017). Letters above boxes indicate significant differences ($p < 0.05$) between the three age classes: classes with the same letter do not differ significantly. n = 20 for each age class. (Figure adapted from Paper I)

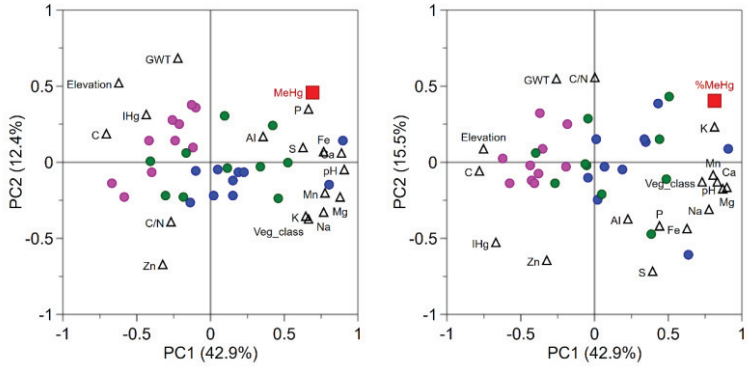

Figure 7. PLS analyses on MeHg (left) and %MeHg (right) in solid peat along the peatland chronosequence. The fifteen peatlands were divided into three age classes according to peatland age, young (blue triangles), intermediate (green boxes) and old (pink dots). (Figure adapted from Paper I)

In solid peat, PLS models showed that both concentrations of MeHg and %MeHg were positively correlated to variables high in young peatlands (e.g. pH, Ca, Fe, S, Mg, Mn) and negatively correlated to those characteristically high for old peatlands (e.g. elevation and C). More details can be found in Paper I.

In porewater, higher concentrations of low molecular weight organic substances (e.g. acetate, lactate) and Fe(III) were all observed in young peatlands compared to old peatlands. Concentrations of both SO_4^{2-} and Fe(III) (potential electron acceptors) were significantly and positively correlated with concentrations of MeHg as well as %MeHg in both peat soil and porewater, with one exception where SO_4^{2-} concentrations did not correlate significantly to %MeHg in peat soil (Figure 8, Figure 9). For more details, see Paper II.

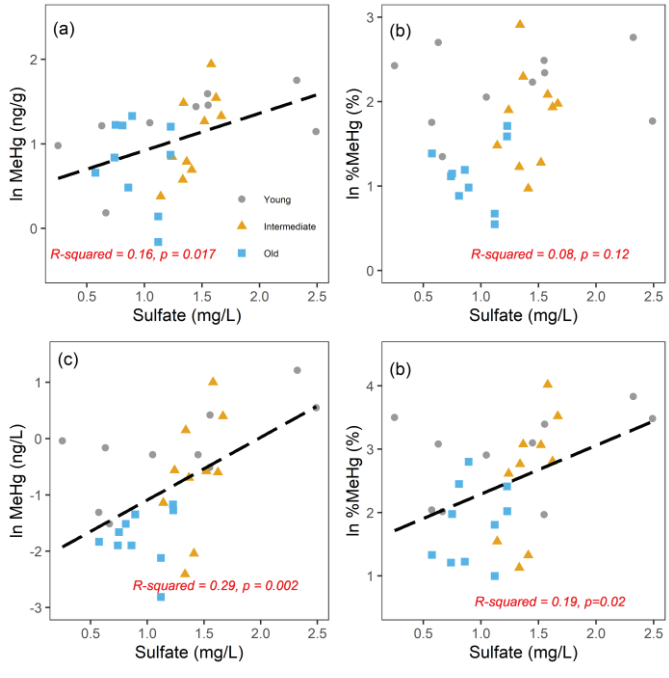

Figure 8. Correlations between sulfate and net MeHg formation (MeHg and %MeHg) in both peat soil (a, b) and porewater (c, d) along the chronosequence. Concentrations of sulfate were determined only for the porewater samples collected in June 2017, n = 30. Concentrations of MeHg and %MeHg in both peat soil and porewater were log transformed. (Figure adapted from Paper II)

