Mercury methylating microbial communities of boreal forest soils

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Abstract

The formation of the potent neurotoxic methylmercury (MeHg) is a microbially mediated process that has raised much concern because MeHg poses threats to wildlife and human health. Since boreal forest soils can be a source of MeHg in aquatic networks, it is crucial to understand the biogeochemical processes involved in the formation of this pollutant. High-throughput sequencing of 16S rRNA and the mercury methyltransferase, *hgcA*, combined with geochemical characterisation of soils, were used to determine the microbial populations contributing to MeHg formation in forest soils across Sweden. The *hgcA* sequences obtained were distributed among diverse clades, including *Proteobacteria, Firmicutes*, and *Methanomicrobia,* with *Deltaproteobacteria*, particularly *Geobacteraceae*, dominating the libraries across all soils examined. Our results also suggest that MeHg formation is linked to the composition of also non-mercury methylating bacterial communities, likely providing growth substrate (e.g. acetate) for the *hgcA*-carrying microorganisms responsible for the actual methylation process. While previous

research focused on mercury methylating microbial communities of wetlands, this study provides some first insights into the diversity of mercury methylating microorganisms in boreal forest soils.

Importance

Despite a global state of awareness that mercury, and methylmercury in particular, is a neurotoxin that millions of people continue to be exposed to, there are sizable gaps in our fundamental understanding of the processes and organisms involved in methylmercury formation. In the present study we shed light on the diversity of the microorganisms responsible for methylmercury formation in boreal forest soils. All the microorganisms identified have a relevant role on the processing of organic matter in soils. Moreover, our results show that the formation of methylation formation is not only linked to mercury methylating microorganisms but also to the presence of non-mercury methylating bacterial communities that contribute to methylmercury formation by the appropriate substrate to the microorganisms responsible for the actual methylation process. This study improves current knowledge on the diversity of organisms involved in methylmercury formation in soils.

INTRODUCTION

Mercury (Hg) is a potent toxin that might cause severe negative effects on wildlife and human health (1). The toxicity of Hg is of such concern that 128 countries have signed the Minamata Convention, a global treaty that entered into force in August 2017 with the explicit objective to reduce Hg emissions and protect human health and the environment. High Hg emissions in the past have led to high present-day Hg levels in different parts of the atmosphere, oceans and terrestrial ecosystems (2, 3). Because Hg has a strong affinity for reduced sulphur or thiol (RSH) functional groups of soil organic matter (OM) (4, 5), the increased atmospheric deposition of Hg during the industrialisation has resulted in high Hg concentrations in organic-rich soils (6). As a consequence, the typically OM-rich soils in the boreal biome has retained Hg deposition from both natural and anthropogenic emissions, and now represent an important global Hg stock (4, 7).

55 Soil OM has also been identified as a main vector of Hg and methylmercury (MeHg) transport from
56 catchments to surface waters in boreal areas (8, 9). Indeed, the mobilisation of inorganic Hg ($Hg^{(II)}$) and,
57 the more harmful, MeHg from soils by means of OM-mediated transport has been linked to MeHg
58 accumulation in lake sediments within catchments (9, 10) and in fish (11). As high MeHg levels in fish
59 have raised much concern in many boreal regions over the past decades (12, 13) and since forest soils
60 are an important site for MeHg formation (14), it is crucial to understand the processes and the
61 organisms involved in MeHg formation in boreal soils.

62 The methylation of $Hg^{(II)}$ to MeHg is biologically mediated (15) and takes place under oxygen deficient
63 conditions typical for wetlands (16), water logged soils (14), sediments (9) and anoxic water columns
64 (17), but can also occur in suspended particles in the aerobic zone of aquatic systems (18, 19). Specific
65 strains of sulphate-reducing bacteria (20, 21), iron reducing bacteria (FeRB) (22, 23), methanogens (24)
66 and Firmicutes (25) have the capability to methylate $Hg^{(II)}$. However, a number of factors controlling
67 bacterial activity and/or the geochemical speciation of inorganic $Hg^{(II)}$ will govern MeHg formation in
68 the environment (9, 26). For example, increases in temperature might lead to increases in biological
69 activity and accordingly also higher $Hg^{(II)}$ methylation rates (27). Redox potential also seems to be a key
70 factor as suboxic and mildly reducing conditions seem to promote high $Hg^{(II)}$ methylation rates,
71 whereas anoxic and strongly reducing conditions might lead to elevated sulphide concentrations that
72 eventually prevent $Hg^{(II)}$ from being available for methylation (28). Sulphur plays a major role in
73 influencing $Hg^{(II)}$ methylation by directly affecting the activity of some methylating bacteria (e.g.
74 sulphate reducing bacteria, SRB) and/or control the availability of $Hg^{(II)}$ for methylation (5). Specific
75 organic matter (OM) compounds can promote $Hg^{(II)}$ methylation by enhancing bacterial activity (9), but
76 also by defining $Hg^{(II)}$ speciation (29) and $Hg^{(II)}$ availability (30, 31). OM can also facilitate $Hg^{(II)}$
77 methylation by inhibiting mercury sulphide (HgS(s)) precipitation or enhance HgS(s) dissolution
78 thereby providing available $Hg^{(II)}$ for methylating microorganisms (32). High OM concentrations might
79 also decrease Hg methylation by formation of high mass molecular mass complexes that hamper $Hg^{(II)}$
80 availability (30). Recently it has been concluded that the availability of $Hg^{(II)}$ depends heavily on the $S^{(-II)}$
81 concentration in porewater and the RSH(aq)/RSH(ads) molar ratio of DOM (29). Together, all these

82 studies highlight that geochemical conditions are key in determining the availability of $Hg^{(II)}$ and the
83 activity of the microbial communities involved in the process.

84 The identification of two functional genes, *hgcA* and *hgcB*, which play essential roles in $Hg^{(II)}$
85 methylation (15), provided the means to more directly characterise the complexity of microbial
86 communities involved in the formation of MeHg in natural ecosystems. This approach has been applied
87 to marshes, sediments and swamps in several geographic regions (33–36); rice paddies in China (37),
88 and water conservation areas of the northern Everglades, USA (38). However, very little work to date
89 has been conducted to reveal the distribution of microbial groups responsible for $Hg^{(II)}$ methylation in
90 forest soils within the vast boreal biome. To the best of our knowledge, no studies have directly
91 described the composition and spatial variation in $Hg^{(II)}$ methylating microbial communities in such
92 forests. Therefore, the primary goal of this paper was to describe $Hg^{(II)}$ methylating microbial
93 communities in various boreal forest soils and identify soil characteristics important for shaping these
94 communities. High-throughput next generation sequencing of amplified 16S rRNA and *hgcA* genes
95 combined with molecular barcoding and detailed soil geochemical characterisations were performed to
96 study the $Hg^{(II)}$ methylating microbial communities in 200 soil samples from three different boreal forest
97 regions in order to shed light on the biogeography of microorganisms responsible for MeHg formation
98 in the boreal landscape.

