Impact of mercury on the forest soil ecosystem

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Summary

Mercury (Hg) is one of the most problematic heavy metals. Over the past century the Hg consumption and the Hg emission have increased dramatically. The main causes of anthropogenic Hg emissions are fossil fuel combustion and the use of Hg in gold mining. Worldwide, various efforts and international cooperation aim to reduce the use of Hg and its emissions. Due to restrictions in the use of Hg and process optimizations, global mercury emissions have been reduced in the past few years. Nevertheless, Hg is still a serious environmental problem and its emission is still very high, especially in Asian countries. About half of the Hg emitted into the atmosphere enters the global atmospheric circulation where its residence time is approximately one year. Thus, it can be distributed globally and may be deposited on parts of the earth’s surface far away from any Hg emission source. Mercury is persistent and is not degradable. It accumulates in living organisms, and can lead to neurological and psychological diseases in humans.

The aim of this study was to improve our understanding about soil contamination with Hg. This thesis is divided into four sections. I) In the first section, we examined the bioaccumulation of Hg and methyl-Hg in mushrooms and earthworms, with a focus on how soil properties relate to the accumulation of Hg and methyl-Hg, and if their accumulation is species specific (Chapter 2). II) Given the high methyl-Hg concentrations found in some earthworms, we hypothesized that the anaerobic conditions in the digestive tract of earthworms lead to a methylation of Hg in the earthworms themselves. This idea was tested in a laboratory experiment. In addition, we were interested in finding out if soil pollution with Hg affects the bacterial community structure in the mutualistic digestive tract of earthworms (Chapter 3). III) The influence of Hg on soil microorganisms has been investigated several times, but mainly for soils with high organic matter contents. The effect of methyl-Hg on soil microorganisms has, however hardly been investigated so far. We conducted laboratory experiments in which we contaminated seven forest soils with Hg or methyl-Hg, and examined the effect of the soil treatment on the soil microbial community structure and soil functions (Chapter 4 and 5). IV) In the last section, we modeled the future Hg (and that of
cadmium and lead) soil concentrations using a dynamic soil model (CHUM-AM). We wanted to see if the soil concentrations of heavy metals in future will decline due to decreasing emissions of heavy metals and if, in the near or distant future, critical limit concentrations in soils are likely to be exceeded (Chapter 6).

We found that the Hg accumulations in mushrooms ($BCF_{Hg} = 7.6$) and earthworms ($BCF_{Hg} = 7.2$) are similar. However, the bioaccumulation of methylated Hg in earthworms ($BCF_{methyl-Hg} = 83.1$) was much higher than in fungi ($BCF_{methyl-Hg} = 19.3$). In general, no correlation was found between the soil Hg concentrations and the concentrations in fungi or earthworms. The Hg and methyl-Hg concentrations differed significantly between the ecological groups of fungi and earthworms. Litter-decomposing mushrooms contained about four times higher Hg concentrations ($2.56 \text{ mg Hg kg}^{-1} \text{ dw}$) than mycorrhizal fungi ($0.81 \text{ mg Hg kg}^{-1} \text{ dw}$), and about 10 times higher concentrations than wood-decomposing fungi ($0.24 \text{ mg Hg kg}^{-1} \text{ dw}$). The methyl-Hg concentrations were also highest in litter-decomposing fungi ($0.060 \text{ mg of methyl-Hg kg}^{-1} \text{ dw}$), followed by mycorrhizal ($0.017 \text{ mg of methyl-Hg kg}^{-1} \text{ dw}$) and wood-decomposing fungi ($0.009 \text{ mg of methyl-Hg kg}^{-1} \text{ dw}$).

Earthworms that inhabit the topsoils (endogeic) contained the highest concentrations of Hg ($1.63 \text{ mg Hg kg}^{-1} \text{ dw}$), and methyl-Hg ($0.153 \text{ mg of methyl-Hg kg}^{-1} \text{ dw}$), followed by earthworms that inhabit deep burrows (anecic) ($0.69 \text{ mg Hg kg}^{-1} \text{ dw}$, $0.055 \text{ mg of methyl-Hg kg}^{-1} \text{ dw}$) and litter-inhabiting organisms (epigeic) ($0.49 \text{ mg Hg kg}^{-1} \text{ dw}$, $0.025 \text{ mg of methyl-Hg kg}^{-1} \text{ dw}$). Up to 10% of the total Hg in earthworms was present in methylated form. We found strong evidence that Hg can be methylated directly into earthworms. Earthworms of the species *Lumbricus terrestris* that grew in sterile, Hg-treated soils for 30 days contained approximately 6 times more methyl-Hg than earthworms from unpolluted soil. We assume that the anaerobic digestive tract of earthworms provide suitable conditions for the methylation of Hg by bacteria, leading to an accumulation of methyl-Hg in earthworm tissue. The bacterial communities in the earthworms were significantly affected if the soil was treated with inorganic-Hg, and even more if it was treated with methyl-Hg. Mercury and methyl-Hg also affect the microorganisms in forest soils. At Hg concentrations
above 3.2 µg Hg g\(^{-1}\) dry soil, the basal respiration of the soil is greatly reduced. At a bioavailable concentration of 0.004 µg Hg g\(^{-1}\) dry soil, we also found significant changes in the bacterial community structure of the soil. In one out of seven soils, the bacterial community structure could already be strongly influenced by adding of 5 µg methyl-Hg kg\(^{-1}\) dry soil. In five soils treated with the average methyl-Hg concentrations found in earthworms (90 µg methyl-Hg kg\(^{-1}\)), the bacterial structures were changed significantly, and in four soils the microbial biomass was reduced significantly. In contrast to soil contaminations with inorganic Hg, the basal respiration of the soils treated with methyl-Hg tended to increase with increasing methyl-Hg levels. In the topsoils of natural Swiss forests without any nearby Hg source, between 0.2 and 2.4% of the Hg are present in their methylated form. According to our model, soil Hg concentrations in unpolluted forest sites are likely to double during the next 1000 years with current levels of Hg deposition. Despite this increase, these higher concentrations will probably not affect the microorganisms in the soils.
Zusammenfassung

Quecksilber (Hg) ist eines der problematischsten Schwermetalle weltweit. Im letzten Jahrhundert nahmen der Verbrauch und die Emissionen von Hg dramatisch zu. Die grössten Verursacher der anthropogenen Emission von Hg sind die Verbrennung fossiler Brennstoffe sowie die Verwendung von Hg bei der Goldgewinnung. Weltweit existieren verschiedene politische Bemühungen und internationale Zusammenarbeiten mit dem Ziel, die Verwendung von Hg zu reduzieren und seine Emissionen zu verringern. Durch Nutzungseinschränkungen und durch Prozessoptimierungen bei der Verwendung von Hg konnte die globale Emission von Hg während den letzten Jahren leicht reduziert werden. Dennoch bleibt Hg ein ernst zu nehmendes Umweltproblem. Die Emission von Hg ist immer noch sehr hoch, besonders in Asien. Etwa die Hälfte des emmitierten Hg erreicht den globalen atmosphärischen Kreislauf wo es im Durchschnitt etwa ein Jahr verweilt, bevor es wieder auf die Erdoberfläche eingetragen wird. Somit kann Hg global verteilt und an emissionsfernen Standorten deponiert werden. Quecksilber reichert sich in Lebewesen an und kann zu neurologischen und psychologischen Erkrankungen beim Menschen führen.

Ziel dieser Arbeit war es grundlegende Erkenntnisse von Bodenbelastungen durch Hg zu generieren. Die Arbeit gliedert sich in vier Themenbereiche. I) In einem ersten Bereich untersuchten wir die Bioakkumulation von Hg und die von Methyl-Hg durch Pilze und Regenwürmer. Wir interessierten uns, ob bestimmte Bodeneigenschaften zu einer erhöhten Hg Akkumulation führt und ob sie speziesabhängig ist (Kapitel 2). II) Aufgrund der teilweise hohen Methyl-Hg Konzentrationen in Regenwürmern vermuteten wir, dass die anaeroben Bedingungen im Verdauungstrakt der Regenwürmer zu einer Methylierung des Hg führt. Diese Idee untersuchten wir in einem Laborexperiment. Zudem interessierte uns, ob Hg-Bodenbelastungen die bakteriellen Gemeinschaftsstrukturen im mutualistischen Verdauungstrakt der Regenwürmer beeinflusst (Kapitel 3). III) Der Einfluss von Hg auf Bodenmikroorganismen wurde bereits mehrfach untersucht, jedoch vorwiegend für Böden welche reich an organischem Material sind. Bisher noch kaum untersucht wurde der Effekt von Methyl-Hg auf die Bodenmikroorganismen. Wir führten Laborexperimente durch, in
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denen wir Proben von sieben Waldoberböden mit Hg oder Methyl-Hg kontaminierten und untersuchten den Einfluss der Schadstoffe auf Bodenfunktionen und die mikrobiellen Gemeinschaftsstrukturen (Kapitel 4 und 5). IV) In einer letzten Untersuchung modellierten wir die zukünftigen Hg (sowie jene von Cadmium und Blei) Bodenkonzentrationen mittels eines dynamischen Bodenmodelles (CHUM-AM). Wir interessierten uns, ob durch die Abnahme der Schwermetalldepositionen auch die Bodenkonzentrationen abnehmen und ob in naher oder ferner Zukunft kritische Bodenkonzentrationen überschritten werden (Kapitel 6).

Wir fanden heraus, dass sich Hg in Pilzen \( \text{BCF}_{\text{Hg}} = 7.6 \) und Regenwürmern \( \text{BCF}_{\text{Hg}} = 7.2 \) durchschnittlich etwa gleich stark anreichert. Methyliertes Hg hingegen zeigte eine wesentlich höhere Bioakkumulation in Regenwürmern \( \text{BCF}_{\text{methyl-Hg}} = 83.1 \) als in Pilzen \( \text{BCF}_{\text{methyl-Hg}} = 19.3 \). Im Allgemeinen bestand kein Zusammenhang zwischen den Bodengehalten von Hg und den Hg Gehalten in den Pilzen und Regenwürmern. Die Hg und Methyl-Hg Gehalte unterschieden sich jedoch signifikant zwischen den ökologischen Gruppen der Pilze und Regenwürmern. Streuabbauende Pilze enthielten etwa viermal höhere Hg-Konzentrationen \( 2.56 \text{ mg Hg kg}^{-1} \text{ dw} \) als Mykorrhiza Pilze \( 0.81 \text{ mg Hg kg}^{-1} \text{ dw} \) und etwa 10 mal höhere Konzentrationen als holzabbauende Pilze \( 0.24 \text{ mg Hg kg}^{-1} \text{ dw} \). Die Methyl-Hg Konzentrationen waren ebenfalls in streuabbauenden Pilzen \( 0.060 \text{ mg Methyl-Hg kg}^{-1} \text{ dw} \) am höchsten, gefolgt von Mykorrhiza Pilzen \( 0.017 \text{ mg Methyl-Hg kg}^{-1} \text{ dw} \) und holzabbauenden Pilzen \( 0.009 \text{ mg Methyl-Hg kg}^{-1} \text{ dw} \). Bei den Regenwürmern wiesen Organismen, welche im Oberboden (endogäisch) leben, die höchsten Hg \( 1.63 \text{ mg Hg kg}^{-1} \text{ dw} \) und Methyl-Hg \( 0.153 \text{ mg Methyl-Hg kg}^{-1} \text{ dw} \) Gehalte auf, gefolgt von tief grabenden Organismen (anektisch) \( 0.69 \text{ mg Hg kg}^{-1} \text{ dw}; 0.055 \text{ mg Methyl-Hg kg}^{-1} \text{ dw} \) und streubewohnenden Organismen (epigäisch) \( 0.49 \text{ mg Hg kg}^{-1} \text{ dw}; 0.025 \text{ mg Methyl-Hg kg}^{-1} \text{ dw} \). Bis zu 10% des Hg in Regenwürmern war in der methylierten Form vorhanden. Wir fanden starke Indizien, dass Hg direkt in den Regenwürmern methyliert werden kann. Wuchsen Regenwürmer der Spezies *Lumbricus terrestris* in sterilen, Hg behandelten Böden auf, wiesen sie nach 30 Tagen einen etwa 6 mal höheren Methyl-Hg Gehalt auf als Regenwürmer aus unbelasteten Böden. Wir vermuten, dass Hg im anaeroben
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Verdauungstrakt der Regenwürmer durch Bakterien methyliert und im Regenwurmgewebe angereichert wird. Die bakteriellen Gemeinschaften in den Regenwürmern wurden durch Hg und insbesondere durch Methyl-Hg Bodenkontaminationen stark beeinflusst.

Quecksilber und Methyl-Hg beeinflussen ebenfalls die Mikroorganismen von Waldböden. Bei Hg Konzentrationen über 3.2 µg Hg g⁻¹ trockener Boden wurde die Basalatmung der Böden stark reduziert. Ab einer bioverfügbaren Konzentration von 0.004 µg Hg g⁻¹ trockener Boden fanden wir zudem signifikante Änderungen in der bakteriellen Gemeinschaftsstruktur der Böden. Die bakterielle Gemeinschaftsstruktur in einem Boden wurde bereits mit einer Methyl-Hg Zugabe von 5 µg kg⁻¹ trockenem Boden stark beeinflusst. In fünf von sieben Böden mit einer Methyl-Hg Konzentration, welche der mittleren Konzentration in Regenwürmern (90 µg Methyl-Hg kg⁻¹) entspricht, waren die bakteriellen Strukturen stark verändert und in vier Böden war die mikrobielle Biomasse signifikant reduziert. Im Gegensatz zu Bodenbelastungen mit anorganischem Hg stieg die Basalatmung der Böden mit Zunahme der Methyl-Hg Gehalte der Böden tendenziell an. In unbelasteten Schweizer Waldoberböden liegen zwischen 0.2% und 2.4% des Hg in methylierter Form vor. Wir berechneten für die Zukunft eine weitere Zunahme der Hg Bodengehalte unbelasteter Waldstandorte um etwa einen Faktor zwei während den nächsten 1000 Jahren, bei gleich bleibenden Hg Depositionen. Trotz dieser Zunahme gehen wir nicht davon aus, dass diese Konzentrationen die Mikroorganismen der Böden stark beeinflussen oder gar beeinträchtigen werden.
Chapter 1

1.1. Introduction and research objectives

The emissions of heavy metals (HMs) into the environment have increased considerably during the past century. Due to atmospheric deposition, HMs may be carried into soils far away from their sources. The United Nations Economic Commission for Europe considers cadmium (Cd), lead (Pb) and mercury (Hg) as the HMs of highest priority. In this PhD thesis, we focused mainly on Hg, but we also kept an eye on the other HMs, Cd and Pb. Mercury is a relative rare element in the earth’s crust, which contains only about 0.5 µg g⁻¹ (Tchounwou et al. 2003). It can be emitted in large quantities into the environment through various natural processes, e.g. volcanic eruptions, forest fires and soil evasion, but anthropogenic processes such as fossil fuel and coal combustion, chlorine production and gold mining have greatly increased the Hg emissions (Schroeder and Munthe 1998; Swain et al. 2007). In contrast to natural emissions, anthropogenic emissions could increase the regional concentrations of Hg in soil and water dramatically. A simplified Hg cycle is shown in Figure 1.1.
Figure 1.1: Mercury cycle. Mercury is emitted from anthropogenic (e.g. industrial processes) and natural (e.g. volcanic eruptions) mainly as Hg\(^0\) and Hg\(^{2+}\) into the atmosphere. Divalent Hg is deposited near its source whereas elemental Hg can be transported globally. In the atmosphere, in water bodies and in soil, Hg can undergo several biotic and abiotic transformations.

The toxicity of Hg is widely known, and the use of Hg has decreased since the 1970s. In Switzerland, the use of Hg is restricted today (BAFU 2013). What is much more problematic than elemental Hg is its organic form monomethylmercury (methyl-Hg) (Boening 2000; Mason and Benoit 2003). The lipophilic nature of methyl-Hg means it can accumulate much more efficiently in organisms than inorganic-Hg. Hg(II) and methyl-Hg strongly binds to thiol groups, and can therefore bind to DNA, enzymes and synapses in cells (Broussard et al. 2002; Syversen and Kaur 2012).

In the middle of the 20\(^{\text{th}}\) century, the Hg problematic in aquatic ecosystems received much attention when thousands of people in Minamata (Japan) became ill and died due to the consumption of methyl-Hg polluted fish (Kurland et al. 1960). In the following years, the Hg cycle in aquatic ecosystems was intensively studied. In contrast, much less is known about the ecotoxicology and bioaccumulation of Hg and its methylated form in terrestrial ecosystems. For research on terrestrial ecosystems, natural forest soils are of special interest. They are not often contaminated with Hg since they are usually far from industrial
areas, but forest canopies increases the inputs of Hg and their organic matter strongly accumulate Hg in top soil layers. Furthermore, forest soils are mainly left undisturbed and not ploughed, unlike agriculture soils, and thus act as archives of depositions and soil-forming processes. In the top soils of Swiss forests, the concentrations of Hg range between 0.05 and 0.55 μg g$^{-1}$ dw soil (Ernst et al. 2008). In general, between 0.5 and 1.5% of total Hg in its methylated form are present in top soils (Boudou and Ribeyre 1997).

Mercury in soil can be bound to soil particles and organic matter, to dissolved organic carbon (DOC) or as free ions in soil solution. The living organisms in soils take up and accumulate Hg from the different soil compartments depending on their mode of life (Figure 1.2). Bacteria, for example, take up Hg presumably from soil solution, fungi from soil solution or by decomposing organic matter and earthworms by ingesting soil particles and organic matter directly. Commonly, Hg dissolved in soil solution (mainly bound on DOC or present as free ions) is called bioavailable Hg.

Figure 1.2: Concept of Hg in soils. Mercury sources (red boxes), Hg existence in the soil (black boxes), Hg release from the soil (blue boxes) and Hg uptake in the biota (green ovals).
Mushrooms are well known to accumulate Hg (Stegnar et al. 1973; Alonso et al. 2000; Falandysz et al. 2002) but studies of methyl-Hg in mushrooms are a few (Stegnar et al. 1973; Bargagli and Baldi 1984). Similarly, studies regarding the accumulation of Hg and in particular of methyl-Hg in earthworms are rare (Burton et al. 2006; Ernst et al. 2008). Most studies concerning the accumulation of Hg and methyl-Hg in mushrooms and earthworms have been conducted in polluted sites. There exist an urgent need for studying the accumulation of Hg and methyl-Hg in mushrooms and earthworms at unpolluted sites for estimating the risk of secondary poisoning.

In this work we wanted to answer the following questions considering the bioaccumulation of Hg and methyl-Hg in terrestrial habitats (Figure 1.2, black and green boxes):

1) Fungi and earthworms take up Hg compounds from different biochemical pathways. Exist there any differences in the accumulation of Hg and methyl-Hg between these two receptors at unpolluted soils?

2) Which soil properties affect the accumulation of Hg in mushrooms and earthworms?

3) Does the bioaccumulation of Hg and methyl-Hg depend on the ecophysiological groups of organisms, and from species-specific differences?

4) Can mushroom species be used as indicator for Hg pollution?

In general, the most problematic Hg species is monomethyl-Hg (CH$_3$-Hg-R; methyl-Hg) due to its toxicity and efficient bioaccumulation. Several groups of organisms, like bacteria and mushrooms, have the capacity to methylate inorganic-Hg (Vonk and Sijpsteijn 1973), and methylation of Hg has been observed under different conditions, but mainly occurs under anoxic conditions (StLouis et al. 1996; Schwesig et al. 1999; Drott et al. 2007; Holloway et al. 2009). Mercury may also be methylated in the digestive tract of higher soil organisms such as earthworms due to the anaerobic conditions in their digestive tract, but this has not been studied so far.

Further research questions are still open considering earthworms in Hg polluted soils:
1) Does the earthworms gut provide suitable conditions for the methylation of inorganic-Hg and are bacteria in the gut responsible for such a methylation?

2) How does soil contamination of Hg or methyl-Hg affect earthworm’s digestive tract bacteria?

The bioavailable amount of Hg is not only dependent on total Hg concentration in soil, but also on soil properties such as organic matter content, pH or humidity (Ranjard et al. 1997; Rasmussen et al. 2000). In soils with an high organic matter content, the Hg solubility and mobility is dominated by the interaction between Hg and organic matter (De Vries et al. 2007). In mineral soils with lower organic matter contents Hg cycling is controlled by other matrices such as iron- and aluminium-oxy/hydroxides and clay particles (De Vries et al. 2007). Laboratory and field studies in Sweden have showed that Hg induced a 10% reduction in microbial activity (basal respiration) with Hg concentrations over 2 – 3 mg kg\(^{-1}\) (Bringmark and Bringmark 2001; Bringmark and Bringmark 2001). The soils in their study were podzolic and contained a high proportion of organic matter (about 85%). Rasmussen and Sorensen (2001) studied the effects of contaminations of Hg on the bacterial community structure and diversity in agricultural soils. They observed a change from predominantly K-strategists in unpolluted soils to r-strategists in polluted soils. Forest soils in Switzerland often contain few amounts of organic matter (<10%) and ecotoxicological studies of Hg in temperate forest soils are very limited. Furthermore only two studies described the impact of methyl-Hg on soil microorganisms (Van Faassen 1973; Kungolos et al. 1999). The HM concentrations below which no adverse effects on soil organisms occur are called the critical limits (e.g. critical metal concentrations) (De Vries and Bakker 1998). Based on the critical limits, critical loads can be calculated (De Vries et al. 2005). These loads are the acceptable levels of HM deposition which never result in the critical limits being reached. To determine the critical limits of Hg compounds in soils, more needs to be known about ecotoxicological effects of the different Hg compounds on different receptors in various environments. However, ecotoxicological assessments of Hg on microorganisms have so far mainly been carried out
in soils rich in organic matter and not in soils comparable to those present in Switzerland. The lack of information about the toxicity of Hg to soil microorganisms in temperate forest soils means that the critical limits, and thus also the critical loads are uncertain. About critical limits we were interested in the questions:

1) Critical limits for Hg were mainly derived from ecotoxicological assessments conducted in organic soils (podzolic soils). Are these critical limits also appropriate for Swiss forest soils containing low organic matter contents?
2) How does the current Hg and methyl-Hg level in natural forest soils in Switzerland affect the soil functions and the microbial community structures?
3) How does an enhanced Hg and methyl-Hg concentration affect the soil microbial communities and soil functions?
4) Which groups of microorganisms are tolerant and which are sensitive to Hg or methyl-Hg pollution and can indicator species for such pollutions be identified?

Tipping et al. (2011) predicted a decrease in concentrations of Hg for soils in UK. There exists no study modeling the long term behavior of Hg for soils in Switzerland. To be able to evaluate the long term impact of Hg to soil organism’s dynamic modeling studies must be conducted. In this PhD thesis we wanted to answer the following questions about the long term behavior of Hg in natural forest soils (Figure 1.2, red boxes):

1) Will the concentrations of Hg in soils increase or decrease in future with current deposition?
2) Assuming a deposition according the proposed critical loads, will the Hg concentrations in soils and in soil solution exceed critical limits during the next 1000 years?
1.2 Background

1.2.1 Heavy metals

1.2.1.1 Definition
Mercury is often described as a heavy metal (HM). The term “heavy metal” is widely used but in the literature no generally accepted definition exists. Heavy metals are often thought to the dense (specific gravity) metals, but there is no consensus about what density can be described as “light” and what as “heavy”. In the IUPAC (International Union of Pure and Applied Chemistry) Technical Report, Duffus (2002) cited literature which used the term “heavy metals” for densities ranging from 3.5 to 7 g cm$^{-3}$, but found it is usually used to describe a metal density of at least 5 g cm$^{-3}$. Other authors define “heavy metals” according to the atomic weight, the atomic number, specific chemical properties, and/or the toxicity of the metals. Heavy metals and their compounds are often thought to have highly toxic properties, but this definitional approach is problematic. Metals can exist in many different compounds with very different properties and toxicities. For example, the oral uptake of elemental Hg is not very toxicity, whereas the oral uptake of methyl-Hg is extremely toxic to most organisms. Furthermore, each metal has certain properties and its own particular physicochemical characteristics. Nevertheless, the term “heavy metals” is still frequently used in the literature. In this thesis, we have mainly considered the “heavy metal” Hg (11.3 g cm$^{-3}$), but have also focused on Cd (8.7 g cm$^{-3}$) and Pb (13.5 g cm$^{-1}$; Table 1.1).

1.2.1.2 Critical limits/loads
Various toxic and carcinogenic effects of different metals on humans and animals have been reported (Lloyd et al. 1997; Fraga and Oteiza 2002; Donaldson et al. 2003; Hengstler et al. 2003). Many studies have also noted that certain metals affect such soil functions as bacterial activity, the soil microbial biomass carbon and the microbial community structures in soils (Van Faassen 1973; Tu 1988; Bringmark et al. 2001; Bringmark et al. 2001; Palmborg et al. 2001; Lazzaro et al. 2006; Lazzaro et al. 2006). Heavy metals such as Fe, Cu, Cd, Cr,
Hg and Ni have been shown to induce the production of radicals in cells, which may damage the DNA, lead to lipid peroxidation and the depletion of proteins (Valko et al. 2005). In addition, metals can directly impact organisms by transforming and occupying proteins or affecting their neural signal transmission (Martelli et al. 2006; Pentyala et al. 2010). Once emitted to the atmosphere, metals may be transported across long distances irrespective of national boundaries and deposited far away from their source. Due to the toxicity of HMs and their transboundary distribution, the United Nations Economic Commission for Europe (UNECE) adopted, in 1998, the protocol on HMs (Cd, Pb, Hg) in the Convention on Long-Range Transboundary Air Pollution (CLRTAP; see section 1.2.2.1.2). In the Manual for Critical Loads and Critical Limits Calculation for HMs, the convention provides guidelines for deriving the critical loads and critical limits for Cd, Pb and Hg (De Vries et al. 1998). Critical loads are the maximal acceptable input levels of particular pollutants (e.g. HMs) into the environment (soil/water), which will never negatively affect organisms (Figure 1.2). The concentration below which no negative effects of a pollutant on the soil and water organisms should occur is called critical limit. The calculation of critical limits/loads depends on the receptor, pollutant and the ecosystem involved (De Vries et al. 1998).

1.2.1.3 Modeling heavy metal concentrations

Mathematical models are often used in the natural sciences to calculate equilibrium conditions and predict the long-term future of ecosystems (e.g. acidification levels and nutrient or pollutant contents). Under the CLRTAP of the UNECE, seventeen parties have participated in modeling the critical loads for HMs in their countries. To calculate these critical loads, a steady-state mass-balance method was used and the loads were calculated according to the critical limits for impact on human health and ecosystem functions (UBA 2004; Slootweg et al. 2005). The choice of the critical limit strongly affects the calculation of the critical loads, and thus the link between chemistry, e.g. the HM concentration in the soil, and its biological impact. The biological impact depends on environmental factors and the receptors considered lead to a relatively large uncertainty in calculating critical loads.
The CLRTAP’s standard model for critical load calculation is the so-called Simple Mass Balance (SMB) model (Sverdrup and Devries 1994; Posch M. et al. 1997; UBA 2004). The SMB considers soil as a single-layer, with homogeneous compartments and a depth equal to the rooting zone. The biogeochemical processes are simplified in the SMB model so that it can be applied on a large scale. Other steady-state models to calculate critical loads are MACAL (De Vries 1991) and PROFILE (Warfvinge and Sverdrup 1992), which are both multi layer models. These models allow concentrations to be calculated at different soil depths, but they require much more input data as the SMB. All steady-state models calculate final situations in the future, but the time it takes for them to reach a steady state is unknown. Therefore, there is a lack of knowledge when the system will reach the steady-state situation and what the current situation is compared with the steady state. To study such aspects, dynamic models have been used (Alveteg et al., 1998).

With dynamic models it is possible to predict the time it will take for a system to reach equilibrium. They also allow quantitative assessments of the impact of long-term emission scenarios (increasing/decreasing) on environments. Dynamic models were developed over 20 years ago to predict soil acidification and surface-water acidification, which are mainly driven by atmospheric deposition of sulphur and nitrogen. The most commonly used dynamic models are SMART, SAFE and MAGIC (Cosby et al. 1985; De Vries et al. 1989; Warfvinge et al. 1993). In contrast to the number of dynamic models developed to predict acidification, those that simulate HM concentrations are rare. A simple dynamic model to predict metal concentrations in soils was developed by Posch and de Vries (2009). The single soil-layer model is characterized by four variables: the initial concentrations of the metals, the current metal input, the time and the concentration of the metal as a function of time.

A more complex dynamic model that can predict metal concentrations in soils and soil solution is the CHemistry of the Uplands Model (CHUM) (Tipping 1996), which has an improved version CHemistry of the Uplands Model – Annual, Metals (CHUM-AM) (Tipping et al. 2006; Tipping et al. 2006). CHUM divides a soil column into three different layers (organic, mineral and bedrock). The model uses mean annual precipitation and deposition values,
Chapter 1

considers biogeochemical processes such as reactions between ions and mineral particles, and organic matter in the soil and in the soil solution. CHUM-AM (and also CHUM) has only been used for soils in the UK so far, which tend to be rich in organic matter.

1.2.1.4 Soil pollution with HM

Soil organisms, such as bacteria, fungi or earthworms, play an important role in decomposing and mineralizing dead organic matter and supplying plants with nutrients. Soil fertility largely depends on the turnover of organic matter. Pollution can affect the biological activity in soils, and suppress the decomposition of organic matter and the supply of nutrients. Heavy metals, such as Cu, Ni, Cd, Zn, Pb and Hg, are an important group of inorganic soil pollutants. Whereas many metals are essential for organisms in trace amounts, e.g. many enzymes have a Cu component (Arnon 1949; Keller et al. 1991), no biological functions are known for Cd, Pb and Hg (Bruins et al. 2000). All three occur in ores and are emitted into the atmosphere through natural and anthropogenic processes.

The highest amounts of natural Cd emissions are the result of volcanic eruptions and forest fires (Nriagu 1979). Cadmium emissions dramatically increased during the 20th century due to anthropogenic processes, such as iron and steel manufacturing and fossil fuel combustion (Nriagu 1990; Järup 2003). The atmospheric deposition of Cd probably increased in the 1930s, and had almost doubled by 2000 (Barbante et al. 2004). Moreover, large quantities of Cd have entered soils through the disposal of sewage sludge on farm land (Hooda and Alloway 1993). Today, Cd is still an important component in the industrial sector and is used in, e.g. steel manufacturing or to produce Ni-Cd batteries. The annual Cd deposition at sites far away from any emission source in Switzerland was about 90 μg m⁻² in the year 2007 (FOEN 2008).

The anthropogenic Pb emission predominate the natural emission. Lead is emitted mainly through industrial processes, such as fossil fuel combustion. After the introduction of leaded gasoline around 1950, the emission of Pb increased dramatically. In the last 20 years, Pb emission have strongly decreased in Switzerland and other countries, mainly through the use
of unleaded gasoline, but also the installation of catalysts in combustion engines, as well as improvements in refuse incineration plants and industrial processes (Davies 1995; Schwikowski et al. 2004; FOEN 2008). Leaded gasoline has been banned in all European petrol stations since the beginning of the year 2000 (Schwikowski et al. 2004). The annual Pb deposition at sites far away from any emission source in Switzerland was about 5 mg m$^{-2}$ in the year 2007 (FOEN 2008).

Mercury is a rare element in the earth’s crust, which contains about 0.5 µg g$^{-1}$ (Tchounwou et al. 2003). The most important sources of natural Hg emissions are volcanic eruption, forest fires and soil evasions (Selin et al. 2007). Anthropogenic Hg emissions to the atmosphere occur through fossil fuel and coal combustion, the chlorine production or through gold mining (Schroeder et al. 1998; Swain et al. 2007). In contrast to natural emissions, the anthropogenic Hg emissions have regionally increased soil and water pollution dramatically. In Switzerland, Hg emissions increased at the beginning of the 20th century due to the introduction of steam railways (coal combustion), but this source of emissions largely stopped with the electrification of the railways. After the Second World War, emissions of Hg in Switzerland increased again due to post-war industrialization, followed by a decrease that has continued since about 1970 (Ross-Barraclough and Shotyk 2003), mainly through improvements in refuse incineration plants and crematories (FOEN 2008). The current annual Hg deposition in rural sites without any nearby Hg source is about 20 µg m$^{-2}$ (Ross-Barraclough et al. 2003). Table 1.1 summarises the main properties and sources of Cd, Pb and Hg described here.
Table 1.1: Main characteristics of cadmium, lead and mercury.

<table>
<thead>
<tr>
<th></th>
<th>Cadmium</th>
<th>Lead</th>
<th>Mercury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symbol</td>
<td>Cd</td>
<td>Pb</td>
<td>Hg</td>
</tr>
<tr>
<td>Atomic number</td>
<td>48</td>
<td>82</td>
<td>80</td>
</tr>
<tr>
<td>Atomic weight [g mol(^{-1})]</td>
<td>112.4</td>
<td>207.2</td>
<td>200.6</td>
</tr>
<tr>
<td>Density at 20°C [g cm(^{-3})]</td>
<td>8.7</td>
<td>11.3</td>
<td>13.5</td>
</tr>
<tr>
<td>Oxidation states</td>
<td>2, 1</td>
<td>4, 3, 2, 1</td>
<td>4, 2, 1</td>
</tr>
<tr>
<td>Earth's crust content [ppm]</td>
<td>0.3</td>
<td>18</td>
<td>0.4</td>
</tr>
<tr>
<td>Anthropogenic sources</td>
<td>Metal-processing industries, Ni-Cd-batteries, refuse incinerator plants, fertilizer, sewage sludge, fossil fuel combustion,</td>
<td>Batteries, ammunition, alloys, gasoline, insecticides, anticorrosive coating,</td>
<td>Chlor-alkali industri, gold-mining, batteries, pesticides, incinerator plants, crematoriums, fossil fuel combustion</td>
</tr>
<tr>
<td>Effects on humans</td>
<td>backache, high blood pressure, kidney failure, cancer, pulmonary oedema,</td>
<td>sickness, intestinal colic, kidney failure, neurotoxic, death</td>
<td>neurological diseases, psychological diseases, death,</td>
</tr>
<tr>
<td>Effects on plants</td>
<td>Reduces plant growth, necrosis, leaf chlorosis, disturbed water balance</td>
<td>Reduces plant growth</td>
<td>(low uptake) Inhibits photosynthesis, reduces seed viability</td>
</tr>
<tr>
<td>Effects on microorganisms</td>
<td>affects activity, changes bacterial community structure</td>
<td>affects activity, changes bacterial community structure</td>
<td>Reduces biomass, affects activity, changes bacterial community structure</td>
</tr>
<tr>
<td>Average global emission from anthropogenic sources [t yr(^{-1})]</td>
<td>2'983</td>
<td>119'259</td>
<td>2'235</td>
</tr>
<tr>
<td>Average emissions from anthropogenic sources in Switzerland [t yr(^{-1})]</td>
<td>1.3</td>
<td>27</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*Data adapted from Van Faassen (1973); Patra and Sharma (2000); Bringmark et al. (2001); Pacyna and Pacyna (2001); Rasmussen et al. (2001); Järup (2003); Clemens (2006); FOEN (2008)*

The behavior of the two HMs, Cd and Pb, in terrestrial ecosystems have been well studied so far, but little is known about the affects of Hg. International concern about Hg emissions to the environments has grown during recent years and new conventions about Hg have been adapted. The aim of this research was to find out more about Hg in terrestrial systems, and the element Hg will therefore be introduced in more detail in the next section.
1.2.2 Mercury

1.2.2.1 Political background

1.2.2.1.1 Swiss legislation

The protection of the environment is enshrined in the Federal Constitution of the Swiss Confederation (art. 2.4) (Constitution 1999). With the Federal Act on the Protection of the Environment (Environmental Protection Act, EPA) (EPA 1983), the Swiss Federation passed regulations to protect humans and the environment against pollutants. Air pollution should be limited at its source (limitation of emissions). If emissions could be expected to be harmful or a nuisance, they are limited more strictly (art.11). Several regulations deal with reducing emissions (art. 12): (1) specification of maximum emission values; (2) regulations on construction and equipment; (3) traffic or operating regulations; (4) regulations on the heat insulation of buildings; and (5) regulations on thermal and motor fuels. Furthermore the Federal Council specifies that depositions of several pollutants should be below the values that negatively affect humans, animals plants and soil biota (art. 13; 14). In the Ordinance on Air Pollution Control (OAPC) (OAPC 1985), maximum acceptable emissions and deposition values for several organic and inorganic pollutants are defined (Table 1.2). In Switzerland the use of Hg is strictly regulated. Table 1.3 summarizes the most relevant restrictions on Hg use in Switzerland.

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>Emission [mg m$^{-3}$]</th>
<th>Deposition [µg m$^{-2}$ day$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>Pb</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Hg</td>
<td>0.1</td>
<td>-</td>
</tr>
</tbody>
</table>

The Federal Office for the Environment (FOEN) commissioned Empa (Empa 2013) to monitor air pollution in Switzerland, which led to the National Air Pollution Monitoring Network (NABEL) being established in 1978. At 16 stations distributed throughout Switzerland, air
quality is measured at typical pollution sites, e.g. city-centre streets, residential areas, and rural farms (NABEL 2013). When Switzerland ratified the Convention on Long Range Transboundary Air Pollution (CLRTAP, 1979), the NABEL became integrated in the European Monitoring and Evaluation Programme (EMEP).

In addition, the Swiss Soil Monitoring Network (NABO) was established in 1984 as an instrument for the early detection of soil pollution and for evaluating the effectiveness of soil protection measures (NABO 2013). The network includes 105 sites distributed across the whole country, and is monitored by Agroscope ART Reckenholz-Tänikon.

Table 1.3: Restrictions on Hg-containing products in Switzerland according to the Chemical Risk Reduction Ordinance (BAFU 2013).

<table>
<thead>
<tr>
<th>Material</th>
<th>Regulation</th>
<th>Exceptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electric equipment</td>
<td>Not allowed if they contain &gt; 0.1% Hg</td>
<td>Military equipment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medicinal equipment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In vitro-diagnostic equipments</td>
</tr>
<tr>
<td>Vehicles</td>
<td>Materials containing &gt; 0.1% Hg are not allowed</td>
<td></td>
</tr>
<tr>
<td>Wood materials</td>
<td>&gt; 25 ppm Hg are not allowed</td>
<td></td>
</tr>
<tr>
<td>Batteries</td>
<td>&gt; 5 ppm Hg are not allowed</td>
<td>Button-cell batteries with at most 20 g Hg kg⁻¹</td>
</tr>
<tr>
<td>Packing material</td>
<td>&gt; 100 ppm Hg are not allowed</td>
<td></td>
</tr>
<tr>
<td>Fertilizers</td>
<td>Organic and inorganic fertilizers may be used if they contain &lt; 1 ppm Hg</td>
<td>Sewage sludge must not be used as fertilizer</td>
</tr>
<tr>
<td>Others</td>
<td>- medicine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- antiques restoring</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- production of some cosmetics</td>
<td></td>
</tr>
<tr>
<td></td>
<td>If no replacement for Hg-containing material is available:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- laboratory material</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- colors for restoration work</td>
<td></td>
</tr>
</tbody>
</table>
1.2.2.1.2 International conventions

The United Nations Economic Commission for Europe (UNECE) was established in 1947 with the main aim to facilitate the economic cooperation of the European countries and of the whole world (UNECE 2013). The UNECE is one of five commissions of the United Nations. The four others are the: (1) Economic Commission for Africa (ECA), (2) Economic and Social Commission for Asia and the Pacific (ESCAP), (3) Economic Commission for Latin America and the Caribbean (ECLAC), (4) Economic and Social Commission for Western Asia (ESCWA) (UNECE 2013). The work of the UNECE includes economic cooperation and integration, environmental policy, forests, housing and land management, population units, statistics, sustainable energy, technical cooperation, trade, transport and climate change. To improve environmental protection and human health, and reduce environmental pollution, the UNECE has set up five environmental treaties, including the Convention on Long-range Transboundary Air Pollution (CLRTAP), which came into force in 1979. The aim of the CLRTAP is to reduce and prevent air pollution through monitoring, research and information exchange. In 1998 the transboundary distribution of HMs was identified as a problem and the Protocol on Heavy Metals was therefore added to the CLRTAP. It was decided to monitor HM pollution, in particular of Cd, Pb and Hg, and to encourage research on their effects on human health and the environment. The aim was where necessary to reduce the emission of these HMs. Switzerland ratified the CLRTAP in 1983 and the HM Protocol in 2000.

In addition to the UNECE, the United Nations Environment Programme (UNEP) has also focused on the Hg problematic since 2003. The UNEP, founded in 1972, is the agency of the United Nations that coordinates its environmental activities. In 2005, the UNEP Global Mercury Partnership was initiated to help countries and private entities work together to reduce the use of Hg and its release into the environment (UNEP 2009). In February 2009, over 140 countries (including Switzerland) agreed at the 25th session of the UNEP to start negotiations on a legally binding instrument for globally Hg pollution. Between 2010 and 2013 five negotiating sessions were conducted with the aim to reduce Hg emissions and the use of Hg in products and industrial processes. The final session took
place in Geneva (Switzerland) in January 2013. The participating countries accepted the Minamata Convention (so named in memory of the thousands of people poisoned in Minamata by Hg-contaminated food) with the goal to reduce Hg production and products containing Hg and to regulate the treatment of waste containing Hg. The convention is due to be signed in October 2013 in Minamata (Japan).

1.2.2.2 The Hg cycle

1.2.2.2.1 Hg emissions

Mercury is a naturally occurring metal in the Earth’s biogeochemical system. Anthropogenic processes, such as coal-fired power plants, fossil fuel combustion, chlorine production or metal smelting have led to an atmospheric Hg concentration about three times higher than the pre-industrial level (Mason et al. 1994). In addition to the direct release of Hg through human activity, natural Hg emission occurs through evasion from land and ocean surfaces, forest fires and volcanic eruptions. These “natural” Hg releases include both primary emissions, e.g. from rock weathering or ocean upwelling, and from the reemission of previously emitted and deposited Hg from anthropogenic activities (Selin et al. 2007). The total current gaseous Hg release into the atmosphere is estimated to be between 4400 Mg yr\(^{-1}\) (Lamborg et al. 2002) and 7000 Mg yr\(^{-1}\) (Mason et al. 1994).

