Stable isotope fractionation of selenium by biomethylation in soil

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Jóse Ortega y Gasset 09.05.1883 - 18.10.1955
spanischer Philosoph, Soziologe und Essayist

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List of abbreviations

AG1-X8	=	Anion exchange resin
AL	=	Alluvial soil
Al_o	=	Oxalate –extractable aluminium
BS	=	Base saturation
CDT	=	Canyon Diablo Troilite (reference material)
C/N	=	Carbon to nitrogen ratio
CO_3-C	=	Concentration ratio of OC and soil N
ECEC	=	Effective cation exchange capacity
DMSe	=	Dimethyl selenide
DMDSe	=	Dimethyl diselenide
DS	=	Double spike
DSMZ	=	Deutsche Sammlung für Mikroorganismen und Zellkuturen
FAO	=	*Food and Agriculture Organization of the United Nations*
Fe_d	=	Dithionite- citrate extractable iron
Fe_o	=	Oxalate –extractable iron
FO	=	Forest soil
GA	=	Garden soil
GLS	=	Gas-liquid separator
HG	=	Hydride generator
MC-ICPMS	=	Multicollector inductively-coupled plasma mass spectrometer
MeOH	=	Methanol
Mn_o	=	Oxalate –extractable manganese
N_{tot}	=	Total nitrogen concentration
NIST	=	National Institute of Standards and Technology
OC	=	Soil organic carbon
PA	=	Park area soil
RS	=	Roadside soil
RSD	=	Relative standard deviation
SGR-1	=	Green river shale (reference material)
S_{tot}	=	Total S concentration
USGS	=	United States Geological Survey

Summary

Selenium is an essential nutrient in small amounts but it becomes toxic at higher concentrations. The fate of Se in the environment is closely linked to redox reactions such as the reduction of Se oxyanions to methylselenides. Volatile methylselenides compounds are commonly observed and are an important flux of Se in the environment.

The overall goal of my thesis was to determine the stable isotope fractionation of Se by biomethylation of different oxidized Se species (Se[IV] and Se[VI]) in soil.

First, a method was established to trap volatile methylselenides quantitatively. Alkaline peroxide solution was suitable for this purpose. The alkaline peroxide trap showed a recovery of 95.6 ± s.d. 5.4% in volatilization experiments with methylselenide standards. The use of the alkaline peroxide trap in closed microcosm experiments proved to result in no Se losses and balanced Se isotope budgets. The mass-weighted mean $\delta^{82/76}$Se values for Se(IV) and Se(VI) microcosm experiments were -0.31 ± 0.05‰ (n = 3) and -0.76 ± 0.07‰ (n = 3) compared with -0.20 ± 0.05‰ and -0.69 ± 0.07‰ in the supplied Se, respectively.

In the second part of my thesis, the fungus species *Alternaria alternata* was incubated with Se(VI) and Se(IV) in closed microcosms for 11-15 d and additionally with Se(IV) for 3 to 5 d at 30°C. In 11-15 d, 2.9-11% of Se(VI) and 21-29% of Se(IV), and in 3 to 5 d, 3-5% of Se(IV) were methylated. The initial $\delta^{82/76}$Se values of Se(VI) and Se(IV) were -0.69 ± 0.07‰, and -0.20 ± 0.05‰, respectively. The $\delta^{82/76}$Se values of methylselenides differed significantly between Se(VI) (-3.97 to -3.25‰) and Se(IV) (-1.44 to -0.16‰) as sources after 11-15 d of incubation. Thus, the $\delta^{82/76}$Se values of methylselenide indicate the source species of methylselenides. The shorter incubation of Se(IV) for 3-5 d, showed a large Se isotope fractionation of at least -6‰ before steady state was reached.

In the third part, I determined Se partitioning among three operationally defined soil pools and the $\delta^{82/76}$Se values of total Se in ten urban topsoils with 0.09-0.52 mg kg^{-1} Se representing five different land use types (alluvial grassland, garden, park, roadside area, and forest). Only a small part of Se occurred in exchangeable and thus readily bioavailable form and in the little reactive residual fraction. Most Se was adsorbed to organic matter and Fe (hydr)oxides (42-77% of total Se). The $\delta^{82/76}$Se values of total Se in the topsoils were close to the bulk Earth composition with an average $\delta^{82/76}$Se value of -0.03 ± 0.38‰. Slighly lower $\delta^{82/76}$Se values of -0.59 to -0.35‰ in mainly forest soils and slightly higher ones of 0.26 to 0.45‰ in mainly alluvial soils were presumably caused by soil/plant-recycling and Se contamination by river water, respectively.

The fourth part comprised a natural attenuation experiment and a microcosm incubation of soil with *A. alternata*. Equilibration of spiked Se(IV) and Se(VI) for 3 d resulted in decreasing water-soluble Se concentrations by 32-44% and 8-14%, respectively, associated with small isotope fractionations of ε = -0.045 to -0.12‰ and -0.05 to -0.07‰, respectively. In two of the incubated soils – moderately acid roadside and garden soils - between 9.1 and 30% of the supplied Se(IV) and 1.7% of the supplied Se(VI) were methylated while in a strongly acid forest soil no Se methylation occurred. The methylselenides derived from Se(IV) were strongly depleted in ^{82}Se ($\delta^{82/76}$Se = -3.3 to -4.5‰) compared with the soil (0.16-0.45‰) and the spiked standard (0.20‰).

My results demonstrate that stable Se isotope ratios provide new insight into Se transformation processes.

Zusammenfassung

Selen ist in geringen Mengen ein essentielles Nährelement, das aber in höheren Gehalten toxisch wird. Der Se-Kreislauf in der Umwelt ist eng mit Redoxreaktionen wie der Reduktion von Se-Oxyanionen zu Methylselenid verknüpft. Flüchtige Methylselenide sind weit verbreitet und stellen einen wichtigen Se-Fluss in der Umwelt dar.