99 ## RESULTS

100 ### Bacterial community composition in boreal forest soils

101 Soil samples were collected from 200 sites in October 2012 and were distributed across eight
102 catchments in three boreal forest regions in Sweden (Table S1, Table S2). A total of 3 321 197 high
103 quality 16S rRNA sequences remained after quality control and chimera removal (7–72 911 reads per
104 sample). The sample with only 7 reads was removed, and we then rarefied the rest of the data to the
105 remaining sample with the fewest reads (1692 reads). The final rarefied sequence dataset (329 940 reads)
106 clustered into 33 158 operational taxonomic units (OTUs) using a similarity threshold of 97 %. In the
107 rarefied dataset, 35 taxa at phyla level, 69 taxa at class level, 119 taxa at order level, and 187 taxa at

family level were detected from all the soil samples across three regions. The overall coverage of the forest bacterial community is reflected in the combined richness detected for random subsets of analysed samples. The logarithmic shape indicated that most of the considerable OTU richness occurring in the forest soils was accounted for in the combined dataset (Fig. S1). Among the dominant phyla across all regions (>5 % relative abundance), *Acidobacteria* was the most abundant, followed by *Proteobacteria, Planctomycetes, Bacteroidetes, Parcubacteria and Verrucomicrobia* (Table 1). Combined, these phyla accounted for 77.5 % of the total sequences (Table 1). Most of the previously identified clades known to contain $Hg^{(II)}$ methylators (25, 39) were detected in the present study, including *Deltaproteobacteria* (3.31 % of the total reads)*, Chloroflexi* (2.60 % of the total reads), *Firmicutes* (0.77 % of the total reads) and *Euryarchaeota* (0.66 % of the total reads) (Table 1). Microbial community composition based on 16S rRNA sequences in the 34 studied MeHg hotspots showed a similar pattern in terms of the dominant phyla (>5 % relative abundance), with *Acidobacteria* and *Proteobacteria* being the most abundant ones. However, *Bacteroidetes* and *Chloroflexi* contributed much more to the total communities at these hotspots compared to the combined dataset across all 200 samples (Table 1).

A non-metric multidimensional scaling (nMDS) plot based on 16S sequences was used to visualise the composition of the bacterial community among samples. *Unclassified Acidobacteriales, Unclassified Ignavibacteriales, Spirochaetaceae, Holophagaceae*, *Anaerolineaceae, Betaproteobacteria and Tepisiphaeraceae* were important contributing families for shaping the differences in bacterial community composition among samples (Fig. 1). Geochemical factors that were correlated (correlation coefficients > 0.5) with the bacterial composition were projected on top with longer vectors implying stronger correlations (Fig. 1). %MeHg, reflected by bubble sizes, presented a strong coupling to the bacterial community composition, which was further confirmed by %MeHg presenting a long vector among all the geochemical factors (Fig. 1). Water content, C%, S% and N% were all found to be the factors that affected the composition of soil bacterial community (Fig. 1), indicating that a supply of organic matter and nutrients in the moist soil shapes the bacterial community. This is in agreement with previous research that pointed out the contribution of nutrients and organic matter to bacterial activities

and $Hg^{(II)}$ methylation (9, 28). Also, S was well correlated with both C and N (Table S3), suggesting that most of the measured sulphur in the sampled soils has likely an organic origin. This has been found as a common feature in boreal soils (40–42).

Unclassified Fibrobacterales, Methanosaetaceae, Unclassified Ignavibacteriales, Spirochaetaceae, Holophagaceae and *Anaerolineaceae* exhibited the highest correlations with %MeHg (Table 2). *Syntrophobacteraceae, Methanosarcinaceae, Methanoregulaceae, Desulfobulbaceae, Syntrophaceae, Desulfobacteraceae* and *Dehalococcoidaceae*, which potentially host $Hg^{(II)}$ methylators (25, 39), were also found relevant to the bacterial community composition in high-%MeHg sites (Table 2).

Figure 1.

Distribution of $Hg^{(II)}$ methylators

The samples with high soil MeHg concentrations and %MeHg > 1% were defined as "MeHg hotspots". In 34 MeHg hotspots (see soils geochemistry descriptors in Table S4, n = 34), the relative abundance of microbial families carrying representatives known to methylate $Hg^{(II)}$ was assessed based on *hgcA* sequences (25, 39). A total of 1 257 577 *hgcA* sequences remained after quality control and chimera removal (11 404–55 461 reads per sample). The *hgcA* dataset was rarefied to the remaining sample with the fewest reads (11 404 reads). The rarefied sequence dataset accounted a total of 387 736 reads that clustered into 573 operational taxonomic units (OTUs) using a similarity threshold of 97 %. As for the 16 rRNA, the logarithmic shape indicated that most of the considerable species richness of $Hg^{(II)}$ methylators occurring in the forest soils was accounted for in the combined dataset (Fig. S1). Representative sequences from 22 families were found in the 34 analysed MeHg hotspots. Of all the *hgcA* sequences, 3.13 % were not taxonomically assigned (Unclassified), 0.28 % were unclassified *Euryarchaeota*, and 7.28 % could not be assigned beyond the rank of Bacteria (Unclassified Bacteria).

The majority of the sequences annotated to the level of family clustered with *Deltaproteobacteria*, making up 85.4 % of all the *hgcA* reads (Table 3). The remaining classified *hgcA* sequences were distributed across diverse families affiliated to *Firmicutes* and *Methanomicrobia*. *Unclassified Deltaproteobacteria* represented up to 56 % of the reads and among the identified families,

Geobacteraceae were the most abundant, contributing up to 40 % in Strömsjöliden. *Ruminococcaceae* (3.21 % of all *hgcA* reads) occurred as another important family in the hotspots in Örebro; while methanogens and syntrophic lineages were less abundant in the hotspots based on *hgcA* sequences (Table 3).

Unclassified Desulfuromonadales, Geobacteraceae, Ruminococcaceae, unclassified Desulfovibrionales, Desulfovibrionaceae, and unclassified Deltaproteobacteria seemed to contribute to differences in the composition of $Hg^{(II)}$ methylators in the studied soils (Fig. 2a). Among the measured geochemical parameters, the S% and the C/S seemed to have an impact on shaping the community composition of $Hg^{(II)}$ methylators (Fig. 2b). Moreover, *Methanoregulaceae, Desulfovibrionaceae, Desulfuromonadaceae, Desulfarculaceae* and *Methanomassiliicoccaceae* correlated positively with S% and negatively with C/S (Table S5). In the studied MeHg hotspots, S was strongly correlated with both C and N (Table S6), suggesting most of the measured sulphur in the hotspots is also likely presented in organic forms.

Phylogenetic analysis of *hgcA* genes

All the *Proteobacteria* families belonged to *Deltaproteobacteria*, a class with which most currently confirmed $Hg^{(II)}$-methylating bacteria are affiliated (43, 44). When combined, the 20 most abundant OTUs accounted for 72 % of the total reads. Noteworthy, phylogenetic analysis revealed that the most abundant $Hg^{(II)}$-methylating OTUs ("OTU_0005", "OTU_0705", "OTU_0008", and "OTU_0012") in the studied forest soils were either taxonomically assigned as *Geobacter sp. or* phylogenetically related to *Geobacter* species (Fig. 3). Among the 20 most abundant OTUs, 17 were taxonomically annotated as *Deltaproteobacteria.* Among these 17 OTUs, 9 were taxonomically annotated as *Geobacter* and 8 were phylogenetically related to *Geobacter* species (Fig. 3). Summing the identified *Geobacter* and the OTUs phylogenetically related to *Geobacter* species, these 17 OTUs accounted for 62 % of the total *hgcA* reads. The 5th most abundant OTU and was taxonomically denoted as *Firmicutes* (*Ethanoligenens*) and the 6th and 7th could not be annotated beyond the bacterial domain.