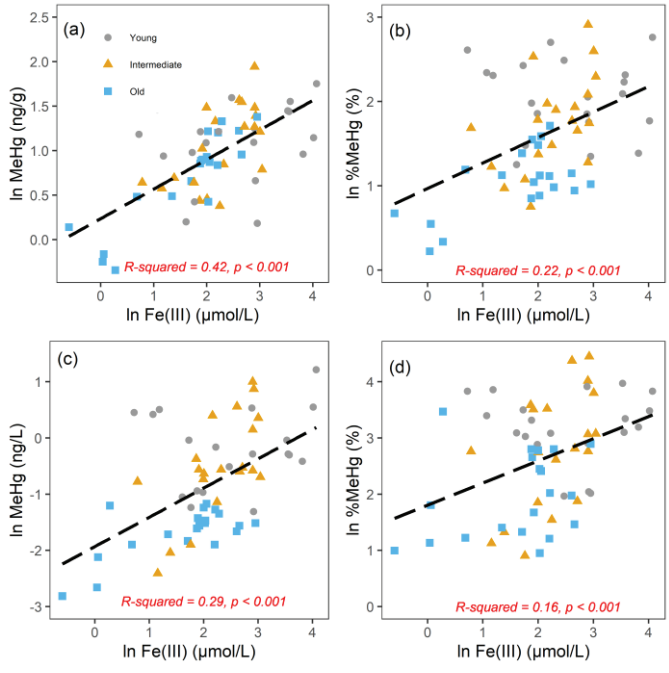

Figure 9. Correlations between Fe(III) and net MeHg formation (MeHg and %MeHg) in both peat soil (a, b) and porewater (c, d) along the chronosequence (n = 60). Concentrations of MeHg and %MeHg in both peat soil and porewater as well as concentrations of Fe(III) were log transformed. (Figure adapted from Paper II)

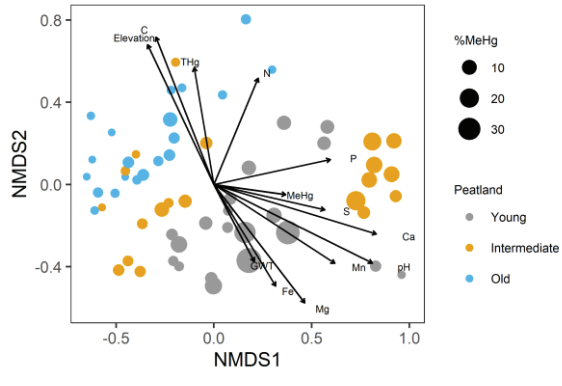

Figure 10. Trophic gradient along the peatland chronosequence shaping the microbial community composition in relation to MeHg formation. Only the 60 samples collected in 2016 were used for this analysis since the geochemical parameters in the solid peat were measured only in samples collected that year. (Figure adapted from Paper IV)

The geochemical features in solid peat were strongly correlated to the microbial community composition along the peatland chronosequence, with pH, elevation as well as total concentrations of C, Mn, Mg, Fe and Ca as the main drivers shaping the microbial community composition (Figure 10). Furthermore, the microbial communities from all the samples collected in 2016 and 2017 were significantly correlated to both MeHg (Mantel test, permutations = 999, n = 120, $r = 0.11$, $p = 0.003$) and %MeHg ($r = 0.28$, $p = 0.001$) in solid peat along the chronosequence. By quantifying the expressions of specific functional genes using qPCR with cDNA as templates, the active microorganisms, including bacteria (16s rRNA), Hg methylating archaea (archaeal *hgcA*), SRB (*dsrA*) and methanogens (*mcrA*), were broadly detected along the peatland chronosequence. The expressions of 16s rRNA, *mcrA* and archaeal *hgcA* were significantly correlated to both MeHg and %MeHg, with one exception, namely the expression of archaeal *hgcA* which was not significant in explaining variation in %MeHg. The expression of *dsrA* was highly variable among the peatlands and not significantly correlated to either MeHg or %MeHg in peat soil (Table 3). More details can be found in Paper IV.

Table 3. Correlations between net MeHg formation and the gene expressions of active microorganisms in peatlands along the chronosequence. Boldface P-values indicate significance at $\alpha = 0.05$. (Table adapted from Paper IV)

Gene	Target	MeHg		%MeHg	
		r	*p*	*r*	*p*
16s rRNA	Bacteria	0.59	**< 0.001**	0.44	**0.016**
dsrA	Sulfate reducing bacteria	0.27	0.200	0.20	0.334
mcrA	Methanogens	0.57	**0.001**	0.41	**0.029**
archaeal *hgcA*	Archaeal methylators	0.37	**0.043**	0.12	0.514

3.5 Solubility and chemical speciation of Hg

The solubility of Hg(II) was slightly higher in young peatlands compared to old peatlands (i.e. a lower Hg(II) log K_d, $p = 0.07$), while the solubility of MeHg was significantly higher in young and intermediate peatlands compared to old peatlands (i.e. lower log K_d MeHg, n = 60, $p < 0.05$). The solubility of Hg(II) was not correlated with MeHg in either peat soil or porewater, but positively correlated with %MeHg in peat soil and negatively

correlated with %MeHg in porewater. The modeling of Hg(II) chemical speciation indicates that the formation of HgS(s) and polysulfides influence the solubility of Hg(II) and thus its subsequent availability for transformation processes, particularly in the young peatland samples (Figure 11). Moreover, higher concentrations of NOM−RS_{TOT} (ads, aq) functional groups in the young peatland samples were predicted to favor the competition for Hg(II) complexation over HS^- in the both aqueous and solid phases of the peat. As a consequence, HgS(s) was less prone to precipitate in the young wetlands (on average, around 5.9% of total Hg(II), as compared to 20.2% in the old and 27.6% in the intermediate peatlands). More detailed information can be found in Paper II.