In the pre-industrial period, natural Hg emissions came from geological sources such as through passive degassing from volcanoes and more importantly through volcano eruptions (Varekamp and Buseck 1986). Mercury has long been mined, for example by the Chinese, and has even been found in ancient Egyptian tombs (Masur 2011). Mercury was widely used to treat syphilis, in addition to use as a general antibiotic (Dayan and Ooi 2005; Masur 2011), as already described in “The Canon of Medicine” published in the year 1025. Cinnabar (HgS) has been used as a red pigment for several thousand years (Clark et al. 1997; Vandenabeele et al. 2005), and since the middle of the 20\(^{th}\) century, Hg (mainly organic Hg compounds) has been used as a fungicide (Bakir et al. 1973; Clarkson 1993).
About half of current anthropogenic Hg emissions came from Asian countries (Pacyna et al. 2006; Selin et al. 2007), where the most Hg-contaminated sites are in China, Japan, the Philippines, India and Kazakhstan. Almost one third of the Hg-polluted sites are associated with the chemical industry, while nearly one-fifth are near Hg-mining areas, with another one-fifth near gold-mining areas (Li et al. 2009). Most Hg mines worldwide have been closed in recent decades and large-scale gold mining using Hg has been largely phased out and is now illegal in many countries (Telmer and Veiga 2009), but the use of Hg in artisanal and small-scale gold mining is still a matter of concern (UNEP 2013). It is assumed, that between 20% and 30% of global gold is produced in artisanal and small-scale gold mines that use Hg for gold extraction and employ about 10 - 15 million miners (UNEP 2008). About a quarter of the Hg used globally is required for artisanal and small-scale gold mining (Figure 1.3), which is one of the main sources of Hg emissions into the atmosphere (Figure 1.4). The number of gold miners is likely to rise since gold prices have dramatically increased in recent years (UNEP 2011). Overall, however, Hg emissions decreased as industrial processes have improved and those processes that used to require Hg have been replaced by non-Hg technologies (Li et al. 2009). Thus, globally anthropogenic Hg emissions declined by about 5% between 1995 and 2000. In the United States, Hg emissions have decreased by about 12%, in Russia by about 46%, in China by about 2%, but in other countries such as India, Brazil, Mexico and Spain, Hg emissions are still increasing (Selin et al. 2007). Due to the overall decline in Hg emissions worldwide, atmospheric concentrations of Hg have decreased by about 20% to 38% since 1996 (Slemr et al. 2011).
Figure 1.3: Estimated global Hg consumption in different sectors. Data source: UNEP (2006).

Figure 1.4: Sources of estimated global anthropogenic Hg emissions into the atmosphere in 2005. Data source: UNEP (2008).
1.2.2.2 Hg in the atmosphere

Mercury emitted by humans can be roughly equally divided into the relatively unreactive form \( \text{Hg}^0 \), the reactive gaseous form [RGM, representing Hg(II) in the gas phase] and the particulate form \( \text{Hg}_p \) (Mason et al. 1994). Once emitted into the atmosphere, about half of the Hg is deposited locally [mainly as Hg(II)] (Mason et al. 1994), whereas the remaining Hg enters into global circulation with a residence time of approximately one year (Fitzgerald and Mason 1997; Seigneur et al. 2004). Atmospheric Hg is mainly present in the two oxidation states \( \text{Hg}^0 \) and Hg(II), where Hg exists primarily in its inorganic form. Methyl-Hg in the atmosphere accounts for less than 3% of the total gaseous Hg (Lin and Pehkonen 1999). Under environmental conditions, the monovalent Hg state [Hg(I)] is rarely stable and is rapidly oxidized to Hg(II) (Gardfeldt and Jonsson 2003). \( \text{Hg}^0 \) in the atmosphere (in the gas or aqueous phase) is oxidized to Hg(II), mainly by ozone and OH (Pleijel and Munthe 1995; Shia et al. 1999; Lin et al. 2006), but these reactions are still not well understood (Calvert and Lindberg 2005). Halogenic compounds could also be important oxidants for \( \text{Hg}^0 \) conversion (Holmes et al. 2006; Lin et al. 2006; Steffen et al. 2008). Other researchers suggest that bromide ions are the most important oxidants of \( \text{Hg}^0 \) in the atmosphere (Donohoue et al. 2006; Seigneur and Lohman 2008).

The lifetime of Hg(II) in the atmosphere is much shorter (several days to a few weeks) than that of \( \text{Hg}^0 \) (approximately 1 year) (Slemr et al. 1981; Lindqvist and Rodhe 1985). The products of \( \text{Hg}^0 \) oxidation are thought to be Hg-oxides (HgO) (Schroeder et al. 1998; Sommar et al. 2001), which are very soluble in water and thus dissolve in clouds and aqueous aerosols (Schroeder et al. 1998). In the aqueous phase, Hg-oxides dissociate to \( \text{Hg}^{2+} \) and bind mainly to Cl' (Pleijel et al. 1995), resulting in \( \text{HgCl}_2 \) (Lin and Pehkonen 1998). Hg(II) may also be reduced through photolysis in the aqueous phase (Pleijel et al. 1995), but how relevant this is for the Hg cycle is not clear (Gardfeldt et al. 2003). In the aqueous phase, Hg(II) can bind to other anions such as sulphate or to particulate matter, or can be deposited via wet deposition on the Earth’s surface. Hg(II) compounds can also volatilize into
the gas phase, and then be deposited on land or sea surfaces where they may be reduced to Hg$^0$. The atmospheric behaviour of Hg is illustrated in Figure 1.5 (Shia et al. 1999).

![Figure 1.5: Atmospheric transformations of Hg according to Shia et al. (1999).](image)

The total Hg concentrations in the atmosphere are generally higher in the Northern Hemisphere, where the most sources of Hg emission are located, than in the Southern Hemisphere (Slemr et al. 2003; Slemr et al. 2011).

It is assumed that the oxidation of Hg$^0$ to Hg(II) is controlled by photochemistry and that the production of Hg(II) is highest at midday (Selin 2009). In Polar regions during springtime, Hg$^0$ in the atmosphere decreases rapidly while RGM spikes (Schroeder et al. 1998; Lindberg et al. 2002; Steffen et al. 2005; Selin 2009). Thus significantly higher amounts of Hg are deposited on the Arctic’s surface (Steffen et al. 2008). Such increased deposition events are called Arctic Mercury Depletion Events (AMDEs). It is estimated that AMDEs can lead to the Hg deposition of up to 300 t yr$^{-1}$ in the Arctic (Ariya et al. 2004; Skov et al. 2004). Such events strongly correlate with ozone destruction in the troposphere due to a rapid oxidation by halogens, especially bromides (Simpson et al. 2007). It is assumed that AMDEs occur when Hg$^0$ is oxidized by reactive halogens (especially Br), which are generated in aquatic regions through a combination of refreezing sea ice and UV radiation (Kaleschke et al. 2004;
Brooks et al. 2006). A schematic cycle for Hg oxidation in Polar regions is given in Figure 1.6 (Steffen et al. 2008).

Background information

Figure 1.6: Mercury cycling in Polar regions according to Steffen et al. (2008).

1.2.2.2.3 Hg in terrestrial and aquatic ecosystems

Whereas the dry deposition of Hg on aquatic surfaces is marginal, the dry deposition on grassland and especially on forests can clearly exceed the wet deposition (St Louis et al. 2001; Lindberg et al. 2007; Gandois et al. 2010). Once deposited on terrestrial surfaces, predominantly as Hg(II), Hg may subsequently be reduced to Hg⁰ and revolatilize into the atmosphere (Hintelmann et al. 2002; Selin et al. 2008), it may be associated with vegetation (Obrist 2007) or with organic matter in the soil (Skyllberg et al. 2003). In forest soils, Hg(II) shows a high affinity to organic matter (Skyllberg and Drott, 2010), especially if the organic matter has a high content of thiol groups. Due to this strong affinity, Hg has a low mobility in soil and tends to accumulate in the top layer (Meili,1991; Yin et al.,1996; Meili et al., 2003). Mercury in soil solutions is assumed to mainly be bound to DOC (De Vries et al., 2007; Tipping et al., 2011).

Mercury in vegetation in the aboveground biomass originates mainly from the atmosphere, i.e. Hg⁰ uptake through gas exchange at the stomata and deposits of Hg(II) on leaves, and in belowground biomass from the soil (Obrist 2007). Through litter and throughfall, Hg bound to
leaves is deposited on the ground (St Louis et al. 2001). It is estimated that more than 90% of Hg in the terrestrial environment resides in soil, where it binds strongly to organic matter, especially to reduced sulphur groups (Skyllberg et al. 2003). Mercury in the soil can be reduced to the volatile Hg species Hg⁰ leading to Hg evasion into the atmosphere (Zhang and Lindberg 1999; Schluter 2000). Evasion depends on numerous soil properties, such as temperature, soil moisture and microbial activity (Zhang et al. 1999; Johnson et al. 2003; Bahlmann et al. 2006; Fritsche et al. 2008; Choi and Holsen 2009). Usually between 0.03 and 1.6% of the total Hg in the soil is emitted per year (Tipping et al. 2011).

In soils, especially in wetlands, Hg can be methylated to methyl-Hg. In surface soils, between 0.5 and 1.5% of the total Hg present is in its methylated form (Boudou et al. 1997). Several groups of organisms, like bacteria and fungi, can methylate Hg (Vonk and Sijpeste 1973; Fischer et al. 1995; Shao et al. 2012). Methylation of Hg has been observed under different conditions, but mainly occurs under anoxic conditions (StLouis et al. 1996; Schwesig et al. 1999; Drott et al. 2007; Holloway et al. 2009). In general, sulphate-reducing organisms are assumed to be responsible for Hg methylation (Barkay and Wagner 2005; Holloway et al. 2009). Both inorganic-Hg and methyl-Hg can accumulate in soil organisms. Mushrooms are known to accumulate Hg (e.g. Stegnar et al. (1973); Byrne et al. (1976); Seeger and Nutzel (1976); Minagawa et al. (1980); Kalac et al. (1991); Kalac et al. (1996); Alonso et al. (2000); Falandysz et al. (2002); Falandysz et al. (2003); Cocchi et al. (2006); Svoboda et al. (2006)), but their accumulation of methyl-Hg has rarely been studied (Stegnar et al. 1973; Minagawa et al. 1980; Bargagli et al. 1984; Fischer et al. 1995). Similarly, inorganic-Hg and methyl-Hg have been found to accumulate in earthworms (Bull et al. 1977; Hinton and Veiga 2002; Burton et al. 2006; Ernst et al. 2008; Zhang et al. 2009). When higher animals consume mushrooms and earthworms, they may also consume Hg and its compounds, which could lead to secondary poisoning (Ernst et al. 2008).

Deposition of Hg in aquatic ecosystems away from point sources is driven by depositions from the atmosphere or by runoff from water bodies (Selin 2009). The most important source of exposure for humans to the highly toxic methyl-Hg is the consumption of contaminated fish.
(Broussard et al. 2002; Mergler et al. 2007). Mercury deposition in aquatic ecosystems is predominated as Hg(II) similar to that on terrestrial surfaces. In water, Hg(II) can be reduced to Hg⁰, which may volatilize into the atmosphere. Another part of Hg(II) can be methylated to methyl-Hg. Methyl-Hg can be photodegraded to Hg⁰ or assimilated by the biota. More important environments for Hg methylation are sediments and wetlands (Selin 2009). In aquatic environments, methyl-Hg strongly bioaccumulates in fish and biomagnificates across the food web, from bacteria through plankton through fishes to fish consuming fishes. Most of the Hg in fish is present in its methylated form (Baeyens et al. 2003; Rolfhus et al. 2011). Mercury contamination of freshwater environments is widespread. In all states in the USA apart from two, residents were advised in 2006 to avoid consuming fish from some water bodies due to the risk of methyl-Hg contamination (Selin 2009).

For adults, WHO (WHO 2004) recommends a maximum weekly intake of Hg of 5 µg kg⁻¹ body weight, of which no more than 3.3 µg kg⁻¹ should be present as methyl-Hg. The Joint FAO/WHO Expert Committee on Food Additives (JECFA 2006) proposed reducing the recommended weekly intake limit of methyl-Hg to 1.6 µg kg⁻¹ body weight to protect embryos and fetuses. Broussard et al. (2002) suggest that a long-term intake of methyl-Hg over 4.3 µg kg⁻¹ body weight will lead to chronic poisoning.

1.2.3 Soil organisms and their responses to HM pollution

Soils provide a home for many organisms. Plants, animals and microorganisms inhabit the soils above and below ground. All living cells can be classified into two groups: prokaryotes and eukaryotes. Prokaryotic organisms are generally smaller and form fewer complexes than eukaryotic organisms. They also differ regarding: 1) DNA: Prokaryotic DNA is not enclosed within a membrane, whereas eukaryotic DNA is present in the nucleus, which separates it from the cytoplasm. The DNA of prokaryotes is not associated with histones in contrast to that of eukaryotes. Further, prokaryotic DNA is arranged mostly in singular circular chromosomes, whereas eukaryotic DNA is found in multiple chromosomes. 2) Membrane-enclosed organelles: eukaryotic cells contain numerous membrane-enclosed organelles, e.g.
mitochondria, endoplasmatic reticulum, lysosomes and Golgi complex, which are missing in prokaryotic organisms. 3) Cell wall: the cell walls of prokaryotic cells almost always contain the macromolecular network peptidoglycan, whereas not all eukaryotic cells have cell walls and when they do, they are much simpler than those of prokaryotic cells and include cellulose and chitin. 4) Cell division of prokaryotic cells usually happens by binary fusion, and of eukaryotic cells by mitosis (Tortora et al. 2011).

Soil organisms are exposed differently to Hg. Earthworms take up organic matter and soil particles directly, so that bound Hg reaches their digestive tract. Microorganisms are exposed mainly to bioavailable Hg in soil solution and fungi, but also through decomposing dead organic matter (Figure 1.2). In the following sections, relevant features of earthworms and soil microorganisms are briefly described.

1.2.3.1 Soil-dwelling invertebrates - earthworms

All animals are totally composed of eukaryotic cells. The dominant macrofauna in soils are usually earthworms, which may constitute up to 80% of the soil fauna biomass (Ireland 1983) and are arguably the most important soil-forming organisms. Earthworms strongly influence the soil’s structure, its water-holding capacity, as well as nutrient transport and availability. Thus they are important for the soil’s fertility (Drake and Horn 2007). More than 8000 species of earthworm are known. They belong to the class Oligochaeta forming about 800 genera inhabiting both terrestrial and aquatic ecosystems. Their length ranges from a few millimeters to about 2 m (Edwards 2004). The occurrence of earthworms in soils depends on many factors, such as soil type, soil moisture-holding capacity, pH, precipitation, temperature and organic matter content (Edwards 2004). Earthworms are usually divided into three groups: (1) epigeic earthworms, e.g. *Lumbricus rubellus*, *Eisenia fetida*, *Dendrodrilus rubidus*, inhabit the litter layer and feed preferentially on litter; (2) endogeic earthworms, e.g. *Aporrectodea caliginosa*, *A. rosea*, *Octolasion lacteum*, inhabit topsoils, ingest high amounts of mineral soil and build horizontal burrows; (3) anecic earthworms, e.g. *L. terrestris*, *A.*
Longa, which inhabit deep burrows and ingest moderate amounts of soil. They feed on litter on the soil surface, which they then drag into their burrows, thereby forming vertical burrows. Earthworms are known to accumulate HMs (Ma 1982; Ireland 1983; Morgan and Morgan 1988) including Hg (Talmage and Walton 1993; Edwards et al. 1998; Burton et al. 2006; Ernst et al. 2008; Zhang et al. 2009). In natural Swiss forest soils, Hg concentrations in the tissue of earthworms of almost 5 mg kg\(^{-1}\) dw have been observed (Ernst et al. 2008). Earthworms consume soil, organic matter and microorganisms directly. Their guts provide varying environmental conditions for the microorganisms they ingest, but they are normally free of oxygen and contain large amounts of nutrients such as glucose or amino acids (Drake et al. 2007). If microorganisms pass through an earthworm’s gut, microbial activity is promoted (Drake et al. 2007). The earthworm’s gut can be described as a mutualistic digestive system (Barois and Lavelle 1986; Brown and Doube 2004) since ingested microorganisms enhance the degradation of organic matter by excreting exoenzymes, which in turn enhance the nutrient supply of earthworms.

### 1.2.3.2 Microorganisms

Bacteria are prokaryotic organisms that can take three basic shapes: coccus, bacillus and spiral. Their diameters range from 0.2 to 2 µm and their lengths from 2 to 8 µm (Tortora et al. 2011). Since soil is spatially heterogeneous, it provides numerous niches and microhabitats for bacteria (Torsvik et al. 2002). In forest soils, it is estimated that about \(4 \times 10^7\) bacterial cells are present per one gram soil in the top 1 m layer (Richter and Markewitz 1995), and one gram of soil may contain \(1 \times 10^3\) to \(1 \times 10^6\) different species (Torsvik et al. 2002; Gans et al. 2005; Tringe et al. 2005). Bacteria are the most abundant organisms in soils (Kirk et al. 2004), and are responsible for many fundamental ecological processes (Balser and Firestone 2005). They are involved in the degradation of organic matter, the release of nutrients into the soil solution, the release of gas, e.g. H\(_2\), CO\(_2\) or N\(_2\)O, to the atmosphere and in N-fixation (Conrad 1996; Balser et al. 2005). Some bacteria can also promote plant growth and are thus called plant growth-promoting rhizobacteria (PGPR). PGPR can weaken
or prevent the growth of roots of phytopathogenic organisms, facilitate the uptake of nutrients through plant roots (Glick 1995), and release phytohormones that stimulate root growth as well as mineral and water uptake in inoculated plants (Ryu et al. 2003; Babalola 2010). Only a few bacterial taxa have been well studied and the ecological characteristics of most soil bacterial taxa remain largely unknown (Fierer et al. 2007).

Members of the Fungi kingdom (fungus) are eukaryotic, unicellular (yeasts) or multicellular (molds) organisms. Unicellular fungi are larger than bacteria and oval in shape. The fruiting bodies of fungi are called mushrooms and may look a bit like plants. In contrast to plants, however, fungal cells have cell walls containing chitin instead of cellulose and fungi cannot carry out photosynthesis. In soils, fungi are mainly inconspicuous. Their thallus consists of long filaments (hyphae) that extend below ground to immense proportions. People perceive primarily the fruit bodies of fungi, whereas their largest part of them is normally hidden in the soils. Most fungi reproduce with sexual and asexual spores.

Most plants live in symbiosis with fungi (Smith and Read 1997) which is than called mycorrhizae. Plants obtain nutrients, such as nitrogen, phosphate, inorganic salts and water, from these fungi and the fungi obtain carbon from plants assimilated through photosynthesis (Hata et al. 2010). In contrast, saprophytic fungi obtain nutrients from decomposing dead litter and wood. Bacteria and fungi are major decomposers of dead organic matter in most terrestrial ecosystems (Verburg et al. 1999; Von Lutzow et al. 2006) and play an essential role in nutrient cycling. Some fungi are also sources of food for humans (mushrooms) and medicine (penicillin) and others. Mushrooms are known to accumulate HMs including Hg (Stegnar et al. 1973; Byrne et al. 1976; Minagawa et al. 1980; Kalac et al. 1991; Kalac et al. 1996; Alonso et al. 2000; Falandysz et al. 2002; Falandysz et al. 2003; Cocchi et al. 2006; Svoboda et al. 2006). This accumulation occurs because fungi have filamentous mode of growth, with branching and extra-cellular release of enzymes and metabolites. In industrial and municipal areas, Hg concentrations over 100 mg Hg kg⁻¹ dw have been found in mushrooms (Kalac et al. 1996; Michelot et al. 1998), whereas in unpolluted forest sites Hg
concentrations in fungal fruiting bodies clearly below 10 mg Hg kg\(^{-1}\) were detected (Alonso et al. 2000).

Most HMs in soil solution are present as divalent or trivalent cations (Nies 1999). Heavy metals can bind to cell walls or enter the cells through specific or unspecific channels (Nies 1999). In general, many fungi are known to tolerate enhanced HM concentrations in soils (Tam 1995; Gupta et al. 2002; Rajapaksha et al. 2004). Metals can disrupt cell membranes, bind to proteins and nucleic acids and damage them (Bruins et al. 2000; Patra et al. 2004), or displace essential cations from their binding site inside the cells (Nies 1999).

Contamination with HMs affects soil functions. High concentrations of HMs in soils have been found to reduce basal respiration (CO\(_2\) efflux from soil into the atmosphere which indicates mineralization activity) (Van Faassen 1973; Landa and Fang 1978; Bringmark et al. 1998; Bringmark et al. 2001; Nwachukwu and Pulford 2011). They may, however, also enhance basal respiration (Landa et al. 1978; Leita et al. 1995; Khan and Scullion 2000; Landi et al. 2000). Bacterial and fungal activity may be affected differently by HM pollution. Whereas bacterial activity may be reduced, the fungal activity may markedly increase (Rajapaksha et al. 2004). Contamination with Hg typically results in an initial decrease in bacterial cell numbers and a subsequent growth of Hg-resistant bacteria (Rasmussen et al. 2001; Holtze et al. 2003). Soil contamination with HMs generally reduces fungal diversity and fungal growth (Kungolos et al. 1999; Gupta et al. 2002), but many bacteria and fungi are also known to tolerate increased HM concentrations (including Hg) in soils (Brunker and Bott 1974; Singh and Sherman 1974; Tam 1995; Gupta et al. 2002; Rajapaksha et al. 2004; Holtze et al. 2006; Kelly et al. 2006; Oregaard and Sorensen 2007).

Bacterial resistance to Hg compounds is usually associated with the presence of the Hg resistance (\textit{mer}) operons (Robinson and Tuovinen 1984; Barkay et al. 2003). The mercuric reductase enzyme (\textit{merA}) catalyzes the reduction of Hg(II) to the volatile form Hg(0), which can diffuse through the cells and finally into the atmosphere. The \textit{merB} enzyme is responsible for some bacteria's tolerance of organic-Hg compounds as it catalyzes their degradation into the less toxic form Hg(II) (Barkay et al. 2003). Mercury methylation could
also explain the resistance of bacteria to higher Hg(II) concentrations (Oregaard et al. 2007). Methyl-Hg-compounds are volatile and may diffuse through the cell membrane, whereby the Hg concentrations in the cytoplasm decrease.

The reason why some fungi may tolerate Hg may be their capacity to exude organic acids, which can complex metals and thus immobilise them (Meharg 2003). Fungi may also detoxify Hg(II) by reducing it to the volatile species Hg\(^0\) or by converting it into sulphide-Hg, which facilitates the precipitation of Hg (Brunker et al. 1974; Aiking et al. 1985; Kelly et al. 2006). Furthermore, Hg(II) may be methylated by some fungi, which could make them more tolerant to inorganic Hg (Vonk et al. 1973).

1.2.4 Methodology for assessing the ecotoxicity of HMs in soils

Analyzing the soil respiration rate (CO\(_2\) efflux from soil) is a useful and well established technique for estimating soil microbial activity. Numerous studies have tested the effect of HM pollution on soil microbial activity (Landa et al. 1978; Bringmark et al. 1998; Lazzaro et al. 2006; Nwachukwu et al. 2011). CO\(_2\) effluxes from soils usually include root- or plant-derived CO\(_2\), soil organic matter-derived CO\(_2\), as well as rhizosphere and microbial respiration (Kuzyakov 2006). In experiments containing sieved soils, i.e. without plants and living roots, respiration mainly involves microbial organisms like bacteria or fungi and is referred to as “basal respiration” (Kuzyakov and Gavrichkova 2010). Basal respiration can be considered as reflecting the microbial activity in the soil, depending on the carbon pool, the soil properties, environmental temperature and the microbial biomass. Basal respiration strongly depends on the microbial activity and also on the total microbial biomass. An easy way to measure the microbial biomass carbon (MBC) is to use the substrate induced respiration (SIR) method of Anderson and Domsch (1978). It involves adding an easily consumable energy source (e.g. glucose solution) to the soils and then measuring repeatedly the resulting CO\(_2\) release. About one hour after adding the nutrient source, the CO\(_2\) release rate will remain constant (plateau) for several minutes. This rate is then the basis for estimating the active MBC in the soils. In experiments with HM-contaminated soils,
MBC appears generally to decrease (Akmal et al. 2005; Baath et al. 2005; Tischer 2005; Stefanowicz et al. 2008). Interestingly, with lower MBC more microbial activity has been reported (Leita et al. 1995; Wang et al. 2007; Dos Santos et al. 2012). The metabolic quotient, i.e. the ratio of respiration to biomass, has been found to be higher in HM-polluted soils than in unpolluted soils (Fliessbach et al. 1994; Giller et al. 1998). The increased metabolic quotients in polluted soils could be the consequence of the maintenance-energy requirement being higher or the microorganisms’ efficiency in using the substrate being lower (Leita et al. 1995; Giller et al. 1998; Renella et al. 2007).

Heavy metal (including Hg) pollution has been shown to change the microbial community structure in soil (Baath et al. 1998; Rasmussen et al. 2001; Lazzaro et al. 2006). Mercury pollution selectively affects microbial communities, sometimes leading to a reduction or disappearance of sensitive populations, but sometimes to the survival and growth of Hg-resistant microbial populations (Holtze et al. 2006). The three most popular techniques for studying microbial community structures (microbial fingerprinting methods) are phospholipid fatty acid (PLFA) profiling, denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). Microbial fingerprinting methods provide an overall profile of the microbial community.

PLFA profiling is based on differences in PLFA between groups of organisms. All biological cell membranes contain phospholipids, but the fractions of PLFA differ between organisms or groups of organisms, and some organisms produce unique types of PLFA (Green and Scow 2000). Therefore extracting and quantifying the PLFA groups provides information about microbial communities, and may identify specific microbial functional groups, e.g. sulfate-reducing bacteria. Using PLFA, fungal and bacterial components Gram-positive and negative bacteria can be distinguished (Baath et al. 1998; Rajapaksha et al. 2004).

In contrast to PLFA profiling, DGGE and T-RFLP fingerprinting methods are DNA- or RNA-based techniques. We only discuss the T-RFLP technique in more detail because it provides a more accurate technique with a higher resolution and a better reproducibility than DGGE (Hartmann et al. 2005). The first step is to extract the total genomic DNA (or RNA) of the
sampled matter, e.g. soil. The DNA of interest is then amplified in a polymerase chain reaction (PCR) to produce numerous copies of all target genes using labeled forward primers. The DNA sequences differ between groups of organism in the variable region of genomic DNA. To study bacterial communities, the small-subunit ribosomal 16S rRNA gene is most often amplified. This gene is present in almost all bacteria (Janda and Abbott 2007), and it contains highly conserved and also variable regions between different groups of bacteria (Chakravorty et al. 2007). To study fungal communities, mainly two regions are amplified, either the ITS region or the 18S rRNA genes (Garde and Bruns 1993; Smit et al. 1999; Borneman and Hartin 2000). After PCR, the DNA mixture is digested with enzymes that cut the DNA at defined parts. This digestion process produces terminally-labelled restriction fragments (T-RFs) which differ in length depending on the organism. The fragments can then be separated by capillary electrophoresis, which results in the characteristic profiles of the community compositions in the samples (Frey et al. 2006). The profiles of different samples, e.g. HM-polluted and non-polluted soils can be compared by conducting similarity analyses (Figure 1.7).
Background information

Figure 1.7: T-RFLP technique, example for bacteria (16S rRNA). T-RFLP profiling provides a tool to compare the microbial communities in different samples. Cloning and sequencing techniques yield taxonomic information about the microbial communities.

After comparing the T-RFLP profiles, it is possible to identify the taxonomic relationship of particular bacterial or fungal T-RFs. Extracted DNA is amplified with unlabelled primers and cloned into vectors, which are then transformed into E. coli cells. The cells are plated out on nutrient media and, after a few days, particular colonies are selected and their DNA amplified using labeled primers. A T-RFLP profile is then performed, which produces ideally in only one fluorescence peak after capillary electrophoresis. This peak can be compared to the T-RFLP profiles of the environmental sample. If this peak targets an interesting peak in the T-RFLP sample, e.g. if the peak is larger in HM-polluted soils than in unpolluted soils, the clone can then be sequenced to identify the organisms.
1.3 Thesis outline

Five papers have been written based on the findings from this PhD thesis. Four have already been published and one is accepted for publication in a peer-reviewed journal.

Accumulation of mercury and methylmercury by mushrooms and earthworms from forest soils (2011) Environmental Pollution 159: 2861 – 2869
Stephan Raphael Rieder, Ivano Brunner, Milena Horvat, Anna Jacobs, Beat Frey

Methylation of mercury in earthworms and the effect of mercury on the associated bacterial communities (2013) PLOS ONE e61215
Stephan Raphael Rieder, Ivano Brunner, Otto Daniel, Bian Liu, Beat Frey

Response of soil bacterial communities to mercury chloride application to various forest soils Soil Biology and Biochemistry 65: 329 – 337
Beat Frey and Stephan Raphael Rieder

Methyl-mercury affects microbial activity and biomass, bacterial community structure but hardly the fungal community structure (2013) Soil Biology and Biochemistry 64: 164 - 173
Stephan Raphael Rieder and Beat Frey

Dynamic modeling of the long term behavior of Cd, Pb and Hg in Swiss forest soils using CHUM (Science of the total Environment, in press)
Stephan Raphael Rieder, Edward Tipping, Stefan Zimmermann, Elisabeth Graf Pannatier, Peter Waldner, Markus Meili, Beat Frey
Chapter 2

Accumulation of mercury and methylmercury by mushrooms and earthworms from forest soils

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Contribution of the authors:
\textsuperscript{1}Experimental design; \textsuperscript{2}sampling; \textsuperscript{3}data analyzing; \textsuperscript{4}paper writing

2.1 Abstract

Accumulation of total and methyl-Hg by mushrooms and earthworms was studied in thirty-four natural forest soils strongly varying in soil physico-chemical characteristics. Tissue Hg concentrations of both receptors did hardly correlate with Hg concentrations in soil. Both total and methyl-Hg concentrations in tissues were species-specific and dependent on the ecological groups of receptor. Methyl-Hg was low accounting for less than 5 and 8\% of total Hg in tissues of mushrooms and earthworms, respectively, but with four times higher concentrations in earthworms than mushrooms. Total Hg concentrations in mushrooms averaged 0.96 mg Hg kg\textsuperscript{-1} dw whereas litter decomposing mushrooms showed highest total Hg and methyl-Hg concentrations. Earthworms contained similar Hg concentrations (1.04 mg Hg kg\textsuperscript{-1} dw) whereas endogeic earthworms accumulated highest amounts of Hg and methyl-Hg.
2.2 Introduction

Mercury (Hg) is released in the atmosphere by natural (e.g. volcanic eruptions, forest fires, evaporation from soils and water) and anthropogenic processes (e.g. fossil fuel combustion, gold mining, ore roasting and processing) (Schroeder et al. 1998; Pacyna et al. 2006; Swain et al. 2007). The annual global Hg input into the atmosphere is above 6000 tons (Swain et al. 2007) whereby the residence time of elemental Hg in the atmosphere is approximately one year (Fitzgerald et al. 1997). Therefore, Hg released to the atmosphere can be distributed over long distances and deposited in areas far away from its source. Higher Hg contents in soils and water usually appear near mining areas (Kalac et al. 1996; Kocman et al. 2004; Swain et al. 2007) or chlor-alkali plants (Zagury et al. 2006; Gibicar et al. 2009).

Mercury and especially the organic form monomethylmercury (methyl-Hg) are highly toxic for microorganisms, animals and humans (Boening 2000; Mason and Benoit 2003). The lipophilic nature of methyl-Hg results in much more efficient accumulation in organisms compared to inorganic Hg. Different groups of organisms like bacteria and mushrooms showed the capacity to methylate Hg (Vonk et al. 1973). Methylation of Hg was observed under different conditions but mainly under anoxic situations (StLouis et al. 1996; Schwesig et al. 1999; Drott et al. 2007; Holloway et al. 2009). The contents of methyl-Hg in soils are low, whereas the percentage of methyl-Hg compared to total Hg contents in soils is nominal in a range between 0.5 and 1.5% (Boudou et al. 1997).

In soils Hg is highly immobile and mainly accumulates in the top layers due to its strong affinity to organic matter and soil minerals thereby reducing the concentrations in the soil solution (Ravichandran 2004). Therefore the bioavailability of Hg in soils is usually low (Tipping et al. 2010). Accumulation of Hg and methyl-Hg in soil organisms is important in particular with respect to the estimation of the risk of secondary poisoning (Ernst et al. 2008). Mushrooms and earthworms are interesting to examine different pathways of exposure to Hg from soil: (1) uptake from soil solution (predominantly mushrooms), (2) decomposition of litter and soil organic matter by mushrooms, and (3) ingestion of soil particles and litter by earthworms (Ernst and Frey 2007). Mushrooms are well known to accumulate Hg (e.g.
Stegnar et al. (1973); Byrne et al. (1976); Seeger et al. (1976); Minagawa et al. (1980); Kalac et al. (1991); Kalac et al. (1996); Alonso et al. (2000); Falandysz et al. (2002); Falandysz et al. (2003); Cocchi et al. (2006); Svoboda et al. (2006)) due to their filamentous mode of growth, branching and extra cellular release of enzymes and metabolites. In contrast, studies on the accumulation of methyl-Hg in mushrooms are a few (Stegnar et al. 1973; Minagawa et al. 1980; Bargagli et al. 1984; Fischer et al. 1995). Similarly, investigations on the accumulation of Hg (Burton et al. 2006; Ernst et al. 2008) and in particular of methyl-Hg in earthworms are also rare (Bull et al. 1977; Hinton et al. 2002; Zhang et al. 2009). However, most studies regarding Hg accumulation in mushrooms and earthworms have been conducted in polluted sites, but investigations in non-contaminated sites, in particular forest soils, are rare (Stegnar et al. 1973; Ernst et al. 2008). Therefore, there is an urgent need to study the bioavailability of Hg and methyl-Hg in mushrooms and earthworms collected from non-contaminated forest soils to estimate the risk of secondary poisoning.

The aim of this study was to determine and compare total and methyl-Hg concentrations in two biological receptors from non-contaminated forest sites. In particular, we were interested on the accumulation of total Hg and methyl-Hg in the different ecophysiological groups of mushrooms (mycorrhizal, wood and litter decomposing saprotrophs) and earthworms (epigeic, endogeic, anecic).

2.3 Materials and methods

2.3.1 Sampling and sample preparation

Earthworms and fruiting bodies of mushrooms were collected at 34 well characterized forest sites (Walthert et al. 2004; Blaser et al. 2005; Zimmermann et al. 2006) distributed over Switzerland (Figure 2.1). Forest sites from the Swiss Alps were excluded due to their special climatic conditions. Except for three sites, all sampling points were non-contaminated forest soils with different physico-chemical characteristics. Smelters were located in the vicinity of two sites (Gerlafingen, Visp) and a shooting range near one site (Zuchwil). 

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All samples were taken around of the described soil profiles from Walthert et al. (2004), Blaser et al. (2005), and Zimmermann et al. (2006), with the soil samples taken within a radius of 2 m, the earthworms within a radius of 5 m, and the mushrooms with a radius of 20 m. Three soil samples of the topsoil (A-horizon) were taken with a soil auger (5 cm in diameter, 20 cm depth) and were pooled for analyses. The soil samples were then sieved (2 mm), dried at 15°C in a forced-draught oven for 5 days (for the determination of physico-chemical parameters) or frozen in liquid nitrogen and freeze-dried (for the determination of Hg and methyl-Hg). Residual moisture contents of the soils were then measured (105°C for 3 days). Homogenization of the soil samples were performed by gently crushing the soils in an acid-washed porcelain pestle and mortar. To collect the earthworms, four randomly plots (0.125 m²) with similar habitats (e.g. soil type, vegetation) at a distance of about 8 to 12 m between plots were chosen. Earthworms were collected up to a soil depth of 0.3 m by digging with a spade. Deeper living earthworms were collected by the formaldehyde method of (Raw 1959) whereby a 0.37% formaldehyde solution was poured on the soil. The solution irritates the skin of worms and brings them to the soil surface after about 20 min. Immediately after sampling the organisms were washed with deionised water to remove remaining soil particles and formaldehyde. The earthworms were classified in accordance with Sims and Gerard (1985) and sorted by site and species. Earthworms were maintained and starved in the laboratory for one week on moistened filter paper at 15°C until their gut contents were emptied. Thereafter earthworms were frozen in liquid nitrogen, lyophilized and stored at -20°C until processing. Fruiting bodies of mushrooms were picked from the soil surface and roughly classified on the genera level. Aliquots were frozen in liquid nitrogen, lyophilized and stored at -20°C until processing. The remaining part of fruiting bodies was dried for further taxonomical classification. Mushrooms and earthworms samples were grounded by a tungstic coated ball mill to a fine powder and the residual moisture content was further examined by heating aliquots to 105°C for 3 days.
Figure 2.1. Sampling sites. 1: Alpthal (8°42'E, 47°02'N); 2: Bettlachstock (7°25'E, 47°13'N); 3: Bodio (8°55'E, 46°22'N); 4: Burgdorf (7°35'E, 47°02'N); 5: Copera (8°59'E, 46°08'N); 6/7: Ermatingen (9°05'E, 47°38'N); 8: Etzwil (8°11'E, 47°34'N); 9: Geissgrat (7°29'E, 47°14'N); 10: Gerlafingen (7°33'E, 47°10'N); 11: Gottschalkenberg (8°40'E, 47°08'N); 12: Jussy (6°17'E, 46°13'N); 13: Krauchthal (7°34'E, 47°02'N); 14: Laufen (7°25'E, 47°23'N); 15: Laura (9°06'E, 46°12'N); 16: Lausanne (6°39'E, 46°34'N); 17: Möhlin (7°52'E, 47°34'N); 18: Morcote (8°54'E, 45°56'N); 19: Neunkirch (8°32'E, 47°41'N); 20: Niederlinsbach (8°00'E, 47°23'N); 21: Novaggio (8°50'E, 46°01'N); 22: Othmarsingen (8°13'E, 47°23'N); 23: Pian d’Arf (9°07'E, 46°13'N); 24: Piotta (8°40'E, 46°30'N); 25: Pura (8°51'E, 45°59'N); 26: Sagno (9°02'E, 45°51'N); 27: Schänis (9°04'E, 47°09'N); 28: Schüpfen (7°21'E, 47°02'N); 29: Sihlwald (8°34'E, 47°14'N); 30: Visp (7°51'E, 46°17'N); 31: Vorderwald (7°53'E, 47°16'N); 32: Zofingen (7°59'E, 47°18'N); 33: Zuchwil (7°34'E, 47°11'N); 34: Zugerberg (8°33'E, 47°06'N). In Ermatingen, two different sites (6: hollow and 7: plateau) with a distance of about 50 m were sampled (Ermatingen D20 and D21).

2.3.2 Analyses

Mercury analyses were only performed if at least three fruiting bodies of the same mushroom species or three adult earthworms of the same species were available per sampling site. For total Hg analyses, all samples were solubilised in HNO₃ (65%) and HF (40%) with a microwave digestion system (MLS Milestone, Perkin–Elmer) and measured by Cold Vapour Atomic Absorption Spectroscopy (CV-AAS: 2000, Perkin-Elmer) with a detection limit of
about 5 µg Hg kg\(^{-1}\) dw. The accuracy of analyses was checked against biological and soil certified reference materials (NIST1547: Peach Leaves; CMI7004 Soil Loam). Regular analysis does not exceed 15% of the certified material. The total Hg of selected samples was in good accordance to measurements with a direct mercury analyser (data not shown).

Methyl-Hg was determined following the procedure described in Bloom (1989), Horvat et al. (1993) and Liang et al. (1994). Approximately 100 mg of sample was weighed in Teflon vials. After addition of 6 ml of mixture comprising 5% H\(_2\)SO\(_4\) and 18% KBr and 1.0 ml 1M CuSO\(_4\) solution, the vials were closed and shaken vigorously for 15 min. Ten ml of CH\(_2\)Cl\(_2\) was added to each vial and the samples were shaken again for 15 min. The samples were then centrifuged for 5 min at 3200 rpm. The organic phase (CH\(_2\)Cl\(_2\)) was separated from aqueous phase. Extraction was repeated with additional 5 ml CH\(_2\)Cl\(_2\). Approximately 35 ml of Milli-Q water was added to the combined CH\(_2\)Cl\(_2\). The organic phase was evaporated on water bath at about 90°C. Thereafter the samples were purged with N\(_2\) for 5 min to remove remaining CH\(_2\)Cl\(_2\). An aliquot of the aqueous sample was added to Teflon reaction vial and pH was adjusted to be 4.6 with addition of 100 µl of acetate buffer. The weight was measured after each step to calculate back the concentrations in solution to concentrations in the solid samples. For ethylating mercury compounds fifty µl of 1% NaBEt\(_4\) (sodium tetraethylborate) was added into the reaction vial at the end and the mixture was left to react at the room temperature for 15 min. Ethylated methyl-Hg as ethylmethyl-Hg was purged onto Tenax trap for 15 min with Hg-free N\(_2\). The Tenax traps were then connected to the flow of Ar and methyl-Hg was thermally desorbed (180°C) onto isothermal GC column. All Hg species were converted to Hg\(^0\) by pyrolysis at 600°C and measured by cold vapour atomic fluorescence detector (CV AFS). The limit of detection was about 0.05 µg MeHg kg\(^{-1}\) dry wt (Bloom 1989; Horvat et al. 1993; Liang et al. 1994). The used certified reference materials (DORM-2, TORT-2) were obtained from the National Research Council of Canada and IAEA-086 was obtained from the International Atomic Energy Agency. Regular analysis does not exceed 10% of the certified material. The recovery for methyl-Hg extractions was determined using the method of standard addition and ranged between 89% and 103%. All samples were
measured in duplicates and all measurements were repeated at least twice. Bioconcentration factors (BCFs) were calculated as the Hg or methyl-Hg concentrations in the tissues divided by the corresponding concentrations in the soils.