DISCUSSION

Community composition of $Hg^{(II)}$ methylators in boreal forest soils

Among the diverse microbial communities seen in the soil samples (Table 1), most of the previously identified $Hg^{(II)}$ methylating groups, e.g., *Deltaproteobacteria, Chloroflexi, Firmicutes and Euryarchaeota* could be detected (Table 3). *Deltaproteobacteria* have been considered a predominant $Hg^{(II)}$ methylating class in anaerobic soils (34, 37, 38). In the present study, *Deltaproteobacteria* were also the predominant $Hg^{(II)}$ methylators at the hotspots with *Geobacteraceae* as the most represented family. This family alone contributed over 30% of all *hgcA* reads, and their importance could be seen at all the sampled sites and particularly in Strömsjöliden (Table 3). Iron reducing bacteria (FeRB) have previously been shown to be important for $Hg^{(II)}$ methylation in some environments (22, 23, 36, 43), and most *Geobacter* tested so far are particularly efficient at MeHg formation in the laboratory (23). This suggests that the ability to methylate $Hg^{(II)}$ is widely distributed and a typical feature among the *Geobacteraceae*. The lack of a specific inhibitor for FeRB have hindered the quantification of the relative contribution of FeRB compared to SRB (i.e molybdate inhibitor) and methanogens (i.e. Bromoethanesulfonate inhibitor) to MeHg formation. The discovery of the *hgcA* pushed the state of the art and made possible to identify $Hg^{(II)}$ methylators in environment (15, 25). Our results combined with previous findings in wetlands and paddy soils (34, 37, 38) highlight the importance of *Geobacteraceae* as $Hg^{(II)}$ methylators in boreal forest soils and evidence their potentially very important roles in a wide range of environments.

While SRB are considered to be the principal $Hg^{(II)}$ methylators in aquatic systems (27, 45–48), not much information is available on $Hg^{(II)}$ methylators in soils. However, identified SRB in the hotspots only accounted for a minor portion of $Hg^{(II)}$ methylators (Table 3). However, it is nevertheless plausible that at least some of the *hgcA* sequences annotated as unclassified *Deltaproteobacteria* (Table 3) could be unknown $Hg^{(II)}$ methylating SRB or even $Hg^{(II)}$ methylating sulphate-reducing syntrophs, capable of syntrophic fermentation of simple organic acids in the absence of sulphate as the terminal electron acceptor (49, 50). Therefore, we cannot discard the possibility that also SRB contribute significantly to $Hg^{(II)}$ methylation in the studied systems. A previous study based on selective inhibitors and rate

measurements indeed suggested SRB played an important role in MeHg formation in boreal forest soils (41). Additionally it has been demonstrated that even when SRB belong to the 'rare biosphere' of peatlands, they contribute significantly to respiration processes (51).

Ruminococcaceae belongs to another newly confirmed representative of $Hg^{(II)}$ methylators, the *Firmicutes* (25). *Firmicutes* contributed to $Hg^{(II)}$ methylating microbial communities at the water conservation areas of the Florida Everglades (38) but were not detected in boreal wetlands (34). In the present study, *Ruminococcaceae* were prominent contributors to the *hgcA* pool in hotspots from Örebro and in all soils from Strömsjöliden (Table 3). They could thus play a role in shaping the composition of $Hg^{(II)}$ methylating community as further indicated by the negative correlation though weak between *Ruminococcaceae* and C/S, a primary geochemical factor shaping $Hg^{(II)}$ methylating communities in the hotspots (Table S5 and Fig. 2b). Not much research has been devoted to the possible relationship between organic S and $Hg^{(II)}$ methylating *Ruminococcaceae*. Considering the abundance of this group in forest soils, further efforts are needed to shed light on the metabolic or physiological pathways of $Hg^{(II)}$ methylating *Ruminococcaceae*.

Methanogens were early on suspected to be responsible for $Hg^{(II)}$ methylation (52), but not until recently were they verified as a significant source of $Hg^{(II)}$ methylators in various environments (24, 34). In the hotspots in the studied soils, they were also detected, though not very abundant in the $Hg^{(II)}$ methylating microbial community. *Chloroflexi* has recently been identified as potential $Hg^{(II)}$ methylators in the water conservation areas, paddy soils and wetlands (34, 38, 53). The *hgcA* data did not confirm any significant role of this group in MeHg production in boreal forest soils (Table 3), even though 16S rRNA data revealed non-$Hg^{(II)}$ methylating *Chloroflexi* (e.g. the class *Anaerolineae*) in soils from all three regions (Table 1).

Previous studies have mainly explored flooded environments such as paddy soils (37), boreal wetlands (34) and the water areas of the Florida Everglades (38). Hence our study provided important new information on the composition and diversity of $Hg^{(II)}$ methylating microbial communities in non flooded boreal forest soils and the boreal landscape, and in doing so identified *Geobacteraceae* as significant $Hg^{(II)}$ methylators in the terrestrial biome. The diversity of $Hg^{(II)}$ methylators described in

this study need to be interpreted cautiously. The *hgcA* gene was only recently discovered and the optimization of the appropriate methods and, in particular the design of primers for the *hgcA* amplification, is still developing (54). Additionally, DNA based methods only reveal the presence of organisms, while alternative approaches based on transcription data, proteomes or rate measurements are needed for verifying their activity. Our data nevertheless provide new insights about $Hg^{(II)}$ methylating microbial communities in boreal forest soils and can as such guide and serve as a resource for future research efforts in this field.

Bacterial communities fuel $Hg^{(II)}$ methylators

%MeHg has previously been used as a proxy for methylation efficiency (55, 56), and high %MeHg has also in a few cases been shown to correlate positively with the abundance of $Hg^{(II)}$ methylators (14, 57). In the current study, sites with high %MeHg featured bacterial communities different from those observed at sites with low % MeHg (Fig. 1). Although, families known to contain $Hg^{(II)}$ methylators (*Syntrophobacteraceae, Methanosarcinaceae, Methanoregulaceae, Desulfobulbaceae, Syntrophaceae, Desulfobacteraceae* and *Dehalococcoidaceae*; 25) were found at sites with high %MeHg, there were also positive correlations between %MeHg and families that are not known to host $Hg^{(II)}$ methylators, such us *Unclassified Fibrobacterales, Methanothrix* (formerly *Methanosaeta*), *Unclassified Ignavibacteriales, Spirochaetaceae, Holophagaceae* and *Anaerolineaceae* (Table 2). This suggests that not only the $Hg^{(II)}$ methylators themselves, but also the supporting and interacting bacterial communities residing in the soil environment may influence MeHg formation across the studied regions. *Anaerolineaceae, Spirochaetaceae* and *Holophagaceae* are for example known to generate acetate by fermentation processes (58). *Fibrobacterales*, have recently been suggested to have an important role in cellulose hydrolysis in anaerobic environments, including soils (59). The *Ignavibacteria* class was recently described (Iino et al., 2010) and the physiology and metabolic capacities of this group is still poorly known, even if a distinctive feature of this group is the ability to grow on cellulose and its derivatives with the utilization of Fe(III) oxide as electron acceptor (60). It may well be that these families, which correlated well with %MeHg (Table 2) and seem to be involved in the degradation of long chain OM compounds (61, 62), promoted MeHg production by providing appropriate substrates