Das übergeordnete Ziel meiner Dissertation war, die Stabilisotopenfraktionierung von Se durch Biomethylierung verschiedener oxidierter Se-Spezies (Se[IV] und Se[VI]) im Boden aufzuklären.

Zunächst wurde eine Methode entwickelt, die es erlaubte flüchte Methylselenide quantitativ zurückzuhalten. Es zeigte sich, dass alkalische Peroxid-Lösung dafür geeignet war. Mit alkalischer Peroxid-Lösung wurde eine Wiederfindung von 95,6 ± Standardabweichung 5,4% in Verflüchtigungsexperimenten mit Methylselenid-Standards erreicht. Bei Einsatz von alkalischer Peroxid-Lösung in geschlossenen Mikrokosmos-Experimenten kam es zu keinen Se-Verlusten und ausgeglichenen Se-Isotopenbilanzen. Die massengewichteten $\delta^{82/76}$Se-Werte lagen für Se(IV) und Se(VI) am Ende der Mikrokosmos-Inkubationen bei -0,31 ± 0,05‰ (n = 3) und -0,76 ± 0,07‰ (n = 3) verglichen mit -0,20 ± 0,05‰ und -0,69 ± 0,07‰ im jeweils zugegebenen Se.

Im zweiten Teil meiner Dissertation wurde die Pilzart *Alternaria alternata* mit Se(VI) und Se(IV) in geschlossenen Mikrokosmen für 11-15 und Se(IV) zusätzlich für 3-5 Tage bei 30°C inkubiert. In 11-15 Tagen wurden 2,9-11% des Se(VI) und 21-29% des Se(IV) und in 3-5 Tagen, 3-5% des Se(IV) methyliert. Die anfänglichen $\delta^{82/76}$Se-Werte von Se(VI) und Se(IV) lagen bei -0,69 ± 0,07‰, und -0,20 ± 0,05‰. Die $\delta^{82/76}$Se-Werte der Methylselenide unterschieden sich nach 11-15 Tagen Inkubation signifikant zwischen Se(VI) (-3,97 bis -3,25 ‰) und Se(IV) (-1,44 bis -0,16‰) als Quellen. Die $\delta^{82/76}$Se-Werte der Methylselenide zeigen also die Quellen der Biomethylierung von Se an. Die kürzere Inkubation von Se(IV) für 3-5 Tage führte zu einer ausgeprägten Se-Isotopenfraktonierung von mindestens -6‰, bevor ein Fließgleichgewicht erreicht wurde.

Im dritten Teil bestimmte ich die Bindungsformen von Se mit drei operativ definierten sequentiellen Extraktionen und die $\delta^{82/76}$S-Werte des gesamten Selens in zehn urbanen Oberböden mit 0,09-0,52 mg kg^{-1} Se, die fünf verschiedene Landnutzungstypen repräsentierten (Überschwemmungsgrünland, Garten, Park, Straßenrand und Wald). Nur ein kleiner Teil des Seleniums lag in austauschbarer und damit direkt bioverfügbarer und in residualer, wenig reaktiver Form vor. Das meiste Se war an die organische Substanz und Fe-(Hydr-)Oxide gebunden (42-77% des gesamten Selens). Der mittlere $\delta^{82/76}$Se-Wert des gesamten Selens in den Oberböden lag mit -0,03 ± 0,38‰ nahe beim Mittelwert der gesamten Erde. Geringfügig niedrigere Se-Isotopensignale von -0,59 bis -0,35‰ v.a. in Waldböden und geringfügig höhere von 0,26 to 0,45‰ in Überschwemmungsgrünland wurden vermutlich durch Boden-Pflanze-Recycling und Se-Kontaminationen durch das Flusswasser verursacht.

Der vierte Teil umfasste ein “Natural Attenuation”-Experiment und Mikrokosmos-Inkubationen von Bodenproben mit *A. alternata*. Die Equilibrierung von zum Boden gegebenem Se(IV) und Se(VI) für drei Tage führte zu abnehmenden wasserlöslichen Se-Gehalten um 32-44% bzw. 8-14, die mit kleinen Isotopenfraktionierung (ε = -0,045 bis -0,12 ‰ and -0,05 to -0,07‰ verbunden waren. In zwei der inkubierten Böden mit mäßig sauren pH-Werten wurden zwischen 9,1 und 30% des zugefügten Se(IV) und 1,7% des zugefügten Se(VI) methyliert während in einem stark sauren Boden keine Methylierung auftrat. Das aus Se(IV) entstandene Methylselenid war deutlich gegenüber dem zugegebenen Se-Standard (0,20‰) an ^{82}Se verarmt ($\delta^{82/76}$Se = -3,3 bis -4,5‰).

Meine Ergebnisse zeigen, dass die stabilen Isotopenverhältnisse von Se neue Einblicke in Se-Transformationsprozesse erlauben.

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This section has been deleted in conformity with the regulation by the internet publisher (ArchiMeD) because of duty of secrecy. I am grateful to all the people for their support, and discussions during my PhD work. Their names are still listed in the printing version of this thesis.

A Summarizing overview

1 Introduction

Selenium was detected in 1817 by the Swedish chemist Jöns Jakob Brezelius and named by him after the Greek moon goddess "*Selene*" because of the close affinity to the earlier detected element tellurium (Lat. "*tellus*" = earth) (Hollemann et al. 1985). It is the 34th element in the periodic table and is one of the chalcogens. In the order of element abundance in the Earth's crust selenium is on the 60th place (Schrauzer, 1997). Selenium is ubiquitous in the Earth's crust, occurs as trace element in sulfidic ores, or is associated with copper. Selenium is used in manufacture of photo- and solar cells, rectifiers, in alloys, and as a pesticide (Daunderer, 2006). Furthermore, Se is used as dye in the glass and ceramics industry, as solvent (selenium oxychloride), coloring agent (HSe) in the processing of Cu, Pb, and Zn, anti-dandruff agents (selenium sulfide), and for material testing (the radionuclide ^{75}Se) (Whitton and Houlder, 1979).