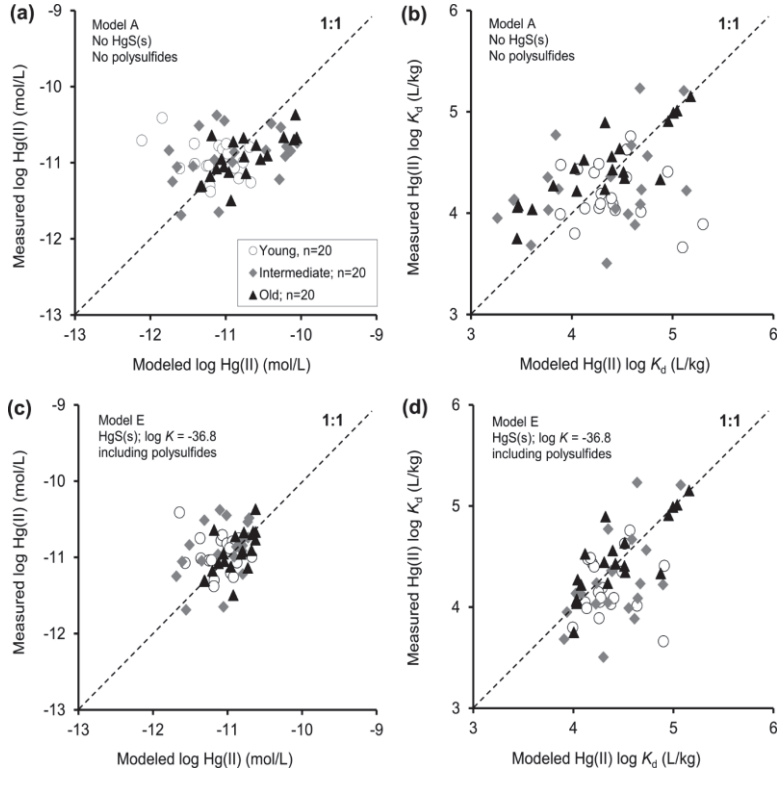

Figure 11. Test of a thermodynamic model for Hg(II) solubility in porewater along the peatland chronosequence without (a, b) and with (c, d) inclusion of HgS(s) formation and polysulfide formation. The left panels (a, c) are for log aqueous phase concentrations of Hg(II) and the right panels (b, d) are the corresponding data for the log K_d. (Figure adapted from Paper II)

3.6 Determination of methylation and demethylation processes

Both methylation and demethylation processes were enhanced in the young peatland in comparison to the intermediate and old ones. While k_m was 26 and 53 times higher in the young peatland compared to the intermediate and old, respectively, k_d was only 2.4 times higher in the young peatland as compared to the intermediate and old peatlands. MeHg degradation along the chronosequence exhibited a gradual shift with peatland age. Biotic processes dominated MeHg degradation in the young peatland; both biotic and abiotic processes were important in the intermediate peatland, and abiotic processes were dominant in the old peatland (Figure 12).

By experimental manipulation with addition of specific microbial inhibitors, the major microbial metabolisms responsible for Hg(II) methylation in peatlands of different chronosequence trophic status and biogeochemistry were identified. In the young peatland, net methylation was largely driven by sulfate reduction but the contribution to k_m declined with decreasing trophic status of the peatlands (i.e. young > intermediate > old peatland). Methanogenic and syntrophic metabolisms became more important for net methylation in intermediate and old peatlands (Figure 13). More detailed information can be found in Paper III.