(e.g. acetate) for the $Hg^{(II)}$ methylators. $Hg^{(II)}$ methylators and non-$Hg^{(II)}$ methylating members of *Desulfobulbaceae*, known to oxidise organic substrates incompletely to acetate (63), might also have provided the necessary substrate to $Hg^{(II)}$ methylators (Table 2). Based on our results, we propose an important role of also the non-$Hg^{(II)}$ methylating bacterial heterotrophs in sustaining the activity of the $Hg^{(II)}$ methylating microorganisms and thereby influencing MeHg formation in boreal forest soils. Moreover, the correlation between *Methanothrix* and %MeHg deserves special attention. It has been shown that *Methanothrix* can establish syntrophic cooperation with *Anaerolineaceae* (61) or *Geobacteraceae* (64) in methanogenic degradation of long chain carbon compounds (alkanes). As our results show that *Geobacteraceae* are major contributors to the $Hg^{(II)}$ methylating microbial community (Table 3), the high correlation found between *Methanothrix* and %MeHg could be the result of the interaction between the non-$Hg^{(II)}$ methylating *Methanothrix* and the $Hg^{(II)}$ methylating *Geobacteraceae.* In brief, we provide novel system-level information on putative trophic interactions between non-$Hg^{(II)}$ methylating and the $Hg^{(II)}$ methylating taxa. We further suggest that more in depth studies with metagenome-level sequencing and metabolic pathway reconstruction will be a logical next step to gain a more complete understanding of how $Hg^{(II)}$ methylating bacterial and archaeal species interact in soils.

CONCLUSIONS

A newly developed strategy that combine high-throughput *hgcA* amplicon sequencing with molecular barcoding revealed diverse clades of $Hg^{(II)}$ methylators in forest soils. This study confirms a predominant role of *Deltaproteobacteria*, and in particular *Geobacteraceae,* as key $Hg^{(II)}$ methylators in boreal forest soils. *Firmicutes*, and in particular *Ruminococcaceae*, were also abundant members of the $Hg^{(II)}$ methylating microbial community. Besides the identified $Hg^{(II)}$ methylators, we suggest that the non-$Hg^{(II)}$-methylating bacterial community (e.g. *Anaerolineaceae, Holophagaceae* and *Spirochaetaceae*) might have contributed to the net MeHg formation (%MeHg) by processing OM and thereby providing low OM compounds as a substrate to $Hg^{(II)}$ methylators (e.g acetate). By revealing linkages between $Hg^{(II)}$ methylators and non- $Hg^{(II)}$ methylators, our results calls for further community-level work on the metabolic interactions in soil microbial communities to understand $Hg^{(II)}$ methylation.

Such studies would need to go beyond the $Hg^{(II)}$ methylating microbial populations. Our findings provide a better understanding of $Hg^{(II)}$ methylating microbial communities in forest soils and the boreal landscape.

MATERIALS AND METHODS

Site description

Soil samples were collected from 200 sites in October 2012 and were distributed across eight catchments in three boreal forest regions in Sweden (Table S1 and S2). Within each of the catchments, 25 samples were collected. The most southern region Örebro (59°10′16.39″N 14°34′3.01″E) includes three catchments and the sampled soils are dominantly Podzol with Histosols (65) in the lower parts of the catchments along the streams. The organic matter (O) horizons were most often thicker than 20 cm. More detailed information is given in Eklöf et al. (66). Two northern regions, Balsjö (64°1′37″N 18°55′43″E) and Strömsjöliden (64°6′48″N 19°7′36″E), are located 600–700 km north of Örebro and around 14 km apart from each other. Balsjö includes three catchments dominated by orthic Podzol, with Histosols along the streams. The O horizons were most often thicker than 10–20 cm in the lower parts and less than 10 cm higher up in the catchments. More details are given in Löfgren *et al*. (2009). Strömsjöliden includes two catchments and the soils are dominated by fine-grained moraine. The organic layers are most often less than a few centimetres deep. The samples with high soil MeHg concentrations and %MeHg > 1% were defined as "MeHg hotspots" (n=34), see a summary of the soil characteristics of "MeHg hotspots" in Table S4.

The daily mean air temperatures during the 9 sampling days in September in 2012 varied between 7 and 12 °C in Örebro catchments and 4 and 11 °C in Balsjö and Strömsjöliden catchments. There were no major rain events during the sampling period and the temperature and precipitation was normal for the time of the year.

Soil sampling

Soil samples were collected with a soil coring tube (Ø=23 mm). In each catchment, around half of the samples (n=12) were collected systematically along the topographic fall line of the hill slope, at set

distances from the stream draining the area. These samples were collected from the upper 6 cm of the O horizons or the whole O horizons if these were less than 6 cm deep. The locations of the remaining sampling sites (n=13) were chosen by actively looking for potential hot spots for MeHg formation, such as wet patches, driving tracks and stump holes. These targeted samples were also collected from various depths, e.g. depths where groundwater levels were most frequently fluctuating were of special interest for potential $Hg^{(II)}$ methylation.

Single-use plastic gloves were used and soil samples for chemical analyses were collected in plastic bags or acid washed Falcon tubes and stored on ice in a cooler during transport to the laboratory (within 8 hours). Soil samples for molecular analyses were collected following adequate aseptic sampling protocols. All sampling equipment was sterilized by washing in 70% ethanol in between samples. Samples were collected in sterilized plastic tubes and frozen in liquid nitrogen directly in the field, and then stored at -80°C until further processing and analyses.

Chemical analyses

Soil samples were analysed for total Hg (THg), MeHg, water content, and mass percentage of carbon (C), nitrogen (N) and sulphur (S). Samples were freeze-dried and ground by hand in a mortar prior to analyses for THg, C%, N% and S%. Wet and dry weights were measured to estimate the water content. Total Hg was measured using a Perkin Elmer SMS100 total Hg analyser in accordance with US EPA method 7473. The method includes a thermal decomposition step, followed by amalgamation and atomic absorption spectrophotometric detection (working range 0.05–600 ng). Reproducibility and accuracy of measurements were checked by analyses of replicate samples and reference standards. Analyses of MeHg were done by using GC-ICPMS (68) on fresh samples immediately after thawing. C, N and S were analysed on dry soils packed tightly in tin capsules (Elemental Microanalysis, 6.4 mm) and subsequently measured by high temperature catalytic oxidation with a COTECH ECS 4010 elemental analyser calibrated with sulfanilamide standard (C 41.84 %, N 16.27 %, H 4.68 %, O 18.58 %, S 18.62 %). Analytical precision was $< \pm 0.3$ % for C, ± 1.5 % for N and ± 3.5 % for S.