The soil pH was measured potentiometrically in 0.01 M CaCl₂ with a soil-extractant ratio of 1:2. Soil samples were further dissolved in 1 M NH₄Cl (ratio 1:10) for 1 h on an end-over-end shaker for measuring exchangeable cations. The extracted cations were examined by Inductive Coupled Plasma Mass Spectrometry (ICP-MS) (Perkin-Elmer OPTIMA 3000). By summing up the molar charge contents of Na, K, Ca, Mg, Mn, Al and Fe the cation exchange capacity (CEC) was calculated. Carbon contents were measured with a CN analyzer (NA 2500, CE Instruments).

2.3.3 Statistic

The statistical calculations were performed with the program STATISTICA (StatSoft). Calculations for statistic significance (p values) were performed using ANOVA or in case of non normal distributed dates with Kruskal-Wallis test. Pearman correlation coefficients were used to evaluate relationships between soil Hg concentration and tissue Hg concentrations. Figures were generated with STATISTICA or Excel (Microsoft Corporation).

2.4 Results

2.4.1 General soil properties and Hg and methyl-Hg concentrations

The investigated sites contained a wide range of soil and forest types (Table 2.1). Measured pH ranged from 2.9 (Krauchthal) to 7.2 (Neunkirch). The total Hg in soils ranged between 0.07 (Jussy) and 0.55 (Visp) mg Hg kg⁻¹ dry soil by an average value of 0.18 mg Hg kg⁻¹ dry soil. Three forest sites were expected to be contaminated because of the vicinity to a smelter industry (Gerlafingen, Visp) or a shooting range (Zuchwil). However, only one site (Visp) showed elevated Hg concentration (0.55 mg Hg kg⁻¹ dry soil) in soils compared to the other sites. Hg concentration of non-contaminated soils showed a very low but significant
correlation ($r^2=0.12; p<0.01; n=34$) related to the soil organic C concentration. There were no correlations between Hg concentration in soils and other soil physico-chemical characteristics such as pH, texture, CEC and base saturation (data not shown). All methyl-Hg concentrations in soils were low and ranged between 0.2 and 2.4% of total Hg (Table 2.1).
TABLE 2.1. Location, collected mushrooms and earthworms, soil type, humus form, dominant tree species, soil properties and Hg and methyl-Hg concentrations (mean ± standard error) of A horizon of the sampling sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>Sampling site properties</th>
<th>Mushroom genera*</th>
<th>Earthworm species**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soil type^b</td>
<td>Humus form^c</td>
<td>Dominant tree species</td>
</tr>
<tr>
<td>Alpthal</td>
<td>Cambisol</td>
<td>Rhizomull</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Bettlachstock</td>
<td>Cambisol</td>
<td>Rhizomull</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Bodio</td>
<td>Cambisol</td>
<td>Rhizomull</td>
<td>Castanea sativa</td>
</tr>
<tr>
<td>Burgdorf</td>
<td>Cambisol</td>
<td>Rhizomull</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Copera</td>
<td>Podsol</td>
<td>Rhizomull</td>
<td>Castanea sativa</td>
</tr>
<tr>
<td>Ermatingen D20</td>
<td>Gley</td>
<td>Rhizomull</td>
<td>Acer platanoides</td>
</tr>
<tr>
<td>Ermatingen D21</td>
<td>Gley</td>
<td>Leptomoder</td>
<td>Picea abies</td>
</tr>
<tr>
<td>Etzwil</td>
<td>Cambisol-pseudogley</td>
<td>Hemimor</td>
<td>Picea abies</td>
</tr>
<tr>
<td>Geissgrat</td>
<td>Leptisol</td>
<td>Rhizomull</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Gerlafingen</td>
<td>Cambisol</td>
<td>Rhizomull</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Gottschalkenberg</td>
<td>Stagnogley</td>
<td>Leptomoder</td>
<td>Abies alba</td>
</tr>
<tr>
<td>Jussy</td>
<td>Cambisol</td>
<td>Mull</td>
<td>Quercus robur</td>
</tr>
<tr>
<td>Krauchthal</td>
<td>Podsol</td>
<td>Hemimor</td>
<td>Picea abies</td>
</tr>
<tr>
<td>Laufen</td>
<td>Leptisol</td>
<td>Vermimull</td>
<td>Quercus petraea</td>
</tr>
<tr>
<td>Laura</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Picea abies</td>
</tr>
<tr>
<td>Lausanne</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Möhlin</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Castanea sativa</td>
</tr>
<tr>
<td>Morcote</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Pinus sylvestris</td>
</tr>
<tr>
<td>Neunkirch</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Quercus ceras</td>
</tr>
<tr>
<td>Niedererinsbach</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Castanea sativa</td>
</tr>
<tr>
<td>Novaggio</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Quercus petraea</td>
</tr>
<tr>
<td>Ohmargingen</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Pian d’Arf</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Picea abies</td>
</tr>
<tr>
<td>Piotta</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Castanea sativa</td>
</tr>
<tr>
<td>Pura</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Castanea sativa</td>
</tr>
<tr>
<td>Sagno</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Castanea sativa</td>
</tr>
<tr>
<td>Schänis</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Schüpfen</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Fraxinus excelsior</td>
</tr>
<tr>
<td>Sihlwald</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Pinus sylvestris</td>
</tr>
<tr>
<td>Vorderwald</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Zofingen</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Zuchwil</td>
<td>Cambisol</td>
<td>Vermimull</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Zugberger</td>
<td>Cambisol</td>
<td>Leptomoder</td>
<td>Picea aebis</td>
</tr>
</tbody>
</table>
A: Amanita; B: Armillaria; C: Boletus; D: Collybia; E: Cortinarius; F: Craterellus; G: Hebeloma; H: Laccaria; I: Lactarius; J: Leccinum; K: Lepista; L: Lycoperdon; M: Macrolepiota; N: Oudemansiella; O: Ramaria; P: Russula; Q: Suillus; R: Tricholoma; S: Tricholomopsis; T: Xerocomus

**a: Aporrectodea caliginosa; b: Aporrectodea longa; c: Aporrectodea rosea; d: Dendrodrilus rubidus; e: Lumbricus rubellus; f: Lumbricus terrestris; g: Octolasion cyaneum; h: Octolasion tyrtaeum**

*standard error between different sampling times (mushroom and earthworm collection); two time points
*according to FAO
*Humus form according to Green et al. (1993)
2.4.2 Mercury and methyl-Hg concentrations in the fruiting bodies of mushrooms

Sufficient mushroom fruiting bodies for analyses were collected at 25 of the 34 sampling sites (Table 2.1). The highest species diversity was found in Vordemwald with six different mushroom genera. The collected mushrooms belonged to 48 different species and representing 20 different genera. *Russula*, *Lactarius* and *Lepista* were the most abundant genera occurring at 16, 6 and 5 different forest sites, respectively (Tables 2.1 and 2.2). *Russula nigricans* was collected at five forest sites. All other species were found in less than three forest sites. The majority of the collected mushrooms live in symbiosis with plants and form mycorrhizas (34 species belonging to 13 genera). The other mushrooms live saprophytically by degrading dead wood (6 species, 4 genera) or litter (8 species, 3 genera) (Table 2.2).
TABLE 2.2. Total Hg and methyl-Hg concentrations in mushrooms (mean ± standard error). Mean concentrations are calculated per each exact classified mushroom species or per genera (*Cortinarius* sp., *Russula* sp.). BCF = Bioconcentration factor.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Ecophysiological group</th>
<th>n*</th>
<th>Hg [mg kg⁻¹ dw]</th>
<th>MeHg [mg kg⁻¹ dw]</th>
<th>% Hg is MeHg</th>
<th>BCF Hg</th>
<th>BCF MeHg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amanita</strong></td>
<td>caesarea</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.35</td>
<td>0.024</td>
<td>6.8</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td><strong>Boletus</strong></td>
<td>erythropus</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.24</td>
<td>0.009</td>
<td>3.6</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td><strong>Cortinarius</strong></td>
<td>3 spp.</td>
<td>Mycorrhizal</td>
<td>3</td>
<td>0.77 ± 0.07</td>
<td>0.011 ± 0.002</td>
<td>1.4 ± 0.2</td>
<td>5.1 ± 2.2</td>
<td>6.8†</td>
</tr>
<tr>
<td><strong>Craterellus</strong></td>
<td>cornucopioides</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.03</td>
<td>0.001</td>
<td>4.7</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Hebeloma</strong></td>
<td>meso phaeum</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.69</td>
<td>0.054</td>
<td>7.8</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td><strong>Laccaria</strong></td>
<td>lacata</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.51</td>
<td>0.012</td>
<td>2.2</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td><strong>Lactarius</strong></td>
<td>deterrimus</td>
<td>Mycorrhizal</td>
<td>2</td>
<td>0.56 ± 0.41</td>
<td>0.002 ± 0.001</td>
<td>0.3</td>
<td>3.7</td>
<td>0.6†</td>
</tr>
<tr>
<td><strong>Lactarius</strong></td>
<td>luteolus</td>
<td>Mycorrhizal</td>
<td>2</td>
<td>0.21 ± 0.02</td>
<td>0.007 ± 0.004</td>
<td>3.1 ± 1.7</td>
<td>2.7 ± 0.8</td>
<td></td>
</tr>
<tr>
<td><strong>Lactarius</strong></td>
<td>scrobiculatus</td>
<td>Mycorrhizal</td>
<td>3</td>
<td>0.54 ± 0.27</td>
<td>0.002 ± 0.000</td>
<td>0.8 ± 0.1</td>
<td>1.6 ± 0.0</td>
<td>2.1†</td>
</tr>
<tr>
<td><strong>Leccinum</strong></td>
<td>griseum</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.28</td>
<td>0.001</td>
<td>0.5</td>
<td>4.1</td>
<td></td>
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<tr>
<td><strong>Ramaria</strong></td>
<td>largenti</td>
<td>Mycorrhizal</td>
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<td>2.61</td>
<td>0.013</td>
<td>0.5</td>
<td>32.2</td>
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<tr>
<td><strong>Russula</strong></td>
<td>artropurpurea</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.88</td>
<td>0.005</td>
<td>0.6</td>
<td>6.3</td>
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<tr>
<td><strong>Russula</strong></td>
<td>aurea</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.28</td>
<td>0.024</td>
<td>8.3</td>
<td>3.7</td>
<td></td>
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<tr>
<td><strong>Russula</strong></td>
<td>cyanoxantha</td>
<td>Mycorrhizal</td>
<td>3</td>
<td>5.61 ± 1.75</td>
<td>0.046 ± 0.016</td>
<td>1.0 ± 0.9</td>
<td>50.4 ± 32.2</td>
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<tr>
<td><strong>Russula</strong></td>
<td>foetens</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.07</td>
<td>0.001</td>
<td>1.5</td>
<td>0.8</td>
<td>1.0†</td>
</tr>
<tr>
<td><strong>Russula</strong></td>
<td>nigricans</td>
<td>Mycorrhizal</td>
<td>5</td>
<td>3.06 ± 0.97</td>
<td>0.011 ± 0.008</td>
<td>0.8 ± 0.6</td>
<td>15.6 ± 4.0</td>
<td>9.0†</td>
</tr>
<tr>
<td><strong>Russula</strong></td>
<td>purpurata</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.03</td>
<td>0.001</td>
<td>2.3</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td><strong>Russula</strong></td>
<td>9 spp.</td>
<td>Mycorrhizal</td>
<td>9</td>
<td>0.26 ± 0.05</td>
<td>0.023 ± 0.010</td>
<td>10.5 ± 2.9</td>
<td>1.5 ± 0.4</td>
<td>11.7 ± 3.2†</td>
</tr>
<tr>
<td><strong>Suillus</strong></td>
<td>grevillei</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.38</td>
<td>0.006</td>
<td>1.6</td>
<td>6.0</td>
<td></td>
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<td><strong>Suillus</strong></td>
<td>luteus</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.51</td>
<td>0.001</td>
<td>0.3</td>
<td>2.4</td>
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<td>0.010</td>
<td>1.6</td>
<td>7.9</td>
<td></td>
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<td>saponaceum</td>
<td>Mycorrhizal</td>
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<td>0.25</td>
<td>0.028</td>
<td>11.3</td>
<td>3.9</td>
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<tr>
<td><strong>Xerocomus</strong></td>
<td>badius</td>
<td>Mycorrhizal</td>
<td>3</td>
<td>0.72 ± 0.08</td>
<td>0.029 ± 0.027</td>
<td>3.6 ± 3.2</td>
<td>2.8 ± 0.5</td>
<td>151.2†</td>
</tr>
<tr>
<td><strong>Xerocomus</strong></td>
<td>chrysenteron</td>
<td>Mycorrhizal</td>
<td>1</td>
<td>0.75</td>
<td>0.035</td>
<td>4.6</td>
<td>4.1</td>
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<tr>
<td><strong>mean</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.81 ± 0.21</td>
<td>0.017 ± 0.004</td>
<td>4.8 ± 1.0</td>
<td>6.1 ± 1.7</td>
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<tr>
<td><strong>Armillaria</strong></td>
<td>cepistipes</td>
<td>Wood decomposing</td>
<td>1</td>
<td>0.09</td>
<td>0.001</td>
<td>1.5</td>
<td>0.5</td>
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<tr>
<td><strong>Armillaria</strong></td>
<td>sp.</td>
<td>Wood decomposing, parasitic</td>
<td>1</td>
<td>0.07</td>
<td>0.001</td>
<td>1.5</td>
<td>0.5</td>
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<td>perlatum</td>
<td>Wood decomposing</td>
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<td>0.58</td>
<td>0.012</td>
<td>2.1</td>
<td>6.3</td>
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<td>pyriforme</td>
<td>Wood decomposing</td>
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<td>0.02</td>
<td>0.002</td>
<td>11.3</td>
<td>0.2</td>
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<td><strong>Oudemansiella</strong></td>
<td>platypylla</td>
<td>Wood decomposing</td>
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<td>0.58</td>
<td>0.029</td>
<td>5.0</td>
<td>2.0</td>
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<td><strong>Tricholomopsis</strong></td>
<td>rutilans</td>
<td>Wood decomposing</td>
<td>1</td>
<td>0.11</td>
<td>0.009</td>
<td>8.5</td>
<td>0.5</td>
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<tr>
<td><strong>mean</strong></td>
<td></td>
<td>Wood decomposing</td>
<td>6</td>
<td>0.24 ± 0.12</td>
<td>0.009 ± 0.005</td>
<td>5.0 ± 1.4</td>
<td>1.7 ± 1.9</td>
<td></td>
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<tr>
<td><strong>Collybia</strong></td>
<td>dryophila</td>
<td>Litter decomposing</td>
<td>1</td>
<td>0.39</td>
<td>0.016</td>
<td>4.0</td>
<td>6.3</td>
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<td><strong>Collybia</strong></td>
<td>maculata</td>
<td>Litter decomposing</td>
<td>1</td>
<td>3.44</td>
<td>0.013</td>
<td>0.4</td>
<td>11.7</td>
<td></td>
</tr>
<tr>
<td><strong>Lepista</strong></td>
<td>inversa</td>
<td>Litter decomposing</td>
<td>1</td>
<td>2.76</td>
<td>0.068</td>
<td>2.5</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td><strong>Lepista</strong></td>
<td>irina</td>
<td>Litter decomposing</td>
<td>2</td>
<td>4.44 ± 0.92</td>
<td>0.113 ± 0.084</td>
<td>2.3 ± 1.4</td>
<td>38.8 ± 0.0</td>
<td></td>
</tr>
<tr>
<td><strong>Lepista</strong></td>
<td>luscina</td>
<td>Litter decomposing</td>
<td>1</td>
<td>2.21</td>
<td>0.077</td>
<td>3.5</td>
<td>35.1</td>
<td></td>
</tr>
</tbody>
</table>

Chapter 2
## Bioaccumulation of Hg and methyl-Hg

<table>
<thead>
<tr>
<th>Species</th>
<th>Litter decomposing</th>
<th>2</th>
<th>2.14 ± 0.94</th>
<th>0.075 ± 0.003</th>
<th>4.4 ± 2.1</th>
<th>18.0 ± 1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lepista nebularis</em></td>
<td></td>
<td>2</td>
<td>4.78 ± 1.71</td>
<td>0.082 ± 0.027</td>
<td>2.1</td>
<td>28.6</td>
</tr>
<tr>
<td><em>Macrolepiota procera</em></td>
<td></td>
<td>2</td>
<td>0.89</td>
<td>0.006</td>
<td>0.7</td>
<td>6.4</td>
</tr>
<tr>
<td><em>Macrolepiota mastoidea</em></td>
<td></td>
<td>1</td>
<td>2.56 ± 0.53 <em>a</em></td>
<td>0.060 ± 0.016 <em>a</em></td>
<td>2.8 ± 0.6 <em>a</em></td>
<td>22.6 ± 5.0 <em>a</em></td>
</tr>
<tr>
<td><strong>mean</strong></td>
<td><strong>Litter decomposing</strong></td>
<td>8</td>
<td>2.56 ± 0.53 <em>a</em></td>
<td>0.060 ± 0.016 <em>a</em></td>
<td>2.8 ± 0.6 <em>a</em></td>
<td>22.6 ± 5.0 <em>a</em></td>
</tr>
<tr>
<td><strong>mean total</strong></td>
<td></td>
<td>48</td>
<td>0.96 ± 0.19</td>
<td>0.021 ± 0.004</td>
<td>4.5 ± 0.8</td>
<td>7.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>49.5 ± 30.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* n = number of forest sites at which at least tree mushrooms per species were collected
** number of different species
1-4 number of analysed mushrooms (1 or 4)

Statistics: different letters (a to c) implicate significant differences (p<0.05) between the particular ecophysiological groups
The concentrations of total and methyl-Hg in fruiting bodies of the mushrooms varied between the different sampling sites (Figure 2.3a) but not on a significant level. Overall there was no correlation ($r^2=0.02; p<0.22; n=63$) between soil Hg and mushroom Hg concentration except for *R. nigricans* ($r^2=0.92; p<0.01; n=5; $Figure 2.2a).

![Figure 2.2](image)

**Figure 2.2:** Correlation between soil Hg concentration and tissue Hg concentration in mushrooms and earthworms. a) data of *Russula nigricans* considered; b) data of *Lumbricus rubellus* considered.

Mushrooms contained on average 0.96 mg Hg kg$^{-1}$ dw and considerably less methyl-Hg (0.021 mg MeHg kg$^{-1}$ dw). Both total and methyl-Hg amounts in tissues were genus specific (Figure 2.3b) but not on a significant level (Hg: $p=0.15$; MeHg: $p=0.09$). The Hg concentrations in fruiting bodies varied highly significant between the ecological groups of mushrooms (Table 2.2). Mycorrhiza forming mushrooms showed a mean Hg concentration of 0.81 mg Hg kg$^{-1}$ dw but the mean Hg content of saprophytic species were 0.24 mg Hg kg$^{-1}$ dw for wood decomposing and 2.56 mg Hg kg$^{-1}$ dw for litter decomposing fungi. Three mycorrhizal mushrooms (*Ramaria largentii, Russula cyanoxantha* and *R. nigricans*) contained elevated Hg concentrations between 2.61 and 5.61 mg Hg kg$^{-1}$ dw. Mercury and methyl-Hg concentrations in mycorrhizal mushrooms were not significantly higher as in wood decomposing mushrooms, but both mycorrhizal and wood decomposing mushrooms contained significantly less Hg and methyl Hg as litter decomposing mushrooms (Table 2.2). A highly significant correlation ($p<0.0001$) was found between total and methyl-Hg concentration in the fruiting bodies. The percentages of methyl-Hg compared to total Hg
Bioaccumulation of Hg and methyl-Hg

ranged between 0.3% \((Lactarius\ deterrimus,\ Suillus\ luteus)\) and 11.3% \((Lycoperdon\ pyriforme,\ Tricholoma\ saponaceum)\) whereas a mean percentage of 4.5% of total Hg present in their methylated form was found.

Bioconcentration factors for Hg ranged between 0.2 \((L.\ pyriforme,\ Russula\ purpurata)\) and 50.4 \((R.\ cyanoxantha)\). Litter decomposing mushrooms showed significantly higher BCFs\textsubscript{Hg} (22.6) than mycorrhizal (6.1) and wood decomposing (1.7) mushrooms. In addition, mycorrhizal mushrooms had significantly higher BCFs\textsubscript{Hg} than wood decomposing mushrooms (Table 2.2). The BCFs\textsubscript{methyl-Hg} ranged between 0.6 \((Craterellus\ cornucopioides)\) and 192.2 \((Macrolepiota\ procera)\). Litter decomposing mushrooms showed significantly higher BCFs\textsubscript{methyl-Hg} (56.5) than wood decomposing (13.9) and mycorrhizal (11.5) mushrooms.

**Figure 2.3:** Total Hg concentration (mean ± standard error) in soils, in mushrooms and the methyl-Hg concentration in mushrooms are listed according to a) sampling sites and b) genera. The mushroom species are grouped, and divided by dashed lines in three ecological categories: wood decomposing, mycorrhizal, and litter decomposing mushrooms.
2.4.3 Mercury and methyl-Hg concentrations in earthworm tissue

Sufficient earthworms for Hg analyses were collected at 26 of the 34 forest sites (Table 2.1). Seven different earthworm species were found representing all three different ecophysiological groups (two epigeic, two anecic, three endogeic). *Lumbricus rubellus* was the most frequent earthworm species and was sampled at 16 forest sites. The other species were found at three to nine sites. The concentrations of total and methyl-Hg in the earthworm tissues varied between forest sites but not on a significant level (Figure 2.4a). In general, no correlation was found between total Hg concentrations in soils and Hg concentrations in earthworm tissues \( (r^2<0.001; p<0.89; n=52) \). A correlation between Hg concentrations in soils and between earthworms \( (r^2=0.35; p<0.02; n=16) \) was only found in *L. rubellus* (Figure 2.2b).

Hg concentrations in tissues averaged 1.04 mg Hg kg\(^{-1}\) dw with much lower levels of methyl-Hg averaging 0.089 mg MeHg kg\(^{-1}\) dw (Table 2.3). Both total and methyl-Hg concentrations in tissues were species-specific at a highly significant level \( (p<0.001, \text{Figure } 2.4b) \). The mean Hg concentrations ranged from 0.20 \( (L. rubellus) \) to 2.12 mg Hg kg\(^{-1}\) dw \( (Octolasion cyaneum) \). Hg concentrations in earthworms varied significantly between all ecological groups (Table 2.3). Endogeic earthworms accumulated highest amounts of Hg \( (1.63 \text{ mg Hg kg}^{-1} \text{ dw}) \) which was about two times higher than in anecic \( (0.69 \text{ mg Hg kg}^{-1} \text{ dw}) \) and three times higher than in epigeic \( (0.49 \text{ mg Hg kg}^{-1} \text{ dw}) \) earthworms (Table 2.3). Total Hg concentrations correlated highly significant \( (p<0.0001; \text{for all species: } r^2=0.52; n=52; \text{for } Aporrectodea caliginosa: r^2=0.87; n=6) \) with methyl-Hg concentrations. The methyl-Hg concentrations ranged between 0.014 \( (L. rubellus) \) and 0.242 mg MeHg kg\(^{-1}\) dw \( (O. cyaneum) \). Percentages of methyl-Hg compared to total Hg were between 5.7\% \( (Aporrectodea longa) \) and 10.1\% \( (Lumbricus terrestris) \) whereas a mean percentage of 7.7\% was found.
TABLE 2.3. Total Hg and methyl-Hg concentrations in earthworms (mean ± standard error). Mean concentrations are calculated per each earthworm species. BCF = Bioconcentration factor.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Ecophysiological group</th>
<th>n*</th>
<th>Hg [mg kg⁻¹ dw]</th>
<th>MeHg [mg kg⁻¹ dw]</th>
<th>% Hg is MeHg</th>
<th>BCF Hg</th>
<th>BCF MeHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendrodrilus</td>
<td>rubidus</td>
<td>Epigeic</td>
<td>7</td>
<td>0.78 ± 0.21</td>
<td>0.043 ± 0.007</td>
<td>6.4 ± 1.2</td>
<td>4.6 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Lumbricus</td>
<td>rubellus</td>
<td>Epigeic</td>
<td>16</td>
<td>0.20 ± 0.03</td>
<td>0.014 ± 0.004</td>
<td>6.2 ± 0.8</td>
<td>1.1 ± 0.2</td>
<td>10.2 ± 3.4³</td>
</tr>
<tr>
<td>mean</td>
<td>Epigeic</td>
<td></td>
<td>2**</td>
<td>0.49 ± 0.29</td>
<td>0.025 ± 0.015</td>
<td>6.3 ± 0.1</td>
<td>2.9 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Aporrectodea</td>
<td>caliginosa</td>
<td>Endogeic</td>
<td>6</td>
<td>1.03 ± 0.32</td>
<td>0.096 ± 0.041</td>
<td>9.1 ± 1.7</td>
<td>4.1 ± 1.2</td>
<td>315.7³</td>
</tr>
<tr>
<td>Aporrectodea</td>
<td>rosea</td>
<td>Endogeic</td>
<td>5</td>
<td>1.74 ± 0.25</td>
<td>0.116 ± 0.053</td>
<td>6.6 ± 2.8</td>
<td>14.5 ± 6.4</td>
<td>66.6 ± 30.3²</td>
</tr>
<tr>
<td>Octolasion</td>
<td>cyaneum</td>
<td>Endogeic</td>
<td>9</td>
<td>2.12 ± 0.48</td>
<td>0.242 ± 0.132</td>
<td>9.8 ± 3.1</td>
<td>14.0 ± 4.5</td>
<td>347.5 ± 202.8²</td>
</tr>
<tr>
<td>mean</td>
<td>Endogeic</td>
<td></td>
<td>3</td>
<td>1.63 ± 0.32</td>
<td>0.153 ± 0.044</td>
<td>8.5 ± 1.0</td>
<td>10.9 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>Aporrectodea</td>
<td>longa</td>
<td>Anecic</td>
<td>6</td>
<td>1.02 ± 0.25</td>
<td>0.067 ± 0.029</td>
<td>5.7 ± 1.7</td>
<td>10.2 ± 4.4</td>
<td>58.7 ± 41.4²</td>
</tr>
<tr>
<td>Lumbricus</td>
<td>terrestris</td>
<td>Anecic</td>
<td>3</td>
<td>0.36 ± 0.10</td>
<td>0.038 ± 0.012</td>
<td>10.1 ± 1.1</td>
<td>1.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>Anecic</td>
<td></td>
<td>2</td>
<td>0.69 ± 0.33</td>
<td>0.055 ± 0.015</td>
<td>7.9 ± 2.2</td>
<td>6.0 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>mean total</td>
<td></td>
<td></td>
<td>7</td>
<td>1.04 ± 0.26</td>
<td>0.089 ± 0.029</td>
<td>7.7 ± 0.7</td>
<td>7.2 ± 2.1</td>
<td>159.7 ± 71.0</td>
</tr>
</tbody>
</table>

* n = number of forest sites at which at least tree earthworms per species were collected
** number of different species
³⁴ numbers of analysed earthworms (1 to 4)

Statistics: different letters (a to c) implicate significant differences (p<0.05) between the particular ecophysiological groups
BCFs for total Hg ranged between 1.1 (L. rubellus) and 14.5 (Aporrectodea rosea). Epigeic earthworms showed lowest BCF$_{\text{Hg}}$ values (2.9). In contrast, BCF$_{\text{Hg}}$ were two times higher (6.0) in anecic earthworms and about four times higher (10.9) in endogeic earthworms than in epigeic earthworms (Table 2.3). The BCFs$_{\text{methyl-Hg}}$ ranged between 15.1 (L. rubellus) and 191.5 (O. cyaneum). Endogeic earthworms showed significantly higher BCFs$_{\text{methyl-Hg}}$ (141.7) than anecic (49.8) and epigeic (28.5) earthworms. The averaged BCF$_{\text{methyl-Hg}}$ was about 11 times higher as the averaged BCF$_{\text{Hg}}$.

Figure 2.4: Total Hg concentration (mean ± standard error) in soils, in earthworms and the methyl-Hg concentration in earthworms are listed according to a) sampling sites and b) genera. The earthworm species are grouped, and divided by dashed lines into the three ecological categories: epigeic, endogeic and anecic earthworms.
2.5 Discussion

2.5.1 Mercury concentrations in forest soils

Former studies examining Hg concentrations in mushrooms and earthworms were mainly carried out in polluted soils. Total Hg concentrations in the top layer of our studied forest soils ranged between 0.07 and 0.55 mg Hg kg\(^{-1}\) dry soil (about 0.001 to 0.156 mg Hg g\(^{-1}\) organic matter), which were in accordance to observations of Byrne et al. (1976) reported from unpolluted sites (range between 0.08 and 0.33 mg Hg kg\(^{-1}\) dry soil) and were about 20 to 40 times lower than in studies from polluted soils (Bull et al. 1977; Zhang et al. 2009). Forest soils are assumed to contain higher Hg concentrations compared to grasslands due to dry deposition on forest canopy (usually 2 to 3 times higher) and subsequent throughfall and litterfall (St Louis et al. 2001). In addition, forest soils which are rich in organic matter show a high affinity to bind the divalent Hg (Hg\(^{2+}\)) to organic matter especially if organic matter contains thiol groups (Skyllberg and Drott 2010). As a consequence, Hg is accumulated in the topsoil (Meili 1991; Yin et al. 1996; Meili et al. 2003) representing the habitat for fungi and most earthworms. Tipping et al. (2010) reported a critical limit of 3.3 mg Hg kg\(^{-1}\) organic matter or 0.13 mg Hg kg\(^{-1}\) soil. Below these concentrations no harmful effects on soil organisms are expected. By applying this definition twenty forest soils in this study (59%) would exceed the critical limit of Hg. We also found a very weak but statistically significant correlation (\(r^2=0.16; p=0.009; n=34\)) between soil Hg and soil organic carbon (C\(_{org}\)), which is in accordance to Pant and Allen (2007) reporting a strong correlation (\(r^2=0.52\)) between Hg and C\(_{org}\) in a contaminated area in Tennessee (USA). Similarly, Obrist et al. (2009) found a strong correlation (\(r^2=0.83\)) between Hg and soil C in unpolluted mineral soils from Sierra Nevada.

2.5.2 Mercury and methyl-Hg concentrations in mushrooms

*Russula* was the most abundant genus found at 16 sites. *Russula* mushrooms belonged to 15 different species but only 6 species could be determined exactly (Table 2.2). *Russula nigricans* was the most collected species of all mushrooms (5 forest sites). All other species
were found at most in three forest sites thereby it renders more difficult to draw conclusions on the accumulation of Hg in the different mushrooms species from non-contaminated sites. Except for *R. nigricans* no correlation was found between soil properties and Hg contents in fruiting bodies. Here, mushrooms contained between 0.03 and 5.61 mg Hg kg\(^{-1}\) dw which was, in general, lower than the Hg concentrations in mushrooms found in other studies. Bargagli et al. (1984) sampled 195 species of higher fungi in a cinnabar mining area around *Siena* (Central Italy) and found near a smelting industry Hg concentrations in mushrooms between 1.6 and 112 mg Hg kg\(^{-1}\) dw and Hg concentrations in soils between 345 and 782 mg Hg kg\(^{-1}\) dry soil. Kalac et al. (1996) studied Hg in fruiting bodies in the vicinity of a Cu - Hg smelter in eastern Slovakia. Collected mushrooms close to the smelter representing the genera *Boletus*, *Lactarius*, *Lepiota* and *Russula* contained Hg concentrations ranging from 14.9 to 119 mg Hg kg\(^{-1}\) dw. Similar concentrations were found in the region of *Paris* (France) reported by (Michelot et al. 1998). They collected fruiting bodies of 92 mushroom species and measured Hg concentrations between 33.8 and 93.9 mg kg\(^{-1}\) dw. Alonso et al. (2000) collected mushrooms of six wild growing species in unpolluted (*Pinus pinaster* forest, nitrified pastures) and polluted sites (main roads) in the province of *Lugo* (Spain). They found Hg concentrations ranging from 0.6 to 8.6 mg Hg kg\(^{-1}\) dw with no clear influence of traffic on Hg tissue concentrations.

Accumulation of Hg in mushrooms was species-specific. In particular, Hg concentrations in fruiting bodies are influenced by the ecological groups of mushrooms. Saprophytic species decomposing litter tended to accumulate more Hg and also more methyl-Hg than wood decomposing and mycorrhizal mushrooms ($BCF_{Hg}$: for litter decomposers 22.6; for wood decomposers 1.7; for mycorrhizal mushrooms 6.1), which is in accordance to others (Alonso et al. 2000; Melgar et al. 2009). The higher Hg contents in saprophytic species are assumed to be the consequence of their higher decomposing activity, e.g. catalase activity (Kojo and Lodenius 1989). On the contrary, the lower Hg concentration in fruiting bodies of wood decomposing mushrooms could be the result of the low level of Hg in the wood and the limited amount of substrate compared to saprophytic species colonizing soils (Melgar et al.
Bioaccumulation of Hg and methyl-Hg

2009). The BCFs for Hg ranged in the present study between 0.2 (R. purpurata) and 55.4 (R. cyanoxantha) with a mean value of about 7.6. These findings were in line with others, whereas also BCF_{Hg} greater than 200 were observed if the sites were polluted (Bargagli et al. 1984; Fischer et al. 1995; Falandysz et al. 2002; Falandysz et al. 2003).

The fraction of methyl-Hg to total Hg in mushrooms were low and ranged between 0.3 and 11.3% (mean: 4.5%) of total Hg concentrations, which is in accordance to Bargagli et al. (1984) studying similar species than ours. Similarly, Minagawa et al. (1980) found between 2.9 and 9.1% of the total Hg in the methylated form, although the Hg concentrations in fruiting bodies were six times higher than we found here. BCF_{methyl-Hg} ranged between 0.6 (C. cornucopioides) and 192.2 (M. procera). The BCF_{methyl-Hg} was up to 45 times higher as BCF_{Hg} (Tricholomopsis rutilans) and on average about 2.5 times higher. Fischer et al. (1995) observed that BCF_{methyl-Hg} was between 3 and 117 times higher than BCF_{Hg}. The higher BCF_{methyl-Hg} can either result from (1) a higher mobilization and uptake of methylated compared to inorganic Hg, (2) a more efficient accumulation in fruiting body, or (3) in possible biological methylation of inorganic Hg in the mushrooms (Vonk et al. 1973; Fischer et al. 1995).

2.5.3 Mercury and methyl-Hg concentrations in earthworms

There are only a limited number of studies investigating Hg and methyl-Hg in earthworms. Data are mainly obtained from polluted sites (Bull et al. 1977; Zhang et al. 2009) or laboratory experiments (Burton et al. 2006; Zagury et al. 2006; Hinton and Veiga 2009). Ernst et al. (2008) studied Hg concentrations in natural Swiss forest soils, however, they did not measure methyl-Hg. Here, we found a large variation of the Hg concentrations in earthworms at our different sampling sites. Except for L. rubellus no correlation was found between soil Hg and Hg in earthworms. Zagury et al. (2006) also did not observe a correlation between soil Hg and tissue Hg contents in earthworms (Eisenia andrei).

Hg and methyl-Hg concentrations in worms were highly species-specific. Ernst et al. (2008) also found that earthworms in forest soils showed a large variability in the Hg concentrations
among species. Epigeic (litter-inhabiting) earthworms showed about 3 times lower Hg and 6 times lower methyl-Hg contents as endogeic earthworms. The Hg concentrations of their habitats might partly explain the differences between the tissue Hg concentrations of the different ecophysiological groups. In fact, the Hg concentrations in litter are usually lower than the Hg concentrations in topsoils (Ernst et al. 2008). Therefore the habitat for epigeic earthworms contains less Hg than the habitat for endogeic earthworms (organic soil layer).

Due to the strong accumulation of Hg in topsoils, the Hg decrease with increasing soil depth whereby the habitat of anecic earthworms (deep burrows-inhibiting) contains less Hg. BCF$_{Hg}$ for epigeic earthworms were about two times lower than for anecic earthworms and about four times lower than for endogeic earthworms. The calculated BCFs for anecic earthworms were low because this organisms spends most of the time in deep burrows and only comes to surface for eating. The analysed soil samples, however, originating from topsoils contain higher Hg concentrations than soils from deeper horizons.

The percentages of methyl-Hg to total Hg were between 5.7 and 10.1%, which is in accordance to Bull et al. (1977) reporting values between 8 and 13% in the tissue of L. terrestris and Zhang et al. (2009) between 3 (Drawida sp.) and 12% (Allolobophora sp.). Our BCF$_{methyl-Hg}$ ranged between 15.1 (L. rubellus) and 191.5 (O. cyaneum). The BCF$_{methyl-Hg}$ were between 6 (A. longa) and 25 (A. caliginosa) times higher than the BCF$_{Hg}$. Zhang et al. (2009) also observed much higher BCF$_{methyl-Hg}$ than BCF$_{Hg}$ in all studied earthworm species (between 20 and 150 times higher). The lipophilic nature of methyl-Hg results in a much more efficient accumulation as inorganic Hg. Potential Hg biomethylation in the earthworm digestive tract (Hinton et al. 2002) might also be responsible for the enhanced BCF$_{methyl-Hg}$.
2.6 Conclusion

Mercury and methyl-Hg concentrations in mushrooms and earthworms from non-contaminated Swiss forest soils were low compared to literature. Both total and methyl-Hg concentrations in tissues were species-specific and dependent on the ecological groups of receptor. *Russula nigricans* and *Lumbricus rubellus* were the only species which showed a significant correlation between Hg concentrations in soils and Hg tissue concentrations. Mushrooms and earthworms contained similar Hg concentrations, whereas methyl-Hg concentrations in earthworms tended to be higher than in mushrooms. Earthworms take up either more methyl-Hg as mushrooms from soil, retain more methyl-Hg in the body, or are able to methylate inorganic Hg in their tissues or organs. The high methyl-Hg concentration in earthworms and their high $BCFs_{\text{methyl-Hg}}$ indicate that earthworms are a relevant source of methyl-Hg, which requires further research to estimate the ecological risk of secondary Hg poisoning in forest ecosystems.

2.7 Acknowledgements

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Chapter 3

Methylation of mercury in earthworms and the effect of mercury on the associated bacterial communities
Stephan Raphael Rieder¹,²,³,⁵, Ivano Brunner¹, Otto Daniel¹, Bian Liu³,⁴, Beat Frey¹,⁵

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Contribution of the authors:
¹Experimental design; ²performing experiments; ³data analyzing; ⁴technical support; ⁵paper writing

3.1 Abstract

Methylmercury compounds are very toxic for most organisms. Here, we investigated the potential of earthworms to methylate inorganic-Hg. We hypothesized that the anaerobic and nutrient-rich conditions in the digestive tracts of earthworm’s promote the methylation of Hg through the action of their gut bacteria. Earthworms were either grown in sterile soils treated with an inorganic (HgCl₂) or organic (CH₃HgCl) Hg source, or were left untreated. After 30 days of incubation, the total-Hg and methyl-Hg concentrations in the soils, earthworms, and their casts were analyzed. The impact of Hg on the bacterial community compositions in earthworms was also studied. Tissue concentrations of methyl-Hg in earthworms grown in soils treated with inorganic-Hg were about six times higher than in earthworms grown in soils without Hg. Concentrations of methyl-Hg in the soils and earthworm casts remained at significantly lower levels suggesting that Hg was mainly methylated in the earthworms. Bacterial communities in earthworms were mostly affected by methyl-Hg treatment. Terminal-restriction fragments (T-RFs) affiliated to Firmicutes were sensitive to inorganic and methyl-Hg, whereas T-RFs related to Betaproteobacteria were tolerant to the Hg treatments. Sulphate-reducing bacteria were detected in earthworms but not in soils.
3.2 Introduction

Mercury (Hg) is a naturally occurring metal, which is released in the environment by numerous natural and anthropogenic processes (Schroeder et al. 1998; Swain et al. 2007). Mercury is relatively stable in the atmosphere and can spread over the entire globe before returning to the earth’s surface. In soil, Hg is highly immobile and accumulates in the top layer, mainly by binding to organic matter especially to thiol groups (Skyllberg and Drott 2010). Tipping et al. (2010) reported a critical limit of 3.3 mg Hg kg\(^{-1}\) organic matter corresponding to 0.13 mg Hg kg\(^{-1}\) soil. At lower concentrations, it is assumed that there are no harmful effects on soils organisms. By applying this definition, 60% of 34 natural forest soils studied in Switzerland would exceed this critical limit (Rieder et al. 2011).

Methylmercury compounds (CH\(_3\)Hg-R; methyl-Hg) are the most toxic Hg compounds for humans (Clarkson and Magos 2006). Methylation of Hg occurs through biotic and abiotic processes, although biotic processes are most important (Shao et al. 2012). Sulphate-reducing bacteria (SRB) under anaerobic conditions seem to be of particular importance to methylate Hg (Barkay et al. 2005; Drott et al. 2007; Holloway et al. 2009). In all SRB, enzymes that catalyze the reduction of sulphite to sulphide were found. Sulphite reductases enzymes (EC 1.8.99.3) consist of at least two polypeptides, encoded by the dissimilatory sulphite reductase genes \(dsrA\) and \(dsrB\) (Klein et al. 2001). The presence of SRB in environmental samples is commonly analyzed by targeting the \(dsrAB\) genes (Dhillon et al. 2003; Wagner et al. 2005; Santillano et al. 2010).

The methylation and bioaccumulation of Hg have been well studied in aquatic ecosystems because consuming Hg-contaminated fish may lead to humans being poisoned. In contrast, studies of Hg, and in particular of methyl-Hg, in terrestrial ecosystems are rare. Over 90% of the invertebrate biomass in soils may consists of earthworms (Ireland 1983). Earthworms play an important role in many soil-forming processes (Brown 1995). They also serve as a substantial food source for several higher organisms, such as birds and moles. Earthworms in forest soils are known to accumulate Hg and methyl-Hg (Ernst et al. 2008; Rieder et al. 2011). Bioaccumulation factors (BAF) of Hg in earthworms were between 1 and 15 whereas
BAF for methyl-Hg ranged from 15 to 191 (Rieder et al. 2011). The lipophilic property of methyl-Hg results in more efficient bioaccumulation than inorganic-Hg, which may explain why there are considerably higher BAF for methyl-Hg than for inorganic-Hg. Another possibility for the high BAF in earthworms is, that inorganic-Hg is methylated in earthworms, e.g. due to the activity of the microbiota in their digestive tracts. In particular, unique conditions prevail in the earthworm gut, which is anaerobic, with large amounts of easily available carbon, and these may favour the anaerobic growth of microorganisms (Horn et al. 2003; Drake et al. 2007).