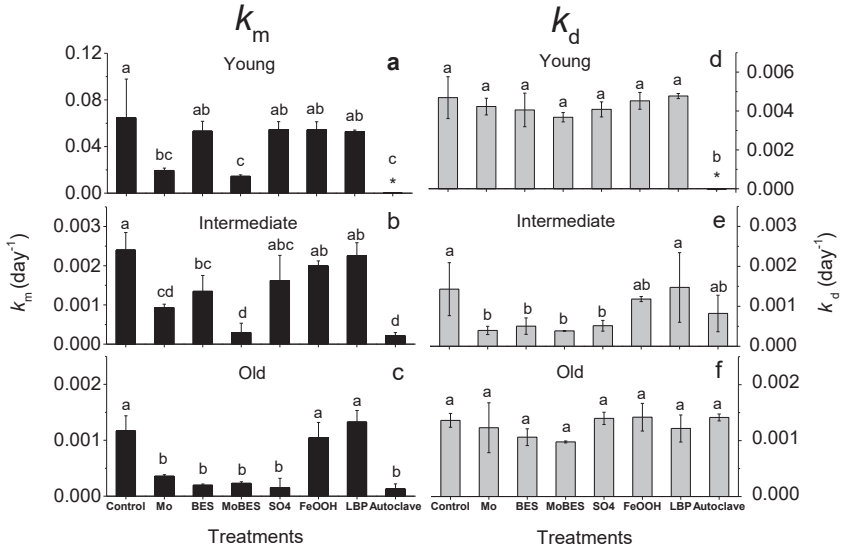

Figure 12. Rate constants for Hg(II) methylation (k_m) (left) and MeHg demethylation (k_d) (right) in the different manipulations of the microcosms from the chronosequence of peatlands. Error bars represent one standard error of the replicate samples from sacrificial incubation bottles (n = 3). LBP = sodium lactate (1 mM) + sodium butyrate (1 mM) + sodium propionate (1 mM), Mo = Na_2MoO_4 (1 mM), BES = sodium 2-bromoethanesulphonate (5 mM), SO_4^{2-} = Na_2SO_4 (1 mM). Autoclave (121°C, 30 min). When values are too low to be visible in the figure, this is indicated by "*". Mean values with the same letter are not significantly different from each other (p = 0.05). (Figure adapted from Paper III)

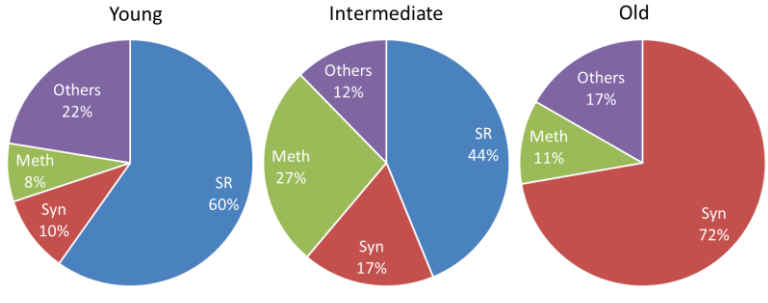

Figure 13. Relative contributions of different microbial metabolisms to k_m across the peatland chronosequence. SR: sulfate reduction; Meth: methanogenesis; Syn: syntrophic interactions. Others: iron reduction, regular fermentation and other metabolisms like syntrophic fermentation by non-SRB. (Figure adapted from Paper III)

4. Discussion

The peatland chronosequence provides a natural gradient of trophic status and hydrogeochemistry due to the successive isolation of the peat surface from mineral substrates caused by the increasing peat depth, increasing lateral extent of peatland area and decreasing weathering rates in the watershed as peatlands age (Figure 2). Along this trophic gradient, both MeHg concentrations and %MeHg in both solid peat and porewater were higher in the young and mesotrophic peatlands compared to the old and oligotrophic peatlands (Figure 4, Figure 6). Similar to trends of MeHg and %MeHg along the peatland chronosequence, both k_m and k_d were higher in the young peatland relative to the intermediate and old ones (Figure 12). These results can be related to the conceptual model for how nutrient status influences net MeHg formation in northern wetlands proposed by Tjerngren *et al.* (2012). The biogeochemical conditions along the chronosequence studied here cover the lower range (acidic part) of the nutrient gradient in this conceptual model, with the maximum in the young peatlands of the chronosequence corresponding to the intermediate trophic status in the conceptual model where net MeHg formation is highest.

In the incubation experiments, Hg(II) methylation was almost completely absent in sterile controls from all studied peatlands (Figure 12). This confirms the findings of others that net MeHg formation in peatlands is generally the result of microbial Hg methylation (Drott *et al.*, 2008; Hammerschmidt & Fitzgerald, 2006). This is also evident from the phylogenetic studies indicating that the microbial communities in peatlands along the chronosequence were significantly correlated to both MeHg and %MeHg in solid peat (see Paper IV). However, microbial Hg methylation is not dependent on any single methylator guild but on complex interactions among microbial communities, including prokaryotes, algae, fungi, and

methanogens (Correia *et al.*, 2012). This is supported by the results of our incubation experiments showing that different microbial metabolisms contributed to Hg(II) methylation to different degrees along the peatland chronosequence (Figure 13), as well as by the results of phylogenetic studies indicating different correlations between net MeHg formation and the gene expressions of specific functional microbial groups that may be involved in Hg methylation (Table 3) and different correlations between net MeHg formation and putative Hg and non-Hg methylators (see Paper IV).

In the phylogenetic studies, the changes in microbial communities along the chronosequence (Figure 10) are in line with previous studies suggesting that microbial community composition is greatly driven by environmental factors (Zhou *et al.*, 2017; Urbanová & Bárta, 2014; Lin *et al.*, 2012). As a consequence of the changes in microbial communities, net MeHg formation varied along the chronosequence (Figure 4, Figure 6). All these confirm the general view that up to a point, higher nutrient availability in peatlands stimulates microorganisms and subsequent Hg methylation. The more interesting question for our study is what limits the rate of net methylation at different points along the peatland nutrient gradient. It should be noted that since this study is carried out along the peatland chronosequence that is within < 10 km of the sea and exposed to a similar climate and atmospheric deposition for a similar period of time (Figure 1, Table 1), temperature/climate differences should not be a factor influencing net MeHg formation in the 10 cm of peat soil sampled immediately below the average growing season GWT.