The average concentration of Se in the Earth crust is 0.1 mg kg^{-1}. Selenium occurs in at least 40 different minerals, most of which are sulfides such as pyrite (FeS_2). Important Se deposits exist in the Sierra Umango in Argentina, Sweden (ores with up to 1000 mg kg^{-1} Se), Nevada, Washington, and Idaho (all U.S.A.) (U.S.Geological Survey, 1996-2000), Uzbekistan, Quebec in Canada (Brown, 1996), Norway (100-300 mg kg^{-1} Se) and Japan (Feiser, 1966). In the Mansfeld and Rammelsberg regions of Germany, Se was mined from copper minerals which contain up to 65 mg kg^{-1} Se (Eckart and Berg, 1958).

The concentrations of Se in soils usually range from 0.1-2 mg kg^{-1} with a worldwide mean concentration of 0.4 mg kg^{-1} Se but some seleniferous soils contain as much as 38 mg kg^{-1} Se. Selenium concentrations in soil depend on type of parent materials, presence of fine particles, distance to the oceans and organic materials in soils (McNeal and Balistrieri, 1989). Soils derived from igneous rocks contain low Se concentrations whereas the weathering of sedimentary rocks from the Creataceous and Paleocene results in seleniferous soils (Girling, 1984). The Se concentration of German soils varies from 0.02 to 2 mg kg^{-1} and shows a north-south gradient with decreasing Se concentrations towards the south (Hartfiel and Bahners, 1988; Hartfiel and Schulte, 1988).

Selenium is an essential trace element for animals and humans with a narrow margin between sufficiency and toxicity. Until 1957, Se was considered a poison that can be only harmful for human beings. In 1973, it was shown that this trace element is essential. As part of the enzyme glutathione peroxidase, it protects cell membranes from destruction. However,

selenium is at higher concentrations toxic due its chemical similarity to sulfur leading to non-specific replacement of sulfur by Se in proteins.

Selenium occurs in four oxidation states (–II; 0; +IV and +VI), similar to sulfur. Under oxidizing conditions the species selenite ($Se[IV]O_3^{2-}$), hydroselenite $HSe[IV]O_3^-$), and selenate ($Se[VI]O_4^{2-}$) dominate. In soils and sediments, Se oxyanions can be adsorbed on different minerals and organic matter. The adsorption of Se in soils and sediments is controlled by the concentration and oxidation state of Se, the reaction time, and soil properties including organic matter and clay concentration, pH, and cation-exchange capacity (Singh et al. 1981). Selenate and hydroselenate (Se[IV]) show a high affinity to iron minerals, whereas Se(VI) is more water soluble and less strongly adsorbed. Consequently, Se(VI) is more easily leached to the groundwater and more bioavailable. The adsorption of Se(IV) on iron (hydr)oxides results in the formation of outer- and inner-sphere surface complexes (Parida et al. 1997).

The abiotic reduction and oxidation of Se is controlled by the pH and Eh values. Myneni et al. (1997) reported that inorganic Se can be reduced abiotically by green rust. The Se(IV) can be reduced to Se(-II) form whereas Se(VI) is only reduced to Se(IV) or Se(0). The abiotic reduction of Se(IV) to Se(0) was observed in laboratory experiments with synthetic montmorillonite (Charlet et al. 2007) and on iron oxide surfaces (Chen et al. 2009).

Besides abiotic transformations, biotic processes play an important role in the environment. Dowdle and Oremland (1998) reported the oxidation of Se(0) to Se(IV) by different microorganisms. However, among the biotic Se transformations the dissimilatory reduction of Se(VI) dominates, while oxidation processes only occur to a small extent. Microbial reduction of Se takes place under both aerobic and anaerobic conditions. Microorganisms use Se oxyanions [Se(VI) and/or Se(IV)] as terminal electron acceptors (Fernandez-Martinez and Charlet, 2009). The capacity to reduce Se(IV) is more common than that to reduce Se(VI) (Doran, 1982).

In bacteria, fungi, and plants Se is metabolized by reduction/oxidation and/or biomethylation. Biomethylation is thought to be a way of detoxifying the environment. Volatile alkylselenides may be produced by microorganisms, plants, and animals. First evidences of biomethylation were detected by Challenger (1945) and a detailed study of biomethylation was conducted by Chasteen (1993) (*Figure A-1*). During the methylation of Se, selenite or selenate is first reduced, followed by an assimilation process to form organic Se intermediates. Through the binding of methyl groups ($-CH_3$), the organic Se intermediates are converted to dimethylselenonium compounds which further react to volatile

organoselenides. Among the volatile Se compounds, dimethyl selenide (DMSe) is the main product and is the most abundant Se compound in the environment. In addition, usually small quantities of dimethyldiselenide (DMDSe) are released. The most important naturally occurring methylating agents are Methylcobalamine, S-adenosyl methionine, and N-Methyltetrahydrofolate.