In this study we tested this possibility and hypothesized that the conditions in earthworms’ digestive tracts favour the methylation of Hg by their gut-inhabiting bacteria. Earthworms \textit{(Lumbricus terrestris L.)} were either grown in sterile soils treated with mercury(II)chloride (HgCl$_2$), with methylmercurychloride (CH$_3$HgCl) or in soils without Hg treatment. The total-Hg (inorganic + organic Hg compounds) and methyl-Hg concentrations in soils and earthworms were analyzed after 30 days. The impact of Hg on the total bacterial community structures and compositions in earthworms were studied by molecular analyses. Because biotic Hg methylation is generally attributed to SRB, we determined the genetic potential for sulphate reduction by analysing the presence of \textit{dsrA} genes in the bacterial communities in soils and earthworms. To the best of our knowledge, ours is the first study to investigate the ability of earthworms to methylate inorganic-Hg under natural conditions (soils).

\subsection*{3.3 Material and Methods}

\subsubsection*{3.3.1 Ethic statement}

The research institute WSL has a general permit to use the area of their surrounding for scientific purposes. No endangered or protected species were involved in the experiment.
3.3.2 Experimental design

Laboratory experiments with *Lumbricus terrestris* L. and soils treated with inorganic-Hg, with methyl-Hg or without any Hg compounds were conducted (Figure 3.1). The experiments were performed in 3.3 L high density polyethylene boxes filled with 2 kg dw sterile soil. In the main experiment (Figure 3.1A) three approaches were used: a) soils without earthworms to examine the abiotic methylation and demethylation of Hg-species in the soil, b) soils without earthworms, but with a suspension obtained from rinsing the earthworms body surface to examine a possible Hg-methylation in the soils driven by microorganisms carried in by earthworms and c) soils with earthworms to examine the methylation of Hg in the earthworms. Sterile soil was necessary to test the ability of the microbes in the earthworm gut to methylate Hg. Otherwise, methyl-Hg in soils produced by microbes would have been taken up directly by earthworms without forming it in the gut of earthworms. Sterile soil is expected to have an impact on the "mutualistic digestive system", however, preliminary experiments showed that growth and mortality of earthworms grown in sterile soil were not affected (data not shown).

In a separate experiment, casts (excreted feces) were used as controls to study a possible methylation of Hg by organisms introduced into the soil by earthworms (Figure 3.1B). We analyzed earthworm cast also, because it offer very different environmental conditions for microbial growth than the surrounding soil (Kizilkaya 2004).

The experimental soil was collected in a forest close to the Swiss Federal Research Institute WSL, Birmensdorf. Soil properties (pH, C/N, clay-silt-sand content) were determined according to FAL (1997) before the soils were treated. The soil was dried at 105°C for two days, sieved (4 mm), homogenized and autoclaved three times. Soil aliquots were incubated for three days on petri dishes containing MMN-Agar media to test the sterility of the soils. After autoclaving, soil aliquots were pooled to three samples for analyses the initial concentrations of Hg and methyl-Hg in the soil. Table 3.1 summarizes the main physico-chemical properties of the soil.
Table 3.1: Soil properties and Hg and methyl-Hg concentrations (mean ± SD) in the soil used in the laboratory experiments.

<table>
<thead>
<tr>
<th>Soil type</th>
<th>pH</th>
<th>C/N</th>
<th>Clay [%]</th>
<th>Silt [%]</th>
<th>Sand [%]</th>
<th>Hg tot [mg kg(^{-1})]</th>
<th>Methyl-Hg [µg kg(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cambisol</td>
<td>7.0</td>
<td>10.8</td>
<td>25</td>
<td>39</td>
<td>36</td>
<td>0.09 ± 0.00</td>
<td>0.32 ± 0.16</td>
</tr>
</tbody>
</table>

One part of the sterile soil was treated with HgCl\(_2\) [Merck (nr. 4419.0050), USA; 30 mmol resp. 6 mg Hg kg\(^{-1}\) soil; named soil+inorganic-Hg] , a second with CH\(_3\)HgCl [Sigma-Aldrich (nr. 33368), USA; 3 mmol resp. 0.75 mg methyl-Hg kg\(^{-1}\) soil; named soil+methyl-Hg] and a third was left untreated (named soil-Hg) (Figure 3.1A, i). The water content of each soil was adjusted to 30% by adding distilled Milli-Q water. During the experiments the water content was determined gravimetrically at each second day and the water loss was compensated by adding sterilized Milli-Q water.

*L. terrestris* was chosen as the model organism as it is very abundant in soils of deciduous forests in Switzerland (Ernst et al. 2008) and the fraction of methyl-Hg compared to the total-Hg in its tissue is high (Rieder et al. 2011). Juvenile earthworms were obtained from a commercial source (National Bait Inc., Canada). Before incubation of the earthworms in sterile soils, the earthworms were grown in the untreated collected forest soil (not sterilized, not Hg treated) for six weeks for adapting the earthworms and their gut-inhabiting bacteria to the new condition. Thereafter, the earthworms were kept in boxes with several layers of moist filter papers without feeding for 6 days at 15°C to let them empty their guts. To clean the body surface of the earthworms and to obtain a suspension of mucus, soil and microorganisms adhering to the earthworm’s body surface, each individual was rinsed in a 0.8% NaCl solution. Thereafter, six earthworms were taken and pooled to three samples for analysing the initial total and methyl-Hg concentrations. The earthworms contained about 0.1 mg Hg kg\(^{-1}\) dw and 5.1 µg methyl-Hg kg\(^{-1}\) dw (4.9% methyl-Hg of total-Hg) at the beginning of the experiments.

In the experiments with earthworms, six individuals were put into each box. Equal experiments were conducted without earthworms and with a suspension obtained from
earthworm surfaces (Figure 3.1A, ii). Freeze-dried lettuce was used as a food source, and 0.5 g was added to each box in all experiments (also in experiments without earthworms). The boxes were incubated at 15°C in the dark for 30 days. We assumed that the soil treatment should not harm the earthworms according to Ernst et al. (2007) and Lock and Janssen (2001). All experiments were performed in four replicates. The soils were sampled at the beginning and at the end of the experiments. The soil in each box was totally mixed before and after incubation and at each time point several aliquots were taken and pooled together. At the end of experiments the earthworms were starved for six days on several layers of moist filter paper until their gut contents had emptied (Figure 3.1A, iii). The six earthworms per each box were pooled (resulting in n=4 per treatment), placed in liquid nitrogen and stored at -20°C until further treatment. Before chemical and microbial analyses, the frozen earthworms were lyophilized and milled.

To study and collect casts, ten earthworms were incubated as described above in soil treated with inorganic-Hg for one week (Figure 3.1B, i). Thereafter, the earthworms were placed into new boxes containing soil treated with inorganic-Hg (Figure 3.1B, ii) and after three days, they were removed (Figure 3.1B, iii). The earthworm-free boxes were kept in the dark with constant water content for 28 days. To observe a potential ageing effect, subsamples of earthworm casts were sampled (about 500 mg) at the day of removing earthworms (cast was excreted between 0 and 3 days ago), after 2, 7, 15 and 28 days (Figure 3.1B, iv). The collected samples were stored at -20°C. The samples were lyophilized, milled and stored in the dark until analysis. The cast experiment was investigated in four replicates.
Figure 3.1: Experimental design. Two experimental assays (A and B) were performed: In the main experiment (A) sterile soil was either treated with inorganic Hg (+inorganic-Hg), methyl-Hg (+methyl-Hg) or without Hg (-Hg) (i). The soils were incubated abiotically, with earthworms or with a earthworm rinsing suspension for 30 days in the dark at 15°C (ii). At the end of incubation, the earthworms were removed from the soil and the Hg and methyl-Hg concentrations in the soil and earthworms were determined (iii). In a separate experiment (B), casts (excreted feces) were used as controls to study a possible methylation of Hg by organisms introduced into the soil by earthworms. Earthworms were incubated for one week in soils treated with inorganic-Hg (i) before they were placed into new boxes containing soils treated with Hg (ii). After three days, the earthworms were removed (iii). Immediately after removing the earthworms a cast sampling period has been started for 28 days (iv).

3.3.3 Mercury analyses

The total-Hg (inorganic + organic Hg compounds) and methyl-Hg concentrations were measured in all samples. The total-Hg concentrations in the samples were determined by using an atomic absorption spectrophotometer according to the manufacturer’s instructions...
(Advanced Hg Analyser; AMA 254, Altec s.r.l., CZ). The methyl-Hg concentrations were determined by Gas Chromatography-Atomic Fluorescence Spectroscopy (GC-AFS) according to Liu et al. (2012). The accuracy of the total-Hg and methyl-Hg analyses was checked against certified reference materials (TORT-2 for biotic and ERM CC580 for soil samples) and its recovery ranged between 95 and 106% for Hg and between 83 and 112% for methyl-Hg.

### 3.3.4 DNA extraction and PCR of 16S rRNA and dsrA genes

Genomic DNA in the earthworms and soils was extracted using a modified bead-beating method described in Frey et al. (2008). Approximately 500 mg of earthworm tissue or soil sample were processed with a BioFastPrep system (ThermoSavant). The extracted DNA was quantified with Pico Green (Invitrogen, Carlsbad, CA, USA) and stored at -20°C. DNA aliquots extracted from earthworm and soil samples (5ng µl⁻¹) were pretreated with BSA at 95°C for 4 min to remove PCR inhibitors. Bacteria in earthworms were amplified by a polymerase chain reaction (PCR) targeting the 16S rRNA genes similarly to that described in (Frey et al. 2006). Twenty ng of pretreated DNA was added to 20 µl PCR reaction mix containing 1x PCR buffer, 0.5 mM MgCl₂, 400 µM dNTP, 0.6 mg ml⁻¹ BSA and 0.05U µl⁻¹ Hot star Taq polymerase (Qiagen), 0.2 µM of the forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and of the reverse primer 1378R (5'-CGGTGTGTACAAGGCCCAGGGAACG-3') were prepared. For T-RFLP analyses the forward primers were fluorescently (FAM) labelled. The PCR reactions were conducted in a Veriti Thermal Cycler (Applied Biosystems, Foster City, USA) and were started by an initial denaturizing step for 15 min at 95°C, followed by 35 cycles of the following steps: 95°C for 45s, 48°C for 45s and 72°C for 2 min. The reaction was finished by an extension step for 5 min at 72°C. SRB were analyzed in soil and earthworm samples using for DNA amplification primers targeting the dsrA subunit (Dhillon et al. 2003) followed by agarose gel electrophoresis similar as described by Yu et al. (2010). Two separate PCR were conducted using two different reverse primers. We used the forward primer DSR1F (5'-ACS CAC TGG
AAG CAC G-3’) and the reverse primers DSR4R (5’-GTG TAG CAG TTA CCG CA-3’) or DSR1334R (5’-TYT TCC ATC CAC CAR TCC-3’) described by Santillano et al. (2010). The PCR reagents were similar to those used previously for the 16S rRNA genes. After an initial step at 94°C for 15 min, 42 cycles of the following steps were performed: 94°C for 45s, 55°C for 1 min and 72°C for 2 min. The reaction was finished at 72°C for 5min and the PCR products were verified by agarose electrophoresis and subsequently analysed under UV illumination.

3.3.5 T-RFLP profiling of total bacterial community in earthworms

16S rRNA amplicons were digested with 0.2U MspI restriction enzymes according to the manufacturer’s recommendations (Thermo Fischer Scientific, Waltham, USA). The digested DNA was purified with the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Billerica, USA) according to the manufacturer’s instructions. For capillary electrophoresis, 1 µl of the purified digestion product was mixed with 12.9 µl HiDi formamide (Applied Biosystems) and 0.1 µl of ROX500 DNA fragment length standard (Applied Biosystems) before heating at 95°C for 2 min. T-RFLP profiles were performed using the ABI Genetic Analyzer 310 (Applied Biosystems) and then analyzed using the software GeneScan V.3.1. and Genotyper V.2.5. (Applied Biosystems) according to Frey et al. (2006). The size and relative abundance were defined for peaks between 50 and 500 bps by applying a threshold value of 100 fluorescence units. The data for the T-RFLP analyses were standardized by calculating the relative abundance of each T-RF as described by Zumsteg et al. (2012).

3.3.6 Cloning and sequencing of 16S rRNA and dsrA genes

One clone library was generated from the 16S rRNA genes collected from earthworms to study the composition of the total bacterial communities, and one clone library was performed with dsrA genes from earthworms. For the clone libraries, the DNA extracted from earthworms from all Hg treatments (soil-Hg, soil+inorganic-Hg, soil+methylHg) was pooled for the PCR of 16S rRNA and dsrA genes separately, similar as described before except for
Methylation of Hg in earthworm using unlabelled primers. The PCR products were purified with Montage PCR Centrifugal Filter Devices (Millipore) and quantified using UV-VIS spectroscopy (NanoDrop Spectrophotometer ND-1000, Wilmington, USA).

The amplified and purified 16S rRNA and dsrA genes were cloned into pGEM-T Easy Vectors according to the manufacturer’s instructions (Promega, Wisconsin, USA). We selected 400 colonies (384 for 16S rRNA genes and 16 for dsrA genes) and conducted PCR using vector-specific primers (M13F and M13R) as described by Frey et al. (2011).

T-RFLP analysis of the colony PCRs was conducted as described above. The T-RFLP profiles of the clones were compared with the profiles of the environmental samples (earthworms). By overlapping the whole community profile with the clone profile, the clone could be assigned to a precise fragment size category. Clones yielding the selected fragments of interest (dominant T-RFs or T-RFs which were affected by the three soil Hg treatments) were sequenced. In total, 136 clones (120 clones for 16S rRNA and 16 clones for dsrA) were sequenced using the 3730XL DNA sequencer (Applied Biosystems) and using the forward primer 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and the reverse primer 907R (5’-CTACGGCTACCTGTAGTACGA-3’) for 16S rRNA genes, or the primer SP6 (5’-ATTAGGGTACACTATAGTTACGA-3’) and T7 (5’-TAATACGACTCAGTACTAGAGG-3’) for dsrA genes.

The sequences were then checked and manually edited in the software BioEdit (V.7.1.3.0 by Tom Hall), chimera checked (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) and a BLAST search was conducted in the NCBI database (http://www.ncbi.nlm.nih.gov) and the ribosomal database project (rdp.cme.msu.edu). The 16S rRNA gene sequences were deposited in Genbank under accession numbers between JX183735 and JX183794, the dsrA gene sequences under the accession numbers between JX461240 and JX461242.

Sequences were aligned with the ClustalW sequence alignment in BioEdit. Phylogenetic trees were calculated using Bayesian inference with the program MrBayes (ver. 3.2) (Huelsenbeck and Ronquist 2001) and the LG+I+G model. A Markov chain Monte Carlo simulation was run for 2,000,000 generations. Trees were visualized using the software FigTree (ver. 1.3.1).
3.3.7 Statistical analyses

The statistical analyses of total-Hg and methyl-Hg concentrations in the samples were performed with the program STATISTICA (StatSoft, Tulsa, USA). Calculations for statistic significance (p values) of total and methyl-Hg concentrations in soils and earthworms from the different experimental approaches and in the cast experiment were performed using the Kruskal-Wallis test (p≤0.05). The effects of the three soil Hg treatments on the abundance of particular T-RFs were tested by ANOVA and Post-hoc Tukey-HSD test using STATISTICA (StatSoft). A Bray Curtis similarity matrix (Bray and Curtis 1957) from square-root-transformed T-RFLP data was calculated and Principal Coordinate Analysis (PCoA) was performed to estimate the Hg treatments (soil-Hg, soil+inorganic-Hg, soil+methyl-Hg) on the bacterial community structure using the software Primer 6 v.6.1.13 and Permanova v.1.0.3 (Primer-E, UK). The significance of the effects of Hg-treatments on the community structures (T-RFLP profiles) was tested by performing Permutational MANOVA analyses.

3.4 Results

3.4.1 Hg and methyl-Hg contents

After the incubation, the concentrations of total-Hg in soils were not significantly different (p<0.05) from the initial concentrations in all experiments. Before incubation, the methyl-Hg concentration in soils treated with methyl-Hg was similar as the total Hg concentration (data not shown). The concentrations of total-Hg and methyl-Hg in soils at the end of the experiments with and without an earthworm rinsing suspension (abiotic experiments) were similar. However, the concentrations of methyl-Hg in soils treated with methyl-Hg decreased by about 60% during the experiments (data not shown).

The concentrations of total-Hg and methyl-Hg in the earthworms increased in all experiments (Table 3.2). The initial concentrations of methyl-Hg in earthworms was about 5 µg methyl-Hg kg⁻¹ soil dw. At the end of the experiments, the concentrations of methyl-Hg in earthworms were significantly higher in experiments with inorganic-Hg (75 µg methyl-Hg kg⁻¹ soil dw) and
with methyl-Hg (16 613 µg methyl-Hg kg⁻¹ soil dw) than without Hg (11 µg methyl-Hg kg⁻¹ soil dw) (p=0.035 and 0.001 respectively; Table 3.2). The concentrations of total-Hg in the casts were about 25% lower than in the surrounding soils, whereas the concentrations of methyl-Hg in the casts were similar to the concentrations in the soils but did not change significantly (p<0.05) over time (Table 3.2).
Table 3.2: Total-Hg and methyl-Hg concentrations (mean ± SD; n=4) in soils, earthworms and in casts.

<table>
<thead>
<tr>
<th>Soil*</th>
<th>Treatment</th>
<th>Hg tot [µg kg(^{-1})]</th>
<th>Methyl-Hg [µg kg(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–Hg</td>
<td>101 ± 5.0</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>–Hg</td>
<td>92 ± 0.2</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>+inorganic-Hg</td>
<td>6 145 ± 547</td>
<td>0.81 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>+inorganic-Hg</td>
<td>5 640 ± 401</td>
<td>1.26 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>+inorganic-Hg</td>
<td>6 035 ± 523</td>
<td>1.00 ± 0.55**</td>
</tr>
<tr>
<td></td>
<td>+methyl-Hg</td>
<td>700 ± 18</td>
<td>250 ± 37</td>
</tr>
<tr>
<td></td>
<td>+methyl-Hg</td>
<td>594 ± 52</td>
<td>301 ± 267</td>
</tr>
<tr>
<td></td>
<td>+methyl-Hg</td>
<td>682 ± 4</td>
<td>288 ± 164</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Earthworms*</th>
<th>Treatment</th>
<th>Hg tot [µg kg(^{-1})]</th>
<th>Methyl-Hg [µg kg(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>–Hg</td>
<td></td>
<td>218 ± 57</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>+inorganic-Hg</td>
<td></td>
<td>24 271 ± 5 531</td>
<td>75 ± 35</td>
</tr>
<tr>
<td>+methyl-Hg</td>
<td></td>
<td>15 582 ± 3 085</td>
<td>16 613 ± 7 770</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Casts***</th>
<th>Age [d]</th>
<th>Hg tot [µg kg(^{-1})]</th>
<th>Methyl-Hg [µg kg(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td></td>
<td>5 248 ± 864</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>2-5</td>
<td></td>
<td>4 563 ± 615</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>7-10</td>
<td></td>
<td>4 630 ± 622</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>15-18</td>
<td></td>
<td>4 165 ± 821</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>28-31</td>
<td></td>
<td>4 619 ± 149</td>
<td>0.9 ± 0.4</td>
</tr>
</tbody>
</table>

* Sampled after 30 days of incubation
** n=3
*** Soil treated with inorganic-Hg

3.4.2 Bacterial community profiling

The bacterial community structures in the earthworms were strongly influenced by the Hg treatments (Table 3.3; Figure 3.2). Bacterial T-RFLP profiles in earthworms grown in soils not treated with Hg are marginally significantly different from those in earthworms grown in soils treated with inorganic Hg (p=0.06; Permutational MANOVA) and were significantly different to those in earthworms from soil treated with methyl-Hg (p=0.03; Permutational MANOVA).
The T-RFLP profiles between the inorganic and methyl-Hg treatments were different but not on a significant level (p=0.12). The numbers of T-RFs in earthworms grown in soil treated with methyl-Hg were lower (26 ± 5) as in experiments with Hg (28 ± 5) and without Hg (41 ± 6; data not shown). Eight T-RFs (11%) in earthworms were significantly decreased in experiments with inorganic-Hg compared to experiments without Hg (Table 3.3).

Table 3.3: Numbers and percentages of total numbers of T-RFs (in parentheses) in earthworms after 30 days which were significantly different (decreasing or increasing) between the soil treatments (-Hg, +inorganic-Hg; +methyl-Hg). Total number of T-RFs in earthworms: n=71.

<table>
<thead>
<tr>
<th></th>
<th>Decreased</th>
<th>Increased</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Hg versus +inorganic-Hg</td>
<td>8 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>-Hg versus +methyl-Hg</td>
<td>11 (16)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>+inorganic-Hg versus +methyl-Hg</td>
<td>0 (0)</td>
<td>2 (3)</td>
</tr>
</tbody>
</table>

In methyl-Hg treated soils, eleven T-RFs (16%) in earthworms were significantly less and two T-RFs (3%) significantly more abundant compared to experiments without Hg (Table 3.3). Overall, the bacterial T-RFLP profiles in earthworms grown in soils without Hg were clearly different from those in earthworms grown in soils with methyl-Hg, according to PCoA analyses (Figure 3.2).
Figure 3.2: PCoA of bacterial T-RFLP profiles in earthworms. The influence of the Hg treatment (symbols) was estimated with Permutational MANOVA.

3.4.3 Bacterial community composition

The T-RF profiles of the earthworm samples were compared to the T-RF profiles of the clones. At least five clones yielding the selected fragments of interest (dominant T-RFs or T-RFs which were affected by the three soil Hg treatments; Figure 3.3) were sequenced. Sequence analysis confirmed that cloned sequences were similar and always matched the same closest relatives. The cloned sequences were related to the phyla Firmicutes, Actinobacteria and Proteobacteria (Figure 3.4). Eight T-RFs (119bp, 145bp, 151bp, 153bp, 166bp, 291bp, 298bp and 319bp) belong to the phylum Firmicutes. The cloned sequence of T-RF 151bp (clone SEst1) was most similar (99%) to a Brevibacillus sp. strain KZ17 (FJ481959) isolated from wasps. In earthworms, the abundance of this T-RF (151bp) tended to decrease in experiments with inorganic-Hg and methyl-Hg (Figure 3.3). Cloned sequences of the T-RFs 153bp (clone SEs2) and 166bp (clone SEs3) were closely related to each other, and were most similar (97%) to a Bacillus sp. strain KZ_AalM_Mm2 (GU726177) isolated from mosquitoes. The abundance of both T-RFs in earthworm samples was significantly lower in experiments with inorganic-Hg and methyl-Hg than in those without Hg. Overall, T-RFs affiliated to the phylum Firmicutes seem to be sensitive to inorganic-Hg and methyl-Hg.
Cloned sequences of three T-RFs (164bp, 278bp and 279bp) are phylogenetically affiliated to *Actinobacteria* (Figure 3.4). Cloned sequences of T-RF 278bp (clone SEs4) were most similar (98%) to a *Microbacterium sp.* strain YT0620 (AB376082). T-RF 278bp was significantly more abundant in earthworms grown in soils without Hg than in soils treated with inorganic-Hg or methyl-Hg.

We found four T-RFs belonging to *Alphaproteobacteria* (396bp), *Betaproteobacteria* (450bp, 482bp) or *Gammaproteobacteria* (87bp) that were more abundant in earthworms from soils treated with inorganic-Hg or methyl-Hg than in soils without Hg. The T-RF of 450bp was of special interest because it was clearly more abundant in earthworms grown in soils treated with methyl-Hg, where it was the most dominant T-RF, than in soils without Hg or with inorganic-Hg. Cloned sequences of T-RF 450bp (clone SEst3) were most similar (99%-100%) to an environmental clone (AJ543436) affiliated to *Acidovorax sp.* and to a clone LT 1 (FJ214171) related to *Verminephrobacter sp.* which were both obtained from earthworms.
We also tested the DNA extracts obtained from earthworm and also from soils to detect the presence of SRB with specific PCRs targeting the *dsrA* genes. In all earthworm but not in soil samples (-Hg, +inorganic-Hg, +methyl-Hg) weak PCR products appeared (data not shown). Cloned sequences were most similar to the *dsrA* subunit of *Desulfovibrio vulgaris* strain RCH1 (similarity between 97 and 99%; CP002297).
Figure 3.4: Phylogram with the phylogenetic relationships of 16S rRNA gene sequences. The phylogenetic tree shows the bacterial strains and environmental clones most closely to the T-RF in Figure 3.3. Whenever possible, closest strains were used for the calculation of the tree but when no strain was available (e.g. many species of Mollicutes could not be isolated so far) the closest clone was used. The tree was calculated by Baysian inference using sequences of 898bp lengths and shows the affiliation between the clones and closest related sequences of NCBI. The clones of our study are bold marked. Only bootstrap values above 0.9 are given. The scale bar represents 0.1 (10%) of sequence divergence.
3.5 Discussion

This study demonstrates that earthworms provide suitable conditions for the methylation of inorganic-Hg. In the biotic experiments with earthworms, the concentrations of methyl-Hg in soils are expected to decrease with time due to the strong uptake and retention of methyl-Hg in earthworms, and due to the decrease of methyl-Hg concentrations in soils measured in experiments without earthworms. Therefore, in these experiments, the concentrations of methyl-Hg in the soils should decrease (initial soil concentration minus loss due to accumulation by earthworms and evasion/demethylation of methyl-Hg) during incubation. This was, however, not observed here. Instead, the concentrations of methyl-Hg in soils were slightly higher (1.2 µg methyl-Hg kg\(^{-1}\) soil dw) at the end of the experiments. Hinton et al. (2002) showed that earthworms are potentially able to methylate Hg. They dissolved Hg\(^0\) in tannic acid, mixed it with silica sand and paper substrate, and let earthworms (Eisenia foetida) grow in it. They analyzed the methyl-Hg concentrations and found a ratio of methyl-Hg to total-Hg up to 160 times higher in earthworm tissues than in the acid and the substrate.

In our study, we have chosen, in contrast, a natural habitat (soil) for the earthworms and Hg\(^{2+}\), the most dominant Hg species in natural soils (Morel et al. 1998). We were able to show, that inorganic-Hg is methylated in the earthworm itself, and not by the bacteria introduced into the soils by the earthworms. This finding is based on the following evidence. Firstly, the earthworms contained about six times higher concentrations of methyl-Hg if they grew in soils treated with inorganic-Hg than in soils without Hg. Secondly, the concentrations of methyl-Hg in earthworm casts were similar to the concentrations in the soils and did not change over time. Thirdly, the earthworm rinsing solution did not enhance the methyl-Hg concentrations in the inorganic-Hg treated soils and thus, microorganisms introduced into the soil (cast/rinsing solution) did not methylate Hg in the soils. Uncertainties of the measurements of methyl-Hg occur, since it renders difficult to separate chromatographically methyl-Hg from inorganic-Hg in samples with very high Hg concentrations. Another uncertainty is that methyl-Hg can be formed artificially during the analytical procedure as pointed out in (Nevado et al. 2008). Despite these uncertainties we were able to demonstrate...
Methylation of Hg in earthworm

evidence that inorganic-Hg is methylated in earthworms itself, and not by the bacteria introduced into the soils by earthworms.

Mercury methylation has been observed in terrestrial invertebrates. Limper et al. (2008) tested the ability of termites (Mastotermes darwinesis) to methylate inorganic-Hg. They found an in vivo methylation of Hg in termites and identified SRB (Desulfovibrio intestinalis) in their intestinal tract as important players in the formation of methyl-Hg. In our study, SRB (D. vulgaris) were also detected in earthworms but not in soils. Therefore, we cannot exclude that SRB may have played a role in the methylation of the Hg in our study. Desulfovibrio vulgaris has also recently been shown to facilitate the methylation of inorganic-Hg in freshwater sediments (Shao et al. 2012).

The occurrence of methylation in earthworms has potentially implications for the Hg cycle in soil ecosystems as it enriches the methyl-Hg pool in soils, and consequently also in the food web. In addition, biomethylation is not only restricted to anaerobic conditions in soils as usually reported (Drott et al. 2007; Holloway et al. 2009), but also to well-drained soils.

The impact of inorganic-Hg in soils on soil bacterial community structures has already been reported (Rasmussen et al. 2001; Holtze et al. 2003; Ranjard et al. 2006; Philippot et al. 2008; Tipping et al. 2010; Vishnivetskaya et al. 2011; Mosher et al. 2012). It is also well known, that Hg pollution changes the gut bacterial community in soil invertebrate (Lapanje et al. 2007; Lapanje et al. 2008; Lapanje et al. 2010). However, as far as we know, the effects of methyl-Hg on the bacterial community structures in soils and earthworms have never been studied. Here, we showed that the bacterial community structures in the earthworms were clearly affected by Hg. Several T-RFs (87bp, 278bp, 279bp, 319bp, 450bp and 482 bp) in the earthworms were affected by Hg and these cloned sequences had closest relatives to sequences retrieved from intestine tracts of earthworms (Schramm et al. 2003; Singleton et al. 2003; Knapp et al. 2009; Nechitaylo et al. 2009; Wüst et al. 2011). T-RF 319bp (clone Et1) was slightly increased with inorganic and methyl-Hg and cloned sequences were phylogenetically affiliated to Mollicutes obtained from earthworms. Mollicutes within the Firmicutes contain no cell walls and contain a reduced genome size as a consequence of a
reductive or degenerative evolution process (Pollack et al. 1997; Razin 2007; Sirand-Pugnet et al. 2007). They obtain nutrients from their host cells (earthworm) parasitically. Therefore, the higher tolerance of *Mollicutes* to Hg could be due to the lower number of genes and enzymes affected by Hg, and/or their supply with nutrients from host cells. When the earthworms were grown in soils with methyl-Hg, T-RF 450bp became dominant (increase from about 7% to 27% of the total abundance). The cloned sequence of T-RF 450bp (clone SEst3) was closely related to sequences affiliated to *Acidovorax* sp. (AJ543436) and *Verminephrobacter* sp. (FJ214171) within the *Betaproteobacteria* retrieved from earthworms. *Verminephrobacter* are Gram-negative bacteria symbiotically colonizing the nephridia of the earthworms *Eisenia foetida*. Interestingly, they have been described as obligate aerobic organisms occurring under low oxygen conditions (Pinel et al. 2008). In contrast, *Acidovorax* sp. are able to grow outside of earthworms (Willems et al. 1990; Schulze et al. 1999; Pinel et al. 2008). Nephridia are the paired excretion organs of invertebrates and are comparable to the kidneys of vertebrates. It was suggested a long time ago that nephridia are symbiotically colonized by bacteria (Pandazis 1931; Schramm et al. 2003), but the functions of the associated bacteria are not well studied. These symbionts promote the degradation of proteins (Pandazis 1931). *Verminephrobacter* sp. may also play a role in the detoxification of inorganic-Hg or methyl-Hg in our earthworms. A wide range of bacteria belonging to the phyla of *Firmicutes*, *Actinobacteria*, and *Proteobacteria* are known to be Hg resistant (Ni Chadhain et al. 2006; Rasmussen et al. 2008). Bacterial resistance to Hg is mainly associated with the presence of mercury resistance (*mer*) operons (Robinson et al. 1984). The mercuric reductase enzyme (*merA*) catalyzes the conversion of Hg(II) to the volatile Hg(0). The *merB* enzyme degrades organic Hg compounds to the less toxic form Hg(II) (Barkay et al. 2003). However, *mer* genes are predominant in aerobic environments and were rarely found in obligate anaerobes (Barkay et al. 2010) assuming that this mechanism is hardly important for this study. Other unknown mechanisms for Hg(II) reduction are suggested under anaerobic conditions (Peretyazhko et al. 2006; Wiatrowski et al. 2006).
Another mechanism for microbial Hg resistance is based on the methylation of Hg(II) (Oregaard et al. 2007). Methyl-Hg-chloride may diffuse through the cell membrane, and as a result the concentrations of Hg in the organisms probably decrease. However, the mechanisms which could be important in this study are not known and need to be further investigated.

In our study, the concentrations of methyl-Hg in soils at the start and after incubation did not vary significantly (p<0.05) in experiments with or without inorganic-Hg. Interestingly, in all soils treated with methyl-Hg, the concentrations of methyl-Hg decreased (initial: 0.75 mg methyl-Hg kg\(^{-1}\) soil dw and end: almost 0.3 mg methyl-Hg soil dw) by about 60% during the experiments (data not shown). Bacteria are able to demethylate Hg-species (Spangler et al. 1973; Walsh et al. 1988; Barkay et al. 2003). Abiotic decomposition of methyl-Hg due to photodegradation has also been reported in surface water (Sellers et al. 1996). In the abiotic experiments with sterile soil treated with methyl-Hg, the concentrations of methyl-Hg decreased by about 60%, even though the boxes were stored in the dark so that a photodegradation of methyl-Hg in soils could be excluded. Likewise, the concentrations of total-Hg in these soils did not decrease, which indicates that methyl-Hg in the soil was degraded by other factors than photodegradation.

**3.6 Conclusion**

We have been able to show that the gut of earthworms provides suitable conditions for the methylation of inorganic-Hg. Control experiments (abiotic; with earthworms rinsing suspension; cast) strongly supported our finding that inorganic-Hg is methylated in earthworms itself, and not by the bacteria introduced into the soils by earthworms. SRB may play a role in the methylation of inorganic-Hg in our earthworms because they were found only in the earthworms and not in the soils. The transformation of inorganic-Hg to the much more toxic form of methyl-Hg may not only occur under anaerobic soil conditions, but also in the anaerobic guts of earthworms inhabiting aerobic soil environments. The occurrence of
biomethylation in earthworms may have implications for the Hg cycle in soil ecosystems. This process may enrich the methyl-Hg pool in soils and, consequently, also may enrich methyl-Hg in the food web.

3.7 Acknowledgement

We thank Beat Stierli, Anita Zumsteg, Stefan Schmutz, Anja Gall and Jan Wiederhold for laboratory assistance. We are also grateful to the linguistic lecturer Silvia Dingwall for improving our text.
Chapter 4

Response of soil bacterial communities to mercury chloride application to various forest soils
Beat Frey and Stephan Raphael Rieder

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Preface: This study was conducted by Beat Frey and Stefan Rieder. The soil was sampled and spiked with HgCl₂ by Beat Frey. Stefan Rieder conducted microbial analyses (PCR, T-RFLP, sequencing), and analyzed the data together with Beat Frey. Both authors were involved in the writing process equally.

4.1 Abstract

This study evaluates the response of the bacterial communities to different mercury (Hg) amendments in temperate forest soils. Seven soils were spiked with increasing amounts of Hg [(0, 0.032, 0.32, 3.2 and 32 µg Hg(II) g⁻¹ dry soil)]. After 30 days, we examined the bioavailable Hg using bacterial biosensors (mer-lux), basal respiration, bacterial community structures and identified indicator OTUs which were responsive to Hg. In soils treated with at least 3.2 µg Hg g⁻¹ dry soil, resulting in bioavailable Hg higher than 0.004 µg Hg g⁻¹ dry soil, the basal respiration was strongly affected. High bioavailable Hg also caused significant changes in the bacterial T-RFLP profiles. Members of the Alphaproteobacteria (Rhodospirillales) and Betaproteobacteria (Burkholderiales) were found to be Hg-tolerant. Here, we propose a critical limit concentration for soluble Hg of 0.004 µg Hg g⁻¹ soil.
4.2 Introduction

Present concentrations of mercury (Hg) over large areas in Europe are increased to levels that may exert some environmental impact (critical limits) (De Vries et al. 2005). Hg is emitted from anthropogenic and natural sources to the atmosphere, mainly in the gaseous form, and can be transported to remote and pristine locations (Pacyna et al. 2006; Harmens et al. 2008). In soils, Hg is highly immobile, bound to the soil organic matter in particular to thiol groups (Obrist et al. 2009; Skyllberg et al. 2010) and has the ability to interact with soil mineral components as well as to form precipitates with sulphide, carbonate, hydroxide, and other anions (Schuster 1991; Skyllberg et al. 2006). Forest soils rich in organic matter can therefore be regarded as an efficient filter between the supply from the atmospheric deposition and soil layers at greater depths (Grigal 2003; Meili et al. 2003; Akerblom et al. 2008). In boreal forest soils, the concentrations of Hg in the litter layer vary from 0.07 µg to 1.0 µg Hg g\(^{-1}\) dry soil (Meili et al. 2003; Akerblom et al. 2008; Klaminder et al. 2008) and may exceed 0.5 µg Hg g\(^{-1}\) dry soil in temperate forests in Germany (Schwesig and Matzner 2000) and in Switzerland (Ernst et al. 2008; Rieder et al. 2011).

Evidence of detrimental effects of Hg to soil microorganisms in general and in particular in forest soils is scarce. Although Hg is known to act as powerful toxicant, ecotoxicological data of Hg in temperate forest soils are very limited. Most of the studies have been performed in boreal forest soils (Bringmark et al. 2001; Bringmark et al. 2001; Akerblom et al. 2010), in agricultural soils (Landa et al. 1978; Ranjard et al. 1997; Ranjard et al. 2000; Müller et al. 2001; Rasmussen et al. 2001; Casucci et al. 2003) or in tropical soils (Harris-Hellal et al. 2009). Soil bacterial communities are responsible for many fundamental ecological processes, such as the biogeochemical cycling of chemical elements or the decomposition of plant and animal residues (Balser et al. 2005). Evaluating the ecotoxicity of Hg in soils can give valuable information about the sensitivity of the indigenous soil microorganisms. However, there is only limited data on critical limit concentrations below which there is no effect on soil bacterial communities and published threshold values were only given as total Hg concentrations in soils. For example, critical limit for total Hg contents to prevent
Ecotoxicity of inorganic-Hg

ecological effects for Hg in organic soils have been set to 0.4 µg Hg g\(^{-1}\) dry soil (or 0.5 µg Hg g\(^{-1}\) organic matter) by an international expert group on effect-based critical limits for HMs working within the framework of UNECE Convention on Long-range Transboundary Air Pollution (Curlic et al. 2000; Meili et al. 2003; De Vries et al. 2005). At lower concentrations, it is assumed that there are no harmful effects on soil organisms. Data obtained for the estimation of these critical limits were mainly derived from podzolic soils with a high organic matter content (≥85%). Soil type is a very important factor to evaluate the response of soil microorganisms to HM pollution (Lazzaro et al. 2006; Lazzaro et al. 2006). Similarly, Tipping et al. (2010) reported a critical limit for total content of 3.3 µg Hg g\(^{-1}\) organic matter or 0.13 µg Hg g\(^{-1}\) dry soil. By applying this definition, 60% of 34 natural forest soils studied in Switzerland would exceed this critical limit (Rieder et al. 2011). In addition, regarding the evaluation of critical limits of Hg in temperate forest soils data of the soluble fraction instead the total content would be more ecotoxicological relevant (Lazzaro et al. 2006; Lazzaro et al. 2006). Therefore, there is an urgent need to study the impact of Hg to soil microbial communities in forest soils with contrasting physico-chemical characteristics (e.g. low in organic matter and a broad pH range from acidic to calcareous soils) and to evaluate critical limit concentrations for soluble Hg in the soil water.

In the present study we determined the effects of increasing Hg concentrations on the soil bacterial communities of natural deciduous, spruce and mixed forests in Switzerland. Seven different forest soils with contrasting physico-chemical characteristics and within a pH range from 4.4 to 7.3 were spiked with a Hg(II) chloride solution in microcosms experiments. Short-term Hg exposure and employed microcosms minimize abiotic variances and enable good standardization (Lazzaro et al. 2008). We tested microbial activity variables (basal respiration) in soils and examined the bacterial community structure by using T-RFLP profiling of the 16S rRNA gene in relation to the increasing Hg concentrations in soils. Our overall aim was to relate effects on the bacterial communities to the bioavailable Hg concentrations and to determine threshold concentrations for soil types with low organic matter content below no negative effects on the bacterial communities emerge.
4.3 Material and Methods

4.3.1 Soil types, location, soil characteristics

Seven forest soils with different physical and chemical properties containing no or only a small litter layer were chosen from a soil profile database (Ernst et al. 2008; Rieder et al. 2011) of the Swiss Research Institute WSL, Birmensdorf, Switzerland (Tables S4.1 and S4.2). Physico-chemical characteristics of the soils tested have been described elsewhere (Lazzaro et al. 2006; Lazzaro et al. 2006; Ernst et al. 2008; Rieder et al. 2011). After removal of the litter layer (less than 1 cm), soil material was collected at a soil depth of 0-10 cm (A-horizon) with a shovel. At each site, ten samples of the mineral soil were collected randomly from an area of approximately 5 × 5 m, then pooled in a plastic bag. The collected soil was stored at 4 °C in the dark for a week before use.

4.3.2 Microcosm setup and mercury additions

Prior to setting up microcosms the soils were sieved (mesh size 2 mm) and air-dried at room temperature (about 15 °C) for three days. The soils were then uniformly rewetted to a moisture content of 58% (dry wt equiv.) with mercuric-chloride (HgCl₂, Sigma-Aldrich, Buchs, Switzerland) dissolved in sterilized Millipore water to obtain concentrations of 0.032, 0.32, 3.2 and 32 µg Hg g⁻¹ dry soil. These concentrations were chosen by compiling data from literature dealing with Hg contaminations and metal effects on soil microbial communities (Ranjard et al. 2000; Müller et al. 2001). After addition of the Hg solution the soils were placed in plastic bags and mixed thoroughly. Control treatments received only water or CaCl₂ to test the ionic strength on the soil microbial communities. The soils were kept at room temperature for 24 h prior to filling in the microcosm to obtain a uniform distribution of the metal and equilibrium between Hg and soil binding sites. Then the soils (100 g) were transferred into 250 mL plastic jars (Stericup filter systems, Millipore, Billerica, MA, USA), and covered with the supplied air permeable caps throughout the experiment to minimize evaporation, but permitting aeration. The microcosms were incubated in the dark at 20 °C and 60% of external humidity in climatic chambers similar as described by (Lazzaro et al.
2006; Lazzaro et al. 2006). Water loss was minimal (determined daily by weight), and, if necessary, was compensated by the addition of sterile water. All experiments were done in triplicate from both Hg-spiked and control soils (water and CaCl₂). The microcosms were harvested after 30 days of incubation. Part of the soil was immediately used for basal respiration and C biomass measurements, while aliquots were frozen at -80 °C for DNA extraction. The remaining soil was stored at 4 °C for determination of pH, DOC, and bioavailable Hg and/or oven-dried for 48 h at 105 °C to determine remaining water content.