Microbiological analyses

16S rRNA gene: Microbial DNA was extracted from soil samples using the Power soil DNA isolation Kit (MoBio Laboratories Inc, CA, USA) and the quality of the extracted DNA was assessed by gel electrophoresis (1% agarose). Bacterial 16S rRNA genes were amplified in two steps polymerase chain reaction (PCR) according to the protocol in Sinclair *et al* (2015). Briefly, non-barcoded primers Bakt_341F and Bakt_805R (Table S7) were used for the 1st PCR step of 20 cycles. The resulting PCR products were diluted 100 times before being used as template in a 2nd PCR step of 10 cycles with similar primers carrying sample-specific 7-base DNA barcodes. All PCRs were conducted in 20 µL volume using 1.0 U Q5 high fidelity DNA polymerase (NEB, UK), 0.25 µM primers, 0.2 mM dNTP mix, and 0.4 µg bovine serum albumin. The thermal program consisted of an initial 95 °C denaturation step for 5 min, a cycling program of 95 °C for 40 seconds, 53 °C for 40 seconds, 72 °C for 60 seconds and a final elongation step at 72 °C for 7 minutes. Amplicons from the 2nd PCR were purified using the Qiagen gel purification kit (Qiagen, Germany) and quantified using a fluorescence-based DNA quantitation kit (PicoGreen, Invitrogen). The final amplicons after two PCR steps were pooled in equal proportions to obtain a similar number of sequencing reads per sample. Amplicon sequencing was carried out following the protocol described in Sinclair *et al* (2015) using the MiSeq instrument. Illumina sequencing was performed by the SNP/SEQ SciLifeLab facility hosted by Uppsala University using 300bp chemistry. Chimera identification and OTU (Operational Taxonomic Unit) clustering by denoising was done using UNOISE (from USEARCH version 9, ref. 70, 71). SINTAX (from USEARCH version 9, ref. (72)) with the SILVA reference database (release 128) was used as a base to taxonomically annotate OTUs. The sequence data has been deposited to the EBI Archive under accession number PRJEB20882.

***HgcA* gene:** Among the 50 samples selected based on having %MeHg >1 %, 34 resulted in positive PCR amplification of the *hgcA* gene. The protein-coding gene *hgcA* which plays an essential role in Hg methylation was amplified with previously published *hgcA* primers (*hgcA_ 261F* and *hgcA_912R*) (Table S7, 34) modified for parallelized high-throughput Illumina sequencing. HPLC-purified primers carrying Illumina adaptors at the 5' end (*hgcA_261F_Adaptor* and *hgcA_912R_Adaptor*, Table S7) were here used for the 1st stage PCR. In the 2nd stage PCR, standard Illumina handles and barcode primers

(Table S7) were used to enable pooling of all the samples for parallelized Illumina sequencing. *HgcA* was first amplified in 50 µL volume with 1x Phusion GC Buffer, 0.2 mM dNTP mix, 5% DMSO, 0.1 µM of each adaptor-linked primer, 7 µg/µL BSA, 4 µL extracted DNA template, and 1.0 U Phusion high fidelity DNA polymerase (NEB, UK) for an initial denaturation of 2 min at 98 °C followed by 35 cycles (10 s at 96 °C, 30 s 56.5 °C and 45 s at 72 °C), and a final extension at 72°C for 7 min. Following this initial step, a 2nd PCR was conducted to add sample-specific molecular barcodes. Reactions were carried out in 20 µL volumes using 1x Q5 reaction buffer, 0.2 mM dNTP mix, 0.1 µM barcode primers, purified 1st PCR products and 1.0 U Q5 high fidelity DNA polymerase (NEB, UK) for an initial denaturation of 30 s at 98 °C followed by 18 cycles (10 s at 98 °C, 30 s 66 °C and 30 s at 72 °C), and a final extension at 72°C for 2 min. The quality and size of the *hgcA* amplicons were assessed by gel electrophoresis and GelRed visualization on a 1% agarose gel (Invitrogen, USA) prior to purification by Agencourt AMPure XP (Beckman Coulter, USA) after both PCR steps. Quantifications of purified amplicons from the 2nd stag PCR were performed using the PicoGreen kit (Invitrogen).

Amplicons were sequenced using the same method as for the 16S rRNA gene. Forward read sequences were only used in data analysis due to long PCR product. Low quality sequences were filtered and trimmed using SICKLE (73) and adapter were removed by using CUTADAPT (74). Subsequent processing of reads were performed by USEARCH and clustered at 60% identity cutoff using cd-hit-est (75). HMMER (76) search was used for taxonomical annotation with manually curated database of *Proteobacteria* and sequences of Podar *et al.* (2015). More details can be found in Bravo *et al.* (2018).

Phylogenetic analysis: A phylogenetic analysis was performed for *hgcA* sequences representative for the OTUs observed for the 34 hotspots and existing *hgcA* entries in our curated database. The sequences were adequately curated and taxonomy homogenized using taxtastic [https://github.com/fhcrc/taxtastic] and the R-package taxize (77). The obtained protein sequences were aligned with MUSCLE (78) (version 3.8.1551). The alignment was trimmed to the size of the amplicon, and a tree was generated using RAxML (79) (version 8.2.4) - with the PROTGAMMLG model and

autoMR to choose the number of necessary bootstrap resamplings (n = 750). This tree and the corresponding alignment were used to generate a reference package for PPLACER (80). The guppy tool of PPLACER was then used to classify the sequences with a likelihood threshold of 0.8.

Statistical analysis

Family-level microbial community composition in the different samples were compared using non-metric multidimensional scaling (nMDS) based on Bray-Curtis similarities and using the software PRIMER 7 (81). Information on the common set of samples from community composition based on Bray-Curtis similarities and that from geochemical variables based on Euclidean distance was presented in one single ordination. A combined nMDS plot with bubble and vector plots of geochemical factors projected on the same ordination of community composition was constructed to reveal the relationships between community compositions and potentially explanatory geochemical variables (81). Pearson's correlation coefficient (R) was assessed to reveal linear relationships between variables using a significance level of alpha < 0.05.

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627 **Table 1.** Comparison of the relative abundances (%) of the most abundant taxa (phylum level) in all the samples
628 (n=200) with the 34 MeHg hotspots based on 16S rRNA sequences. Relative abundances of classes under phylum
629 *Proteobacteria* are listed with indent (SD: Standard deviation)

Most abundant taxa	Mean ± SD		Maximum		Minimum	
	All samples	Hotspots	All samples	Hotspots	All samples	Hotspots
Acidobacteria	36.11 ±10.53	25.57 ±8.77	73.64	49.29	8.10	9.40
Proteobacteria	13.99 ±4.03	16.56 ±2.96	28.13	27.60	2.90	8.87
Alphaproteobacteria	6.83 ±3.01	7.13 ±2.81	16.43	13.95	1.77	2.66
Deltaproteobacteria	3.31 ±1.69	3.56 ±1.38	13.36	7.15	0.71	1.30
Gammaproteobacteria	2.06 ±1.33	1.48 ±0.76	7.15	3.66	0.24	0.35
Betaproteobacteria	1.78 ±2.13	4.14 ±2.47	11.11	10.46	0.00	0.65
Epsilonproteobacteria	0.01 ±0.03	0.03 ±0.06	0.30	0.24	0.00	0.00
Planctomycetes	8.18 ±4.21	5.82 ±2.77	24.82	11.64	1.36	1.95
Bacteroidetes	6.61 ±5.24	11.38 ±7.92	51.60	51.60	0.41	1.60
Parcubacteria	6.35 ±4.19	9.01 ±5.14	26.36	24.47	0.06	2.13
Verrucomicrobia	6.28 ±2.78	5.30 ±2.31	14.89	10.64	0.65	0.65
Thaumarchaeota	3.96 ±2.77	2.53 ±2.44	18.44	14.83	0.00	0.00
Actinobacteria	3.11 ±2.38	2.94 ±1.62	19.86	6.03	0.47	0.89
Chlamydiae	2.83 ±2.56	1.31 ±1.08	22.87	3.71	0.24	0.30
Chloroflexi	2.60 ±3.18	7.16 ±5.18	17.79	15.19	0.00	0.12
Others	9.97 ±0.89	12.41 ±1.66	17.14	8.98	0.00	0.00