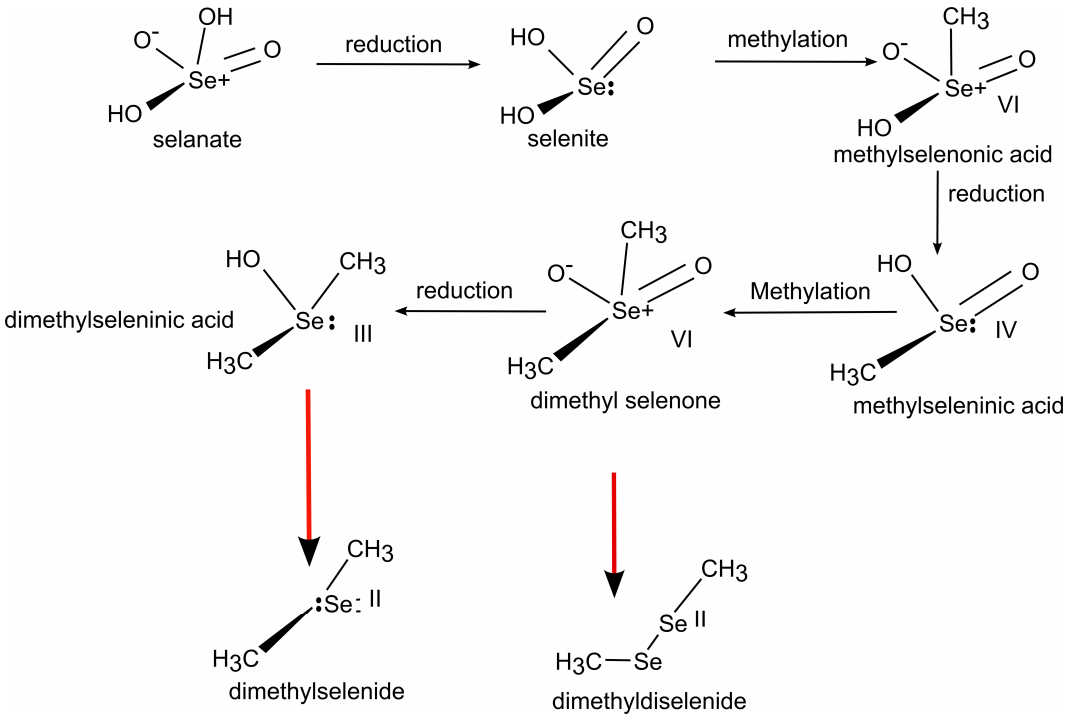

Figure A-1. Challenger mechanism about the transformation of volatile organoselenides (modified after Chasteen & Bentley, 2003)

Stable Se isotope ratios are useful indicators of processes that control Se distribution and speciation. Selenium has six naturally occurring stable isotopes with the masses ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, and ^{82}Se and relative abundances of 0.89, 9.37, 7.64, 23.78, 49.61 and 8.73%, respectively (Wachsmann and Heumann, 1992). The $\delta^{82/76}$Se ratios of igneous rocks have a homogeneous Se isotope composition and therefore are used as proxy for the bulk earth composition (Rouxel et al. 2002). In contrast, hydrothermal sulfides, terrestrial and marine sediments and soils vary in $\delta^{82/76}$Se values by up to 12.8‰ (Hagiwara, 2000; Rouxel et al., 2002; Wen et al., 2007; **Section D**, p. 63).

The fractionation of stable Se isotopes during reduction of Se(VI) or Se(IV) is already established as a useful tool to track redox changes in natural and experimental systems (Johnson, 2004). Isotope fractionation of Se was observed by dissimilatory microbial

reduction of Se(VI) and Se(IV) showing fractionation factors (ε values) of -4.5‰ to -6.5‰ whereas the reduction of Se (IV) to Se(0) resulted in ε values of -6‰ to -9‰ depending on bacterial species (Herbel et al. 2000). Johnson (2004) summarized various processes resulting in the fractionation of stable Se isotopes (*Figure A-2*).

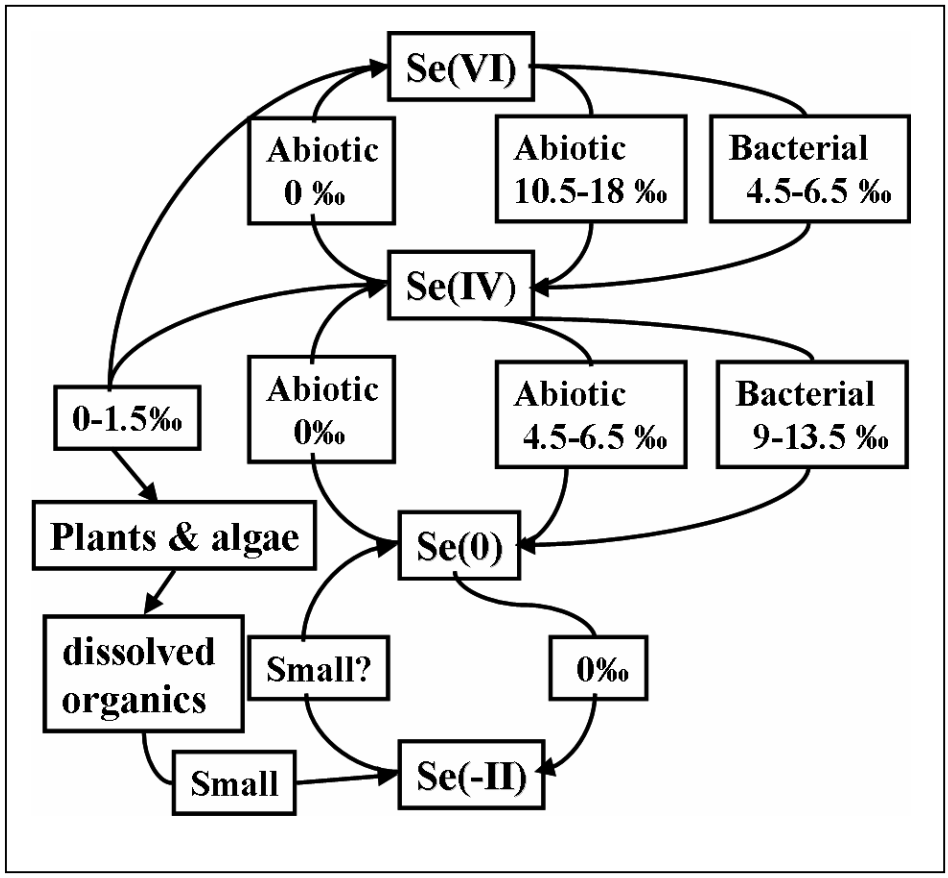

Figure A-2. Se-Isotope fractionation of different natural processes after Johnson (2004)

1.1 Objectives

In my thesis, I addressed the following research questions:

(1) Which procedure provides a quantitative trapping of organoselenides volatilized from microcosms in the laboratory to determine the stable Se isotope ratios with high precision (**Section B**)?