4.3.3 Soil chemical characteristics

Water extractions were performed by shaking 20 g of fresh soil mixed with 200 mL of Millipore water for 16 h at 20 °C on an overhead shaker (Lazzaro et al. 2006). The soil slurry was then centrifuged for 10 min at 1200 x g in a Sigma 6-15 centrifuge (Sigma-Aldrich, St. Louis, MO, USA), and subsequently vacuum-filtered through a 0.45 μm membrane filter (Millipore Stericup systems). Each extract was divided into two portions for pH and DOC analyses. DOC concentrations in the water extracts were measured with a Shimadzu TOC-500 apparatus (Shimadzu, Kyoto, Japan), while pH was measured with an Orion 520A pH meter (Thermo 5 Electron, Waltham, MA, USA).

The bioavailable Hg in soil samples was measured in duplicate by a mer-lux assay, applying a whole-cell bacterial biosensor as described previously (Lappalainen et al. 2000). The mer-lux biosensor has been shown to be a useful and sensitive tool for the estimation of bioavailable Hg in soil (Rasmussen et al. 2000; Ivask et al. 2002; Petänen and Romantschuk 2002). Briefly, 1 g of soil in 10mL of sterile water was shaken at 20 °C in polyethylene tubes for 24 h on an overhead shaker. Soil particles were removed by centrifugation for 10 min at 12 000 x g. Appropriate soil leachate dilutions (100 μL) were mixed with 100 μL of a cell suspension of the biosensor strain Escherichia coli MC1061 (pTOO11) obtained from Aboatox Oy (Turku, Finland), which bioluminesces in the presence of Hg (Lappalainen et al. 2000). Light emission in soil samples, Hg standards, and blank assays were recorded as relative light units using a Luminescan (Labsystems, Waltham, MA, USA) luminometer. A
standard curve was established using the regression equation for the relationship between the amount of bioavailable Hg and the expression factors obtained from a standard assay of known concentrations of Hg (Lappalainen et al. 2000). The detection limit was equal to 0.1 µg Hg L\(^{-1}\). We calculated the bioavailable Hg concentration of the soil extract (g L\(^{-1}\)) back to the amount of bioavailable Hg per g soil (in µg Hg g\(^{-1}\) soil).

4.3.4 Basal respiration
Basal respiration (CO\(_2\) evolution) was measured using the method described by Frey et al. (2008) by incubating moist soil samples (approximately 20 g dry soil) for 3 days in gas tight vessels. The percentage of basal respiration by the Hg treatments, relative to the controls, was taken as an index of activity of the soil microbial communities.

4.3.5 Extraction of DNA
DNA extraction from each of the replicate microcosms (0.5g of fresh soils) was performed using a bead-beating method described by Frey et al. (2006). DNA was quantified using Pico Green (Molecular Probes, Basel, Switzerland), and herring sperm DNA standards (Invitrogen, San Diego, CA, USA).

4.3.6 PCR amplification and T-RFLP analysis
Before PCR, 5 ng µL\(^{-1}\) of DNA were pretreated with 3 µg µL\(^{-1}\) of bovine serum albumin (BSA, molecular biology grade, Sigma, Buchs, Switzerland) and heated for 2 min at 95 °C to bind PCR inhibiting substances such as humic acids (Burgmann et al. 2001). Twenty nanogramm of pretreated DNA were then added to the PCR reaction mix [1X MgCl\(_2\)-containing PCR buffer, 0.5 mM additional MgCl\(_2\), 400 µM of dNTP mixture (Catalys, Wallisellen, Switzerland), 0.3 µg µL\(^{-1}\) BSA, 0.2 µM of each primer (Microsynth, Balgach, Switzerland), 2 U of HotStarTaq® polymerase (Qiagen, Hilden, Germany)] in a 50 µL volume. The bacterial 16S rRNA genes were amplified with forward primer 27F (AGA GTT TGA TCM TGG CTC AG, 5’ labelled with 6-FAM) and reverse primer 1378R (CGG TGT GTA CAA GGC CCG CCG GGA ACG)
PCR was carried out in a PTC-100 thermocycler (MJ Research, Waltham, MA, USA), with an initial activating step for HotStarTaq® polymerase (15 min at 95 °C) followed by 35 cycles with denaturation (94 °C for 45 s), annealing (48 °C for 45 s) and extension (72 °C for 2 min). A final extension step (72 °C for 5 min) was applied to complete the amplification cycles. The PCR amplicons were purified with the Montage PCR purification cleanup kit (Millipore, Billerica, MA, USA) and digested for 3 h at 37 °C with an equal volume of digestion mix [10 U (each) MspI, 1% Tango buffer (Fermentas, Burlington, ON, USA)]. Prior to the T-RFLP analyses the digestions were desalted with Montage SEQ96 sequencing reaction cleanup kit (Millipore), according to the manufacturer's instructions.

Capillary electrophoresis was performed on 2 µL of the digestion products in 11.9 µL of HiDi formamide (Applied Biosystems) and 0.1 µL of ROX500 DNA fragment length standard (Applied Biosystems). The samples were first denatured for 2 min at 95 °C and immediately chilled on ice. Fragments were analysed by capillary electrophoresis with an ABI Genetic Analyzer 310 (Applied Biosystems) as previously described (Frey et al. 2006). The lengths of the labelled fragments (T-RFs) were determined by using GeneScan V.3.1. and Genotyper V.2.5. (Applied Biosystems). Operational taxonomic units (OTUs) were defined as peaks (T-RFs) with a size of x±0.5 bp and a height of at least 150 fluorescence units between 50 and 500 bps in all the replicates in at least.

### 4.3.7 Identification of OTUs responsive to Hg

Samples from soils treated with the highest Hg concentrations (32 µg Hg g⁻¹ dry soil) or from untreated soils were chosen for amplification based on high peak intensity (relative fluorescence) of the OTUs of interest to maximize their recovery. Certain target T-RFs increased or decreased with the addition of Hg. Sequence information from T-RFLP analysis was recovered with adaptor-ligation, fragment size selection, and reamplification with adaptor size-specific PCR to obtain a desired T-RF fraction as described previously by Widmer et al. (2006). Cloning of the size-selected T-RF fraction allowed clones specific to the T-RF to be isolated efficiently.
Briefly, T-RFs were characterized by a specific PCR primer sequence (27F) at the 5’ end and a specific restriction site (MspI) at the 3’ end. The double-stranded MspI-adapter structure was prepared and ligated into restriction fragments as described previously by Widmer et al. (2006). The size selection of T-RFs of interest was done by gel electrophoresis with an SEA 2000 electrophoresis apparatus (Elchrom Scientific, Inc., Switzerland) using precast Spreadex gels (EL 400, 600, 800, or 1200; Elchrom Scientific, Inc., Switzerland). DNA was eluted from the gels and purified as described previously by Widmer et al. (2006). Eluted DNA was used as a template for PCR amplification with primer 27F and the MspI-adapter-primer construct. PCR products were purified with Montage PCR Centrifugal Filter Devices (Millipore). Purified products were ligated into the pGEM-T easy vectors (Promega, Wisconsin, USA) and transformed into competent Escherichia coli JM109 cells according to the manufacturer’s recommendations. Clones were screened by colony PCR with primers 27F (FAM-labeled) and MspI-adapter-primer. PCR products were digested with MspI and T-RFs sizes were analysed as previously described (Widmer et al. 2006). Nine target T-RFs (significantly increasing or decreasing in the highest Hg treatment compared to the controls in the different forest soils) could be isolated and were sequenced as previously described (Frey et al. 2008; Frey et al. 2011). Several clones (≥ 5) of these target T-RFs were sequenced and sequence analysis revealed identical or highly similar sequences between the clones. The sequences were then checked and manually edited in the software BioEdit (V.7.1.3.0), and chimera checked using Bellerophon (Huber et al. 2004). Sequence analysis was performed with Sequence_Match and BLAST searches in the Ribosomal Database Project (RDP-10.30) and GenBank (http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequences were deposited in Genbank under accession numbers between KC111961 and KC112015.

4.3.8 Statistics
The raw data from the T-RFLP profiles were normalized by converting the peak height of each OTU to the percentage value of the total peak height, to obtain relative abundances
Statistical analyses were carried out using ANOVA (p≤0.05) using Statistica 5.1 (Statsoft inc., Tulsa, OK, USA). Average (three microcosm replicates) relative abundances of each OTU were compared for significant differences between controls and the different Hg treatments by one-way ANOVA corrected with the post hoc Tukey-HSD test (Statistica). For community structure analyses, the standardized T-RFLP data were transformed (square root), resembled (similarity P/A) and analysed using the software Primer 6 v.6.1.13 and Permanova v.1.0.3 (Primer-E, Ivybridge, UK). The effects of Hg treatments on the community structures (T-RFLP profiles) were tested by permutation multivariate analysis of variance (PERMANOVA) and canonical analysis of principal coordinates (CAP) (Anderson and Willis 2003) on Bray-Curtis distances of transforms of T-RFLP abundance data. PERMANOVA was conducted using default settings with 9999 unrestricted permutations, while CAP was conducted using default settings. No effect concentrations were estimated on either basal respiration (% of inhibition taken as positive values) or on bacterial community structure (% OTUs affected) in all the soils.

**4.4 Results**

**4.4.1 Soil chemical characteristics**

The soils chosen in this study represent a range of soil types in Switzerland which vary in their physico-chemical characteristics (Table S4.2). Soil texture varied considerably with sand contents ranging from 6% to 66% and clay contents ranging from 9% to 55% across the soils investigated (Lazzaro et al. 2006; Lazzaro et al. 2006; Ernst et al. 2008; Rieder et al. 2011). Carbon content was low and was between 2% and 8%. HNO₃-extractable Hg concentrations were between 0.08 and 0.32 µg Hg g⁻¹ dry soil. These low concentrations indicate that our forest soils were not subjected to a direct anthropogenic Hg sources in their proximity (Ernst et al. 2008; Rieder et al. 2011). In all soils, pH remained unaffected by the Hg treatments. The average concentrations of water-extractable DOC measured in the water extracts ranged from values around 170 µg C g⁻¹ dry soil in Schänis to concentrations...
exceeding 700 µg C g\(^{-1}\) dry soil in Burgdorf (Table 4.1). Hg treatments especially the highest Hg treatment (32 µg Hg g\(^{-1}\) dry soil) increased or tended to increase DOC in the water extracts of most of the soils in particular from Burgdorf, Lausanne, Piotta (P < 0.01) and from Schänis (P < 0.05).

Table 4.1. Hg treatments, bioavailable Hg measured with the reporter biosensor strain *Escherichia coli* MC1061, dissolved organic carbon (DOC) and pH in the water extracts after 30 days of incubation (mean ± standard deviation; n = 3).

<table>
<thead>
<tr>
<th>Location</th>
<th>Hg treatment [µg Hg(II) g(^{-1}) dry soil]</th>
<th>Bioavailable Hg [µg Hg g(^{-1}) dry soil]</th>
<th>Bioavailable Hg of total Hg (%)</th>
<th>DOC [µg C g(^{-1}) dry soil]</th>
<th>pH (H(_2)O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerlafingen</td>
<td>0</td>
<td>&lt; DL</td>
<td>590 ± 120</td>
<td>4.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>0.032</td>
<td>&lt; DL</td>
<td>460 ± 70</td>
<td>4.3 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.32</td>
<td>0.002 ± 0.001</td>
<td>0.6</td>
<td>390 ± 110</td>
<td>4.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.011 ± 0.006</td>
<td>0.3</td>
<td>360 ± 70</td>
<td>4.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.36 ± 0.13</td>
<td>1.1</td>
<td>550 ± 50</td>
<td>4.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Burgdorf</td>
<td>0</td>
<td>&lt; DL</td>
<td>710 ± 70</td>
<td>4.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>0.032</td>
<td>&lt; DL</td>
<td>780 ± 110</td>
<td>4.4 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.32</td>
<td>0.002 ± 0.002</td>
<td>0.6</td>
<td>770 ± 140</td>
<td>4.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.009 ± 0.005</td>
<td>0.3</td>
<td>820 ± 120</td>
<td>4.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.10 ± 0.04</td>
<td>0.3</td>
<td>870 ± 100</td>
<td>4.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Lausanne</td>
<td>0</td>
<td>&lt; DL</td>
<td>230 ± 100</td>
<td>4.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>0.032</td>
<td>&lt; DL</td>
<td>230 ± 30</td>
<td>4.5 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.32</td>
<td>0.004 ± 0.001</td>
<td>1.3</td>
<td>280 ± 90</td>
<td>4.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.008 ± 0.004</td>
<td>0.3</td>
<td>230 ± 110</td>
<td>4.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.032 ± 0.013</td>
<td>0.1</td>
<td>390 ± 130</td>
<td>4.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Piotta</td>
<td>0</td>
<td>&lt; DL</td>
<td>380 ± 60</td>
<td>4.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>0.032</td>
<td>&lt; DL</td>
<td>350 ± 40</td>
<td>4.6 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.32</td>
<td>0.003 ± 0.002</td>
<td>0.9</td>
<td>330 ± 80</td>
<td>4.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.014 ± 0.011</td>
<td>0.4</td>
<td>220 ± 60</td>
<td>4.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.28 ± 0.09</td>
<td>0.9</td>
<td>510 ± 130</td>
<td>4.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Schänis</td>
<td>0</td>
<td>&lt; DL</td>
<td>170 ± 20</td>
<td>5.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>0.032</td>
<td>&lt; DL</td>
<td>170 ± 60</td>
<td>5.7 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.32</td>
<td>0.002 ± 0.002</td>
<td>0.6</td>
<td>160 ± 30</td>
<td>5.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.013 ± 0.009</td>
<td>0.4</td>
<td>110 ± 50</td>
<td>5.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.24 ± 0.05</td>
<td>0.8</td>
<td>240 ± 90</td>
<td>5.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Laufen</td>
<td>0</td>
<td>&lt; DL</td>
<td>240 ± 60</td>
<td>7.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>0.032</td>
<td>&lt; DL</td>
<td>230 ± 40</td>
<td>7.1 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.32</td>
<td>&lt; DL</td>
<td>220 ± 90</td>
<td>7.1 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.008 ± 0.003</td>
<td>0.3</td>
<td>240 ± 50</td>
<td>6.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.027 ± 0.015</td>
<td>0.1</td>
<td>250 ± 110</td>
<td>6.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Sihlwald</td>
<td>0</td>
<td>&lt; DL</td>
<td>170 ± 40</td>
<td>7.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>0.032</td>
<td>&lt; DL</td>
<td>180 ± 40</td>
<td>7.3 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.32</td>
<td>&lt; DL</td>
<td>170 ± 30</td>
<td>7.3 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.004 ± 0.002</td>
<td>0.1</td>
<td>180 ± 30</td>
<td>7.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.015 ± 0.009</td>
<td>0.0</td>
<td>180 ± 50</td>
<td>7.1 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

*<DL not detected = below detection limit; detection limits for bacterial sensors was set to 0.1 µg Hg L\(^{-1}\) soil extracts*
4.4.2 Bioavailable Hg concentrations

In all of the soils not spiked with Hg or with the lowest Hg treatment (up to 0.032 µg Hg g⁻¹ dry soil), bioavailable Hg concentrations were below detection limit (0.1 µg Hg L⁻¹, Table 4.1). Bioavailable Hg concentrations in the second lowest Hg treatments (up to 0.32 µg Hg g⁻¹ dry soil) were ≤0.004 µg Hg g⁻¹ dry soil. The highest bioavailable Hg concentrations were mainly found in acidic soils (pH < 5.0) with a clay content ranging between 9% and 24% (Tables 4.1 and S4.2). Lower available Hg concentrations up to 0.027 ± 0.015 µg Hg g⁻¹ dry soil were found in calcareous soils (pH > 6.5) (Tables 4.1 and S4.2). The concentration of bioavailable Hg measured in the different treatments represents in general a small fraction of the total additions, ranging from less than 0.31% for the acidic soils such as Burgdorf and Gerlafingen, to less than 0.07% for the calcareous soils from Laufen and Sihlwald (data not shown).

4.4.3 Basal respiration

In general, the basal respiration in the two control (H₂O- and CaCl₂-treated) treatments did not differ (Table S4.3) but the basal respiration in Hg treated soils were lower than in the controls in all soils. The highest Hg treatment (32 µg Hg g⁻¹ dry soil) significantly reduced the basal respiration in all the soils. Strongest reduction of basal respiration was found in soils from Gerlafingen (64% reduction, P < 0.001), Schänis (48% reduction, P < 0.001) and Piotta (39% reduction, P < 0.001) compared to the controls (Table 4.2). Basal respiration was also significantly reduced (between 11% and 30% reduction) in the second highest Hg treatment (3.2 µg Hg g⁻¹ dry soil) compared to the controls in the soils from Gerlafingen, Burgdorf, Piotta and Laufen (Table 4.2) but not in soils with lower Hg concentrations. The highest soluble Hg concentrations below which there was no impact on the bacterial communities nor the basal respiration was 0.004 µg Hg g⁻¹ dry soil measured in soils from Lausanne treated at 0.32 µg Hg g⁻¹ dry soil (Table 4.1). Therefore, we set the critical limit concentrations for soluble Hg in the soil water to be 0.004 µg Hg g⁻¹ dry soil.
Table 4.2. Percentages of basal respiration (CO₂ evolution) significantly changing (decreasing) between the control (water) and the different Hg treatments (mean ± standard deviation; n = 3). The absolute values of all treatments are given in Table S4.3 in the supplementary matter.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Hg treatment (µg g⁻¹ dry soil)</th>
<th>% basal respiration decreased compared to the control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.032</td>
<td>0.32</td>
</tr>
<tr>
<td>Gerlafingen</td>
<td>4 ± 1</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>Burgdorf</td>
<td>1 ± 1</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Lausanne</td>
<td>3 ± 1</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>Piotta</td>
<td>1 ± 0</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Schänis</td>
<td>0 ± 0</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Laufen</td>
<td>7 ± 2</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Sihlwald</td>
<td>9 ± 2</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

Significance level: *p<0.05 **p<0.01 ***p<0.001

4.4.4 Changes in the structure of the bacterial communities

T-RFLP analysis in the size range between 50 and 500 bps revealed no differences of the bacterial communities in all soils between the controls (H₂O- and CaCl₂-treatments; data not shown) but large differences between the soils from the different forest sites. The largest number of OTUs scored was 66 in the soil from Burgdorf, followed by the soils from Lausanne (56), Gerlafingen (54), and Sihlwald (54). The lowest number of OTUs detected was 39 in the soils from Schänis (data not shown). The relative abundance of the OTUs varied with Hg treatments. These variations consisted of both decreases and increases, whereas OTUs decreasing in abundance were more prominent (Table 4.3). More than 50% of the OTUs differed significantly in abundance at the highest Hg treatment in soil samples from Schänis (77%), Lausanne (71%) and Gerlafingen (63%), while soils from Sihlwald
(13%) and Laufen (10%) appeared less affected. The soils from Burgdorf (42%) and Piotta (37%) were intermediately affected. At lowest Hg treatments (up 0.32 µg Hg g\(^{-1}\) dry soil), the adverse effects in all soils were absent (from 0% to 3% of OTUs changing in abundance; Table 4.3).

Table 4.3. Numbers of bacterial OTUs significantly (P < 0.05) decreasing or increasing in abundance by Hg compared to the control (water) (mean ± standard deviation; n = 3).

<table>
<thead>
<tr>
<th>Soil</th>
<th>n*</th>
<th>OTUs significantly decreasing</th>
<th>OTUs significantly increasing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hg treatment (µg g(^{-1}) dry soil)</td>
<td>Hg treatment (µg g(^{-1}) dry soil)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.032</td>
<td>0.32</td>
</tr>
<tr>
<td>Gerlafingen</td>
<td>54</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Burgdorf</td>
<td>66</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Lausanne</td>
<td>56</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Piotta</td>
<td>41</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Schanis</td>
<td>39</td>
<td>0 ± 0</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>Laufen</td>
<td>50</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Sihlwald</td>
<td>54</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

* n= total numbers of OTUs

PERMANOVA analyses performed with the T-RFLP profiling data revealed that Hg significantly (p<0.05) affected the bacterial community structures of all the soils (Figure 4.1). The CAP analyses showed clear clusters of bacterial communities within the Hg treatments in all soils, in particular the bacterial communities from the highest Hg treatments (32 µg Hg g\(^{-1}\) dry soil) were significantly (p<0.05) separated from the other treatments (Figure 4.1). In addition, at the second highest Hg treatment (3.2 µg Hg g\(^{-1}\) dry soil) marked structural changes in the bacterial communities were only evident in the soils from Lausanne (34% of OTUs affected, p<0.01). On the contrary, the structures of the bacterial communities at the lowest Hg treatments (0.032 and 0.32 µg Hg g\(^{-1}\) dry soil) were not significantly (p>0.05) affected compared to the controls.
Figure 4.1: Canonical analysis of the principal coordinates (CAP) ordinations of bacterial communities. The CAP analyses based on the relative abundances of OTUs were grouped among the Hg treatments [symbols; \( \mu g \text{Hg(II) g}^{-1} \text{dry soil} \)] for all seven soils. The bioavailable Hg concentrations (\( \mu g \text{Hg g}^{-1} \text{dry soil} \)) of the corresponding Hg treatment are given to the right of the arrow whenever they were above the detection limit of the method. The p-values of the statistical analysis of how the Hg treatment relates to community composition were calculated with PERMANOVA.
Selected OTUs that were responsive to the Hg treatments were targeted for phylogenetic affiliation using a combination of adapter ligation, fragment size selection with gel electrophoresis, and reamplification with adapter specific PCR. In this way, we were able to isolate seven Hg-tolerant (significantly increasing with Hg) and two Hg-sensitive (significantly decreasing with Hg) OTUs. 16S rRNA gene sequences dominated by members of the *Alphaproteobacteria* (order *Rhodospirillales*) and *Betaproteobacteria* (order *Burkholderiales*) were found to be Hg-tolerant in the different soils. In Burgdorf and Gerlafingen, two OTUs (140bp and 437bp) were similar (100% identity) in both soils and were more abundant in Hg treated soils than in the controls (Table 4.4). Cloned sequences of OTU 140bp were most similar (98 - 100%) to *Burkholderia* sp. clones retrieved from polluted soils (Lazzaro et al. 2008; Kunito et al. 2012). Cloned sequences of OTU 437bp in Burgdorf and Gerlafingen were closely related (99%) to an environmental clone WR8142 (AJ292833) within the *Rhodospirillales* isolated from a PCB polluted soil (Nogales et al. 2001). Closest cultured relative of OTU 437bp was a *Telmatospirillum sibirense* strain 26-4b1 (AF524854) isolated from a Sphagnum peat bog. In Piotta and Sihlwald, one OTU with similar length (401bp) but only 87% sequence identity between the different soils was more abundant in Hg treated soils than in the controls. The closest relatives (98% identity) of the sequences of OTU 401bp in Piotta was a *Sphingomonas fennica* type strain K101 T (AJ009706) isolated from contaminated boreal groundwater and an environmental clone NC34_c6_14807 (JQ386164) obtained from soil samples collected from a free air CO$_2$ enrichment experiment. Closest relatives (99% identity) of the sequences of OTU 401bp in Sihlwald were a *Mesorhizobium* strain R-46209 (FR774180) and a clone Elev_16S_999 (EF019808) recovered from trembling aspen rhizosphere. In contrast, two OTUs (175bp and 306bp) isolated were sensitive to Hg and were phylogenetically related to *Gamma-* and *Deltaproteobacteria* (Table 4.4). Cloned sequences of OTU 175bp retrieved from the Laufen soil were phylogenetically related to unclassified *Nannocystineae* from the order *Myxococcales* and had closest relative (100%) to an environmental clone (clone p7o10ok: FJ478973) obtained from uncontaminated prairie soils. The closest relative (100%) of OTU 306bp in Piotta was an environmental clone
BOM_h03 (HM488736) within the order of *Xanthomonadales* obtained from natural forest soils.
Table 4.4. Phylogenetic affiliation of specific OTUs which were indicative for the Hg treatments.

<table>
<thead>
<tr>
<th>T-RF (bp)</th>
<th>Soil</th>
<th>Clone name</th>
<th>Accession number</th>
<th>Closest relatives</th>
<th>Accession number</th>
<th>Identity (%)</th>
<th>Phylogenetic affiliation</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hg-tolerant OTUs</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>Burgdorf</td>
<td>BUR_Hg_mt_140_C9</td>
<td>KC111963</td>
<td>Clone WD2114</td>
<td>AJ292647</td>
<td>100</td>
<td>Betaproteobacteria Burkholderia sp.</td>
<td>PCB-polluted soil</td>
</tr>
<tr>
<td>140</td>
<td>Gerlafingen</td>
<td>GER_Hg_mt_140_F8</td>
<td>KC111964</td>
<td>Clone FB49-9b</td>
<td>AY527792</td>
<td>100</td>
<td>Betaproteobacteria Burkholderia sp.</td>
<td>Uranium-contaminated soil</td>
</tr>
<tr>
<td>401</td>
<td>Piotta</td>
<td>PIO_Hg_mt_401_B12</td>
<td>KC112001</td>
<td>Clone NC34_c6_14807</td>
<td>JQ386164</td>
<td>98</td>
<td>Alphaproteobacteria Sphingomonas sp.</td>
<td>FACE- soil</td>
</tr>
<tr>
<td>401</td>
<td>Sihlwald</td>
<td>SIW_Hg_mt_401_B3</td>
<td>KC112007</td>
<td>Clone Elev_16S_999</td>
<td>EF019808</td>
<td>99</td>
<td>Alphaproteobacteria Rhizobiales</td>
<td>Trembling aspen rhizosphere</td>
</tr>
<tr>
<td>437</td>
<td>Burgdorf</td>
<td>BUR_Hg_mt_437_F12</td>
<td>KC111962</td>
<td>Clone WR8142</td>
<td>AJ292833</td>
<td>99</td>
<td>Alphaproteobacteria Rhodospirillales</td>
<td>PCB-polluted soil</td>
</tr>
<tr>
<td>437</td>
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<td>GER_Hg_mt_437_B10</td>
<td>KC111966</td>
<td>Clone WR8142</td>
<td>AJ292833</td>
<td>99</td>
<td>Alphaproteobacteria Rhodospirillales</td>
<td>PCB-polluted soil</td>
</tr>
<tr>
<td>447</td>
<td>Laufen</td>
<td>LAF_Hg_mt_447_E8</td>
<td>KC111982</td>
<td>Clone B02-Fw</td>
<td>EF455081</td>
<td>100</td>
<td>Gammaproteobacteria Xanthomonadales</td>
<td>Mercury-contaminated soil</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hg-sensitive OTUs</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>Laufen</td>
<td>LAF_Hg_ms_175_F11</td>
<td>KC111974</td>
<td>Clone P7o10ok</td>
<td>FJ478973</td>
<td>100</td>
<td>Deltaproteobacteria Myxococcales</td>
<td>Soil under tall grass prairie</td>
</tr>
<tr>
<td>306</td>
<td>Piotta</td>
<td>PIO_Hg_ms_306_C6</td>
<td>KC111994</td>
<td>Clone BOM_h03</td>
<td>HM488736</td>
<td>100</td>
<td>Gammaproteobacteria Xanthomonadales</td>
<td>Forest soil</td>
</tr>
</tbody>
</table>
4.5 Discussion

The study clearly showed that enhanced Hg treatments (≥ 3.2 µg Hg g\(^{-1}\) dry soil) strongly affect microbial activities and bacterial community structures in forest soils varying in physico-chemical properties. No significant effects (basal respiration; T-RFLP profiling) could be observed at lower doses (0.032 and 0.32 µg Hg g\(^{-1}\) dry soil) in all forest soils with no detectable or only minor bioavailable Hg concentrations (≤ 0.004 µg Hg g\(^{-1}\) dry soil) measured. Therefore, we propose a critical limit concentration for soluble Hg of 0.004 µg Hg g\(^{-1}\) dry soil. As far as we know, ecotoxicological assessments with respect to soil bacterial communities have only been reported for total Hg contents in agricultural soils (Holtze et al. 2003; Ruggiero et al. 2011) or soils rich in organic matter (Curlic et al. 2000; Meili et al. 2003; De Vries et al. 2005). However, evidence of detrimental effects of Hg to bacterial communities in forest soils with contrasting physico-chemical characteristics is scarce and in particular, regarding the evaluation of critical limits of Hg in temperate forest soils, data of the soluble fraction which are more ecotoxicological relevant (Lazzaro et al. 2006; Lazzaro et al. 2006) than total contents have not been published so far. The soils chosen in this study demonstrate a wide variation with respect to parent material, and composition of the soil (C\(_{org}\), 2–8%; clay, 9–55%; pH 4.4–7.3) and represent most common forest soil types in Switzerland (Lazzaro et al. 2006; Lazzaro et al. 2006; Ernst et al. 2008; Rieder et al. 2011). Hg was supplied to the microcosms as aqueous Hg-chloride solutions similar to previous studies (Ranjard et al. 2000; Bringmark et al. 2001; Bringmark et al. 2001; Müller et al. 2001). The amounts of Hg added to our soils were spanning a wide range of concentrations from environmentally relevant concentrations up to levels of extreme pollution. The lowest Hg rates applied in this study (up to 0.32 µg Hg g\(^{-1}\) dry soil) were within the range that has been detected in soils without any Hg source in its proximity (Schwesig et al. 2000; Grigal 2003; Rieder et al. 2011). Higher Hg rates applied (up to 3.2 µg Hg g\(^{-1}\) dry soil) were within the range of contaminated soils (Wiersma et al. 1986). The highest level (32 µg Hg g\(^{-1}\) dry soil) given in our study corresponded to the amounts supplied by others in microcosm experiments with agricultural soils (Ranjard et al. 2000; Rasmussen et al. 2001; Holtze et al.
2003) or a tropical soil (Harris-Hellal et al. 2009) and have been found in heavily contaminated field studies [e.g. chloralkali smelting industries up to 14 µg Hg g\(^{-1}\) dry soil (Zhang et al. 2009)] or gold mining sites [up to 40 µg Hg g\(^{-1}\) dry soil (Gosar and Tersic 2012)]. Our results showed that the effects of Hg on the soil bacterial communities in these forest soils depend on the Hg level applied. No significant effects (basal respiration; T-RFLP profiling) could be observed at lower doses (0.032 and 0.32 µg Hg g\(^{-1}\) dry soil) in all forest soils with no detectable or only minor bioavailable Hg concentrations (≤ 0.004 µg Hg g\(^{-1}\) dry soil) measured. These low bioavailable Hg concentrations might be the reason for the absence of detrimental effects of the supplied Hg to the soil bacterial communities.

A significant reduction between 11 and 30% of the basal respiration was, however, observed at the second highest Hg treatments (3.2 µg Hg g\(^{-1}\) dry soil) in four soils with bioavailable Hg concentrations between 0.008 µg Hg g\(^{-1}\) and 0.014 µg Hg g\(^{-1}\) dry soil. These findings are in accordance to the 10% inhibition of microbial activity at soils treated with rates between 2 and 3 µg Hg g\(^{-1}\) dry soil in a forest soil with a high organic matter (around 85%) reported in Bringmark et al. (2001). Akerblom et al. (2010) found a 5% reduction in heterotrophic respiration at 0.8 µg Hg g\(^{-1}\) dry soil in the F-layer of boreal forests. In contrast, no negative effect of Hg on basal respiration and the bacterial community structure in soils was observed in a tropical soil where they added 1 µg Hg g\(^{-1}\) dry soil (Harris-Hellal et al. 2009). Unfortunately no data on the soluble Hg concentrations in these soils were reported.

High soluble Hg concentrations in soils (up to 0.36 µg Hg g\(^{-1}\) dry soil) were detected in the highest Hg treatments (32 µg Hg g\(^{-1}\) dry soil), where also the major effects the on microbial-mediated processes were observed. The basal respiration was inhibited up to 64% compared to the controls. In addition to the effects on the basal respiration, the highest bioavailable Hg concentrations in the particular soils (between 0.015 µg Hg g\(^{-1}\) and 0.36 µg Hg g\(^{-1}\) dry soil) also caused significant changes in the T-RFLP profiles in all of the seven soils. This indicates that Hg affects both bacterial community structure and microbial activity, which is globally in accordance with previous studies performed in temperate (Müller et al. 2001; Rasmussen et al. 2001) and tropical soils (Harris-Hellal et al. 2009).
The T-RFLP profiles from the highest Hg amendments were generally characterized by the appearance and dominance of some OTUs over the whole community profile, as well as reduction in abundance of other OTUs. Similar effects on the bacterial communities reported in this study are in accordance with that reported by Ranjard et al. (2000) on decreases in the fraction of the most abundant phylotypes in the bacterial community of an agricultural soil exposed to Hg (10 μg Hg g⁻¹ dry soil) compared with the corresponding non-contaminated soil. The changes in bacterial community structure observed after 30 days of incubation are likely to be due to the replacement of Hg sensitive populations by ones more tolerant to Hg stress. Exposure of microbial communities to Hg stress typically results in an initial decline in the bacterial numbers, followed by a rapid growth of Hg-resistant populations (Müller et al. 2001; Rasmussen et al. 2001; Holtze et al. 2003). We isolated several OTUs which were tolerant to Hg. Interestingly, we found OTUs with similar lengths in different soils. In Burgdorf and Gerlafingen, OTUs of 140bp and 437bp became dominant in Hg treated soils. Cloned sequences of OTU 140bp were most similar (98 - 100%) to a Burkholderia sp. strain isolated from acidic forest soils (Kunito et al. 2012) or uncultured Burkholderia sp. clone obtained from a Cd-contaminated acid forest soil (Lazzaro et al. 2008). Cloned sequences of OTUs (401bp and 437bp) which were tolerant to Hg belong to the order Rhodospirillales and Sphingomonadales and closest relatives were found in polychlorinated biphenyl-polluted soils (Nogales et al. 2001).

Hg resistance to cope with high Hg contamination in soil has been described in several eubacterial phyla (Firmicutes, Actinobacteria, and Proteobacteria) and in several archaea genomes (Hart et al. 1998; Holtze et al. 2006; Oregaard et al. 2007). All Hg tolerant OTUs identified in our study were closely related to Alpha- and Beta-proteobacteria. The resistance of bacteria to inorganic Hg is associated with the presence of the Hg resistance (mer) operons (Barkay et al. 2003). The mercuric reductase enzyme (MerA) is responsible for the tolerance to inorganic Hg as it catalyses the reduction of Hg(II) to the volatile Hg(0) which passively diffuses out of the cell (Barkay et al. 2003).
The bioavailability of HMs is essential for its impact to soil microorganisms (Lazzaro et al. 2006; Lazzaro et al. 2006; Frey et al. 2008). The largest part of the supplied Hg (98.7 – 99.9%) was bound to the solid phase and thus was not bioavailable (Table 4.1). The recovery of the spiked Hg in soil solution was generally low which was also reported by others (Rasmussen et al. 2000; Ivask et al. 2002; Petänen et al. 2002). In particular in the calcareous soils (Laufen and Sihlwald) bioavailable Hg was minor or below the detection limit of the method (0.1 µg Hg L\(^{-1}\)) and the negative effects of the supplied Hg on the soil bacterial community structures were less pronounced. This confirms the strong adsorption capacity of soils for Hg and its relation with soil characteristics such as clay, organic matter or pH, as already reported by others (Ranjard et al. 1997; Rasmussen et al. 2000; Ivask et al. 2002; Petänen et al. 2002). We found a small but not significant negative relationship between pH and bioavailable Hg (R\(^2\)=0.35; p=0.16) and a small positive relationship between DOC and bioavailable Hg (R\(^2\)=0.08; p=0.54; for soils spiked with 32 µg Hg g\(^{-1}\) dry soil). Forest soils with high organic matter content show a high affinity to bind Hg(II) (Yin et al. 1996; Meili et al. 2003; Skyllberg et al. 2010). Pant et al. (2007) and Obrist et al. (2009) reported strong correlations (r\(^2\)=0.52; r\(^2\)=0.83) between Hg concentrations and C\(_{org}\) in a contaminated and uncontaminated soils.

The impact of Hg on the bacterial communities was site specific and thus depended on soil properties. In general, with reducing pH values more metallic cations (e.g. Hg) are released into the soil solution (Schuster 1991), and thereby enhancing their bioavailability. Furthermore, the strong affinity of Hg to DOM facilitates its solubility but the complexation of Hg with DOM limits its bioavailability at the same time (Ravichandran 2004). Consequently, the bacterial communities and the basal respiration in soils from Laufen and Sihlwald were the least affected by Hg treatments. In these soils, the measured bioavailable Hg concentrations were lowest which mainly correspond to the low DOC concentrations. Besides the soil type also the speciation and form of the Hg in soil after amendment is important with regard to the ecotoxicity of Hg. Schuster (1991) stated that chloride may be regarded as one of the most mobile and persistent complexing agents for Hg. Thus, HgCl\(_2\)
added into soils might persist in its original form or, more likely, will be bound as an Hg-chloride complex or other reactive Hg$^{2+}$ species to the soil matrix. Han et al. (2003) studied the speciation of Hg and indicated that very little Hg existed in the free ionic form, while a large proportion existed in the form of HgCl$_2$ that was lipophilic and consequently bioavailable (Bollen et al. 2008). Therefore, most Hg in the soil is suggested to be matrix-bound HgCl$_2$ or bound to organic substances (Skyllberg et al. 2000; Skyllberg et al. 2003).

4.6 Conclusions

The study clearly showed that enhanced Hg treatments ($\geq$ 3.2 $\mu$g Hg g$^{-1}$ dry soil) strongly affect microbial activities and bacterial community structures in soils. Critical limit concentrations for soluble Hg were estimated to be 0.004 $\mu$g Hg g$^{-1}$ dry soil. The impact of Hg on soil bacterial communities is likely to be dependent on the soil type where the different soil physico-chemical factors directly influence the metal mobility, bioavailability and the toxicity, and consequently acts on microbial activities and community structures. The soluble fraction of Hg was low with Hg of 98.7 - 99.9% remained sorbed to the solid phase and thus was not available to the soil bacteria. In particular in the calcareous soils bioavailable Hg was minor and the negative effects of the supplied Hg on the soil bacterial community structures were less pronounced.

4.7 Acknowledgements

We are grateful to Andreas Rüdt and Stefan Schmutz for laboratory assistance. Financial support was provided by the Federal Office for the Environment (09-0084.PJ/I235-1899 and 10-0022.PJ/K314-0658).
4.8. Supplementary matter

Supplementary Table S4.1. Coordinates, elevation, geology and vegetation types of the forest soils sampled.

<table>
<thead>
<tr>
<th>Location</th>
<th>Coordinates</th>
<th>Elevation (m)</th>
<th>Soil type</th>
<th>Dominating tree species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerlafingen</td>
<td>7° 33’ 28” E- 47° 10’ 27” N-</td>
<td>477</td>
<td>Dystric Cambisol</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Burgdorf</td>
<td>7° 35’ 49” E- 47° 02’ 16” N-</td>
<td>625</td>
<td>Dystric Cambisol</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Lausanne</td>
<td>6° 39’ 28” E- 46° 34’ 54” N-</td>
<td>800</td>
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<td>Fagus sylvatica</td>
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<tr>
<td>Piotta</td>
<td>8° 40’ 23” E- 46° 30’ 44” N-</td>
<td>1150</td>
<td>Dystric Cambisol</td>
<td>Picea abies</td>
</tr>
<tr>
<td>Schänis</td>
<td>9° 04’ 04” E- 47° 09’ 51” N-</td>
<td>730</td>
<td>Eutric Cambisol</td>
<td>Fagus sylvatica</td>
</tr>
<tr>
<td>Laufen</td>
<td>7° 25’ 35” E- 47° 23’ 28” N-</td>
<td>678</td>
<td>Eutric/Gleyic Cambisol</td>
<td>Quercus petraea</td>
</tr>
<tr>
<td>Sihlwald</td>
<td>8° 34’ 06” E- 47° 14’ 51” N-</td>
<td>503</td>
<td>Eutric/Gleyic Cambisol</td>
<td>Fraxinus excelsior</td>
</tr>
</tbody>
</table>

Supplementary Table S4.2. Physico-chemical characteristics of the soils tested have been presented by Ernst et al. (2008). CEC = cation exchange capacity, BS = base saturation.

<table>
<thead>
<tr>
<th>Location</th>
<th>BS (%)</th>
<th>C (%)</th>
<th>CEC (cmolc kg⁻¹)</th>
<th>Sand (%)</th>
<th>Silt (%)</th>
<th>Clay (%)</th>
<th>Hg content (µg g⁻¹ dry soil) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerlafingen</td>
<td>41</td>
<td>4.1</td>
<td>100</td>
<td>36</td>
<td>40</td>
<td>24</td>
<td>0.197</td>
</tr>
<tr>
<td>Burgdorf</td>
<td>51</td>
<td>2.1</td>
<td>66</td>
<td>66</td>
<td>23</td>
<td>11</td>
<td>0.183</td>
</tr>
<tr>
<td>Lausanne</td>
<td>29</td>
<td>3.5</td>
<td>77</td>
<td>62</td>
<td>25</td>
<td>13</td>
<td>0.077</td>
</tr>
<tr>
<td>Piotta</td>
<td>91</td>
<td>8.2</td>
<td>130</td>
<td>66</td>
<td>25</td>
<td>9</td>
<td>0.130</td>
</tr>
<tr>
<td>Schänis</td>
<td>96</td>
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<td>118</td>
<td>43</td>
<td>35</td>
<td>22</td>
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<tr>
<td>Laufen</td>
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<td>5.0</td>
<td>383</td>
<td>6</td>
<td>39</td>
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<td>0.114</td>
</tr>
<tr>
<td>Sihlwald</td>
<td>100</td>
<td>4.4</td>
<td>297</td>
<td>14</td>
<td>33</td>
<td>53</td>
<td>0.128</td>
</tr>
</tbody>
</table>

* HNO₃-extractable Hg concentrations measured with cold-vapour AAS (Ernst et al. 2008; Rieder et al. 2011)
**Supplementary Table S4.3:** Absolute values of basal respiration (µg CO$_2$ g$^{-1}$ h$^{-1}$; mean ± standard deviation).