Vascular plants have been found to affect the decomposition of peat (Zeh *et al.*, 2020) and to be a main driver of microbial community composition in peatlands through the labile C released in root exudates (Bragazza *et al.*, 2015). That labile C could also serve as a good source of electron donors for methylators. However, the vascular plant removal during two consecutive growing seasons in 2016 and 2017 in this study had no discernible effect on net MeHg formation in both solid and aqueous phases (Figure 4) as well as the formation of simple organic acids (e.g. acetate, lactate) or any other geochemical parameter we measured. Although there were no significant differences in concentrations of simple organic acids between the control and VPR plots, there were higher concentrations of short-lived organic acids but lower C/N ratios in the young peatland porewaters compared to the old peatland porewaters (Figure 3). However, the availability of these electron

donors along the chronosequence do not appear to be controlling net MeHg formation. Our incubation experiments corroborate this since adding low molecular weight organic substances (i.e. lactate, butyrate and propionate) as electron donors did not stimulate methylation (Figure 12). Therefore, all these results suggest that the availability of organic electron donors along the chronosequence is abundant enough so as not to limit net MeHg formation in relation to other factors, such as the availability of electron acceptors and Hg.

A common explanation of changes in net methylation in wetlands along nutrient gradients is the difference in the presence of electron acceptors (e.g. SO_4^{2-} and Fe(III)) for some of the major microorganisms that are known to methylate mercury (Bravo *et al.*, 2018; Johnson *et al.*, 2016; Åkerblom *et al.*, 2013). Our study focused on the 10 cm of peat soil sampled immediately below the average growing season GWT. This is an area where water table fluctuations create redox oscillations likely to favor microbial processes such as reduction of sulfate and ferric iron, by providing suboxic conditions for such processes alternating with oxic conditions that allow for reoxidation of reduction products. Along the peatland chronosequence, even though there were no differences in concentrations of total S in peat soil or SO_4^{2-} in porewater, total concentrations of S were significantly correlated to both MeHg and %MeHg in peat soil (Figure 7). There were also significant positive correlations between concentrations of SO_4^{2-} and MeHg as well as %MeHg in both peat soil and porewater (Figure 8). Moreover, both the concentrations of total Fe in peat soil (Figure 2) and Fe(III) in porewater (Figure 3) were higher in young peatlands. These higher concentrations of potential electron acceptors for known methylators (i.e. FeRB) could therefore stimulate Hg methylation, leading to the higher MeHg and %MeHg in both peat soil and porewater of the young peatlands (Figure 4, Figure 6). This is further supported by the significant and positive correlations between total Fe concentrations and MeHg as well as %MeHg in peat soil (Figure 7) and between concentrations of Fe(III) and MeHg as well as %MeHg in both peat soil and porewater (Figure 9). All these results indicate the importance of Fe(III) and SO_4^{2-} as sources of electron acceptors for Hg methylating microbes along the peatland chronosequence. Thus the abundance of electron acceptors in porewater is a factor that is more strongly related to the differences in net methylation along the chronosequence than any differences in availability of electron donors.

The negative relation between MeHg and THg in solid phase peat (Figure 6) suggests that if Hg is a limitation on methylation, it must be the bioavailable Hg(II) instead of the total Hg pool in the soil and pore water that limits net methylation (Zhu *et al.*, 2018; Jonsson *et al.*, 2014). And indeed there was a correlation between modeled concentrations of polysulfides, indicative of bioavailable Hg(II), and net MeHg formation. However, given the less consistent correlations between the observed Hg(II) solubility and indicators of net MeHg formation, together with the negative relationship of the total soil Hg pool to net MeHg formation, the abundance of bioavailable Hg(II) is not a factor that is as clearly influencing net MeHg formation along the chronosequence as the abundance of electron acceptors.

There is a possibility, though, that bioavailability of Hg(II) might become more important in the future if global Hg emissions remain at current levels or decline further. The lower concentration of THg in the solid peat of the younger and intermediate peatlands (Figure 6) could just be an indication that less Hg was accumulated in the younger peatlands during the last century than in the older peatlands (Paper I). The demonstration of Hg evasion back to the atmosphere at a rate several times higher than current wet deposition of Hg at the Degerö mire 50 km inland from the chronosequence (Osterwalder *et al.*, 2017), however, raises the possibility of an alternative explanation of the findings, namely that Hg is currently evading at even greater rates in younger peatlands. The estimated difference in evasion rates between young and old chronosequence peatlands based on this explanation (5.4 $\mu g/m^2$/year more evasion from the younger peatlands, relative to the older peatlands) is on a par with the annual rate of evasion measured from the older (established over 4000 years ago), more inland Degerö peatland (Paper I). While further research is needed to test the Hg evasion hypothesis, it would suggest that reductions in atmospheric emissions of Hg may eventually reduce the amount of Hg available for methylation.