(2) What are the controls on Se isotope fractionation during biomethylation of inorganic Se to methylselenides by the fungus species *Alternaria alternata* (**Section C**)?

(3) Does the Se isotope ratio vary in soils with different land use and is it possible to infer Se transformation processes from the Se isotope ratios (**Section D**)?

(4) How do different soil properties influence the extent of biomethylation of Se, which was spiked to the soil prior to incubation, by the fungus species *Alternaria alternata* and the Se isotope ratios ($\delta^{82/76}$Se) in methylselenides? Which effect does the natural attenuation of the spiked Se have on the Se isotope ratios? (**Section E**)?

2 Materials and methods

2.1 Study sites and soil sampling

The study sites are located in the city of Bayreuth in Bavaria, SE Germany (49°94´ N, 11°57´ E). The surface horizons (0–5 cm) of ten soils in the urban and peri-urban area, which are affected by anthropogenic activity, were sampled. The samples included alluvial grasslands (AL1 and AL2), forests (FO1 and FO2), house gardens (GA1 and GA2), parks (PA1 and PA2), and grass-covered roadsides (RS1 and RS2, *Figure D-2*, p. 70). The soils were selected in order to represent different typical urban land types and biogeochemical conditions in an area which is prone to Se deficiency. The soil morphology was described in the field according to FAO (2006). Physico-chemical soil properties are described in detail in *Tables D-1 and E-1*, p. 83 and 97 (**Sections D-E**).

2.2 Microcosm experiments

To study the extent of biomethylation of Se by the fungus species *Alternaria alternata* and the associated Se isotope fractionation, 1 ml of fungal inoculum was incubated in 20 ml of malt-glucose-peptone medium in a serum bottle containing 5 mg L^{-1} Se(IV) or Se(VI) (*Figure A-3*, **Section C**). To explore the influence of different soils on Se biomethylation and associated Se isotope fractionation, two grams of soil were added to 20 ml of the malt-glucose-peptone-medium (**Section E**). During incubation, the bottles were horizontally shaken at 100 rpm at 30°C. The serum bottles were covered with aluminum foil to suppress abiotic photochemical formation of methylselenides (Guo et al. 2003). The incubation lasted 3-5 or 11-15 days.

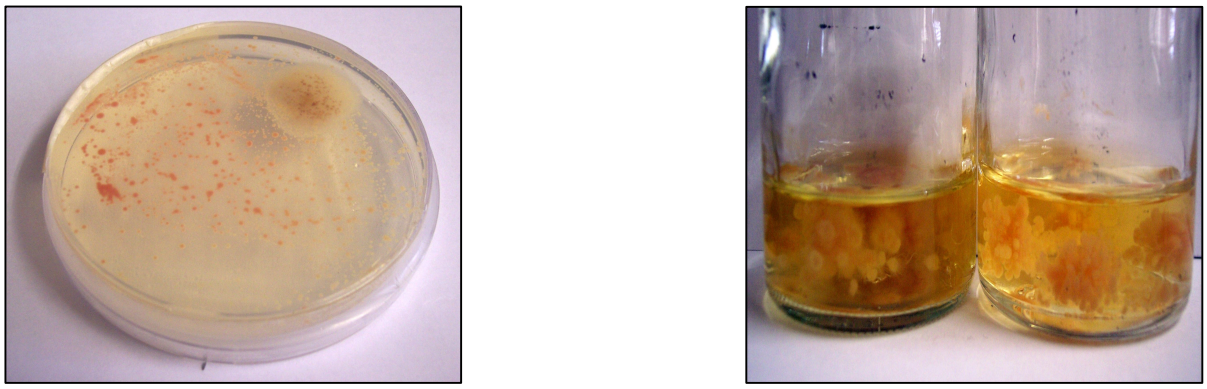

Figure A-3: Aspect of the fungus species Alternaria alternata after incubation with 5 mg L^{-1} of Se(IV). The reddish color of the fungus suggests the transformation to Se(0)on plate (left) and liquid medium (right).

2.3 Sample preparation

For Se isotope analysis, the soil samples were air-dried, sieved to <2 mm, and stored in closed Al containers before analysis (**Sections D-E**). The samples were digested in a microwave oven (MARS5Xpress, CEM Corp., Matthews, NC, USA) at 200°C under pressure in a 10 ml HNO_3 solution. The digested samples were diluted with deionized water and subjected to Se concentration analysis with inductively-coupled plasma-mass spectrometry (ICP-MS, ELEMENT 2, Thermo Scientific, Waltham, MA) by monitoring masses 77, 78 and 82 (**Sections D-E**).

2.3.1 Sequential extraction

The soils were sequentially extracted with the method of Martens and Suarez (1997) to characterize operationally defined Se forms (**Section D**). Five grams of each air-dried soil sample were treated as follows.