<table>
<thead>
<tr>
<th>Location</th>
<th>controls CaCl$_2$ (µg g$^{-1}$ dry soil)</th>
<th>H$_2$O (µg g$^{-1}$ dry soil)</th>
<th>Hg treatment (µg g$^{-1}$ dry soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.032</td>
</tr>
<tr>
<td>Gerlafingen</td>
<td>4.92 ± 0.16</td>
<td>4.99±0.37</td>
<td>4.79±0.14</td>
</tr>
<tr>
<td>Burgdorf</td>
<td>14.99±0.5</td>
<td>14.58±0.7</td>
<td>14.42±0.6</td>
</tr>
<tr>
<td>Lausanne</td>
<td>3.06±0.5</td>
<td>3.05±0.6</td>
<td>2.97±0.4</td>
</tr>
<tr>
<td>Piotta</td>
<td>15.91±0.3</td>
<td>16.07±0.5</td>
<td>15.95±0.3</td>
</tr>
<tr>
<td>Schänis</td>
<td>1.78±0.5</td>
<td>1.80±0.7</td>
<td>1.78±0.3</td>
</tr>
<tr>
<td>Laufen</td>
<td>13.14±0.6</td>
<td>13.05±0.6</td>
<td>12.10±0.8</td>
</tr>
<tr>
<td>Sihwald</td>
<td>10.76±1.6</td>
<td>10.91±0.7</td>
<td>9.97±0.9</td>
</tr>
</tbody>
</table>
Chapter 5

Methyl-mercury affects microbial activity and biomass, bacterial community structure but hardly the fungal community structure
Stephan Raphael Rieder¹,²,³,⁴ and Beat Frey¹,⁴

This chapter is published in Soil Biology and Biochemistry (2013) 64: 164 – 173

Contribution of the authors:
¹Experimental design; ²performing experiments; ³data analyzing; ⁴paper writing

5.1 Abstract

Monomethylmercury is one of the most toxic compounds. Methylation of Hg usually appears under anoxic conditions. In Swiss forest soils, methyl-Hg concentrations of up to 3 µg kg⁻¹ soil dw have been observed, but the impact of methyl-Hg on soil microorganisms have rarely been examined so far. In this study, we investigated the effect of increasing concentrations of methyl-Hg (0, 5, 20, 90 µg kg⁻¹ soil dw) on the microbial communities in various forest soils differing in their physicochemical properties. Experiments were conducted in microcosms under controlled conditions and the basal respiration (BR), the microbial biomass carbon (MBC) and the bacterial and fungal community structures using T-RFLP-profiling were investigated. BR was significantly affected by methyl-Hg. In general, the BR increased with increasing methyl-Hg concentrations, whereas the MBC was significantly reduced. Bacterial communities were more sensitive to methyl-Hg than fungal communities. In five out of seven soils, the bacterial community structures differed significantly between the treatments whereas the fungal communities did not. The impact of methyl-Hg on the soil bacterial communities was site specific. In one soil, a methyl-Hg concentration of already 5 µg kg⁻¹ soil dw significantly affected the relative abundance of 13% bacterial operational taxonomic units (OTU), whereas in other soils concentrations of even 90 µg kg⁻¹ soil dw rarely affected the abundance of OTUs. In this study, for the first time, the impact of methyl-Hg on soil bacterial
and fungal communities in forest soils was assessed. We showed that its impact strongly depends on the physico-chemical conditions of the soil and that bacterial communities were more sensitive to methyl-Hg than fungi.

5.2 Introduction

Mercury (Hg), and in particular monomethylmercury (CH$_3$Hg$^+$; methyl-Hg), are very toxic for most organisms. Mercury is a naturally occurring metal, which is emitted from natural and anthropogenic sources to the atmosphere (Schroeder et al. 1998; Swain et al. 2007). Once emitted into the atmosphere, about half of the Hg is deposited locally [mainly as Hg(II)] (Mason et al. 1994) whereas the remaining Hg (mainly as Hg$^0$) enters to the global circulation with a residence time of approximately one year (Fitzgerald et al. 1997; Seigneur et al. 2004). Hg$^0$ in the atmosphere can be oxidized to Hg(II) with a much shorter lifetime in the atmosphere (days to weeks) than of Hg$^0$ (Slemr et al. 1981; Lindqvist et al. 1985), and oxidized Hg can be subjected to fall out and accumulate in the topsoil or photoreduced to Hg$^0$ again (Selin 2009). Thus Hg can be widely distributed over the globe before returning to the earth’s surface, where it mostly accumulates in the top soil layer. The concentrations of Hg in the top layer of forest soils in Switzerland have been found range between 0.07 and 0.55 mg kg$^{-1}$ soil dw (Ernst et al. 2008), with methyl-Hg percentages between 0.2 and 2.4% of total Hg (Boudou et al. 1997; Rieder et al. 2011). In soils, methyl-Hg strongly binds to the thiol-groups of organic matter (Qian et al. 2002). Mercury is methylated mainly through biotic processes in the soils, involving different groups of organisms, whereby sulphate-reducing bacteria (SRB) living under anoxic conditions are assumed to be mainly responsible for the methylation of Hg (Drott et al. 2007; Holloway et al. 2009). In addition, methyl-Hg may be transported into the soils through rain and litterfall (St Louis et al. 2001).

The toxicity of Hg strongly depends on its chemical species, with methyl-Hg compounds as the most poisonous species (Shao et al. 2012), mainly due to its affinity of Hg for thiol groups of proteins and enzymatic co-factors, Hg ions (Hg$^{2+}$) may affect proteins in three different
ways: (1) by binding to proteins (e.g. to thiol groups) and changing their functions, (2) by replacing essential ionic elements on proteins or (3) by inhibiting the refolding of denaturised proteins (Sharma et al. 2001). Several studies have investigated the effect of Hg(II) pollution on soil microbial processes and community structures. Van Faassen (1973) observed that very high concentrations of Hg-compounds (HgCl$_2$, phenyl-Hg-acetate) in soils inhibited the basal respiration (BR), nitrification and the dehydrogenase activity. Previous research has shown that microbial respiration is reduced in soils treated with inorganic Hg (Tu 1988; Bringmark et al. 2001; Palmborg et al. 2001), but still little is known about how toxic methyl-Hg is for soil microorganisms. We are aware of only three prior studies that described the impact of methyl-Hg on soil microorganisms. Bacterial cell numbers decreased on plating media containing methyl-Hg (Van Faassen 1973) and fungal cell growth was reduced in soils with methyl-Hg twice as much as with HgCl$_2$ (Kungolos et al. 1999). Soils contaminated with methyl-Hg largely affected the bacterial community structures in earthworms (Rieder et al. 2013). The mercuric reductase enzyme $MerB$ is known to be responsible for organic Hg tolerance in bacteria but no organic Hg detoxification mechanism is currently known for organic Hg in fungi. The $MerB$ enzyme catalyzes the degradation of organic Hg compounds to the less toxic form Hg(II) (Barkay et al. 2003). Both bacteria and fungi are important players in the decomposition of organic matter and thus in nutrient cycling and soil fertility (Berg and Mc Claugherty 2007).

Here, we report on a study of the impact of increasing concentrations of methyl-Hg on soil microbial activities and community structures. Seven different forest soils were spiked with four rates of methyl-HgCl (0, 5, 20, 90 $\mu$g methyl-Hg kg$^{-1}$ soil dw) in microcosms. The concentrations were chosen according to methyl-Hg concentrations in the top layers of Swiss forest soils (Rieder et al. 2011). We examined the impact of methyl-Hg by analyzing the BR, the microbial biomass carbon (MBC) and bacterial and fungal community structures using T-RFLP profiling. As far as we know, this is the first study of the effects of increasing concentrations of methy-Hg on bacterial and fungal communities in forest soils differing in soil physico-chemical characteristics.
5.3 Materials and methods

5.3.1 Study sites

Seven forest soils with a broad range of physical and chemical properties containing, with no or a thin litter layer (1 cm) were chosen from a soil profile database (Ernst et al. 2008) of the Swiss Federal Research Institute WSL, Birmensdorf, Switzerland (Table 5.1). After removal of the litter layer, soil was collected at a soil depth of 0-10 cm (A-horizon) with a soil corer (diameter 7 cm). At each site, ten samples of the mineral soil were collected at random locations from an area of approximately 5 × 5 m, then pooled in a plastic bag and returned to the laboratory. The collected soil was sieved (2 mm mesh) and stored at 4°C for a week before use.

Table 5.1: Physico-chemical properties of the seven investigated forest soils (Lazzaro et al. 2006; Rieder et al. 2011).

<table>
<thead>
<tr>
<th>Location*</th>
<th>pH</th>
<th>BS [%]</th>
<th>CEC** [cmolc kg⁻¹]</th>
<th>C [%]</th>
<th>N [%]</th>
<th>DOC [mg C l⁻¹]</th>
<th>Sand [%]</th>
<th>Silt [%]</th>
<th>Clay [%]</th>
<th>Hg*** [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burgdorf</td>
<td>4.5</td>
<td>51</td>
<td>66</td>
<td>2.1</td>
<td>0.1</td>
<td>71</td>
<td>66</td>
<td>26</td>
<td>11</td>
<td>0.08</td>
</tr>
<tr>
<td>Gerlafingen</td>
<td>4.6</td>
<td>41</td>
<td>100</td>
<td>4.1</td>
<td>0.3</td>
<td>59</td>
<td>36</td>
<td>40</td>
<td>24</td>
<td>0.19</td>
</tr>
<tr>
<td>Laufen</td>
<td>6.8</td>
<td>100</td>
<td>383</td>
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<td>24</td>
<td>6</td>
<td>39</td>
<td>55</td>
<td>0.10</td>
</tr>
<tr>
<td>Lausanne</td>
<td>4.6</td>
<td>29</td>
<td>77</td>
<td>3.5</td>
<td>0.2</td>
<td>23</td>
<td>62</td>
<td>25</td>
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<td>0.08</td>
</tr>
<tr>
<td>Piotta</td>
<td>5.0</td>
<td>91</td>
<td>130</td>
<td>8.2</td>
<td>0.5</td>
<td>38</td>
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<td>9</td>
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<tr>
<td>Schänis</td>
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<tr>
<td>Sihlwald</td>
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<td>297</td>
<td>4.4</td>
<td>0.3</td>
<td>17</td>
<td>14</td>
<td>33</td>
<td>53</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* Burgdorf (7°35'E, 47°02'N); Gerlafingen (7°33'E, 47°10'N); Laufen (7°25'E, 47°23'N); Lausanne (6°39'E, 46°34'N); Piotta (8°40'E, 46°30'N); Schänis (9°04'E, 47°09'N); Sihlwald (8°34'E, 47°14'N)

** CEC = cation exchange capacity

*** HNO₃ extractable concentrations

5.3.2 Experimental design

Four mg CH₃HgCl was dissolved in 100 ml sterile Milli-Q-water. The soils from each sampling site were filled in sterile plastic bags and small portions of a methyl-Hg solution
(1ml) were mixed with the soil to reach soil concentrations of 0, 5, 20 and 90 µg methyl-Hg kg\(^{-1}\) soil dw. During the treatment, the plastic bags were gently shaken several times to mix and homogenize the soils. The water content of each soil was adjusted to 35% by adding sterile Milli-Q water and the soils were gently shaken again. We chose these concentrations (0, 5, 20 and 90 µg methyl-Hg kg\(^{-1}\) soil dw) because forest top soils in Switzerland contain between <1 and 3 µg\(^{-1}\) methyl-Hg kg\(^{-1}\) soil dw, whereas soil biota may contain much higher methyl-Hg concentrations (Rieder et al. 2011). Soil microorganisms involved in the decomposing of dead fungi and earthworms are assumed to be exposed to such high concentrations of methyl-Hg. For this study, we therefore chose mean concentrations of methyl-Hg in mushrooms (20 µg kg\(^{-1}\) dw) and in earthworms (90 µg kg\(^{-1}\) dw) as the highest methyl-Hg rates. We also conducted control experiments with soils treated with CaCl\(_2\) to examine the effect of Cl on the microbial communities. However, we did not observe any differences between the two control treatments (CaCl\(_2\) versus water; data not shown).

A mixture of lyophilized powder of needle and leaf litter was mixed into the soils (1.5% of weight) as the food source for the microorganisms. 30g soils of each methyl-Hg treatment were filled in air-permeable boxes (diameter 55mm) and incubated at 15°C in the dark. Each treatment was prepared in three replicates. During the incubation, the water content was determined gravimetrically every second day and, if necessary, any loss was compensated by adding sterilized Milli-Q water to retain the initial humidity. Soil aliquots of the microcosms were sampled at the beginning and at the end of the incubation (28 days). In a preliminary study, we found that approximately 30% of the methyl-Hg added to the soils was decomposed during a 28 day incubation period but that the total amount of Hg did not change (data not shown).

### 5.3.3 Basal respiration

The BR was measured in a closed soil-chamber system at 5 time points (after 1, 2, 8, 15, 28 days) during the incubation. The soil-boxes were connected to the CO\(_2\)-free air source, the red-y smart gas flow meter GSM-B5SA-BM00 (Vögtlin Instruments AG, Aesch, Switzerland).
and the Li-8100 infrared gas analyzer (LI-COR Inc., Lincoln, NE, USA). CO$_2$-free air flowed at a rate of about 0.16 L min$^{-1}$ through the boxes, and entrained the CO$_2$ just released from the soil to the infrared gas analyzer. After reaching a steady state situation (after about 10 minutes), the gas flow and the CO$_2$ concentration were noted and the BR was calculated according equation (1). At the beginning of analyses and after every 10$^{th}$ measurement, two boxes without soil were connected to the system to measure the background signal (blank).

$$\text{CO}_2\text{-efflux} = \text{airflow}_{\text{sample}} \times \text{CO}_2{\text{sample}} - \text{airflow}_{\text{blank}} \times \text{CO}_2{\text{blank}} \times 22.4^{-1} \times 12000 \times 10^{-6}$$  \hspace{1cm} (1)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$-efflux</td>
<td>Basal respiration (BR)</td>
<td>[mg CO$_2$-C min$^{-1}$]</td>
</tr>
<tr>
<td>Airflow</td>
<td>Flow rate in the steady state situation</td>
<td>[l min$^{-1}$]</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>CO$_2$ concentration in the steady state situation</td>
<td>[ppm]</td>
</tr>
<tr>
<td>22.4$^{-1}$</td>
<td>Volume Mol air</td>
<td>[L]</td>
</tr>
<tr>
<td>12000</td>
<td>Molar mass of C</td>
<td>[mg]</td>
</tr>
</tbody>
</table>

The cumulative BR was calculated through linear interpolation between the measurements, and represents the total amount of CO$_2$-C released from the soil.

### 5.3.4 Substrate induced respiration

At the end of the experiments the MBC was assessed according to Anderson et al. (1978) by the substrate-induced respiration (SIR) method. The MBC was calculated from the maximal respiration rate after adding an easily consumable energy source (6 ml of 0.3M glucose) to the soils. The current concentrations of CO$_2$ released from the soils were repeatedly analyzed until a plateau in the CO$_2$ releasing rate was achieved (about 1 hour after adding the glucose). The MBC was then estimated from this CO$_2$ release and the gas flow rate at the time of the plateau situation according to equation (2). Empty boxes were used as in the BR measurements for blank analyses.
Ecotoxicity of methyl-Hg

\[ MBC = 40.04 \times (\text{airflow}_{\text{sample}} \times \text{CO}_2\text{sample} - \text{airflow}_{\text{blank}} \times \text{CO}_2\text{blank}) \times 60 \times 10^{-6} \times \text{g}^{-1} \text{ soil} + 0.0037 \quad (2) \]

- **MBC** = Microbial C content of active organisms [mg C g\(^{-1}\) soil]
- **Airflow** = Flow rate when plateau was reached [ml min\(^{-1}\)]
- **CO\(_2\)** = CO\(_2\) concentration when plateau was reached [ppm]

### 5.3.5 DNA extraction and PCR amplification

Genomic DNA from the soils was extracted by using a bead-beating method (Frey et al. 2008). For each assay, approximately 400 mg of soil was treated with the FastPrep-24 system (MP Biomedicals, California, USA). The extracted DNA was quantified with PicoGreen (Invitrogen, San Diego, CA, USA) and stored at -20°C until processing.

DNA aliquots (about 5 ng µl\(^{-1}\)) from the soil samples were pre-treated (1:5) with BSA at 95°C for 4 min. Bacterial DNA was amplified with a polymerase chain reaction (PCR) targeting the 16S rRNA genes (Frey et al. 2006), and fungal DNA with a PCR targeting the *ITS* region (Gardes and Bruns 1995). 5µl of pre-treated DNA (corresponding to 15 ng DNA) were mixed with 20 µl PCR reaction mix containing 1x PCR buffer, 0.5mM MgCl\(_2\), 400µM dNTP, 0.6mg ml\(^{-1}\) BSA and 0.05U µl\(^{-1}\) Hot star Taq polymerase (Qiagen, Hilden, Germany) and 0.2µM of the fluorescently (FAM) labelled forward primer and unlabelled reverse primers were used.

To targeting the 16S rRNA genes, the forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 1378R (5'-CGGTGTGTACAAAGCCGGAACG-3') (Heuer et al. 1997) were used, while to target the *ITS* region, the forward primer ITS1F (5'-CTTGGTTCCTTAGGAGGAATGAA-3') (Gardes et al. 1995) and the reverse primer ITS4 (5'-TCCTCCGGCTTATTGATAGC-3') (White et al. 1990) were used. The PCR reactions were conducted in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The reactions were started with an initial denaturizing step at 95°C for 15 min. Afterwards, 35 cycles of the following steps were conducted: (a) for 16S rRNA genes: (1) 95°C for 45s, (2) 48°C for 45s and (3) 72°C for 2 min and (b) for the
ITS region: (1) 95°C for 60s, (2) 56°C for 60s and (3) 72°C for 2 min. A final extension step at 72°C for 5 min completed the reactions.

5.3.6 T-RFLP profiling of bacterial and fungal communities

Bacterial and fungal community structures were studied by using T-RFLP profiling (Frey et al. 2006; Lazzaro et al. 2008; Zumsteg et al. 2012). For the bacterial community structures, the 16S rRNA gene amplicons were digested with 0.1U of two different restriction enzymes (HaeIII or MspI) separately, according to the manufacturer's recommendations (Thermo Fischer Scientific, Waltham, USA). For fungi, the ITS amplicons were digested with TaqI (0.1U). The digested DNA was cleaned with the Montage SEQ96 sequencing reaction cleanup kit (Millipore, Billerica, USA) according to the manufacturer’s instructions. For capillary electrophoresis, 1 µl of digested DNA was mixed with 12.9 µl HiDi formamide (Applied Biosystems) and with 0.1 µl of the fragment length standard ROX500 (Applied Biosystems). This mixture was heated at 95°C for 2 min before analysis using the ABI Genetic Analyzer 310 (Applied Biosystems). T-RFLP profiles were analysed using the software packages GeneScan V.3.1 and Genotyper V.2.5 (Applied Biosystems) according to Frey et al. (2011). The bacterial and fungal T-RFLP profiles were analyzed separately for each soil. The sizes of the operational taxonomic units (OTU) were defined for peaks between 50 and 500 base pairs (bp) using a threshold value of 100 fluorescence units. T-RFLP profiling data were normalized by calculating the relative abundance of each T-RF according to Zumsteg et al. (2012).

5.3.7 Statistical analyses

The effects of the soil methyl-Hg treatments on the BR was tested with repeated measures ANOVA (5 time points), the MBC and the relative abundance of T-RFs were tested with ANOVA and Post-hoc Tukey-HSD test using the software STATISTICA (v. 8.0; StatSoft, Tulsa, USA). A Bray Curtis similarity matrix (Bray et al. 1957) from square-root-transformed T-RFLP data were calculated using the software Primer 6 v.6.1.13 and Permanova v.1.0.3.
(Primer-E, UK) for studying microbial community structures. To visualize any differences in the bacterial and fungal soil community structures between the soils with different levels of methyl-Hg, canonical analysis of principal coordinates (CAP) analyses were performed (Anderson et al. 2003). The constrained ordination technique CAP reveals differences in community structure according to the methyl-Hg levels in the soils. Statistical differences between the methyl-Hg treatments on the bacterial and fungal community structures were estimated through Permutational MANOVA analyses with Primer 6 v.6.1.13. Figures were generated with Permanova and Excel (Microsoft Corporation, Berkshire, USA).

5.4 Results

5.4.1 Basal respiration

The BR in unpolluted soils varied significantly (p<0.0001) among the different sites. During the first day of incubation, soils from Burgdorf (5.2 ± 0.4 mg CO₂-C) and Sihlwald (4.8 ± 0.3 mg CO₂-C) released the largest amount of carbon to the atmosphere. The CO₂ release in the other five soils was between 2.3 and 2.9 mg CO₂-C (data not shown). In all soils, the BR decreased in the following 24 hours by about 6% (Piotta), 23% (Lausanne), 28% (Schänis), 43% (Sihlwald), 47% (Burgdorf, Laufen) and 52% (Gerlafingen). The cumulative BR of the seven soils is shown in Figure 5.1.

BR was significantly affected (p<0.01) by the methyl-Hg treatments in four out of seven soils (Figure 5.1). In general, soils (Gerlafingen, Laufen, Lausanne, Sihlwald) treated with methyl-Hg showed a higher BR compared to the non-methyl-Hg treated soils. However, in Piotta the two highest methyl-Hg treatments showed the lowest BR.
Figure 5.1: Mean cumulative basal respiration (BR) of the seven forest soils during incubation at different methyl-Hg levels (µg methyl-Hg kg⁻¹ dry soil). Treatments: 0: no methyl-Hg added; 5: 5 µg methyl-Hg kg⁻¹ dry soil; 20: 20 µg methyl-Hg kg⁻¹ dry soil; 90: 90 µg methyl-Hg kg⁻¹ dry soil. **Note**: the y-axis scale varies. † Overall effects of either time or methyl-Hg treatment or their interactions obtained from MANOVA with repeated measures. ‡ Effects of treatment at the particular timepoints. Significance levels: p<0.05; ** p<0.01; n.s. p>0.05.
5.4.2 Microbial biomass carbon

The MBC concentration in untreated forest soils differed significantly (p<0.05) (Figure 5.2). The highest MBC levels were observed in Burgdorf (0.95 ± 0.04 mg C g⁻¹ soil dw) and Laufen (0.85 ± 0.05 mg C g⁻¹ soil dw), and the lowest in Lausanne (0.34 ± 0.05 mg C g⁻¹ soil dw) and Piotta (0.45 ± 0.03 mg C g⁻¹ soil dw). Methyl-Hg pollution significantly (p<0.05) affected the MBC levels in five soils (Burgdorf, Gerlafingen, Lausanne, Piotta, Schänis). Concentrations of 5 µg methyl-Hg kg⁻¹ soil dw did not affect the MBC concentration in Gerlafingen (p=0.955), Piotta (p=0.978) and Schänis (p=0.995), increased MBC in Burgdorf (p=0.575), Laufen (p=0.056) and Sihlwald (p=0.495) or decreased MBC in Lausanne (p=0.167). Concentrations of 20 µg methyl-Hg kg⁻¹ soil dw decreased the MBC in five out of seven soils but only in Burgdorf (p=0.045) on a significant level. In the soils with the highest methyl-Hg concentrations (90 µg methyl-Hg kg⁻¹ soil dw), MBC was significantly (p<0.05) reduced in five soils. In Lausanne (p=0.012), Piotta (p=0.004) and Schänis (p=0.002), the MBC decreased by more than 30%, in Burgdorf by about 12% and in Gerlafingen by about 25% (Figure 5.2).

Figure 5.2: Microbial biomass carbon (MBC) [mean ± SD] calculated from the substrate induced respiration of the seven forest soils after 28 days of incubation at different methyl-Hg levels (µg methyl-Hg kg⁻¹ dry soil). Different letters (a, b, c) mark significant (p<0.05) differences in MBC between the treatments. No letters mean that no significant differences in MBC exists between the treatments at the particular site.
5.4.3 Bacterial community structure

Between 95 and 123 OTUs (HaeIII) and between 99 and 141 OTUs (MspI) were identified in the T-RFLP profiles of the different forest soils. Since the analysis of the T-RFLP profiles was similar independently of the restriction enzymes (data not shown) we only presented the data of HaeIII (Table 5.2). The abundance of several OTUs were significantly different in soils with 5 µg methyl-Hg kg\(^{-1}\) soil dw than in soils without methyl-Hg, except for Sihlwald (Table 5.2). In Sihlwald, the relative abundance of 14 OTUs (13%; digestion with HaeIII) were significantly affected at 5 µg methyl-Hg kg\(^{-1}\) soil dw compared to the controls. In general, the higher methyl-Hg levels in soils the more OTUs were affected (Table 5.2). The abundance of 27 OTUs (24%, HaeIII) in Sihlwald were significantly inhibited or enhanced with 90 µg methyl-Hg kg\(^{-1}\) soil dw.
Table 5.2: Numbers of bacterial and fungal OTUs significantly (p<0.05) increased or decreased in abundance by methyl-Hg compared to controls. Bacterial T-RFLP profiles (16S rRNA genes digested with HaeIII) and fungal T-RFLP profiles (ITS region digested with TaqI) were used.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Increased</th>
<th>Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 µg kg⁻¹</td>
<td>20 µg kg⁻¹</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burgdorf</td>
<td>105</td>
<td>4</td>
</tr>
<tr>
<td>Gerlafingen</td>
<td>97</td>
<td>2</td>
</tr>
<tr>
<td>Laufen</td>
<td>123</td>
<td>2</td>
</tr>
<tr>
<td>Lausanne</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>Piotta</td>
<td>97</td>
<td>1</td>
</tr>
<tr>
<td>Schänis</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>Sihlwald</td>
<td>111</td>
<td>6</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burgdorf</td>
<td>70</td>
<td>1</td>
</tr>
<tr>
<td>Gerlafingen</td>
<td>75</td>
<td>1</td>
</tr>
<tr>
<td>Laufen</td>
<td>66</td>
<td>2</td>
</tr>
<tr>
<td>Lausanne</td>
<td>76</td>
<td>1</td>
</tr>
<tr>
<td>Piotta</td>
<td>86</td>
<td>0</td>
</tr>
<tr>
<td>Schänis</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>Sihlwald</td>
<td>69</td>
<td>0</td>
</tr>
</tbody>
</table>

*n=total number of OTUs
Permutational MANOVA analyses of T-RFLP profiles of the different soils revealed that methyl-Hg significantly (p<0.05) affected the bacterial community structures of four soils [(Gerlafingen (p=0.028), Laufen (p=0.024), Schänis (p=0.021) and Sihlwald (p=0.0004)]. In Burgdorf, the bacterial community structure was affected by methyl-Hg at a significant level of p=0.051 (Figure 5.3). In particular, CAP ordinations showed clear differences between the highest methyl-Hg concentrations and the non-treated controls in Laufen and Sihlwald, whereas in the other soils the changes in the bacterial community structures between the methyl-Hg treatments were less distinct (Figure 5.3).
Figure 5.3: Canonical analysis of the principal coordinates (CAP) ordinations of bacterial communities (16S rRNA) based on the relative abundances of OTUs. These constrained ordinations show maximized differences among the different methyl-Hg levels (µg methyl-Hg kg⁻¹ dry soil; symbols). The p-values indicate the effects of Hg treatments on the bacterial community structure using Permutational MANOVA analysis.
5.4.4 Fungal community structure

Between 66 and 90 OTUs were identified in the T-RFLP profiles of the different forest soils after digestion of the ITS amplicons with TaqI (Table 5.2). The abundance of only a few (up to five) OTUs were significantly affected at a level of 5 µg methyl-Hg kg\(^{-1}\) soil dw compared to controls (Table 5.2). Higher concentrations of methyl-Hg in soils did not further affect the abundance of more OTUs. The CAP analyses of the T-RFLP profiles showed a separation between the methyl-Hg treatments in Burgdorf and Gerlafingen, but not in the other soils (Figure 5.4). Nevertheless, the statistical analyses using Permutational MANOVA of the different T-RFLP profile revealed that the fungal community structures were not significantly different between the treatments (Figure 5.4).
Figure 5.4: Canonical analysis of the principal coordinates (CAP) ordinations of fungal communities (ITS) based on the relative abundances of OTUs. These constrained ordinations show maximized differences among the different methyl-Hg levels (µg methyl-Hg kg\(^{-1}\) dry soil; symbols). The p-values indicate the effects of Hg treatments on the fungal community structure using Permutational MANOVA analysis.
5.5 Discussion

In this experiment, methyl-Hg was supplied as a methyl-Hg-chloride solution to seven forest soils, representing the most common forest soil types in Switzerland (Lazzaro et al. 2006; Ernst et al. 2008).

The BR in microcosms without methyl-Hg differed significantly in the seven soils. BR can be considered as microbial activity (carbon mineralization) in soils that predominantly depend on the active microbial biomass, the quantity and quality of the organic material, the C and N ratio, the soil texture, the soil moisture and temperature. In our study, the initial BR from soils was between 50 mg C m\(^{-2}\) h\(^{-1}\) and 100 mg C m\(^{-2}\) h\(^{-1}\), which is in line with measurements at similar temperatures in other forest soils (Zimmermann and Frey 2002; Subke et al. 2003; Brumme et al. 2009) and laboratory incubation experiments (Frey et al. 2006; Lazzaro et al. 2006; Kammer et al. 2009). The BR (and also the MBC) may be underestimated due to the dissolution of produced CO\(_2\) in the soil water, in particular in alkaline soils. According to Henry’s law, we estimated that less than 5% of produced CO\(_2\) is dissolved in the soil water and has not been released. During the experiments, the BR decreased in all soils, due to exhaustion of easily degradable C pools. The high activity during the first days of incubation was caused by the mobilization of easily degradable C when sieving and mixing the soil, and the later decrease in activity during incubation is mainly due to the depletion of the carbon pools (Bringmark et al. 1998; Lazzaro et al. 2006).

The cumulative BR differed between the methyl-Hg treatments depending on the sampling site. In five soils, Gerlafingen, Laufen, Lausanne, Schänis and Sihlwald, the most contaminated soils showed the highest cumulative BR, and the soils without methyl-Hg the lowest. In contrast, in Burgdorf and Piotta, the highest methyl-Hg treatment inhibited the BR. Our findings can not be well compared to others. Generally, soils recently spiked with HM solutions reduce BR (Renella et al. 2002) but most of the information is available for cationic metals (e.g. Cd, Cu, Ni, Pb, Zn), although the impacts may be different for bacteria and fungi (Rajapaksha et al. 2004). In contrast, Hg is known to increase the BR (Landa et al. 1978) and Bringmark et al. (1998) observed a slight initial increase in BR in soils contaminated with
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Hg, followed by a clear reduction after 20 days of incubation. In contrast, Hg is also known to increase the BR (Landa et al. 1978). In soils treated with Pb, Zn or Ti, Leita et al. (1995) observed a significant increase in CO₂ evolution with all three metals. Rajapaksha et al. (2004) showed that bacterial and fungal activities were affected differently in soils polluted with the HMs Zn and Cu. They reported a slight decrease in bacterial activity in HM polluted soils in the first week of incubation. In contrast, the fungal activity in most contaminated soils increased 3 to 7 times compared to the control, which indicates that bacteria are more sensitive to HMs than fungi.

Methyl-Hg significantly affected the MBC in five soils. With increasing concentrations of methyl-Hg, the MBC decreased in all sites except for Laufen and Sihlwald, and by over 30% in Lausanne, Piotta and Schänis with methyl-Hg concentrations of 90 µg kg⁻¹ soil dw. Nevertheless, the BR was higher in soils treated with methyl-Hg than in the non-treated controls.

As a result of higher BR and lower MBC, the metabolic quotient (ratio of respiration to biomass, qCO₂) increased with the methyl-Hg treatments in all soils. Stressors such as HMs often increase the qCO₂ values (Fliessbach et al. 1994; Giller et al. 1998), but the contrary has also been found (Bååth et al. 1991). An explanation for the increased qCO₂ values in our study could be that methyl-Hg was toxic and some of the microorganisms died and were decomposed by microorganisms more tolerant to methyl-Hg. The increased qCO₂ values in soil treated with methyl-Hg could also be the consequence of a higher maintenance-energy requirement or a lower metabolic and substrate use efficiency (Leita et al. 1995; Giller et al. 1998; Renella et al. 2007).

Our findings could not be compared to others because we are not aware of previous studies that describe the impact of methyl-Hg on the bacterial and fungal community structure. Rasmussen et al. (2001) showed that the microbial community structure in soils may be altered at relatively low concentrations of inorganic Hg (250 µg Hg kg⁻¹ soil dw). We found that methyl-Hg in the soils we studied can significantly change the bacterial community structure at much lower concentrations (5 µg methyl-Hg kg⁻¹ soil dw). In five out of seven
forest soils, the bacterial community structures were significantly affected by methyl-Hg. In contrast, the fungal communities were less sensitive to methyl-Hg in all soils. Contamination with Hg typically results in an initial decrease in bacterial cell numbers and a subsequent growth of Hg-resistant bacteria (Rasmussen et al. 2001; Holtze et al. 2003).

Bacteria resistance to Hg compounds is associated with the presence of the Hg resistance (mer) operons (Robinson et al. 1984; Barkay et al. 2003). The mercuric reductase enzyme (MerA) is responsible for tolerance to inorganic Hg as it catalyzes the reduction of Hg(II) to the volatile Hg(0), whereas the MerB enzyme is responsible for organic Hg tolerance as it catalyzes the degradation of organic Hg compounds to the less toxic form Hg(II) (Barkay et al. 2003). Another mechanism thought to explain the resistance of bacteria to increased Hg concentrations is related to the methylation of Hg(II) (Oregaard et al. 2007). Newly formed methyl-Hg-compounds may diffuse through the cell membrane, so that the concentrations of Hg in the cells decrease.

Unlike for bacteria, no methyl-Hg detoxification mechanism is known for fungi, and the effect of methyl-Hg on fungi has also been less studied. Enhanced concentrations of HMs in soils generally reduce fungal growth and species diversity (Gupta et al. 2002), and methyl-Hg may reduce the cell growth of fungi twice as much as inorganic HgCl₂ (Kungolos et al. 1999). Many fungi are also known to tolerate increased HM concentrations in soils (Singh et al. 1974; Tam 1995; Gupta et al. 2002; Rajapaksha et al. 2004), including Hg concentrations (Brunker et al. 1974; Kelly et al. 2006). Meharg (2003) suggests that the organic acids exuded by fungi may complex metals in soils, which then immobilises them and reduces their toxicity. Detoxification of Hg(II) by fungi might be either due to the reduction in the volatile Hg⁰ species or the conversion of Hg species to beta-HgS (sulphide mineral), which facilitates the precipitation of Hg (Brunker et al. 1974; Aiking et al. 1985; Kelly et al. 2006). A methylation of Hg by fungi has also been described (Vonk et al. 1973) and may also favour the tolerance of inorganic Hg, but the detoxification of methyl-Hg by fungi has so far not been studied.
The impact of methyl-Hg on soil functioning and soil bacterial community structures in our study was site specific. In Laufen and Sihlwald, methyl-Hg significantly changed the bacterial community structure, but the MBC in these soils was not affected. In Sihlwald, the lowest methyl-Hg treatment significantly affected the relative abundance of 13% bacterial OTUs, whereas in Burgdorf, Lausanne and Piotta, the highest methyl-Hg treatments rarely affected the abundance of particular OTUs. These site-specific differences might be explained by their different bioavailabilities of methyl-Hg in soils.

Heavy metal bioavailability is the main factor causing the impact on soil microorganisms (Lazzaro et al., 2006a, b). Heavy metals can be adsorbed on clay particles, associated with carbonates, Fe- and Mn oxides, sulphides and organic matter (David and Leventhal 1996), and this metal partition may influence the impact of HMs to soil microbial activity and diversity (Adamo et al. 2006; D'Ascoli et al. 2006). Mercury and methyl-Hg strongly bind to organic matter and dissolved organic matter (DOM) (Hintelmann et al. 1997), and Tipping et al. (2011) reported that most of Hg in the soil solution can be considered as bound to DOM. A key factor governing Hg and methyl-Hg solubility in soils is the pH. In general, the lower the pH values, the more metallic cations are released into the soil solution (Schuster 1991; David et al. 1996), which enhances their bioavailability. Due to the strong affinity of Hg and methyl-Hg to organic matter, the release of Hg and methyl-Hg into the soil solution is associated with the DOM solubilization. This release strongly depends on the pH in the soil solution. A protonation of organic matter usually reduces the solubility of DOM, whereby metal-organic complexes are released into the soil solution with increasing pH values (Kalbitz et al. 2000). The strong affinity of methyl-Hg to DOM mediates its solubility, but at the same time the complexation of methyl-Hg with DOM limits its bioavailability (Ravichandran 2004). In this study we analyzed soils with pH values of 6.8 and 7.4, the soils from Laufen and Sihlwald were the only soils studied with neutral or slightly alkaline pH values. Nevertheless, given the low DOC values in Laufen and Sihlwald, less methyl-Hg was presumably in solution in these soils than in the soils from Burgdorf, Gerlafingen and Piotta. However, methyl-Hg still strongly affected the bacterial community structure in soils from
Sihlwald and the BR in soils from Laufen, but neither of the sites was the MBC in the soils affected by methyl-Hg treatments.

### 5.6 Conclusion

This short-term microcosm experiment showed the impact of methyl-Hg on soil bacterial and fungal community structures but no long-term prediction (chronic exposure) is possible (Renella et al. 2002). Methyl-Hg have differently influenced the bacterial community structure, the BR and the MBC in the different soils. Shifts in bacterial community structure do not necessarily affect the MBC. Similarly, a reduction in the MBC does not necessarily lead to a decrease in BR and a change in the microbial community structure. In general, methyl-Hg treatments increase the BR but decrease the MBC. As shown in this study, already very low concentrations of methyl-Hg (5 µg methyl-Hg kg\(^{-1}\) soil dw) did affect the soil bacterial community structure and soil functioning. Nevertheless, most investigated soils were only affected at higher methyl-Hg concentrations (20 and 90 µg kg\(^{-1}\) soil dw). Thus, we suggest a critical limit of about 12 µg methyl-Hg kg\(^{-1}\) soil dw but further studies are warranted to examine the effect of methyl-Hg on microorganisms in other soils. In this investigation only a short-term assay was conducted and there is a need for long-term studies to examine chronically exposure of microorganisms to methyl-Hg in soils. Swiss forest soils contain between <1 and 3 µg methyl-Hg kg\(^{-1}\) soil dw, which corresponds to 0.2 - 2.4% of total Hg. However, soil organisms such as earthworms or mushrooms may contain considerably higher concentrations of methyl-Hg (> 200 µg methyl-Hg kg\(^{-1}\) dw) (Rieder et al. 2011). When they die and decompose, methyl-Hg will be released into the soil, which might affect the soil bacterial community. We therefore cannot exclude that the actual concentrations of methyl-Hg in Swiss forest soils may negatively affect the soil bacterial communities and soil functioning.
5.7 Acknowledgements

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Chapter 6

Dynamic modelling of the long term behaviour of Cd, Pb and Hg in Swiss forest soils using CHUM
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This chapter is in press in Science of the Total Environment

Contribution of the authors:
¹Study design; ²sampling; ³data analyzing; ⁴modelling; ⁵paper writing

6.1 Abstract

The applicability of the dynamic soil model CHUM-AM was tested to simulate concentrations of Cd, Pb and Hg in five Swiss forest soils. Soil cores of up to 50 cm depths were sampled and separated into two defined soil layers. Soil leachates were collected below the litter by zero-tension lysimeters and at 15 and 50 cm soil depth by tension lysimeters over two years. The concentrations of Cd, Pb and Hg in the solid phase and soil solution were measured by ICP-MS (Cd, Pb) or CV-AFS (Hg). Measured metal concentrations were compared with modelled concentrations using CHUM-AM. Additionally we ran the model with three different deposition scenarios (current deposition; maximum acceptable deposition according to the Swiss ordinance on Air Pollution Control; critical loads according to CLRTAP) to predict metal concentrations in the soils for the next 1000 years. Assuming current loads concentrations of Cd and Pb showed varying trends (increasing/decreasing) between the soils. Soils rich in organic carbon or with a high pH value showed increasing trends in Cd and Pb concentrations whereas the concentrations in the other soils decreased. In contrast Hg concentrations are predicted to further increase in all soils. Critical limits for Pb and Hg will partly be exceeded by current loads or by the critical loads proposed by the CLRTAP but the critical limits for Cd will rarely be reached within the next 1000 years. In contrast, maximal acceptable deposition will partly lead to concentrations above the critical limits for Pb in soils within the next 400 years, whereas the acceptable deposition of Cd will not lead to
concentrations above the proposed critical limits. In conclusion the CHUM-AM model is able to accurately simulate heavy metal (Cd, Pb and Hg) concentrations in Swiss forest soils of various soil properties.

### 6.2 Introduction

Contamination of terrestrial ecosystems with heavy metals (HMs) has increased in the last century as a consequence of human activities. Research regarding emission, deposition and the behaviour of HMs in soil has been intensified due to negative impacts of HMs on biota and human health. The pollutants emitted into the atmosphere can be transported over large distances before being deposited on the ground. The rising awareness of problems related to transboundary pollution with HMs led to the *Protocol on Heavy Metals* agreed in 1998 in the frame of the convention on Long-Range Transboundary Air Pollution (LRTAP) of the United Nations Economic Commission for Europe (UNECE). The Working Group on Effects (WGE) coordinates the international co-operative research and compiles information on major pollutant effects and geographical extent of pollutions. The WGE considers three harmful HMs: cadmium (Cd), lead (Pb) and mercury (Hg). The involved parties decided to monitor the HM pollution, to encourage research on relevant effects on human health and the environment and whenever necessary to reduce the emission of these three HMs.