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Table 2. Moderate (0.5≤R<0.7) to weak (0.3≤R<0.5) Pearson correlations between families and %MeHg in all samples based on 16S rRNA. Families potentially involved in Hg methylation were marked in bold.

Families	Correlations with %MeHg
Unclassified Fibrobacterales	0.56
Methanothrix	0.54
Unclassified Ignavibacteriales	0.52
Spirochaetaceae	0.52
Holophagaceae	0.50
Anaerolineaceae	0.41
Lentimicrobiaceae	0.40
Syntrophobacteraceae	0.39
Unclassified Phycisphaerales	0.37
Methanosarcinaceae	0.37
Methanoregulaceae	0.35
Desulfobulbaceae	0.35
Porphyromonadaceae	0.35
Rhodobiaceae	0.33
Unclassified Clostridiales	0.32
Gemmatimonadaceae	0.30
Syntrophaceae	0.30
Unclassified Omnitrophica	0.30
Nitrosomonadaceae	0.30
Desulfobacteraceae	0.30
Dehalococcoidaceae	0.30
Unclassified Obscuribacterales	-0.30
Unclassified Solibacterales	-0.33
Tepidisphaeraceae	-0.38

636 **Table 3.** Relative abundance of families involved in $Hg^{(II)}$ methylation based on *hgcA* sequences in 34 hotspots.

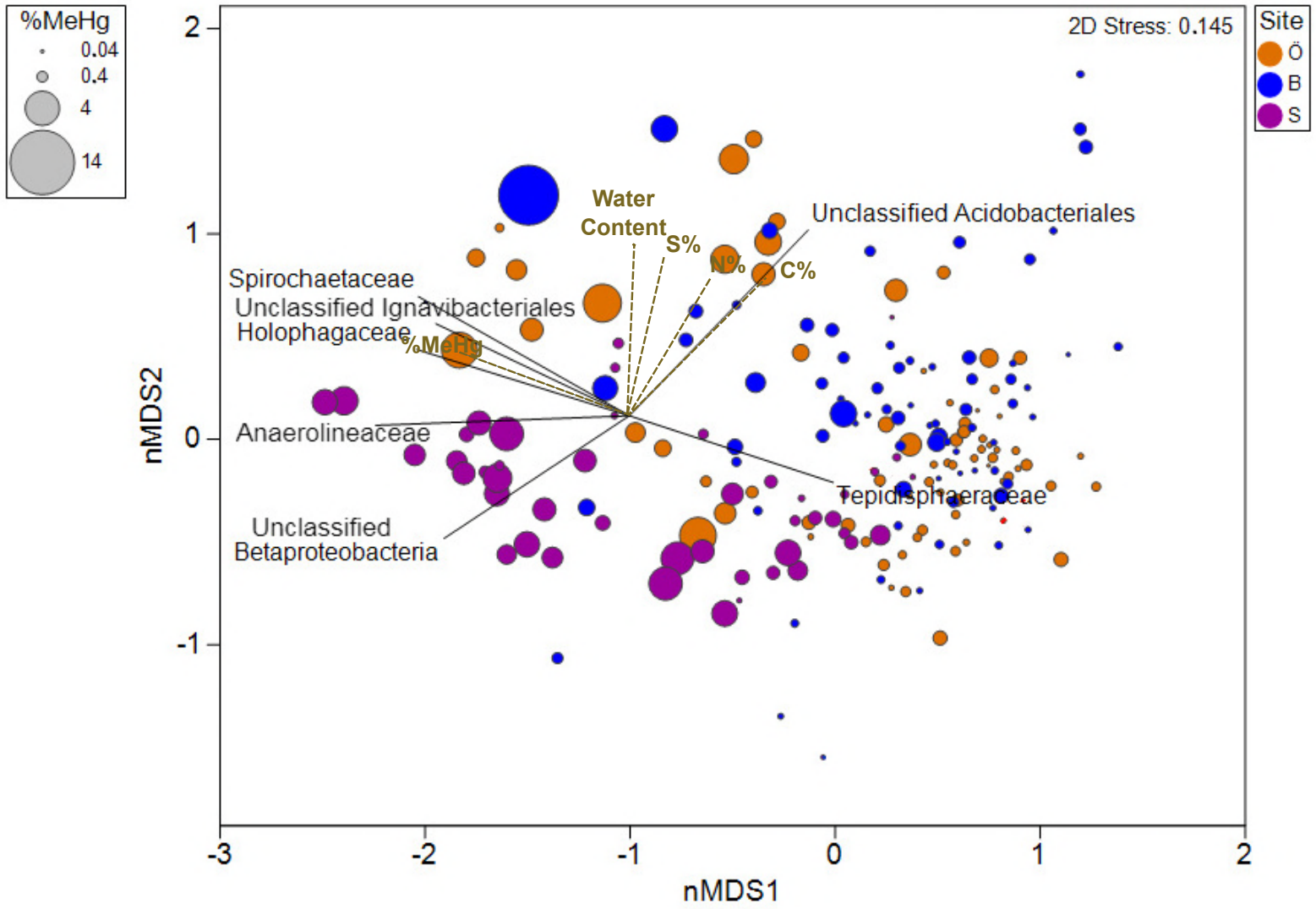
Families	**Örebro**	**Balsjö**	**Strömsjöliden**
	% of *hgcA* reads	% of *hgcA* reads	% of *hgcA* reads
Unclassified Deltaproteobacteria	43.24±37.11	44.85±30.09	55.69±18.23
Geobacteraceae	26.79±31.09	24.62±22.22	39.40±18.96
Unclassified Bacteria	10.72±17.45	25.58±33.67	1.43±1.02
Ruminococcaceae	9.12±18.23	1.52±2.30	0.15±0.04
Unclassified	6.62±8.65	2.37±3.86	1.27±2.98
Unclassified Euryarchaeota	0.84±2.22	0.02±0.02	0.01±0.02
Desulfovibrionaceae	0.83±1.28	0.16±0.03	0.02±0.04
Unclassified Methanomicrobiales	0.49±1.21	0.06±0.09	0.03±0.12
Syntrophaceae	0.35±0.45	0.05±0.00	0.00±0.00
Methanomassiliicoccaceae	0.31±0.53	0.02±0.00	0.13±0.05
Methanoregulaceae	0.20±0.03	0.06±0.03	0.00±0.01
Syntrophomonadaceae	0.17±0.13	0.02±0.03	0.13±0.04
Unclassified Desulfovibrionales	0.14±0.15	0.02±0.05	0.03±0.04
Unclassified Clostridiales	0.06±0.22	0.51±0.19	0.08±0.07
Unclassified Firmicutes	0.06±0.02	0.00±0.00	0.00±0.00
Unclassified Desulfuromonadales	0.03±0.00	0.10±0.03	0.39±0.32
Desulfobulbaceae	0.02±0.02	0.01±0.00	0.00±0.01
Desulfuromonadaceae	0.01±0.01	0.00±0.04	0.00±0.04
Syntrophorhabdaceae	0.01±0.00	0.00±0.02	0.00±0.06
Unclassified Deferrisoma	0.01±0.00	0.00±0.00	1.18±0.98
Desulfarculaceae	0.00±0.02	0.00±0.00	0.00±0.02
Pelobacteraceae	0.00±0.01	0.01±0.01	0.07±0.03