Fraction 1 (*soluble, ligand-exchangeable Se fraction*): 25 mL of 0.1 mol L^{-1} K_2HPO_4-KH_2PO_4 buffer (pH = 7), two hours

Fraction 2 (*iron and organic-bounded Se fraction*): 25 mL of 0.05 mol L^{-1} NaOH, two hours

Fraction 3 (*residual Se fraction*): 5 mL of concentrated nitric acid heating at 90°C for 30 minutes, then adding of 20 mL deionized water and heating at 90°C for two more hours

Fraction 1 was extracted at room temperature whereas Fractions 2 and 3 were extracted at 90°C. For all extraction steps the samples were centrifuged at 11.400 g (=10.000 rpm) (Beckman J2-MC, Beckman Coulter; Brea, Ca USA) for 30 min and the supernatant removed.

2.3.2 Preparation for isotope measurement

The soil total digests and sequential extracts were purified in two steps (*Figure A-4*, **Section D**).

First, I used a hydride generation system to volatilize Se (GILSON Miniplus3; Middleton, WI- USA) and then I poured the redissolved Se through an anion-exchange resin. This two-step purification was necessary because of the presence of germanium (Ge) and arsenic (As), which also form hydrides and therefore are not separated from Se by hydride generation, causes major interferences with Se during mass spectrometry. The anion-exchange resin served to both further reduce matrix effects and pre-concentrate Se.

Prior to hydride generation all Se in the extracts must be converted to Se(IV). To produce H_2Se in the hydride generation system $NaBH_4$ served as reductant. The hydride generation apparatus consists of a peristaltic pump to mix sample and reductant (~7:1) and introduce the mixture to the fritless gas-liquid separator (GLS). To increase the recovery, the GLS drain tubing was clamped and the GLS filled to ~2/3 with sample : reductant. Subsequently, peristaltic pumping was stopped and the solution was bubbled for 1 minute. The SeH_2 generated by GLS was captured in borosilicate tubes containing 5 mL of 1.0 M NaOH + 1 mL of 30% H_2O_2. Thereby, Se(IV) in the trap solution was converted to Se(VI). Finally, the remaining H_2O_2 was removed by heating to 100°C and maintaining this temperature until all of the H_2O_2 was reacted.

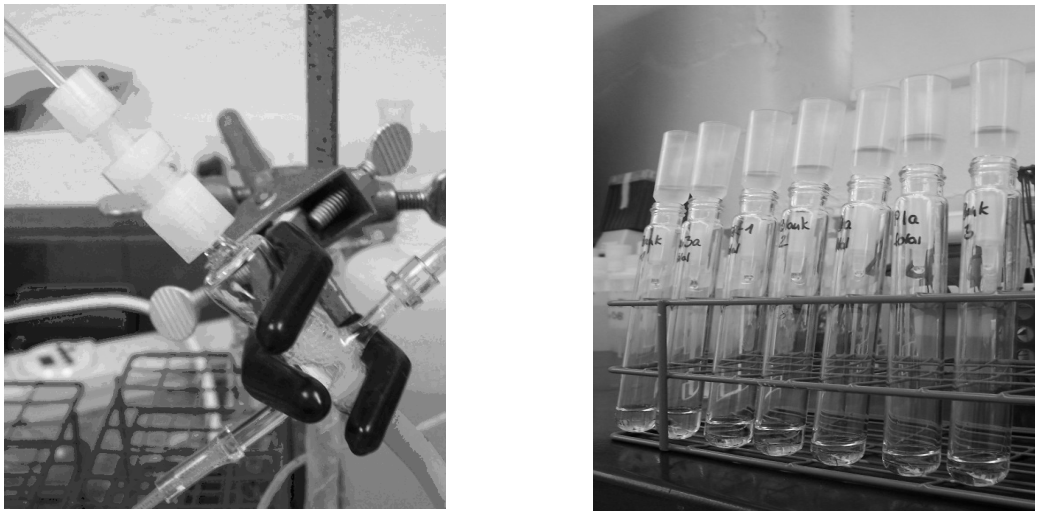

Figure A-4: Sample preparation for the analysis of Se isotopes. The two steps of purification: hydride generator (left) and anion exchange (right).

For the anion-exchange purification step, a column filled with 1 mL of AG1-X8 anion-exchange resin was used for separation and enrichment of Se. After cleaning the resin in three steps with methanol (MeOH) and HCl, the sample was loaded on the column. The selenate ion is retained by the anion-exchange resin, together with sulfate and nitrate. The column was eluted with 10 mL of deionized water until pH was neutral. In the next step, the oxyanions of P, As, and Ge and other weak acids were eluted with 10 mL of 0.1 M HCl. To elute the selenate, I used 5 mL of 6 M HCl and collected the elute in borosilicate tubes. The tubes were held at 105°C in an aluminum block for 90 minutes to convert Se(VI) to Se(IV).

2.4. Selenium isotope ratio measurements

The Se isotope ratios were measured with multicollector inductively-coupled plasma mass spectrometry (Nu Instrument, Nu Plasma, Wrexham, UK, *Figure A-5*) coupled with a hydride generator (GILSON Miniplus3; Middleton, WI- USA) (**Sections B-E**).

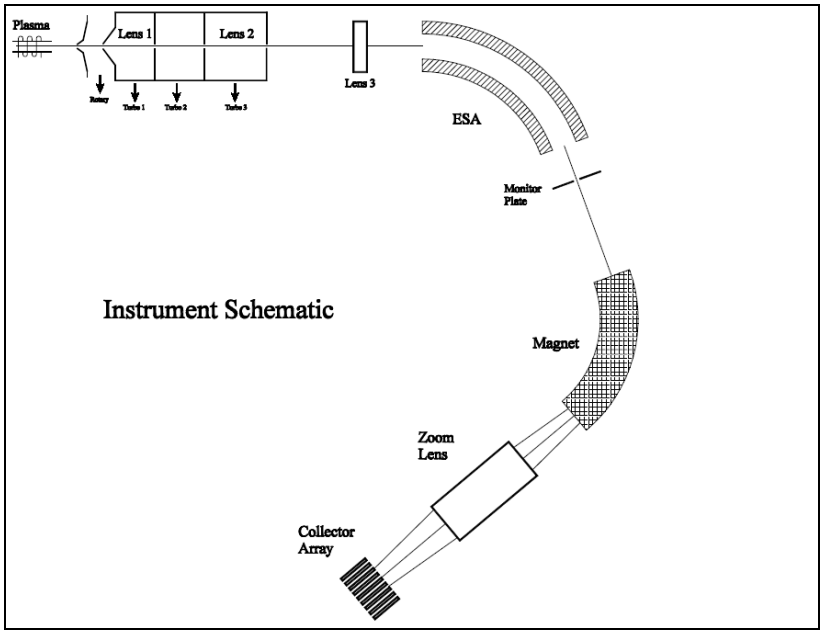

Figure A-5. Schematic sketch of the design of the multicollector inductively-coupled mass spectrometer Nu Instrument (Nu Plasma, Wrexham, UK).