5. Conclusions and future perspectives

5.1 Conclusions

In this thesis, the biogeochemical gradients along a chronosequence of peatlands from younger than 500 years to over 4000 years old was the basis for an investigation of the relative influence of different factors on net MeHg formation. The scientific exploitation of the natural gradient, enhanced by vascular plant removal, and complemented by laboratory incubation studies, led to a number of rather clear conclusions.

One was that vascular plant removal from the 70 × 70 cm plots over the course of two growing seasons did not lead to any discernible changes in net MeHg formation, major chemical features or microbial community composition in peatlands along the chronosequence. Thus we conclude, with additional support from the laboratory incubation studies, that shortages of electron donors were not contributing to the patterns in net MeHg formation along the chronosequence. That conclusion is contingent upon the assumption that the removal of vascular plants had the intended effect of reducing the abundance of electron donors.

A second clear finding, from analysis of both the natural patterns along the chronosequence and the incubation studies, was that availability of electron acceptors, in the form of both Fe(III) and SO_4^{2-}, positively influence net MeHg formation along the chronosequence.

A third, but more indirect finding inferred from the Hg(II) speciation modeling is that the differences in abundance of bioavailable Hg(II) for methylating microorganisms also contributes to the differences in net MeHg formation along the chronosequence. The inferred differences in Hg(II) bioavailability are attributed to formation of HgS(s) and polysulfides as well

as the competition of NOM-RS_{TOT} (ads, aq) functional groups for Hg(II) complexation with HS^- in both the aqueous and solid phases of the peat.

These three findings could almost fit into a relatively simple paradigm that net MeHg formation is limited by the abundance of one of three geochemical factors: either electron donors, electron acceptors or bioavailable Hg(II) in the presence of methylating microorganisms. If the thesis had not gone into the microbial community structures and pathways, the only unresolved feature of that simple paradigm of a single limiting geochemical factor would be a more conclusive evaluation of the limitation posed by bioavailable Hg(II) in relation to the availability of electron acceptors.

The microbial investigations, however, point to a more complex picture. Microbial community composition was significantly correlated to net MeHg formation and influenced by the environmental conditions across the peatland chronosequence. That in itself does not involve a challenge to the idea of finding a single geochemical control in any given environmental setting. However, the different microbial pathways revealed by the incubation studies and supported by the expressions of different genes along the chronosequence, suggest a much more complex set of interactions where SRB, FeRB and methanogenic archaea all play different roles at different locations along the chronosequence nutrient gradient. This complexity is exemplified by the fact that microbes not known to be methylators correlated more strongly to net MeHg formation than known methylators. Another aspect of this complexity is that not all SRB, or FeRB are methylators.

5.2 Future perspectives

While the thesis did manage to arrive at answers for many of the scientific questions that it posed, those answers have identified several new directions for future research that could be helpful for better understanding of Hg cycling, particularly in peatland-rich landscapes. One such area to be pursued is the correlation between bioavailable Hg(II) and net MeHg formation that emerged from the Hg(II) speciation modeling. Tests could be devised for the hypothesis that the abundance of bioavailable Hg(II) is in fact limiting net MeHg formation. This might best be done in laboratory experiments.

A second area for further research is to improve the genomic identification of microbes which do and do not methylate mercury. This is

particularly important since microbial Hg methylation is not dependent on any single guild but on complex interactions among microbial communities, including Hg and non-Hg methylators. That improved identification includes development of better primers for specific microorganisms. The greater challenge, however, will be to resolve the different ways in which Hg methylators and relevant non-Hg methylating consortia interact across the boreal landscape to influence net MeHg formation. Daunting as this challenge is, technologies that can address this are developing rapidly, such as genome-resolved metagenomics and metatranscriptomics.

Appealing as it is to look forward to answering new scientific questions, the question remains how existing knowledge, and specifically the studies in this thesis, can contribute to the evidence base supporting management efforts to control the threat from mercury pollution in landscapes where peatlands are an important source of the MeHg bioaccumulating in food webs. Earlier work has alerted us to the fact that not all wetlands are sources of MeHg to aquatic ecosystems, since nutrient-rich wetlands such as swamps can be sinks of MeHg. In the more nutrient poor (oligotrophic) areas where the rates of net MeHg formation are relatively low, decreases in S-deposition and associated decreases in potential electron acceptors may be helping to mitigate net MeHg formation. This would be a co-benefit from controlling other forms of air pollution. Given the large reductions in S-deposition over Europe and large parts of North America in recent decades, it should be possible to discern that effect, if it is indeed there.