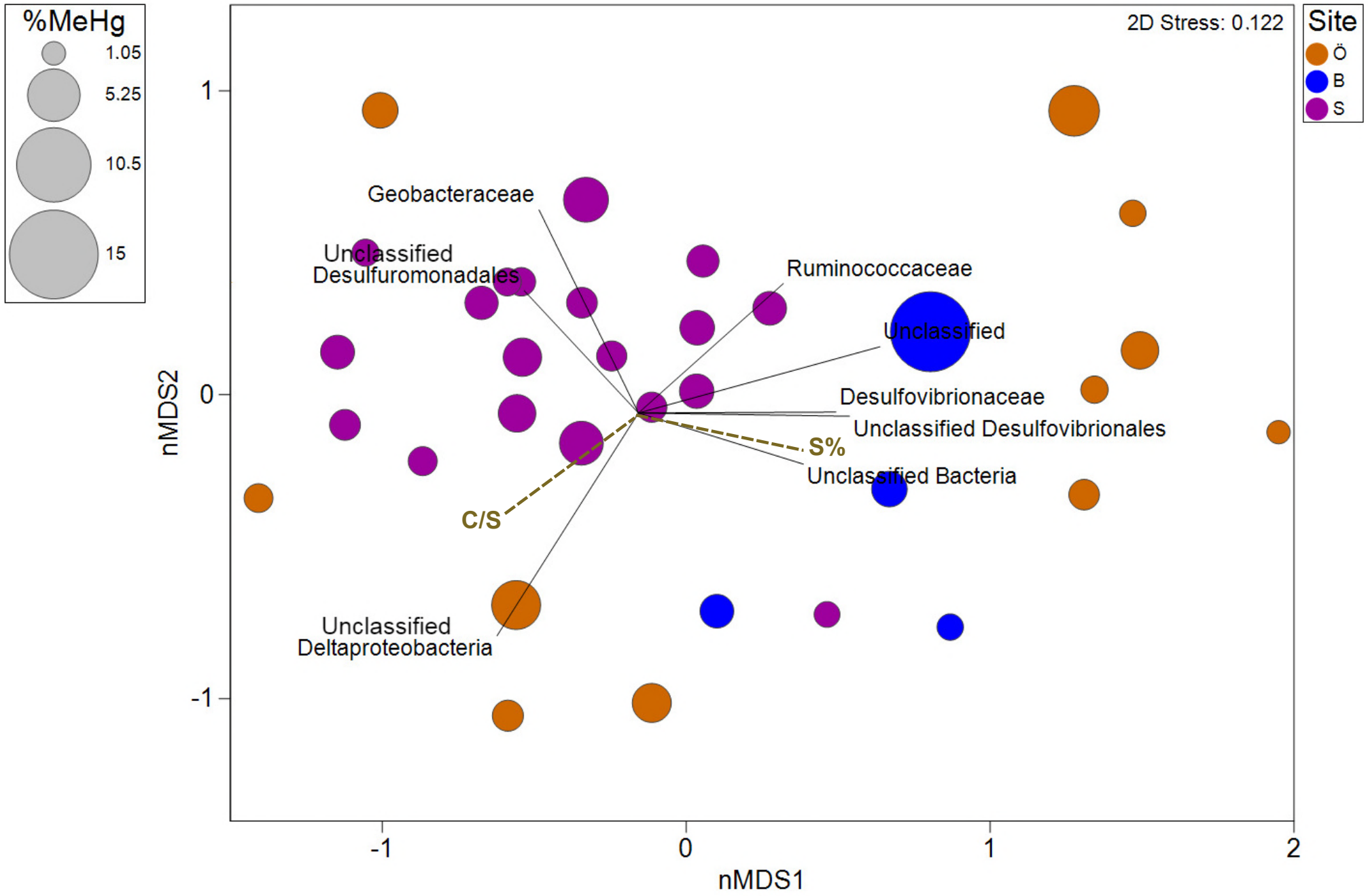
637

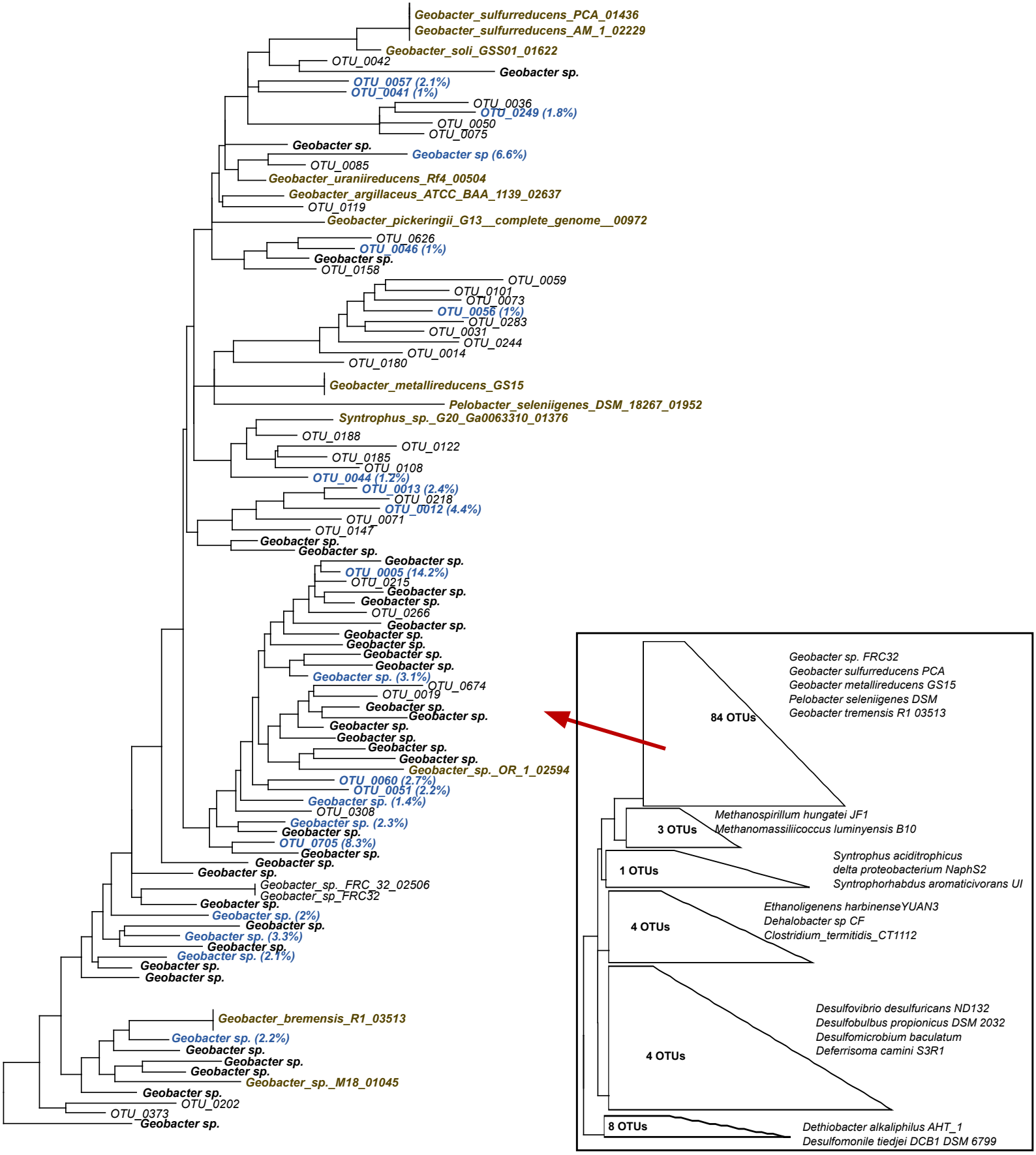
Figure 1. Non-metric multidimensional scaling (nMDS) of microbial community composition of all samples (family level based on 16S rRNA) overlaid with families (black lint) and geochemical factors (dotted brown line) moderately correlated with biotic ordination (correlation coefficients > 0.5) (%MeHg: MeHg/THg). Relative dissimilarities (or distances) among the samples were computed according to the resemblance matrix calculated on fourth rooted family reads.