Instrumental mass bias was corrected with the double spike (^{74}Se + ^{77}Se) technique, which is used for analyte elements with four or more isotopes. The double spike (DS) method was previously described in detail by Johnson et al. (1999). During the mass spectrometric measurement of Se, a significant but almost constant mass bias in favor of the heavier isotopes relative to lighter isotopes occurs which must be corrected. The advantage of mass-

bias correction with the DS method is the opportunity to directly correct the measurement of each individual sample and furthermore to account for isotope fractionation during hydride generation and sample injection even if the analyte is not fully recovered. The double spike was added as early as possible to the samples in the same oxidation state. Prior to purification steps (HG-purification and anion-exchange) and analysis with multicollector ICP/MS, the samples were spiked with the a ^{74}Se + ^{77}Se double-spike solution in the Se(IV) or in Se(VI) form for the trapped methylselenide (oxidized to Se(VI)). As proposed by Zhu et al. (2008) I used a sample/spike ratios of 1.5 to 3.5, because Zhu et al. (2008) reported that in this range of sample:spike ratios, no effect of interferences on the precision of the isotope ratio measurement was detected. The Se of the samples and standards was introduced to the ICP torch as hydride SeH_2 produced by the hydride generator system coupled to the MC-ICP/MS.

Selenium isotopes and possible interferences were simultaneously determined by nine Faraday cups (listed in *Table A-1*).

Table A-1. Simultaneously measured Se isotopes and the used Faraday collectors of the Nu-Plasma MC-ICP-MS device at the University of Illinois in Urbana-Champaign, USA.

Measured masses	**^{74}Se**	**^{76}Se**	**^{77}Se**	**^{78}Se**	**^{80}Se**	**^{82}Se**	**^{75}As**	**^{79}Br**	**^{81}Br**
Faraday cups	L5	L3	L2	Ax	H4	H6	L4	H2	H5

The Se isotope composition is reported as δ value ($\delta^{82/76}Se$ or $\delta^{82/78}Se$) relative to a NIST 3149 standard solution (Eq. A-1, **Sections B-E**):

$$\delta^{82/76}Se\ (‰) = \left[\frac{\left({}^{82/76}Se_{sample} \right)}{\left({}^{82/76}Se_{NIST\,3149} \right)} - 1 \right] * 1000 \qquad \text{(A-1)}$$

Furthermore, I measured the $\delta^{82/78}Se$ and $\delta^{78/76}Se$ ratios because recent published data report $\delta^{80/76}Se$ (Johnson et al., 1999, 2000; Herbel et al., 2000, 2002; Ellis et al., 2003) as well as $\delta^{82/76}Se$ values (Zhu et al., 2008; Rouxel et al., 2002; 2004; **Sections B-E**). Blank correction was performed by the "measure zero" method of the Nu Plasma software before the routine run started. To correct for ^{73}Ge interferences, ^{73}Ge was determined via measuring by an ion counter (IC0).

Besides the Se isotope ratio, I also calculated Se concentrations in the extracts via isotope dilution against the DS solution. (**Sections C-D**). This possible, if $\left(\frac{^{77}Se_{spike}}{^{total}Se_{spike}}\right)$ and $\left(\frac{^{82}Se_{sample}}{^{total}Se_{sample}}\right)$ are constant for all analyzed samples, which can be safely assumed. The reason is I added a known amount of spike to a known amount of sample, and the spike is very well calibrated i.e. we know what the ratios of $^{74/77}$Se (DS), $^{82/77}$Se, $^{82/74}$Se etc. Consequently, the calculation constant *K* can be derived from Eq. A-2.

$$K = \frac{\left(\frac{^{77}Se_{spike}}{^{82}Se_{standard}}\right)}{\left(\frac{m_{spike}}{C_{sample}m_{sample}}\right)} \qquad \text{(A-2)}$$

where *C* is the Se concentration in the sample and *m* is the mass of spike and sample. The values of $\frac{^{77}Se_{spike}}{^{82}Se_{sample}}$, m_{spike}, and m_{sample} were all known and *K* was calculated.

3 Discussion of uncertainty

To check for contaminations, I regularly ran blank analyses. In the blanks, I consistently found less than 16.4 µg Se (n = 10). Given the very low contamination, the analytical results were corrected by subtracting the blank. The detection limit for Se was calculated using the 3σ (3 standard deviation) criterion of the blank and amounted 0.27 µg L^{-1}. To account for possible drift of the measurements with ICP-MS and GF-AAS, I used repeated measurements of 25 µg L^{-1} Se standard. In ICP-MS measurement, yttrium (^{89}Y) or rhodium (^{103}Rh) were used as internal standard (**Sections B-E**).