Since syntrophic interactions between methylating microorganisms are the major pathway for net MeHg formation in more nutrient depleted boreal peatlands, a better understanding of syntrophy might lead to insights about how MeHg exposure could be mitigated, such as the potential for altering the hydrology of wetlands through raising or lowering water tables. Genomic monitoring of peatlands might also provide some information on how the metabolic pathways influencing net MeHg formation are changing over time and space in relation to human influences such as management efforts, land-used change, and climate change, as well as natural landscape development or fluctuations in weather patterns.

Controlling inputs of Hg from the atmosphere has long seemed like a less promising pathway to reducing Hg levels in boreal freshwater fish, due to the store of Hg already in peatlands. The results of this study, however,

support earlier suggestions from studies near the chronosequence that Hg might evade from peatlands when atmospheric Hg levels are reduced.

While there are possibilities for improved scientific understanding to motivate management efforts (e.g. pollution emission reductions) and guide the follow-up of these efforts, the time perspective still appears long compared to the common ambition of achieving mitigation in a matter of years.

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Popular science summary

Mercury occurs naturally in the environment in three different forms: elemental mercury (Hg(0)), inorganic mercury (Hg(II)) and organic methylmercury (MeHg). All Hg species can pose health hazards, but methylmercury is the species of most concern compared with elemental and inorganic forms because of its high bioaccumulation in biota and biomagnification through food chains, risking the health of humans and wildlife. In Sweden, where fish Hg concentrations commonly exceed thresholds for safe consumption, about 10% of children are born with a Hg concentration in their hair that indicates some risk for health impairment. Therefore, despite the great strides taken to limit the use and spread of Hg globally, and by Sweden in particular, there remains a need to continue efforts to reduce exposure to Hg. Since peatlands are a source of MeHg to downstream aquatic ecosystems, and peatlands are a major component of the Swedish landscape, a better understanding of the factors influencing the formation of MeHg in peatlands may be able to help guide efforts to further reduce Hg in the freshwater fish of Sweden and other countries with extensive peatlands.

Since most Hg is deposited from the atmosphere in its inorganic forms, *in situ* transformations of Hg must play a crucial role in the distribution and toxicity of Hg. Microbial Hg methylation is limited by two classes of factors: One class relates to the activity of microorganisms involved in Hg(II) methylation. This involves factors such as availability of electron donors and availability of electron acceptors. The second class of factors concerns the bioavailability of Hg(II) to methylating microorganisms, which is controlled by factors such as inorganic sulphide concentration, natural organic matter functional groups, and pH.

The principal aim of this thesis was to investigate the production of MeHg along the biogeochemical gradient created by a peatland chronosequence to better determine the factors influencing net MeHg formation in boreal peatlands. This is intended to build the evidence-base for guiding strategies to limit exposure of people and wildlife to Hg in northern landscapes with a large component of peatlands. The thesis focused on three types of geochemical influence: 1) electron donors like high-quality root carbon exudates from vascular plants, 2) electron acceptors such as sulfate and ferric iron, and 3) the bioavailability of Hg(II). Recent breakthroughs in genomics were also used to define the microbial communities involved in the formation of MeHg.

Along the chronosequence trophic gradient, MeHg concentrations and %MeHg in both solid peat and porewater were higher in the younger, more nutrient-rich peatlands compared to the older, more nutrient-poor peatlands. Genomic studies also found changes in microbial communities along the chronosequence. This, and our experimental manipulations revealed different microbial metabolism pathways contributing to Hg(II) methylation at different points along the chronosequence. This is consistent with the emerging evidence that Hg methylation does not depend on a single methylating guild or geochemical control, but on complex interactions among microorganisms, including prokaryotes, algae, fungi, and methanogens.

This thesis arrived at answers for many of the scientific questions that it posed, but those answers have identified several new directions for future research. A key challenge is resolving the different ways in which Hg methylators and relevant non-Hg methylating consortia interact across the boreal landscape to influence net MeHg formation. Daunting as this challenge is, appropriate technologies for addressing it are developing rapidly, such as genome-resolved metagenomics and metatranscriptomics.

While there are possibilities for improved scientific understanding to motivate management efforts and guide the follow-up of these efforts, the time perspective still appears long, much longer than a matter of years.

Populärvetenskaplig sammanfattning

Kvicksilver förekommer naturligt i miljön i olika former så som elementärt kvicksilver (Hg(0)), oorganiskt kvicksilver (Hg(II)) och organiskt metylkvicksilver. Alla kvicksilverföreningar kan medföra hälsorisker, men metylkvicksilver är särskilt bekymmersamt på grund av dess höga biotillgänglighet och förmåga att anrikas över födovävar, vilket riskerar att skada djur och människor i toppen av dessa födovävar. I Sverige, där kvicksilverkoncentrationer i fisk vanligtvis överskrider gränsvärden för säker konsumtion, föds cirka 10% av barnen med en kvicksilverkoncentration i håret som indikerar en viss risk för negativ påverkan på hälsan. Trots stora ansträngningar för att begränsa användningen och spridningen av kvicksilver globalt, och i synnerhet i Sverige, finns det fortfarande ett stort behov av åtgärder som ytterligare kan begränsa djur och människors exponering för kvicksilver. Då torvmarker är en källa till metylkvicksilver i nedströms vattenekosystem, och torvmarker är en viktig komponent i det svenska landskapet, kan en bättre förståelse för de faktorer som påverkar bildningen av metylkvicksilver i torvmarkerna utgöra en viktig kunskapsbas vid skapandet av åtgärdsplaner för att ytterligare minska kvicksilver i sötvattensfisk i Sverige och i andra länder med stor andel torvmarker.