Figure 2. Non-metric multidimensional scaling (nMDS) of potential Hg methylators (family level based on *hgcA*) in 34 hotspots overlaid with geochemical factors that were moderately correlated with the biotic ordination positions (correlation coefficients > 0.5)

Figure 3. Phylogenetic relationships of *Deltaproteobacterial hgcA* sequences in the studied forest soils. The 20 most abundant *Deltaproteobacteria* are in blue. The OTUs taxonomically assigned as *Geobacter* are indicated in the plot "*Geobacter sp.*". OTUs non-taxonomically assigned are presented as "OTU". Reference genomes are marked in brown. The tree was generated using RAxML (version 8.2.4) with the PROTGAMMLG model and the autoMR to choose the number of necessary bootstraps (750). Please see details of the collapsed tree in the Fig. S2.


%MeHg
0.04
0.4
4
14
2D Stress: 0.145
Site
Ö
B
S
Water Content
S%
N%
C%
%MeHg
Unclassified Acidobacteriales
Spirochaetaceae
Unclassified Ignavibacteriales
Holophagaceae
Anaerolineaceae
Tepidisphaeraceae
Unclassified Betaproteobacteria
nMDS2
nMDS1
2
1
0
-1
-3
-2
-1
0
1
2


%MeHg
1.05
5.25
10.5
15
2D Stress: 0.122
Site
Ö
B
S
Geobacteraceae
Unclassified
Desulfuromonadales
Ruminococcaceae
Unclassified
Desulfovibrionaceae
Unclassified Desulfovibrionales
S%
Unclassified Bacteria
C/S
Unclassified
Deltaproteobacteria
nMDS2
nMDS1
1
0
-1
-1
0
1
2


Geobacter_sulfurreducens_PCA_01436
Geobacter_sulfurreducens_AM_1_02229
Geobacter_soli_GSS01_01622
OTU_0042
Geobacter sp.
OTU_0057 (2.1%)
OTU_0041 (1%)
OTU_0036
OTU_0249 (1.8%)
OTU_0050
OTU_0075
Geobacter sp.
Geobacter sp (6.6%)
OTU_0085
Geobacter_uraniireducens_Rf4_00504
Geobacter_argillaceus_ATCC_BAA_1139_02637
OTU_0119
Geobacter_pickeringii_G13__complete_genome__00972
OTU_0626
OTU_0046 (1%)
Geobacter sp.
OTU_0158
OTU_0059
OTU_0101
OTU_0073
OTU_0056 (1%)
OTU_0283
OTU_0031
OTU_0244
OTU_0014
OTU_0180
Geobacter_metallireducens_GS15
Pelobacter_seleniigenes_DSM_18267_01952
Syntrophus_sp._G20_Ga0063310_01376
OTU_0188
OTU_0122
OTU_0185
OTU_0108
OTU_0044 (1.2%)
OTU_0013 (2.4%)
OTU_0218
OTU_0012 (4.4%)
OTU_0071
OTU_0147
Geobacter sp.
Geobacter sp.
Geobacter sp.
OTU_0005 (14.2%)
OTU_0215
Geobacter sp.
Geobacter sp.
OTU_0266
Geobacter sp.
Geobacter sp.
Geobacter sp.
Geobacter sp.
Geobacter sp.
Geobacter sp. (3.1%)
OTU_0674
OTU_0019
Geobacter sp.
Geobacter sp.
Geobacter sp.
Geobacter sp.
Geobacter sp.
Geobacter sp.
Geobacter_sp._OR_1_02594
OTU_0060 (2.7%)
OTU_0051 (2.2%)
Geobacter sp. (1.4%)
OTU_0308
Geobacter sp. (2.3%)
Geobacter sp.
OTU_0705 (8.3%)
Geobacter sp.
Geobacter sp.
Geobacter sp.
Geobacter_sp._FRC_32_02506
Geobacter_sp_FRC32
Geobacter sp.
Geobacter sp. (2%)
Geobacter sp.
Geobacter sp. (3.3%)
Geobacter sp.
Geobacter sp. (2.1%)
Geobacter sp.
Geobacter sp.
Geobacter_bremensis_R1_03513
Geobacter sp. (2.2%)
Geobacter sp.
Geobacter sp.
Geobacter sp.
Geobacter_sp._M18_01045
Geobacter sp.
OTU_0202
OTU_0373
Geobacter sp.
84 OTUs
Geobacter sp. FRC32
Geobacter sulfurreducens PCA
Geobacter metallireducens GS15
Pelobacter seleniigenes DSM
Geobacter tremensis R1 03513
3 OTUs
Methanospirillum hungatei JF1
Methanomassiliicoccus luminyensis B10
1 OTUs
Syntrophus aciditrophicus
delta proteobacterium NaphS2
Syntrophorhabdus aromaticivorans UI
4 OTUs
Ethanoligenens harbinenseYUAN3
Dehalobacter sp CF
Clostridium_termitidis_CT1112
4 OTUs
Desulfovibrio desulfuricans ND132
Desulfobulbus propionicus DSM 2032
Desulfomicrobium baculatum
Deferrisoma camini S3R1
8 OTUs
Dethiobacter alkaliphilus AHT_1
Desulfomonile tiedjei DCB1 DSM 6799