To predict future concentration of pollutants in ecosystems, the past, present and future inputs of HM have to be considered. The past atmospheric emission and deposition of HM in Europe have been investigated with peat cores (Weiss et al. 1999; Ross-Barraclough et al. 2003), lake sediments (Petit et al. 1984; Farmer et al. 1996), alpine ice cores (Barbante et al. 2004; Schwikowski et al. 2004), and tree rings (Watmough and Hutchinson 2002). Atmospheric deposition of Cd increased since industrialization and was in 1970 about 36 times higher than before industrialization (Barbante et al. 2004). The deposition increased especially since 1930 and was almost doubled by 2000 (Barbante et al. 2004). The annual Cd deposition at sites far away from any emission source in Switzerland was about 90 μg
Cd m\(^{-2}\) in the year 2007 (FOEN 2008). Cadmium is important in the industrial sector and is used e.g. in steel and iron manufacturing or in Ni-Cd batteries. Emissions of Pb in Europe increased since the beginning of the 20th century mainly due to the introduction of leaded gasoline around 1950, which drastically decreased since 1980 through the drop of use of leaded gasoline and its interdiction at all European gasoline stations in the year 2000 (Schwikowski et al. 2004). The annual Pb deposition at sites far away from any emission source in Switzerland was about 5 mg Pb m\(^{-2}\) in the year 2007 (FOEN 2008). During the last century, the Hg concentrations in the atmosphere have increased by about a factor of three (Mason et al. 1994). It is assumed that half of the emitted Hg enters the global atmospheric cycle whereas the other half is deposited near the emission source (Mason et al. 1994). In 1880, the anthropogenic Hg emissions in Switzerland increased due to the introduction of steam railways with Hg-containing coal as fuel for the locomotive until the electrification of the railways started at the beginning of the 20th century. After the Second World War, the emissions of Hg in Switzerland increased again due to post war industrialization followed by a decrease since about 1970 (Ross-Barraclough et al. 2003). The current annual Hg deposition in rural sites without any nearby Hg source is about 20 μg Hg m\(^{-2}\) (Ross-Barraclough et al. 2003). In the frame of the LRTAP convention, the concept of critical loads (CL) has been introduced and defined as the maximum atmospheric depositions below which no concentrations of pollutants in soil and water will ever be reached leading to adverse effects on the soil biota according to current knowledge (De Vries and Groenenberg 2009). These concentrations are called critical limits and are based on ecotoxicological studies. The standard model for calculation of CL is a steady state model named Simple Mass Balance (SMB) model (Sverdrup et al. 1994; Posch M. et al. 1997; UBA 2004). To be able to predict when the steady state will be reached, dynamic models must be used (Alveteg et al. 1998). Dynamic models were developed more than 20 years ago mainly to predict soil acidification and surface water acidification. A simple dynamic model to predict metal concentrations in soils was developed by Posch et al. (2009). Tipping (1996) developed a more complex dynamic soil model – CHUM (CHEmistry of the Uplands Model) – which can be used to
predict metal concentrations in soil and in soil water. Tipping further adapted CHUM to CHUM-AM (CHEmistry of the Uplands Model - Annual, Metals).

In Switzerland, maximal deposition values have been defined by the Swiss Federal Council (OAPC, 1985, Annex 7). Swiss ‘critical limits’ for HMs in the solid phase of the soil have been introduced in 1998 (OIS, 1998) based on an assessment of health risks related with all major pathways (e.g. food chains) and the relevant known toxicological uptake rates (Hämmann and Gupta 1997). Jauslin et al. (2004) suggested a refinement of the risk assessment procedure. However, the effect of deposition of Cd, Pb and Hg on the future accumulation of these HMs in the soils of Switzerland has never been tested with a comprehensive dynamic model such as CHUM-AM. CHUM-AM (and also CHUM) has only been used for soils rich in organic matter in the UK so far. The model considers the organic matter content as the main factor controlling HM mobility in soils. In contrast, European and in particular Swiss forest soils usually do not contain large amounts of organic matter and the HM mobility is suggested to be largely controlled by other factors such as the soil type and clay content. Therefore it would be highly relevant to assess if CHUM-AM is also able to simulate HM concentrations in forest soils with different soil properties.

In this study, we tested the applicability of CHUM-AM to model Cd, Pb and Hg concentrations in five Swiss forest soils varying in physicochemical properties. Additionally we ran the model with three different deposition scenarios (I. current deposition; II. maximum acceptable deposition according to the Swiss ordinance on Air Pollution Control; III. site specific CL according to the WGE of the convention on LRTAP) and compared the simulated concentrations to critical limits proposed in the literature and the Swiss legislation on soil protection.

6.3.1 Site description

This study was carried out on five well characterized forest soils (Beatenberg, Lausanne, Novaggio, Schânis, Vordemwald) of the Swiss long-term forest ecosystem research programme (LWF) that were selected due to their geographical location, soil properties and
available data sets (Walthert 2003; Heim and Frey 2004; Thimonier et al. 2005; Graf-Pannatier et al. 2011). All soils were from natural forest sites distributed over the different geographical regions in Switzerland. Soils have been characterized as Podzol, dystric and eutric Cambisol and dystric Planosol (Table 6.1).

**Table 6.1:** Site characteristics and soil properties of the two surface soil layers (L1 and L2) of studied forest sites and collection depth of soil water for both soil layers (Walthert 2003; Thimonier et al. 2005; Graf-Pannatier et al. 2011).

<table>
<thead>
<tr>
<th></th>
<th>Beatenberg</th>
<th>Lausanne</th>
<th>Novaggio</th>
<th>Schänis</th>
<th>Vordemwald</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altitude (m a.s.l.)</td>
<td>1510</td>
<td>807</td>
<td>950</td>
<td>733</td>
<td>480m</td>
</tr>
<tr>
<td>Mean annual temperature (°C)</td>
<td>4.6</td>
<td>7.6</td>
<td>9.7</td>
<td>7.9</td>
<td>8.4</td>
</tr>
<tr>
<td>Mean annual precipitation (mm)</td>
<td>1305</td>
<td>1210</td>
<td>2022</td>
<td>1801</td>
<td>1106</td>
</tr>
<tr>
<td>Soil type (FAO 1997)</td>
<td>Podzol</td>
<td>Dystric Cambisol</td>
<td>Dystric Cambisol</td>
<td>Eutric Cambisol</td>
<td>Dystric Planosol</td>
</tr>
<tr>
<td>Humus form</td>
<td>Raw Humus</td>
<td>Mull</td>
<td>Moder</td>
<td>Mull</td>
<td>Moder</td>
</tr>
<tr>
<td>Dominant trees</td>
<td>Picea abies</td>
<td>Fagus sylvatica</td>
<td>Quercus cerris</td>
<td>Fagus sylvatica</td>
<td>Abies alba</td>
</tr>
<tr>
<td>Horizons</td>
<td>L1</td>
<td>L2</td>
<td>L1</td>
<td>L2</td>
<td>L1</td>
</tr>
<tr>
<td>Thickness [cm]</td>
<td>17</td>
<td>27</td>
<td>10</td>
<td>40</td>
<td>9</td>
</tr>
<tr>
<td>Organic C [%]</td>
<td>41</td>
<td>0.5</td>
<td>2.8</td>
<td>1.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Dissolved organic C [mg l⁻¹]</td>
<td>31</td>
<td>29</td>
<td>3.9</td>
<td>1.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Clay content [%]</td>
<td>0.1</td>
<td>4.6</td>
<td>13</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Bulk density [g cm⁻³]</td>
<td>1.6</td>
<td>1.8</td>
<td>1.3</td>
<td>1.4</td>
<td>0.7</td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td>3.8</td>
<td>4.1</td>
<td>4.5</td>
<td>4.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Suction cups buried [cm]</td>
<td>15</td>
<td>50</td>
<td>15</td>
<td>50</td>
<td>15</td>
</tr>
</tbody>
</table>
6.3.2 Model description

CHUM-AM (CHemistry of the Uplands Model - Annual, Metals) was developed by Tipping et al. (2006) and is an improvement from CHUM (Tipping 1996). In contrast to CHUM, CHUM-AM runs on annual and not on daily time steps. Furthermore, the water movements in CHUM-AM are simplified and biogeochemical processes are included. Originally, CHUM-AM considers a soil column composed of three homogenous layers. In our more simplified approach, we only used the first two layers (L1, L2) up to 50 cm varying in their soil properties (e.g., organic matter content). Highest microbial activity, bioturbation, root growth, and organic matter turnover are suggested to be in the first 50 cm of soil depth. Furthermore, heavy metals strongly accumulate in the topsoils. The two soil layers are described in Table 6.1 for each soil site. In this study, the simulation of stream water was neglected and only the soil and soil solution are considered in contrast to studies in the UK (Tipping et al. 2006; Tipping et al. 2006).

The model uses mean annual precipitation and deposition values and provides mean annual concentrations of elements in the solid phase of the soil and in the soil water. CHUM-AM assumes that the soils were in steady state with respect to atmospheric metal deposition before industrialization. Therefore, CHUM-AM calculates 50 times the years between 1400 and 1600 (data for 10,000 calculating years) to reach steady state, which is necessary for the initial conditions of the model. The carbon content in the solid phase and in the soil solution is specified in the input file because CHUM-AM does not include a carbon cycling model. CHUM-AM aims to consider the main processes to calculate soil acidity and solution chemistry and the metal behavior in ecosystems. As mentioned before, CHUM-AM considers the organic matter content (especially content of humic and fulvic acids) as the key factor controlling HM mobility in soils. The competitive interactions of cations (including H\(^+\)) with organic matter in the solid phase and in soil solution and also with mineral cation exchanger mainly determine the fate of metals in soils. Thus, the simultaneous prediction of soil solution chemistry and especially soil acidity is mandatory to predict the fate of metals in
soils. CHUM-AM calculates the reactive HM pools in soil. Therefore the calculated HM concentrations are assumed to be lower than the total HM concentrations in the soils. The species on which the chemical reactions are based are the major cations H\(^+\), Na\(^+\), Mg\(^{2+}\), Al\(^{3+}\), K\(^+\), Ca\(^{2+}\), Fe\(^{3+}\), NH\(_4\)\(^+\); the trace cations Ni\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Pb\(^{2+}\), Hg\(^{2+}\); the anions OH\(^-\), Cl\(^-\), NO\(_3\)\(^-\), SO\(_4^{2-}\), F\(^-\), CO\(_3^{2-}\) and the neutral species Si(OH)\(_4\). The solutes may bind to minerals and organic matter (humic and fulvic acids) whereby the organic matter can be in solid phase, dissolved in soil water, or in suspension. The chemical interactions were calculated by the soil version of WHAM/Model VI (Tipping et al. 1998; Tipping et al. 2003) which has been implemented in CHUM-AM. Cycling in vegetation, seasonal variability of soil properties, flow pathways and depositions and also bioturbation were not considered in CHUM-AM.

6.3.3 Input data
The data used for modelling were measured in the field (LWF data; time frame of measurements 1997 – 2011) or estimated from models or literature (Table 6.2). Soil properties were previously determined (Walthert 2003; Walthert et al. 2004; Blaser et al. 2005; Lazzaro et al. 2006; Lazzaro et al. 2006; Zimmermann et al. 2006; Graf-Pannatier et al. 2011) except for the HM concentrations in the solid phase and in the solution. The soil profiles were divided into different soil layers due to changing soil properties (e.g. organic matter content). In this study we only considered two soil layers (L1 and L2) for which mean soil properties were calculated (Table 6.1). The soil depths and main properties of L1 and L2 are given for each soil site in Table 6.1. Annual average precipitation data, data of total and wet deposition (for cations, N and S) have been determined on these five LWF sites with continuous sampling of bulk (1 to 3 samplers in a nearby open field) and throughfall deposition (4 to 16 samplers below forest canopy) in bi-weekly to monthly intervals since 1997 (Thimonier et al. 2005; Thimonier et al. 2010). In the few cases with overflow of the samplers, precipitation has been reconstructed using data of unheated tipping buckets or of the nearest meteorological station of the Swiss meteorological institute MeteoSwiss.
There were several assumptions made for the input variables. Depositions of Cd were not measured at the LWF sites. The current depositions in Switzerland were obtained from the Swiss country report 2007/2008 of EMEP (European Monitoring and Evaluation Programme; http://www.emep.int) documenting Cd, Pb and Hg depositions derived from modelling with a spatial resolution of 50 x 50 km. In addition, data from the Swiss National Air Pollution Network (NABEL 2011) and from moss analyses were used (FOEN 2008). Depositions were derived from concentrations of several elements (including Cd, Pb) in mosses by FOEN (2008) by transforming the elemental concentrations in mosses with measured deposition (Bergerhoff analyses) data according to Thöni (1996). Historical Cd depositions were estimated from peat bog record data according to Shotyk et al. (2002) and from ice cores (Barbante et al. 2004). The historical deposition of HM was estimated for each site separately by using the current deposition values which were then back calculated with the historical trends in HM depositions.

Similar as for Cd, no date was available for Pb depositions at the selected LWF forest sites. Current Pb depositions (between 1990 and 2008) were obtained from EMEP modelled data, from deposition analyses in Switzerland (NABEL 2011), and from moss analyses (FOEN 2008). Historical Pb deposition data were obtained from five peat profiles (Weiss et al. 1999). These studies calculated depositions of Pb in time steps between 2 and 10 years back to at least 1871. Between the particular time points, we assumed a linear change of the deposition. We have chosen the peat profiles from Weiss et al. (1999) located closest to our forest sites [profile SwM for Beatenberg, Vordemwald and Schänis; profile PRd for Lausanne; profile GdL for Novaggio, profile abbreviations according to Weiss et al. (1999)].

Hg depositions were also not available for the selected LWF forest sites. Current Hg depositions were estimated by EMEP modelled data. Historical Hg depositions (since 1400) were obtained from analyses of peat cores of two bogs from the Swiss Jura Mountains (Ross-Barraclough et al. 2003). Hg depositions modelled by EMEP (2008) for this region were, with values between 16 and 18 µg Hg m\(^{-2}\) year\(^{-1}\), similar to the Hg depositions of 10 – 25 µg Hg m\(^{-2}\) year\(^{-1}\) estimated by Ross-Barraclough et al. (2003) for 1990. Historical Hg
depositions were calculated by approximating the peat core analyses to the current depositions. In contrast to Cd and Pb, Hg is not only lost from soil by leaching, Hg is also lost by reduction of $\text{Hg}^{2+}$ to the volatile Hg species $\text{Hg}^{0}$ followed by evasion (Zhang et al. 1999; Schluter 2000). Evasion is dependent on numerous abiotic and biotic factors such as temperature, soil moisture or microbial activity (Zhang et al. 1999; Johnson et al. 2003; Bahlmann et al. 2006; Fritsche et al. 2008; Choi et al. 2009). During calibration we tested various evasion rates [between 0.03 and 0.1% of total Hg; selected from Tipping et al. (2011)], and have applied a rate of 0.03% in the model runs presented here.

The deposition rates derived from EMEP, from moss analyses, NABO data and peat and ice core studies intend to cover all land use types and we thus assumed that deposition at selected LWF sites are higher due to the filtering effects of the forest canopy depending on e.g. the dominant tree species (Driscoll et al. 1994; De Vries et al. 1998; St Louis et al. 2001; Lindberg et al. 2007; Perez-Suarez et al. 2008; Gandois et al. 2010). Therefore, we increased deposition values from literature by a factor of 2 (deciduous forests) and 2.5 (conifer forest) for Cd and Hg, and 2 (deciduous forests) and 3 (conifer forest) for Pb. The increasing factors were defined by literature values and fine adjustment during the calibrations of the model.

The N depositions were measured in each forest site. The model simulates the acidifying effect on N deposition, i.e. pH changes but not the uptake of N into vegetation and soil. However, the measurement of total deposition also includes amounts of N that are directly taken up by vegetation or washed out as surface runoff without having an acidifying effect. Therefore we estimated the remaining acidifying N depositions out of the N concentrations in the soil solution. We assumed that the current $\text{NO}_3$ and $\text{NH}_4$ concentrations in the soil solution influence the soil pH. All deposited N compounds which were immediately washed out or taken up by plants and thus not influencing soil pH were neglected. We calculated the current depositions for $N_{\text{dep}}$ according to equation (1).

$$N_{\text{dep}} = [N] \times \frac{\text{precipitation-evapotranspiration}}{\text{precipitation}} \tag{1}$$
where \( N \) is the concentration of \( \text{NO}_3 \) or \( \text{NH}_4 \) in soil solution (mean concentration of the soil solution collected in 15 and 50 cm depths). The historical \( \text{NO}_3 \) and \( \text{NH}_4 \) emissions were calculated relative to the year 2000 (\( N_{\text{emission \ year \ 2000}} = 1 \)) and their depositions were estimated by multiplying the \( N_{\text{dep.}} \) value for \( \text{NO}_3 \) and \( \text{NH}_4 \) with the relative \( N \) emission values in Switzerland (FOEN 1995).

The current \( \text{SO}_2 \) and \( \text{SO}_4 \) depositions were measured in all sites whereas the historical depositions were estimated from the emission of \( \text{SO}_2 \) (FOEN 1995). Therefore the normalized historical \( \text{SO}_2 \) emissions were calculated relative to the year 2000 (\( S_{\text{emission \ year \ 2000}} = 1 \)) and the historical deposition of \( \text{SO}_2 \) was estimated by multiplying the normalized emission value with the current \( \text{SO}_2 \) emission value (FOEN 1995). The \( \text{SO}_4 \) deposition was calculated by multiplying the \( \text{SO}_2 \) depositions with a site specific relation factor of \( \text{SO}_2 \) to \( \text{SO}_4 \), calculated from a 10 year deposition observation in each forest site.

The weathering inputs of major and trace metals through chemical weathering of the mineral soil fraction were calculated according to equation (2) (Schnoor and Stumm 1986).

\[
\text{weathering rate} = kw a_{H^+}^{nw} \tag{2}
\]

where the \( kw \) value was estimated from weathering rates calculated by using the model SAFE (Alveteg et al. 1998) and was adjusted to match observations (calibration parameter). The \( a_{H^+} \) was the activity of protons in soil solution and the exponent \( nw \) was set to 0.7 for Al and Fe and 0 for Si, Mg and Ca (Stidson et al. 2002).
<table>
<thead>
<tr>
<th>Input parameter</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Annual wet deposition of:</strong> Na, Mg, K, Ca, Cl, F</td>
<td>measured for 10 years; mean precipitation used as historic inputs</td>
</tr>
<tr>
<td></td>
<td>-&gt; charge balancing calculated</td>
</tr>
<tr>
<td><strong>Annual total deposition of:</strong> Al, Si, Fe, Ni, Cu, Zn</td>
<td>measured for 10 years; mean precipitation used as historic inputs</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_4$, NO$_3$</td>
<td>estimated from soil concentrations, deposition measurements and historical emission data</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>SO$_4$, SO$_2$</td>
<td>estimated from deposition measurements and historical emission data</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total annual deposition of Cd</strong></td>
<td>modelled values from EMEP 2008; moss monitoring (FOEN 2008); peat core (Shotyk et al. 2002) and ice core drilling values (Barbante et al. 2004) for historical data</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total annual deposition of Pb</strong></td>
<td>modelled values from EMEP 2008; moss monitoring (FOEN 2008); peat core values (Weiss et al. 1999) for historical data</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total annual deposition of Hg</strong></td>
<td>modelled values from EMEP 2008; peat core values (Ross-Barraclough et al. 2003) for historical data</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Annual mean precipitation</strong></td>
<td>measured for 10 years; mean precipitation was used as historic annual inputs</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Annual mean evapotranspiration</strong></td>
<td>modelled for each site by using the one dimensional CoupModel (Jansson and Karlberg, 2004) based on soil and plant properties and meteorological data; mean evapotranspiration was used as ancient annual evapotranspiration</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Occult deposition factor</strong></td>
<td>estimated</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fraction covered by grass, heather, trees</strong></td>
<td>estimated</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Soil temperature</strong></td>
<td>mean annual value; measured</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Density of soil solids</strong></td>
<td>measured</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fine earth bulk density</strong></td>
<td>measured</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fraction of volume is rock</strong></td>
<td>measured</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>% water saturation of soil</strong></td>
<td>estimated a mean annual saturation</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>OC content of soil</strong></td>
<td>measured</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Humic and fulvic acid fraction of OM</strong></td>
<td>default values by Tipping et al. (2006)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Clay content of soil</strong></td>
<td>measured</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Weathering inputs:</strong> Na, Mg, Al, K, Ca, Fe, Ni, Cu, Zn, Cd, Pb, Hg, Si,</td>
<td>modelled by SAFE and adjusted during calibration; input of metals due to mineral weathering</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dissolved organic carbon (DOC)</strong></td>
<td>measured</td>
</tr>
</tbody>
</table>
6.3.4 Soil sampling and determination of metal concentrations

To calibrate the model, measured concentrations of Cd, Pb and Hg throughout the soil profile were needed. To measure the total contents of HMs in the soils three soil cores were taken around the soil profiles described in Walthert (2003). The soil cores were sampled with a soil auger (5 cm in diameter) up to about 60 cm soil depth whenever possible (bedrock) within a radius of 2 m from each of the soil profiles described by Walthert (2003). Thereafter the soil cores were separated in two clear defined soil layers (L1, L2) according to previous studies (Walthert, 2003; Walthert et al., 2004; Zimmermann et al., 2006). The soil samples were then sieved (2 mm) to remove stones and litter, air-dried at 15°C in a forced draught oven for 5 days and homogenized by crushing in an acid-washed porcelain pestle and mortar. For determining the residual moisture contents, aliquots of the soils were dried at 105°C for 3 days. The contents of Cd and Pb of each soil horizon were determined by extracting 2.5 g of the dried soil sample in 25 ml of 2 M HNO₃ for 2 h at 95°C (100 rpm) (Ernst et al. 2008). All soil extracts were filtered through filter paper (No. 790 1/2; Schleicher & Schuell). The concentrations of Cd and Pb in the extracts were measured by inductively coupled plasma-mass spectrometry [ICP-MS: ELAN 6000, Perkin–Elmer; detection limits: 0.01 mg Cd kg⁻¹ dw; 0.02 mg Pb kg⁻¹ dw; Ernst et al. (2008)]. The Hg contents in the soils were determined by a direct mercury analyzer [(DMA), AMA 254 Mercury Analyzer, LECO Corporation; detection limit: 0.001 mg Hg kg⁻¹ dw]. Mean concentrations of HMs were calculated for both soil layers (L1, L2).

Soil solutions were collected at a soil depth of 15 cm and 50 cm between 2007 and 2009 (n=8 per site and depth). The soil solution was sampled by tension lysimeters using ceramic suction cups (Graf-Pannatier et al., 2011) and collected bi-weekly in 1 L glass bottles. Elemental concentrations of Cd and Pb in soil solution were determined by ICP-MS (Perkin-Elmer, OPTIMA 3000; detection limits: Cd < 0.02 µg L⁻¹; Pb < 0.05 µg L⁻¹). For Hg analyses, aliquots of the soil leachates were filled in 125 mL PTFE Teflon bottles (acid washed) and the Hg concentrations were determined by cold vapour atomic fluorescence spectroscopy (CV-AFS; detection limit < 0.0003 µg L⁻¹) according to Akerblom et al. (2008).
6.3.5 Model Calibration

Firstly, we calibrated the pH and the concentrations of Al, Ca, K, Mg and Na in the soil solution for both soil layers (L1, L2). Therefore, we adjusted the initial weathering rates of each soil cation, until the measured and calculated concentrations were in good accordance, which means $r<5$ according to equation (3). The initial weathering rates were calculated by using the model SAFE (Alveteg et al. 1998).

$$ r = \sum \left( \frac{[M]_{\text{measured}} - [M]_{\text{calculated}}}{[M]_{\text{measured}}} \right)^2 $$

(3)

where $[M]$ is the concentration of element M (H$^+$, Al, Ca, K, Mg, Na) in soil solution. We further adjusted the input parameter within the range of analyses (e.g. HM deposition, precipitation, DOC concentration), modelled values [evapotranspiration according to Jansson and Karlberg (2004)] and evasion rates for Hg according to determined rates for deciduous forest soils (Tipping et al. 2011) to improve the accordance of modelled and measured concentrations.

6.3.6 Scenarios

After calibration we ran the model with different deposition scenarios for the next 1000 years starting at the year 2010. The three scenarios were as follows:

1) Current deposition (2010 deposition)
2) Maximum acceptable deposition of Cd and Pb according to the Swiss ordinance on Air Pollution Control [OAPC (1985), Annex 7]
3) Critical loads (CL) according to the Working Group on Effects (WGE) of the convention on LRTAP (Slootweg et al. 2005) as deposition input parameters (carried out with the lowest site specific values)
When modelling the different scenarios, we manipulated only the deposition of the studied HM and maintained all other deposition parameters at their current level. The different deposition scenarios are shown in Table 6.3.

**Table 6.3**: Deposition scenarios for Cd, Pb and Hg.

<table>
<thead>
<tr>
<th>Site</th>
<th>2010 deposition [mg m$^{-2}$ a]$^1$</th>
<th>Swiss OAPC$^2$ [mg m$^{-2}$ a]</th>
<th>Critical loads (CL)$^3$ [mg m$^{-2}$ a]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cd</td>
<td>Pb</td>
<td>Hg</td>
</tr>
<tr>
<td>Beatenberg</td>
<td>0.09</td>
<td>4.5</td>
<td>0.04</td>
</tr>
<tr>
<td>Lausanne</td>
<td>0.09</td>
<td>3.0</td>
<td>0.04</td>
</tr>
<tr>
<td>Novaggio</td>
<td>0.28</td>
<td>5.6</td>
<td>0.05</td>
</tr>
<tr>
<td>Schânis</td>
<td>0.07</td>
<td>1.5</td>
<td>0.04</td>
</tr>
<tr>
<td>Vordemwald</td>
<td>0.07</td>
<td>3.0</td>
<td>0.04</td>
</tr>
</tbody>
</table>

$^1$ Estimated current deposition in the forests (wet deposition + throughfall + litterfall)
$^2$ Maximal acceptable HM depositions in Switzerland according the OAPC (ordinance on Air Pollution Control, 1985)
$^3$ Critical loads (CL) were obtained from the WGE report 2005 (Slootweg et al. 2005). The WGE report presents a range for deposition for 277 forest soils in Switzerland. We chose the lowest CL of this range as deposition value.
$^4$ No maximal deposition values for Hg are defined in the Swiss OAPC

We compared the measured and modelled HM concentrations with several critical limit concentrations based on total contents of HM g$^{-1}$ soil (OAPC 1985; Tipping et al. 2010); on total HM per g$^{-1}$ SOM (Meili et al. 2003; Lofts et al. 2004; Tipping et al. 2010); or on HM concentrations per L$^{-1}$ soil solution (WHO 2004; Lazzaro et al. 2006; Lazzaro et al. 2006; DeVries et al. 2007) (Table 6.4). The critical limits were calculated per site and per soil layer as they depend on soil properties.
**Table 6.4**: Critical limits for Cd, Pb and Hg.

### Critical limits for soil water

<table>
<thead>
<tr>
<th></th>
<th>Cd [µg L$^{-1}$]</th>
<th>Pb [µg L$^{-1}$]</th>
<th>Hg [µg L$^{-1}$]</th>
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</thead>
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<tr>
<td>WHO guideline for drinking water</td>
<td>3</td>
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<td>1</td>
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<tr>
<td>Lazzaro et al. (2006a, b)</td>
<td>1.2</td>
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<tr>
<td>De Vries et al. (2007)</td>
<td>-</td>
<td>-</td>
<td>0.035</td>
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### Critical limits for soil solid phase

#### Cd [µg g$^{-1}$]

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<thead>
<tr>
<th>Location</th>
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<th>L2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beatenberg*</td>
<td>21.0</td>
<td>0.3</td>
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<tr>
<td>Lausanne</td>
<td>1.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Novaggio</td>
<td>6.6</td>
<td>5.0</td>
</tr>
<tr>
<td>Schänis</td>
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<td>3.1</td>
</tr>
<tr>
<td>Vordemwald</td>
<td>3.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

#### Pb [µg g$^{-1}$]

<table>
<thead>
<tr>
<th>Location</th>
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<td>30.5</td>
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</tr>
<tr>
<td>Lausanne</td>
<td>7.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Novaggio</td>
<td>17.0</td>
<td>15.1</td>
</tr>
<tr>
<td>Schänis</td>
<td>28.6</td>
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<tr>
<td>Vordemwald</td>
<td>11.3</td>
<td>3.5</td>
</tr>
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</table>

#### Hg [µg g$^{-1}$]

<table>
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<th>Location</th>
<th>L1, L2$^2$</th>
<th>L1$^3$</th>
<th>L2$^3$</th>
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<th>L2</th>
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<tr>
<td>Beatenberg*</td>
<td>0.13</td>
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<td>0.03</td>
<td>0.41</td>
<td>0.01</td>
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<td>Lausanne</td>
<td>0.13</td>
<td>0.2</td>
<td>0.07</td>
<td>0.03</td>
<td>0.01</td>
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<td>0.6</td>
<td>0.43</td>
<td>0.10</td>
<td>0.07</td>
</tr>
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<td>0.03</td>
<td>0.02</td>
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<tr>
<td>Vordemwald</td>
<td>0.13</td>
<td>0.3</td>
<td>0.05</td>
<td>0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*critical limits for soil protection according to Swiss Ordinance relating to impacts on the soil (OIS, 1998):
- for Beatenberg: Cd: 0.32 µg g$^{-1}$, Pb: 20 µg g$^{-1}$, Hg: 0.2 µg g$^{-1}$
- for all others: Cd: 0.8 µg g$^{-1}$, Pb: 50 µg g$^{-1}$, Hg: 0.5 µg g$^{-1}$

1 based on organic carbon content and pH conditions.
2 based on total Hg content in the soils
3 based on the SOM content in the soils
6.4 Results

6.4.1 Cd, Pb and Hg concentrations in forest soils

Contents of Pb and Hg in the solid phase were highest in the top layer (L1) of the soils (Table 6.5). Similarly, the contents of Cd were highest in the top layer (L1) but varied largely. At two sites the total Cd contents were below the detection limit (0.01 mg kg⁻¹). No top layer (L1) reached the critical limit for Cd set by the OIS (0.8 mg Cd kg⁻¹ soil or 0.32 mg Cd kg⁻¹ soil for Beatenberg). Soils in Beatenberg and Novaggio exceeded the critical limit set by OIS for Pb (20 and 50 mg kg⁻¹, respectively) and all top layers (L1) exceeded the critical limit for Pb proposed by Lofts et al. (2004). Contents of Hg in the top layer of Beatenberg exceeded the critical limit set by OIS (0.2 mg kg⁻¹). The Hg contents in the top layer (L1) of Beatenberg, Schänis and Vordemwald exceeded the critical limit (0.13 mg kg⁻¹) proposed by Tipping et al. (2010a) but only in Schänis also the SOM based limit (0.17 mg Hg kg⁻¹) is exceeded. Proposed critical limits by Meili et al. (2003), also based on SOM, are exceeded in the top layers (L1) of Lausanne, Schänis and Vordemwald.

Table 6.5: Measured Cd, Pb and Hg concentrations (mean ± std.dev.) in the two soil layers (L1 and L2) in the solid phase (n=3) and in the soil solution (n=8).

<table>
<thead>
<tr>
<th>Metal concentration in the solid phase*</th>
<th>Cd [µg g⁻¹]</th>
<th>Pb [µg g⁻¹]</th>
<th>Hg [µg g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L1</td>
<td>L2</td>
<td>L1</td>
</tr>
<tr>
<td>Beatenberg</td>
<td>&lt;d.l.¹</td>
<td>60 ± 1</td>
<td>&lt;d.l.</td>
</tr>
<tr>
<td>Lausanne</td>
<td>0.08 ± 0.09</td>
<td>23 ± 3</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Novaggio</td>
<td>0.39 ± 0.06</td>
<td>51 ± 17</td>
<td>21 ± 0</td>
</tr>
<tr>
<td>Schänis</td>
<td>0.25 ± 0.35</td>
<td>34 ± 3</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Vordemwald</td>
<td>&lt;d.l.</td>
<td>29 ± 1</td>
<td>13 ± 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metal concentration in soil solution**</th>
<th>Cd [µg L⁻¹]</th>
<th>Pb [µg L⁻¹]</th>
<th>Hg [µg L⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L1</td>
<td>L2</td>
<td>L1</td>
</tr>
<tr>
<td>Beatenberg</td>
<td>0.02 ± 0</td>
<td>0.07 ± 0.02</td>
<td>2.3²</td>
</tr>
<tr>
<td>Lausanne</td>
<td>0.06 ± 0.03</td>
<td>0.18 ± 0.07</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Novaggio</td>
<td>0.09 ± 0.07</td>
<td>0.02 ± 0.01</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Schänis</td>
<td>&lt;d.l.</td>
<td>&lt;d.l.</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Vordemwald</td>
<td>0.07 ± 0.05</td>
<td>0.32 ± 0.25</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

¹ <d.l.=below detection limit
² Only one measurement available
* Detection limits per g⁻¹ soil: Cd: 0.01 µg; Pb 0.02 µg; Hg: 0.001 µg
** Detection limits per L⁻¹: Cd: 0.02 µg; Pb 0.05 µg; Hg: 0.0003 µg
The concentrations of Cd in the soil solution showed a large variation and tended to increase with soil depth (Table 6.5). The concentrations of Pb in the soil solution tended to increase with soil depth. The concentrations of Hg in the soil solution strongly decreased with increasing depth (Table 6.5). Concentrations of Cd, Pb and Hg in the soil solution were below the WHO guideline for drinking water protection and the Hg concentrations in Vordemwald exceeded the ecotoxicological value (0.035 µg L\(^{-1}\)) given by De Vries et al. (2005).

### 6.4.2 Model applicability for Swiss forest soils and calibration

The calculated pH values in the soil solution were in good accordance with the measured pH values. In Lausanne, Vordemwald and Novaggio, the measured and calculated pH values were similar (±0.2 pH units), in Beatenberg the calculated pH values were too high (+0.5) and in Schânis too low (-1.2), whereby in Schânis, the pH measurements varied widely (5.0 and 7.6; data not shown).

In general, the modelled Cd, Pb and Hg concentrations in the soil matrix and the soil solution were in line with the measured concentrations (Figure 6.1). In the top layer (L1), the measured total HM contents were somewhat higher than the calculated contents, whereas the opposite is true for the concentrations in soil solutions (Figure 6.1 a, b). In the second soil layer (L2), the modelled HM contents were less accurate (Figure 6.1 c) whereas the modelled concentrations in soil solution were in line with the measured concentrations (Figure 6.1 d).
Figure 6.1: Model calibration: measured (mean value per site) versus modelled HM concentrations (Cd, Pb and Hg) in the solid phase (a, c) or in the soil solution (b, d) in L1 (a, b) or L2 (c, d). In samples below the detection limit, the value of the detection limit was used.

6.4.3 Scenarios

6.4.3.1 Current deposition

**Cadmium:** The total contents of Cd will still increase in the top soil layers up to 70% or decrease up to 80% during the next 1000 years depending on the forest site (Figure 6.2; Table 6.6). Concentrations of Cd will never exceed the Swiss guide value for soil protection (OIS; 0.8 or 0.32 mg Cd kg\(^{-1}\)) nor the critical limit estimated by Lofts et al. (2004) for both soil horizons (L1, L2).
The Cd concentrations in soil solution will also increase or decrease (up to 70%) in the topsoils (Figure 6.2; Table 6.6). The concentration of Cd in soil solution will never reach the critical limit.

**Lead:** The total contents of Pb will further increase in two soils (Beatenberg, Schânis) and decrease in the other soils (Figure 6.3; Table 6.6). The OIS guide value (0.5 resp. 0.2 mg Pb kg\(^{-1}\) soil) has already been exceeded in the top layer (L1) of Beatenberg and Novaggio but in Novaggio the Pb contents will decrease. In Schânis the OIS guideline value of Pb will be exceeded in the next 1000 years.

The Pb concentration in soil solution will further increase (Figure 6.3; Table 6.6). The calculated Pb concentrations are overestimated but based on measured values and the slope in future Pb concentrations in soil solution, we assume that the WHO guideline for groundwater protection (10 µg Pb L\(^{-1}\)) and the critical limit proposed by Lazzaro et al. (2006b) (1.8 µg L\(^{-1}\)) will never be reached at each site.

**Mercury:** Total Hg contents will further increase in all studied forest soils (Figure 6.4; Table 6.6). In 1000 years, the Hg contents in the top layer (L1) are about twice as high as the present-day contents (Table 6.6). The Swiss guide value for soil protection (OIS; 0.5 mg Hg kg\(^{-1}\) soil and 0.2 mg Hg kg\(^{-1}\)) in top layers (L1) will never be reached in each soil except for Beatenberg where this value has already been exceeded (Table 6.5). The critical limit for total Hg contents (0.13 mg kg\(^{-1}\) soil) proposed by Tipping et al. (2010) has already been exceeded or will be reached in Beatenberg, Lausanne Novaggio and Schânis in the next 1000 years. The critical limit based on SOM by Tipping et al. (2010) is already exceeded in Schânis and will be reached in Lausanne and Vordemwald in the current millennium. The critical limit proposed by Meili et al. (2003) is already exceeded or will be exceeded in the top layers (L1) of all soils in the current millennium.

The Hg concentrations in soil water will further increase at all forest sites (Figure 6.4). In 1000 years, the Hg concentrations in soil solution will be about three times higher than the present-day Hg concentrations (Table 6.6). The WHO guideline for drinking water protection (1 µg Hg L\(^{-1}\)) (WHO, 2004) will never be reached whereas the ecotoxicological default value
(0.035 µg Hg L\(^{-1}\)) proposed by De Vries et al. (2005) will be exceeded in the top layer (L1) of three soils during the next 1000 years.

6.4.3.2 OAPC deposition

**Cadmium:** The maximum acceptable Cd deposition in Switzerland (OAPC, 1985) is about eight times higher than the current deposition at our forest sites. Such a deposition would substantially increase the Cd content in the soil matrix and also in the soil solution at all forest sites (Figure 6.2; Table 6.6). In the top layer (L1) of soils in Beatenberg and Schănis the contents of Cd in the solid phase will exceed the OIS guide value (0.32 or 0.8 mg Cd kg\(^{-1}\) soil) in less than 300 years but the proposed critical limit by Lofts et al. (2004) for total HM concentration will never be reached in neither of the two soils. In 1000 years the contents of Cd will be about 10 times higher than the current concentrations in Beatenberg and Schănis but only somewhat higher in the other soils (Table 6.6).

In soil solution the WHO guideline for drinking water (3 µg L\(^{-1}\)) for Cd will never be reached in any soil but the critical limit proposed by Lazzaro et al. (2006a) (1.2 µg L\(^{-1}\)) will be reached in Lausanne and Vordemwald within the next hundred years.

**Lead:** Similar to Cd the maximum acceptable Pb deposition in Switzerland (OAPC, 1985) is much higher (about ten times) than the current deposition at our forest sites and would lead to a dramatic increase in the Pb contents in the solid phase except for Lausanne and Novaggio (Figure 6.3; Table 6.6). The OIS guide value (0.5 resp. 0.2 mg Pb kg\(^{-1}\) soil) will be exceeded in the top layers (L1) of Beatenberg and Schănis in less than 400 years and shortly in Novaggio.

In soil solution the Pb concentrations will increase in all soils in particular in the second soil layers (Figure 6.3; Table 6.6). The WHO guideline (10 µg Pb L\(^{-1}\)) will be exceeded in Beatenberg, Lausanne, Novaggio and Vordemwald within the next 100 years.
6.4.3.3 Critical Loads (CL) deposition

**Cadmium:** The CL for Cd were between 8 and 16 times higher than the current Cd depositions. By depositions according to the CL, the Cd contents in the solid phase will largely increase in all soils and in the soil water of four out of five soils (Figure 6.2; Table 6.6). The OIS guide value (0.8 resp. 0.32 mg Cd kg\(^{-1}\) soil) will partly be exceeded in the next 1000 years. The Cd contents in the upper layer (L1) will rise up to 19 times and in the lower layer (L2) up to almost 40 times the present-day contents. The critical limit for Cd in soil solution proposed by Lazzaro et al. (2006a) (1.2 µg L\(^{-1}\)) will be exceeded in four soils within the next 1000 years.

**Lead:** The CL for Pb are smaller than the current depositions in Lausanne, Novaggio and Vordemwald. In Beatenberg and Schânis the current loads are similar than the CL (Figure 6.2 a-e; grey dots). The CL scenario usually will lead to similar or lower Pb concentrations in the soil matrix and in soil water compared to the current deposition.

**Mercury:** Mercury concentrations in the soil matrix and in the soil solution will further increase in all forest soils (Figure 6.4; Table 6.6). The Swiss guideline of the OIS (1998) (0.5 mg Hg kg\(^{-1}\) resp. 0.2 mg Hg kg\(^{-1}\) soil) will never be reached except for Beatenberg (Table 6.5). The critical limit (0.13 mg Hg kg\(^{-1}\) soil) proposed by Tipping et al. (2010a) is already exceeded (Beatenberg, Schânis, Vordemwald) or will be reached within the next 1000 years (Lausanne, Novaggio) (Table 6.5, Figure 6.4). The SOM based critical limit proposed by Tipping et al. (2010) is already exceeded or will be reached during the next 1000 years in Lausanne, Schânis and Vordemwald. In top layers (L1), the critical limit proposed by Meili et al. (2003) which is already exceeded (Lausanne, Schânis, Vordemwald), will be exceeded in Novaggio during the next 1000 years but it will never be reached in Beatenberg. The Hg concentration in soil solution will never exceed the WHO guideline (1 µg Hg L\(^{-1}\)) for drinking water (WHO, 2004), and the critical limit (0.035 µg Hg L\(^{-1}\)) proposed by De Vries et al. (2007) in any of the studied soils.
Table 6.6: Ratio ([M\textsubscript{3000}]/[M\textsubscript{2010}]; M= Cd, Pb or Hg) between the concentrations in the year 3000 and the current (measured) concentrations in the solid phase and in the soil solution. The ratios are shown for both modelled soil layer (L1, L2) and for all three deposition scenarios [1) Current: current (2010) deposition; 2) OAPC: maximal acceptable deposition according to OAPC (1985); and 3) CL: critical loads (CL) according to the Working Group on Effects (WGE) of the convention on LRTAP (Slootweg et al. 2005)].