Eftersom atmosfäriskt kvicksilver främst tillförs terrestra ekosystem i sin oorganiska form (Hg(II)) så spelar omvandlingen av kvicksilver i terrestra och akvatiska miljöer en stor roll för hur kvicksilver mobiliseras i landskapet och hur biotillgängligt det är. Den biologiska produktionen av MeHg är begränsad av två huvudfaktorer; (i) aktiviteten av de mikroorganismer som kan metylera kvicksilver, vilket tex styrs av tillgänglighet av elektrondonatorer och elektronacceptorer som används av dessa mikroorganismer, samt (ii) biotillgängligheten av oorganiskt kvicksilver (Hg(II)) för kvicksilvermetylerande mikroorganismer, som styrs av faktorer såsom oorganisk sulfidkoncentration, funktionella grupper av naturligt organiskt material och pH.

Syftet med denna avhandling var att studera bildningen av metylkvicksilver längs en biogeokemisk gradient av torvmarker som bildar en kronosekvens, för att

bättre förstå de faktorer som påverkar netto-bildningen av MeHg i boreala torvmarker. Detta för att kunna bygga en kunskapsbas för vägledande av strategier med målet att begränsa exponering av kvicksilver för människor och djur i skogslandskap med stor andel torvmark. Avhandlingen fokuserade på tre typer av biogeokemiska faktorer som kan påverka bildningen av metylkvicksilver: (1) tillgänglighet på högkvalitativa kolföreningar som avges från växters rötter och används för mikrobiell energiutvinning, (2) tillgång till elektronmottagare för denna energiutvinning, såsom sulfat eller järn, samt (3) det oorganiskt kvicksilvrets biotillgänglighet för kvicksilvermetylerande mikroorganismer. Arbetet drar även nytta av nya DNA-baserade metoder för att beskriva de mikrobiella samhällen som är involverade i bildandet av MeHg.

Längs den trofisk gradient av torvmarker som ingår i kronosekvensen var metylkvicksilver-koncentrationer och andelen metylkvicksilver av totala koncentrationen kvicksilver, både i fast torv och porvatten, högre i de yngre och mer näringsrika torvmarkerna jämfört med i de äldre och mer näringsfattiga ekosystemen. Det fanns även tydliga skillnader i de mikrobiella samhällen som återfanns längs kronosekvensen av torvmarker. Dessa resultat, tillsammans med experimentella inkubationsstudier i laboratoriet, visade på hur olika grupper av mikroorganismer som använder sig av olika typer av energikällor kan bidra till kvicksilvermetylering i de olika torvmarkerna. Detta stämmer väl överens med nya belägg för att kvicksilvermetyleringen inte beror endast på en enskild grupp av kvicksilvermetylerande bakterier eller enskilda geokemiska faktorer, utan på komplexa interaktioner mellan bakterier, arkéer och deras omgivande miljö.

Denna avhandling kunde svara på många av de vetenskapliga frågeställningar som definierades då studierna inleddes, och bidrar med nya idéer för framtida forskning. En viktig utmaning blir exempelvis att förstå betydelsen av både kvicksilvermetylerande mikroorganismer och de övriga mikroorganismer som inte metylerar kvicksilver men genom samverkan, konkurrens och antagonism kan påverka netto-bildningen av metylkvicksilver i det boreala landskapet. Trots utmaningar med att utföra denna typ av studier så har det etablerats nya kraftfulla tekniker för att studera dessa frågeställningar, där storskalig metagenomik som möjliggör en mer komplett bild av arvsmassan hos samtliga organismer i ekosystemet ter sig särskilt lovande. Trots att det finns goda möjligheter till förbättrad vetenskaplig förståelse för utforma åtgärdsplaner för att begränsa djur och människors exponering till kvicksilver, så är tidsperspektivet för att lösa detta miljöproblem fortfarande mycket långsiktigt.

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Acta Universitatis Agriculturae Sueciae

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Peatlands are an important source of methylmercury (MeHg) in boreal surface waters, increasing the risk of human and wildlife exposure to this neurotoxic compound that bioaccumulates in fish. The thesis resolved the influence of biogeochemical factors on net MeHg formation along a peatland chronosequence. The results show the importance of microbial community interactions with geochemistry in controlling the amount of MeHg that peatlands can deliver to surface waters.

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