<table>
<thead>
<tr>
<th></th>
<th>solid phase</th>
<th>soil solution</th>
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<tbody>
<tr>
<td></td>
<td>Cd</td>
<td>Hg</td>
</tr>
<tr>
<td>1) Current</td>
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<td>Beatenberg</td>
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<tr>
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</tr>
<tr>
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<td>1.1</td>
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</tr>
<tr>
<td>L2</td>
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<td>1.2</td>
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</tbody>
</table>

*No maximal deposition values according to the OAPC exists for Hg*
Figure 6.2: Long-term total Cd concentrations in the solid phase (left) and the soil solutions (right) for L1 (line) or L2 (dashed line) simulated with three deposition scenarios: 1) Current: current (2010) deposition represented by bold black lines; 2) OAPC: maximal acceptable deposition according to OAPC (1985) represented by thin black lines; and 3) CL: critical loads (CL) according to the Working Group on Effects (WGE) of the convention on LRTAP (Slootweg et al. 2005) represented by grey thin lines.
Figure 6.3: Long-term total Pb concentrations in the solid phase (left) and the soil solutions (right) for L1 (line) or L2 (dashed line) simulated with three deposition scenarios: 1) Current: current (2010) deposition represented by bold black lines; 2) OAPC: maximal acceptable deposition according to OAPC (1985) represented by thin black lines; and 3) CL: critical loads (CL) according to the Working Group on Effects (WGE) of the convention on LRTAP (Slootweg et al. 2005) represented by grey thin lines.
Figure 6.4: Long-term total Hg concentrations in the solid phase (left) and the soil solutions (right) for L1 (line) or L2 (dashed line) simulated with two deposition scenarios: 1) Current: current (2010) deposition represented by bold black lines; and 2) CL: critical loads (CL) according to the Working Group on Effects (WGE) of the convention on LRTAP (Slootweg et al. 2005) represented by grey thin lines.
6.5 Discussion

6.5.1 Exceedances of critical metal concentrations

The concentrations of Cd in the soil solution (<0.02 – 0.18 µg Cd L\(^{-1}\)), based on the current findings, are ecotoxicologically not critical. Soluble Cd concentrations in all soils were below the WHO guidelines for drinking water (3 µg Cd L\(^{-1}\)) (WHO, 2004) and the critical limit (1.2 µg Cd L\(^{-1}\)) proposed by Lazzaro et al. (2006a). A similar result was observed for Pb in the soil solution (<0.05 – 6.5 µg Pb L\(^{-1}\)). We did not find any concentration exceeding the WHO guidelines for Pb in drinking water (10 µg L\(^{-1}\)) but at three sites (Beatenberg, Lausanne, Novaggio) the critical limit (1.8 µg Pb L\(^{-1}\)) concentrations estimated by Lazzaro et al. (2006b) were exceeded in at least one soil layer. The Hg concentrations in the soil solution (<0.001 – 0.037 µg Hg L\(^{-1}\)), as far as we know, were also ecotoxicologically not critical (0.035 – 1 µg Hg L\(^{-1}\)).

The current depositions will not lead to ecotoxicologically critical concentrations of Cd and Pb in soils [according to the OIS (1998) guidelines, the WHO guidelines (WHO, 2004) and the critical limits proposed by Lazzaro et al. (2006a); Lazzaro et al. (2006b)]. In contrast, the concentrations of Hg will increase in all soils and will exceed the critical limits for Hg (De Vries et al., 2007; OIS, 1998; Tipping et al., 2010a) but the concentrations will never exceed the WHO guideline (1 µg Hg L\(^{-1}\)) for Hg in drinking water (WHO, 2004). The critical limit proposed by the WHO was mainly based on studies conducted in organic rich soils in Northern Europe and only considers Hg concentrations in the humus layer (O horizon). Forest soils in Switzerland often contain a very small humus layer. If we estimate critical concentrations for soluble Hg in surface soils (L1) according to the Mapping Manual (LRTAP, 2004) and De Vries and Bakker (1998) and taking into account the low DOC concentrations of Swiss forest soils (A-horizons), the critical limits for Hg were substantially lower (between 0.004 and 0.008 µg Hg L\(^{-1}\)) than the WHO guideline (1 µg Hg L\(^{-1}\)) and the critical limit (0.035 µg Hg L\(^{-1}\)) proposed by De Vries et al. (2007). In three out of four soils (Beatenberg not considered because of the large organic layer), this critical limit would be below the estimated preindustrial Hg concentrations in the soil solution (data not shown). As a
consequence we assume that the estimation of critical limits for Hg according to others [Mapping Manual of the convention on LRTAP (2004), De Vries and Bakker (1998); De Vries et al. (2005)] is not appropriate for forest soils in Switzerland and needs to be re-evaluated.

6.5.2 Uncertainties of input data and model

The modelled HM concentrations are highly dependent on the input data. Large uncertainties exist about the HM depositions. Historical HM inputs were assessed by peat and ice core studies conducted in Switzerland (Barbante et al., 2004; Ross-Barraclough and Shotyk, 2003; Shotyk et al., 2002; Weiss et al., 1999). In addition, the tree species also influences the HM inputs (Perez-Suarez et al., 2008; St Louis et al., 2001) which have been taken into account in our estimations with different correction factors for conifer and deciduous forests. Uncertainties are also related to the sampling of the soil matrix and the soil solution at the same spot. To avoid any disturbance of the lysimeters, the soil samples had to be collected several metres from the lysimeters. Depending on the soil heterogeneity, it might lead to discrepancies between the soil and the soil solution properties. This was the case in Schänis where the soil solutions were collected in more alkaline soils than the soil cores. The ceramic cups used for the soil solution sampling might also have adsorbed metals, in particular Pb in acidic and alkaline conditions and Cd at alkaline pH (Rais et al., 2006). This might explain the higher calculated concentrations of heavy metals in the soil solution (Figure 6.1b). There are also uncertainties related to the model itself. CHUM-AM calculates concentrations on an annual timescale and neglects seasonal variations. Furthermore, CHUM-AM neglects erosion, bioturbation or changes in the vegetation (Tipping et al., 2010b). In general, the calculated HM contents in the solid phase of the topsoils were lower than the measured concentrations. These calculated values are comparable to the reactive HM pools in soils (Tipping et al., 2003) which could be estimated in soil samples using a diluted (0.43 M) HNO₃ extraction followed by ICP-MS or CV-AAS detection (Tipping et al., 2003). In our study, we measured the HM concentrations in the soil matrix using a 2 M HNO₃ extraction (Cd, Pb) or by DMA (Hg) resulting in higher measured than calculated concentrations. The measured Hg
concentrations using DMA were also in a good accordance to a 2M HNO$_3$ extraction followed by CV-AAS detection (Rieder et al., 2011).

Nevertheless, the modelled Cd, Pb and Hg concentrations in the soil matrix and the soil solution were in line with the measured concentrations. Therefore CHUM-AM can be used to model HM in for wide range of soils over Europe and not only for organic soils in UK.

6.5.3 Long term trends in HM concentration

The long-term behaviour of HM in Swiss forest soils is site (soil)-specific. Assuming a current deposition, the Cd and Pb concentrations will increase in two soils whereas in the other soils the concentrations will decrease or remain constant. Soils rich in organic carbon or with a high pH will increase in Cd and Pb concentrations whereas the concentrations in the other soils decrease. In contrast, the Hg concentrations will increase in all soils. The deposition of Cd largely decreased during the last 50 years (Shotyk et al., 2002). At that time, the Cd deposition was about twice as high as in 1990 and since then the Cd deposition further decreased (FOEN, 2008). A similar trend was observed for Pb deposition. We assume that in Lausanne and Novaggio the former Pb deposition was much higher for many years as in the other soils according to the peat profiles reported in Weiss et al. (1999) and that at these sites the Pb deposition in the last decades decreased more as in the other sites. In Novaggio, the deposition of Pb is still about three times higher than in the other sites (FOEN, 2008). The strong decrease in Pb depositions in Lausanne and Novaggio and their soil properties lead to the trend of decreasing Pb concentrations in soils. In contrast to Cd and Pb, the Hg depositions decreased only a little during the last decades. Therefore and because of Hg being highly immobile in soils, the Hg concentrations in the soils will further increase in all soils. In our calculations we used an Hg evasion rate of 0.3% per year. Higher evasion rates will decrease the Hg concentrations in soils but they hardly will affect the long-term trends (data not shown). Tipping et al. (2011) modelled the long term behaviour of Hg in three UK soils. Two of these soils were located near a former Hg source. In these two soils, the highest Hg depositions were about nine times higher as the current depositions. Tipping
et al. (2011) estimated that the concentrations of Hg in these soils will decrease whereas in the third soil no decrease was estimated, indicating that the historical Hg deposition strongly influences the Hg dynamics in future.

The maximum tolerable deposition in Switzerland [according to the OAPC (1985)] will substantially increase the Cd and Pb contents in soils and will exceed the critical limit concentrations (Lazzaro et al., 2006a; Lazzaro et al., 2006b; OIS, 1998; WHO, 2004). With the OAPC (1985) the Swiss regulations aim to protect human, animals, plants and the soil biota from air pollutants. Maximal acceptable deposition values are determined for Cd and Pb but not for Hg (OAPC, supplement 7, Art. 2 Abs. 5). These maximal acceptable depositions in Switzerland were comparable to the CL of the UNECE. The OAPC (OAPC, 1985) deposition values, for both Cd and Pb, should be reduced to maintain soil fertility with an unconfined microbial activity. The CL will lead to concentrations rarely exceeding the critical limits, except for the critical limit for Hg as proposed by Tipping et al. (2010a). In contrast, critical limits for Cd are exceeded in most soils by a deposition according to the CL (Lazzaro et al., 2006a; OIS, 1998).

6.6 Conclusion

In conclusion we found that the CHUM-AM model was able to accurately simulate HM (Cd, Pb and Hg) concentrations in Swiss forest soils. Thus, it was shown that CHUM-AS, which has only been applied in UK before current study, may be applicable for a wider range of soils throughout Europe. Long-term trends of Cd and Pb in soils differ between the sites whereas Hg tends to increase in all soils by the current HM depositions. The concentrations of Cd and Pb in soils rich in organic carbon or with a high pH value will further increase whereas the concentrations in the other soils will decrease. The maximal acceptable depositions for Cd and Pb in Switzerland according to the OAPC (OAPC, 1985) are too high and we suggest that they should be reduced in order to maintain long-term soil fertility with an unconfined microbial activity. The critical loads (CL) will lead to concentrations rarely
exceeding the critical limits for Pb and Hg. In contrast, critical limits for Cd are exceeded in most of the soils by a deposition according to the CL. Critical limits for Cd and Pb are based on ecotoxicological studies whereas little is known with respect to Hg. Furthermore, the CL for Cd defined in the frame of the LRTAP convention are also too high according to the current findings whereas the CL for Pb seem to be fairly accurate. More research is needed to validate the CL of Hg to better understand the ecotoxicity of Hg in temperate forest soils.

6.7 Acknowledgements

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Chapter 7

Final discussion

The main aims of this study were i) to examine the bioaccumulation of Hg and methyl-Hg in mushrooms and earthworms in natural forest soils and test whether soil properties increase their accumulations ii) to improve our understanding of how Hg affects microbial communities in soils; iii) to assess the Hg and methyl-Hg concentrations in natural Swiss forest soils according to the results of the ecotoxicological studies; (iv) to examine and improve the critical limits of Hg for Swiss forest soils; v) to study the digestive tracts of earthworms to see if they lead to a methylation of Hg, and finally vi) to predict the long-term behavior of Hg in forest soils in order to be able to assess critical loads.

7.1 Bioaccumulation of Hg and methyl-Hg

Soil organisms take up Hg and methyl-Hg from different Hg pools (Figure 7.1). In soils, Hg is largely bound to organic matter and soil particles. Microorganisms take up Hg mainly from the soil solution. Plants and mushrooms take up Hg from the soil solution or from the “reactive pool”. In contrast, earthworms take up Hg by ingesting soil and OM particles directly (Figure 7.1). The reactive pool includes all soluble or matrix-bound Hg species that are able to be rapidly released into the soil solution (De Vries et al. 1998). The accumulation of Hg by organisms is affected by the bioavailability of Hg and the organism's bioenergetics (Kidd et al. 2012). The Hg concentrations in organisms are often negatively correlated with the growth rate. Fast growing organisms with a high food conversion efficiency and low activities have more dilute Hg in their tissues in general (Trudel and Rasmussen 2006). This process is known as growth dilution. Long living organisms may contain higher concentrations of Hg. For example, the concentrations of Hg increase in fishes with age and size in general (Jackson and Somers 1993; Sonesten 2003). Furthermore, long-living, slowly growing
species of high tropic levels such as fish, birds, and mammals are particularly susceptible to bioaccumulate methyl-Hg (Scheuhammer et al. 2007; Kidd et al. 2012)

![Figure 7.1: The mercury cycle in forest soils. Mercury reaches the soils (light blue) and exists as unreactive, reactive or soluble Hg in the soils (black boxes). Organisms (green ovals) take up Hg from the different Hg pools (red arrows) and Hg is released from the soils by leaching or by evasion (dark blue).]

We found that mushrooms of litter decomposing fungi contained the highest levels of Hg (2.56 mg kg\(^{-1}\) dw) and wood decomposing fungi the lowest (0.24 mg kg\(^{-1}\) dw). Alonso et al. (2000) and Melgar et al. (2009) also observed higher Hg concentrations in saprophytic than in mycorrhizal fungi. It is assumed that the higher Hg levels in saprophytic species are the consequence of greater decomposing activity, e.g. catalase activity. In contrast, the lower Hg concentration in fruiting bodies of wood decomposing mushrooms could be the result of the low level of Hg in the wood and the limited amount of substrate compared to saprophytic species colonizing soils (Melgar et al. 2009).
We were interested in how soil properties are related to the accumulation of Hg in mushrooms. The concentrations of Hg in the mushrooms correlated weakly with the concentrations of Hg in the soils. Therefore, we assume that the total concentrations are not important for bioaccumulation, but rather for the reactive Hg pool or the concentrations in soil solution. Thus, mushrooms cannot be used as indicators for soil pollution with Hg. No other soil properties could be identified which enhance the accumulation of Hg in mushrooms. In Figure 7.2, the Hg and methyl-Hg concentrations in mushrooms are shown depending on the soil pH and organic carbon contents of the soils.

![Figure 7.2: Mercury (a, c) and methyl-Hg (b, d) concentrations in fungal fruiting bodies depending on soil pH (a, b) and soil organic matter (c, d). Data from all mushroom species were used.](image)

In earthworms, the highest concentrations of Hg were found in endogeic earthworms (1.63 mg kg\(^{-1}\) dw) and the lowest in epigeic earthworms (0.49 mg kg\(^{-1}\) dw). It is known that the accumulation of Hg in earthworms is species specific (Ernst et al. 2008) but to our knowledge, the effect of the ecophysiological group on Hg accumulation has never been studied thus far. The concentrations of Hg in different soil components may explain the
species specific accumulation of Hg by earthworms. Usually the Hg concentrations in the litter layer (habitat of epigeic earthworms) are lower than in topsoils (habitat of endogeic earthworms). Furthermore, the concentrations of Hg in soils decrease with soil depth, thus the habitat of anecic earthworms (deep burrow inhabiting) contains less Hg than the habitat of endogeic earthworms. Furthermore, epigeic earthworms are described as r-strategists, and endogeic and anecic earthworms as K-strategists (Edwards et al. 1996). Endogeic and anecic earthworms have a high regeneration potential and can reach much larger sizes (>30 cm) than epigeic earthworms (10-30 mm). Long living organisms contain higher amounts of Hg and methyl-Hg in their bodies as described above.

We observed (in contrast to mushrooms) that the total Hg and methyl-Hg concentrations in earthworms tended to increase with soil pH. Similar to mushrooms, the C\text{org} concentrations of the soils did not influence the Hg accumulation in earthworms (Figure 7.3).

The increase in Hg concentrations in earthworms with increasing pH can be accounted for by the organisms collected in the different environments. Epigeic earthworms were mainly found
in acidic soils whereas endogeic earthworms were found in neutral soils. Since endogeic earthworms contain about three times higher Hg concentrations than epigeic earthworms, the Hg accumulation by earthworms appears to depend on soil pH.

In fungal fruiting bodies, about 4.5% of the Hg was present in a methylated form (0.021 mg methyl-Hg kg\(^{-1}\) dw). The same as for the total Hg, the highest concentrations of methyl-Hg were detected in mushrooms of litter decomposing fungi (0.06 mg methyl-Hg kg\(^{-1}\) dw). In earthworms, about 8% of the Hg was present as methyl-Hg (0.089 mg kg\(^{-1}\) dw). The highest methyl-Hg contents were found in endogeic earthworms (0.153 mg kg\(^{-1}\) dw). These high concentrations of methyl-Hg in earthworms could either be the result of a strong methyl-Hg accumulation in earthworms, as suggested by several authors (Bull et al. 1977; Zhang et al. 2009) or of a methylation inside the earthworms. In laboratory experiments, we found strong evidence that inorganic-Hg was methylated in earthworms themselves. Thus, the high concentrations of methyl-Hg found in earthworms results not only from an efficient bioaccumulation but rather from a transformation of inorganic Hg to methyl-Hg in the earthworms as well.

Mushrooms are popular food sources in many countries. The WHO (WHO 2004) recommend a maximum tolerated weekly intake of 5 µg Hg kg\(^{-1}\) body weight whereby no more than 3.3 µg kg\(^{-1}\) should be present as methyl-Hg. The intake of a 70-kg person should therefore not be over 0.35 mg Hg or 0.25 mg methyl-Hg per week. Thus, with a mean concentration of 0.96 mg Hg kg\(^{-1}\) tissue dw in mushrooms, a long-term weekly consumption of about 300 g dried mushrooms may be acceptable according to the WHO. Considering only edible mushrooms, a large variation in Hg concentrations exists. For example, *Boletus erythropus* contains low concentrations of Hg (0.24 mg kg\(^{-1}\) dw) and methyl-Hg (0.009 mg kg\(^{-1}\) dw), and a weakly intake of almost 1.5 kg dried fruiting bodies is acceptable for a 70-kg adult. In contrast, several popular edible mushrooms contain enhanced concentrations of Hg. *Lepista irina*, *Macrolepiota procera* and *Russula cyanoxantha* contain between 4.44 and 5.61 mg Hg kg\(^{-1}\) tissue dw and 0.046 and 0.113 mg methyl-Hg kg\(^{-1}\) tissue dw. Their weekly consumption should not be over 60 g for a long period of time, where the total Hg and not the
methyl-Hg concentrations are limiting. Over 85% of a mushroom’s weight is accounted for by its water content (Manzi et al. 1999). Thus, a 70-kg adult can intake about 400 g of fresh mushrooms containing the highest concentrations of Hg found in Switzerland, without exceeding the tolerable weekly intake value for Hg according to the WHO.

### 7.2 Assessing the ecotoxicity of Hg in forest soils

The concentrations of Hg in Swiss forest topsoils dw ranged between 0.07 mg kg\(^{-1}\) dw and 0.55 mg kg\(^{-1}\) dw, rapidly decreasing with soil depth, and correlated to OC concentrations. In soil solutions of up to 15 cm soil depth, the concentrations of Hg varied between 0.004 and 0.03 µg L\(^{-1}\). The lowest Hg concentration in soils, which affected the bacterial community structure or the basal respiration was 3.2 mg Hg kg\(^{-1}\) soil dw and a soluble concentration of 0.004 µg g\(^{-1}\) dry soil. With a Hg supply of 3.2 mg Hg kg\(^{-1}\) soil dw, the basal respiration was reduced by about 13 % on average, with 30 % at maximum. Furthermore, at this level of Hg supply, the bacterial community structure was changed in one soil compared to non Hg-treated soils. Unfortunately, we conducted the experiments with steps which were too large between the Hg treatments to identify critical limits for Hg. Especially between the treatments with 0.32 and 3.2 mg Hg kg\(^{-1}\) soil dw, additional treatments should be conducted. In Figure 7.4, a dose response plot between bioavailable Hg and the decrease in basal respiration is shown. At about 0.008 µg Hg g\(^{-1}\) soil dw, clear effects on the basal respiration were observed.
3.2 mg Hg kg\(^{-1}\) soil dw is clearly higher than the measured concentrations of Hg in natural Swiss forest soils. Unfortunately, no comparable data for bioavailable Hg were available for Swiss forest soils. We found that the impact of inorganic Hg and methyl-Hg on soil bacteria depends on the soil type. Soil properties affect the metal mobility, and bioavailability and consequently, their own toxicity. In the two calcareous soils, we measured minor bioavailable Hg concentrations leading to smaller changes in bacterial community structures as in the other five soils.

The soil concentrations of Hg in Switzerland partly exceed critical limits from the literature (Meili et al. 2003; De Vries et al. 2005; Tipping et al. 2010). Mercury in soils strongly binds to thiol groups of OM (Skyllberg et al. 2003). In soils with an OM content of over 10%, Hg adsorption is dominated by the interaction between Hg and OM (De Vries et al. 2007). Therefore, it is suggested to prefer critical limits which are based on the OM content in soils (Meili 1991; Tipping et al. 2010). In mineral soils with a low OM content (<10%), Hg solubility is controlled by other matrices such as iron- and aluminium-oxy/hydroxides and clay particles (De Vries et al. 2007). Thus, it is debatable whether the critical limits for Hg should be based

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**Figure 7.4**: Dose-response plot between bioavailable Hg and a decrease in basal respiration (n=35). For samples below detection limit (0.001 µg Hg g\(^{-1}\) soil dw) the value 0.0005 µg Hg g\(^{-1}\) soil dw was used.
on the total concentrations of Hg or on the OM content of the soils. In Table 7.1 several critical limit concentrations of Hg are shown.

Table 7.1: Proposed critical limit concentrations of Hg in soils and soil solution

<table>
<thead>
<tr>
<th></th>
<th>Total content [µg Hg g(^{-1}) soil]</th>
<th>SOM based [µg Hg g(^{-1}) org]</th>
<th>Free ion concentration [µg g(^{-1})]</th>
<th>Soluble Hg concentration [µg L(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tipping et al. 2010</td>
<td>0.13</td>
<td>3.3</td>
<td>0.04 to 1.2 [µg g(^{-1})]</td>
<td>-</td>
</tr>
<tr>
<td>Meili et al. 2003</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>De Vries et al. 2007</td>
<td>-</td>
<td>0.5</td>
<td>&lt;1 [ng L(^{-1})]</td>
<td>0.035 [µg L(^{-1})]^3</td>
</tr>
<tr>
<td>This study</td>
<td>0.32 - 3.2</td>
<td>-</td>
<td>-</td>
<td>0.004 [µg g(^{-1})]^4</td>
</tr>
</tbody>
</table>

1 Depending on OM and pH and were calculated in this table for soils containing between 1% and 100% OM and pH values between 7.0 and 4.0.
2 Calculated by dividing the SOM based critical limit by the apparent distribution coefficient for Hg (10\(^6\) L kg\(^{-1}\)).
3 Total critical dissolved Hg concentrations in soil solution, which is calculated from the SOM based critical limit and the dissolved organic matter (DOM) concentrations. In this table, the critical limits were calculated for a DOM concentration of 70 mg L\(^{-1}\).
4 Bioavailable Hg in soil samples, determined by using a bacterial biosensor which display bioluminescence in the presence of Hg.

The determined critical limit based on the total Hg concentration in this PhD thesis (between 0.32 - 3.2 µg Hg g\(^{-1}\) soil) is higher than the corresponding critical limit proposed by Tipping et al. (2010) (0.13 µg Hg g\(^{-1}\) soil). Tipping et al. (2010) proposed three approaches to express the ecotoxicity of Hg in soils. The first method considers only the total amount of Hg in soils, the second considers also the OM content and the third considers the free ion concentration in solution. The ecotoxicological data of these critical limits are based on 52 chronic endpoints of which 42 are for microbes. Thus, the critical limits from Tipping et al. (2010) are based on a much larger dataset than the critical limits derived in this PhD thesis.

In this study, we were also interested in the ecotoxicity of methyl-Hg. To the best of our knowledge, only two prior studies consider the effect of methyl-Hg on soil microorganisms. Van Faassen (1973) observed a decrease in bacterial cell numbers on plating media
containing methyl-Hg and Kungolos et al. (1999) observed that fungal cell growth may be reduced in soils containing methyl-Hg twice as much as in soils containing HgCl₂. Methyl-Hg was present in trace amounts (<0.003 mg kg⁻¹) in the soils, resulting in methyl-Hg fractions between 0.2 and 2.4% of the total Hg. This range is slightly larger than previously observed in forest soils (Boudou et al. 1997). We spiked seven forest soils with increasing rates of methyl-Hg and studied soil functions (basal respiration), microbial biomass and bacterial community structure in the soils of the different treatments. In one soil, we already observed effects at concentrations of 0.005 mg methyl-Hg kg⁻¹ soil, but in most soils, significant effects occurred at concentrations of 0.02 mg methyl-Hg kg⁻¹ soil, which is approximately 10 times higher than the concentrations observed in forest soils. Methyl-Hg treatments of the two calcareous soils lead to changes in bacterial community structures similar to those in most other soils, however, the microbial biomass in these two soils was not affected by methyl-Hg treatments. We assume that less methyl-Hg was bioavailable in these two soils compared to the others. Despite the fact that methyl-Hg and also Hg mobility is strongly correlated with DOM solubilization and that DOM solubilization increases with pH, the two soils with the highest pH appear to contain lowest bioavailable Hg and methyl-Hg concentrations. This could be evidence that methyl-Hg, which is adsorbed in DOM, is not easily bioavailable. This is in line with Ravichandran (2004) and that understanding that the bioavailability of Hg and methyl-Hg in soils is predominantly increased with increasing soil pH.

Interestingly, basal respiration in Hg contaminated soils was lower compared to the non-contaminated soils, whereas in most of the methyl-Hg contaminated soils, basal respiration was enhanced. This could indicate different strategies in the detoxification of these pollutants.

We found that bacterial community structures were more sensitive to methyl-Hg pollution than fungal communities. It is known that fungi may tolerate increased concentrations of Hg (Brunker et al. 1974; Kelly et al. 2006) and that bacteria may be more sensitive to heavy metals than fungi (Rajapaksha et al. 2004). While the bacterial resistance to Hg and methyl-Hg is mainly associated with the presence of mer operons (Robinson et al. 1984; Barkay et
al. 2003), no methyl-Hg detoxification mechanism is known for fungi. Detoxification of inorganic Hg by fungi might be either due to the exudation of organic acids which may form metal-complexes, the reduction of Hg(II) to the volatile form Hg\(^0\), a methylation of Hg by the fungi themselves or by the conversion of Hg species to beta-HgS (sulphide mineral), which facilitates the precipitation of Hg (Vonk et al. 1973; Brunker et al. 1974; Aiking et al. 1985; Meharg 2003; Kelly et al. 2006).

Furthermore, soil polluted with methyl-Hg affected the bacterial community structure in earthworm digestive tracts much more than soil polluted with inorganic-Hg. Alterations of the bacterial community in earthworm digestive tracts may negatively affect the performance of earthworms in decomposing organic matter as well as the earthworms’ nutrient supply. Methylation of inorganic-Hg mainly occurs under anoxic conditions. Thus, the concentrations of methyl-Hg in soils may increase because of increasing soil moisture (e.g. through increasing precipitation or soil compaction) and increasing soil temperature (higher biological activity). Nevertheless, the concentrations of methyl-Hg in natural soils will be very low compared to the concentrations found in soil organisms. With the dying and decomposing of such organisms, the concentrations of methyl-Hg (and also of total Hg) can largely increase on a small-scale in soils and affect the microorganisms living there.

The worldwide emission of Hg has decreased in recent years (Selin et al. 2007; Li et al. 2009). Tipping et al. (2011) predicted a decrease in future soil concentrations of Hg for two out of three soils in the UK. In contrast, we have modeled an increase in soil concentrations by a factor between 2 and 3 over the next thousand years. The two soils from the study in the UK were exposed to historical Hg sources (Figure 7.5). Tipping et al. (2011) assumed that the deposition of Hg largely increased in 1850 for about 100 years, stayed at a high level before decreasing for about 30 years until the year 2000. The highest Hg depictions in these two soils in the UK were about 9 times higher than the current depictions (Figure 7.5).
In contrast, Hg emission in Switzerland increased at the end of the 19th century due to introduction of coal combusting railways in 1880, until the electrification of the railways started at the beginning of the 20th century leading to a decline in Hg emission. In Switzerland, the emissions of Hg increased again after the Second World War, due to post war industrialization, and have decreased since about 1970 (Ross-Barraclough et al. 2003). The current annual Hg deposition in rural sites without any nearby Hg source is about 20 μg m\(^{-2}\) (Ross-Barraclough et al. 2003) and the highest historical Hg depositions were about twice as high as the current depositions (Figure 7.6).

Figure 7.5: Historical deposition at two soil sites in UK relative to 2000. Source: (Tipping et al. 2011).

Figure 7.6: Historical deposition in Swiss forest soils relative to 2000.
Therefore, the deposition of Hg in the UK decreased much more than in Switzerland. We assume that the difference in the level of Hg deposition reduction between the UK and Switzerland leads to the different results regarding future Hg levels. By assuming a constant total evasion rate of Hg from the soils (percentage of total Hg), the greater the Hg releases in UK soils (due to the high historical deposition) will appear compared to those in Swiss forest soils. We tested this hypothesis by adapting the historical depositions - related to the year 2000 - for the soils in the UK (Figure 7.5) to the Swiss forest soils and then modeled the future soil concentrations. By doing so, we observed similar results for Swiss forest soils as Tipping et al. (2011) noted for the soils in UK. The concentrations of Hg will stay at current levels in three soils, then decrease or further increase in each individual soil in the future. Therefore, we conclude that the long-term behavior of Hg in soils largely depends on the past Hg depositions. Besides the uncertainties of the historical deposition data, there are several additional uncertainties concerning model inputs (e.g. variations in measurements, assumptions) and model neglections, with regards to the modeling of soil concentrations of Hg. However, CHUM-AM was able to adequately predict the current concentrations of Hg in soils with $R^2$ values of 0.21 for measured vs. modeled concentrations for Hg pool concentrations and 0.44 for Hg in the soil solution.

In this PhD thesis, we predict that the concentrations of Hg in about 1,000 years in Swiss forest soils (up to about 0.5 µg Hg g$^{-1}$) will be in the range of the determined ecotoxicological critical limit (0.32 to 3.2 µg Hg g$^{-1}$). Nevertheless, the ecotoxicological studies were short term exposure experiments (Frey and Rieder 2013) which do not allow prediction of chronic ecotoxicity of long-term pollution (Renella et al. 2002). In contrast, the critical limits for chronic Hg exposure, supposed by Tipping et al. (2011) (0.13 µg Hg g$^{-1}$ soil), will be exceeded in four out of five forest soils in Switzerland over the next 1,000 years.

### 7.3 Evaluation of the methods used

To study ecotoxicity of pollutants several attempts are used. In classical laboratory studies, bacterial cultures were incubated in heavy metal solutions or in growth media containing
heavy metals. This allows for the standardization of the experiments and the manipulation of incubation conditions, however, the force of their expression into natural habitats is limited. Although experiments which were conducted in the environment directly consider real environmental conditions, these experiments are not well reproducible and consequently present a standardization problem. In addition, pollutants were introduced into the environment. In this thesis, we used a laboratory-based microcosm approach. We sampled forest surface soils which we treated with an inorganic or a methyl-Hg solution. This permits the replication and standardization of the experiments and provides effects of soil treatment in “natural” conditions. In this way, we were able to study the relationship between the physico-chemical soil characteristics and the level of Hg or methyl-Hg pollution. A disadvantage of this technique is that the soil structure is destroyed by sampling and homogenizing of the soils. Furthermore, the soil treatments (e.g. drying, sieving) before adding inorganic or methyl-Hg may affect soil functions and microorganisms. In general, laboratory experiments with soil incubation show larger effects than one would observe in the field, where environmental conditions, the bioavailability and the exposure to heavy metals are different (Smolders et al. 2004). Therefore, one must be careful when extrapolating the results from laboratory studies to natural habitats. A second weakness of microcosm experiments as performed in this thesis is the relatively short incubation time of several weeks. With longer incubation times, the decay in carbon supply due to the absence of plants and soil animals may affect soil microorganisms. Therefore, based on the ecotoxicity experiments in this study, we can also assess the short term (acute) response of soil microorganisms and soil functions on the soil treatments with Hg compounds. Renella et al. (2002) attempted to model chronic toxicity in soils with data from short term incubation experiments with HM salts and concluded that short term experiments are not useful to predict chronic toxicity.

T-RFLP profiling – as done in this PhD thesis – is a good and simple technique to study the effect of soil treatment on microbial community structures but T-RFLP analysis still does not provide a complete profile of microbial community diversity (Kirk et al. 2004). T-RFLP
profiling relates to uncertainties in the qualitative and quantitative analysis of the data. The method used for extracting DNA from soil samples already affects the results of studying microbial communities. The quantity of extracted DNA can depend on organisms (e.g. gram-positive vs. gram-negative bacteria). As a control, we tested several commercial DNA extraction kits and the chloroform extraction technique – used in this thesis – and compared the T-RFLP profiles of extracted DNA. The highest numbers of OTUs were observed in the samples from the chloroform extraction technique and almost all OTUs obtained from the extraction kits were also found by this method. When extracting DNA, we always included a negative control. With each additional step (e.g. PCR amplification, purification, T-RFLP profiling), we included a negative and a positive control as quality control. If one control failed, the step was repeated. In addition, we only compared the results of the same soils (or earthworms) which were treated with different Hg compounds among themselves. Determining the complete microbial community was not as important. We were mainly interested in the level of Hg supply at which the microbial communities were affected. Another weakness with T-RFLP profiling is that different bacterial or fungal species may produce the same peak (OTU) in the T-RFLP profile. As a control, we sequenced OTUs of interest (e.g. increasing abundance in earthworms developed in methyl-Hg treated soils) at least three times. In each case, the identified organisms of the particular OTU were identical which indicates that the studied OTU was dominated by this organism. T-RFLP profiling offers a limited resolution of bacterial or fungal community structures (up to 141 OTUs). Pyrosequencing may provide many times the amount of OTUs than T-RFLP profiling and has revolutionized microbial community analyses in recent years. Pyrosequencing provides a sufficient depth to resolve meaningful biological patterns (Margulies et al. 2005) and may provide indicator species for Hg and methyl-Hg tolerance and sensitivity. The DNA extracted from the soils during for this PhD thesis are stored at -20°C and would be available for further analyses e.g. for pyrosequencing analysis. We observed that an acute soil concentration between 0.32 and 3.2 µg Hg g⁻¹ soil led to significant changes in bacterial community structures and activities. New experiments with soil concentrations between 0.32 and 3.2 µg
Hg g$^{-1}$ soil should be performed for estimating more precisely critical soil concentrations for Hg.

Besides the ecotoxicity experiments, the methylation of Hg in earthworms is another important topic in this thesis. Today it is assumed that Hg is mainly methylated by sulphate reducing bacteria (SRB) under anaerobic conditions. Methylation of Hg has already been observed in fungi (Vonk et al. 1973; Fischer et al. 1995) and termites (Limper et al. 2008).

The dominant macro fauna in soils are usually earthworms. We assumed that Hg could also be methylated in earthworms due to the high concentrations of methyl-Hg in earthworms, the mutualistic digestive tract of earthworms with highly active bacteria and the anaerobic and nutrient rich conditions in earthworm guts. This assumption can be proved in two ways. In the first approach, isolated bacteria from earthworm guts were incubated in pure culture media containing inorganic-Hg under anaerobic conditions. Thereafter, the methyl-Hg concentrations in the media are to be determined. The advantage with this approach is that it is possible to prove to the methylation potential of individual bacterial species naturally living in earthworm digestive tracts. One disadvantage of such studies is that a positive methylation detected in the laboratory does not imply that a methylation also occurs in earthworms due to different environmental conditions and the competitive behavior of the various bacteria species. Furthermore, this technique is very time consuming and isolation of bacteria is very problematic. Less than 10% of bacterial species are culturable in the laboratory today (Hirsch and Valdés 2010). An alternative method – which we have chosen in this thesis – is to incubate earthworms in Hg treated soils for several weeks and measure the methyl-Hg concentrations in the earthworms' tissue. Bacteria living in the soils may also form methyl-Hg which could immediately be absorbed by earthworms. Thus, the soils must be sterilized first. Growing in sterile soil may affect microbial communities and the earthworms' health. Furthermore, it is not possible to sterilize earthworms without harming them. Therefore, we cleaned the earthworms by starving them for 6 days on moist filter paper to empty their guts and we rinsed the earthworms with a 0.8% NaCl solution. Starving the earthworms on moist filter paper before incubation may already
disturb the bacterial gut community. Nevertheless, the earthworms were not sterile and excreted microorganisms which potentially could methylate Hg in the soils, which could be assimilated by the earthworms thereafter. Thus, three control experiments were conducted with Hg treated soils containing no earthworms: i) abiotic incubation ii) incubation with bacteria solution collected from earthworms and iii) incubation of excreted earthworms cast. These experiments showed that Hg is methylated in the earthworms’ bodies but we do not know how Hg is methylated or by which organisms. We therefore tested the bacteria living in earthworms for SRB and detected a bacterial strain closely related to a SRB (*Desulfovibrio vulgaris*) which has been recently shown, to facilitate the methylation of Hg in freshwater sediments (Shao et al. 2012). While we know that Hg is methylated in earthworms and we also know that there are candidates (*D. vulgaris*) which are potentially able to methylate Hg, further research is needed to determine the bacteria which is responsible for the methylation. For this, bacteria (especially *D. vulgaris*) must be isolated from earthworm digestive tracts and incubated solely in Hg containing the media as described before.

### 7.4 Outlook

In this PhD thesis, we have showed that the current levels of Hg and methyl-Hg in natural forest soils were low in general and do not affect microbial communities. All studies were performed in soils without any Hg source in their proximity and we therefore do not know how Hg and methyl-Hg affect ecosystems containing higher Hg concentrations.

We found bacteria classes which were tolerant (*Alpha- and Betaproteobacteria*) and others which were sensitive (*Firmicutes*) to inorganic and methyl-Hg soil pollution. Resistance to high Hg concentrations in soil has been identified in several eubacterial phyla (*Firmicutes, Actinobacteria, and Proteobacteria*) and in several archaea genomes (Hart et al. 1998; Holtze et al. 2006; Oregaard et al. 2007). The applied molecular biological studies (e.g. T-RFLP analysis) in this PhD thesis permit only a limited resolution of the microbial community structure. By using the latest technologies, such as pyrosequencing, one would be able to study the total bacterial or fungal community structure in soils, and even identify rare and not
only the most abundant organisms. Mercury and methyl-Hg tolerant and sensitive species and indicator species for Hg pollution could be identified. The soil samples of our laboratory experiments were stored in an extraction buffer at -20°C. Conducting pyrosequencing analyses of these samples would largely clarify the effect of Hg and methyl-Hg on microbial communities.

In this study, we investigated the effect of methyl-Hg on microbial communities and soil functions for the first time in detail. To be able to predict the effect of methyl-Hg on soil microorganisms in more detail, one should expand such experiments to other soils, especially to soils rich in organic matter. Furthermore, in this PhD thesis, only short term incubation experiments were performed to study the ecotoxicity of methyl-Hg. Chronic exposure to pollutants can be more problematic than acute exposure. In our study, fungal communities were not affected by methyl-Hg soil treatments whereas in chronic exposure, the fungi may be affected as well and bacterial communities may be affected at much lower concentrations. Hot spots with methyl-Hg pollution in soils may occur when soil organisms die. Organisms living in this proximity are exposed to acute high concentrations of methyl-Hg. Furthermore, if we assume that the methylation of Hg in soils will increase through higher soil moisture and temperature, the soil organisms would also be exposed to higher methyl-Hg chronically. To assess the long term effect of methyl-Hg to microorganisms, long-term experiments with low methyl-Hg concentrations should be conducted, since the critical limits for methyl-Hg are still inaccurate.

All ecotoxicological tests were performed in soils with low concentrations of Hg and methyl-Hg. In soils with a higher concentration of Hg or methyl-Hg, the ecosystem’s response to soil pollution with Hg or methyl-Hg could be different compared to our studied soils. In such soils, the organisms were already exposed to higher Hg or methyl-Hg concentrations and the microbial communities may have already changed and adapted to such conditions. Earthworms appear to provide a suitable habitat for the formation of methyl-Hg. Nevertheless, the processes (biotic, abiotic) and the organisms involved are still unknown. Sulphate reducing bacteria were detected in earthworms which have already been shown to
be able to methylate Hg in sediments, however, it is not clear whether these bacteria also methylate inorganic-Hg in earthworms. Other bacteria inhabiting earthworms appeared to be tolerant to Hg pollution (especially Beta-Proteobacteria). It should be tested whether particular bacterial species inhabiting earthworm guts are able to methylate inorganic-Hg. This could prove that Hg is already methylated in earthworms themselves. Furthermore, while we also observed that soil pollution with Hg or methyl-Hg largely affects the microbial community structure in earthworm guts, the effect of this change on the earthworms’ health and their capacity for OM degradation is still unknown.

We modeled the long term behavior of Hg (and also of Cd and Pb) for only five Swiss forest soils. In all soils, the concentrations of Hg will further increase in future. One should expand this modeling approach to more soils, especially to those of the LWF sites due to the existence of a good dataset. All soils were natural forest soils without any Hg source in their proximity. We did not study the concentrations of Hg in polluted soils, for example in urban areas or areas near roads in Switzerland. The long-term behavior of Hg in such soils is still unclear and should be clarified to assess Hg pollution in Switzerland more accurately. In addition, the critical loads should be newly calculated for mineral soils in Switzerland based on the higher critical limit concentrations proposed in this thesis.
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Publications


**Rieder, S., Brunner, I., Horvat, M., Jacobs, A., Frey, B. (2011)** Accumulation of mercury and methylmercury by mushrooms and earthworms from forest soils *Environmental Pollution 159: 2861 – 2869*


Selected Presentations

12th Joint Expert Group on Dynamic Modelling, 24-26th October 2012, Sitges (Spain). Oral presentation: Dynamic modeling of Hg in Swiss forest soils - effect of evasion rates and historical depositions on the course of future concentrations

14th International Symposiumon Microbial Ecology (ISME), 19-24th August 2012 Copenhagen (Denmark). Poster presentation: Biomethylation of mercury by earthworms and its influence on ingested bacteria

11th Joint Expert Group on Dynamic Modelling, 26-28th October 2011, Sitges (Spain). Oral presentation: Dynamic modeling of the long term behavior of Hg, Pb and Cd in Swiss forest soils by using CHUM