The accuracy of the whole analytical procedure to determine total Se concentrations was checked with the help of the reference material USGS SGR-1 (green river shale). Replicate analysis of this SGR-1 standard showed a good accuracy of my results (3.27 ± standard deviation 0.03 mg kg^{-1}) with certified values (3.5 to 3.51 mg kg^{-1} – compiled values n = 3). The sum of Se concentrations of the sequential extractions was close to the total Se concentrations determined by microwave digestion with concentrated HNO_3 (slope of the

regression line of the sum of Se concentrations in the sequential extraction on those in the digests: 0.78, r = 0.96) (**Section D**). The precision of my Se determinations was determined by duplicate extraction, purification, and ICP-MS measurement of each sample both, for total digestion and sequential extraction. The relative standard deviations between the two determinations were consistently less than 9.2% (except FO1 with 21.8% because of the low Se concentration) for total Se and less than 16.7% for the sequential extraction.

In order to determine accurate isotope ratios of Se (**Sections B-E**), isobaric interferences must be removed. As shown in *Table A-2*, all Se isotope masses are affected by interferences from argides, elemental ions, and oxides. The minimum acceptable signal for standards and samples was 0.7 V on m/z = 78. To minimize the effect of some isobaric interference, prior to the isotope analysis, Se was chemically separated from Ge and As. Usually, the Ge interference is small with $5x10^{-5}$ V but nevertheless potentially important. If the Ge signal of the analyzed sample is greater than 1x10-4 V, it causes an error of up to ±0.1‰ on $^{78/76}$Se and ±0.4‰ on $^{82/76}$Se. Arsenic can interfere with the Se isotope measurement because of the formation of AsH^+ by hydride generation; AsH^+ can cause an error of up to 0.1‰ on $\delta^{82/76}$Se and $\delta^{76/78}$Se, if the ^{75}As signal has the same size as ^{76}Se. Usually, the ^{75}AsH/As ratio produced by the used hydride generator is similar as the SeH/Se ratio but it is generally small (Clark and Johnson, 2008).

A number of interferences on various Se isotopes are caused by argon (Ar) used as plasma gas. Polyatomic Ar ions such as $^{40}Ar^{40}Ar$, $^{38}Ar^{36}Ar$, $^{40}Ar^{36}Ar$, and $^{40}Ar^{38}Ar$ affect the measurement of ^{80}Se (the most abundant Se isotope), ^{74}Se, ^{76}Se, and ^{78}Se, respectively. The Ar background was removed by subtracting the background signal from the Se signals. However, to account for the ArAr interference caused by intensity drift, an additional correction was necessary. I estimated the contributions of $^{40}Ar^{36}Ar$, $^{40}Ar^{38}Ar$, and $^{40}Ar^{40}Ar$ to the total signal at masses 76, 78, and 80 by measuring the Se intensity and assuming the mean isotope distribution of Se. The resulting Se signal was then subtracted from the total signal to estimate the Ar contribution. Furthermore, the polyatomic interference of $^{40}Ar^{37}Cl^+$ produced from HCl, a component of the extracts, affects the mass ^{77}Se. This effect was controlled by producing a background HCl concentration of 2.0 ± 0.1 M in all solutions, which was much higher than the expected HCl concentration in the solutions to ensure a constant size of the $^{40}Ar^{37}Cl^+$ interference in samples and blanks.

A further interference on ^{82}Se can be produced by krypton (^{82}Kr), which is a trace contaminant in the Ar gas used and also generated from Kr dissolved into the sample solution from the atmosphere (Clark and Johnson, 2008). I removed this interference by subtracting

the on-mass zeroes and monitoring ^{84}Kr to make sure that the supplied argon gas is not unusally contaminated with Kr. Another source of Kr is air entering the GLS of the hydride generator. This source of bias was controlled by equilibrating all blanks, standards, and samples with air to guarantee a constant effect of Kr on the Se isotope measurement in all solutions. If the samples were not equilibrated with air, the $\delta^{82/76}Se$ values are measured by 0.1‰ too low if the intensity of ^{78}Se is about 400 mV.

Finally, borohydrides $^{81}BrH^+$ and $^{79}BrH^+$ produced by the hydride generator during purification, which are readily transferred to the plasma, cause interferences on ^{82}Se and ^{80}Se. To eliminate the Br interferences, N_2 was bubbled through all samples for 15 minutes to drive volatile Br compounds out of the solutions. The success of this measure was controlled by measuring mass 79 which allows for estimating the amount of $^{81}BrH^+$.

Table A-2. Measured Se isotopes and their possible interferences.

Isotopes masses	^{74}Se	^{76}Se	^{77}Se	^{78}Se	^{80}Se	^{82}Se
Isobaric interferences	$^{38}Ar^{36}Ar^+$ $^{74}Ge^+$ $^{58}Ni^{16}O^+$	$^{40}Ar^{36}Ar^+$ $^{38}Ar^{38}Ar^+$ $^{76}Ge^+$ $^{75}AsH^+$ $^{60}Ni^{16}O^+$	$^{40}Ar^{37}Cl^+$ $^{40}Ar^{36}ArH^+$ $^{76}SeH^+$ $^{76}GeH^+$ $^{61}Ni^{16}O^+$	$^{40}Ar^{38}Ar^+$ $^{77}SeH^+$ $^{62}Ni^{16}O^+$	$^{40}Ar^{40}Ar^+$ $^{80}Kr^+$ $^{79}BrH^+$ $^{64}Zn^{16}O^+$	$^{82}Kr^+$ $^{81}BrH^+$ $^{66}Zn^{16}O^+$

The degree of instrumental mass discrimination can be quantified by plotting $\delta^{82/76}Se$ vs. $\delta^{82/76}Se$. The slope of a linear regression line in a plot of the $\delta^{82/76}Se$ vs. $\delta^{82/76}Se$ values for standards and samples may display significant variability during the runs. Thus, for each analytical session, it is crucial to assess the respective $\delta^{82/76}Se$ - $\delta^{82/76}Se$ relationship in order to ensure the accurate mass bias correction of raw data (*Figure A-6*).

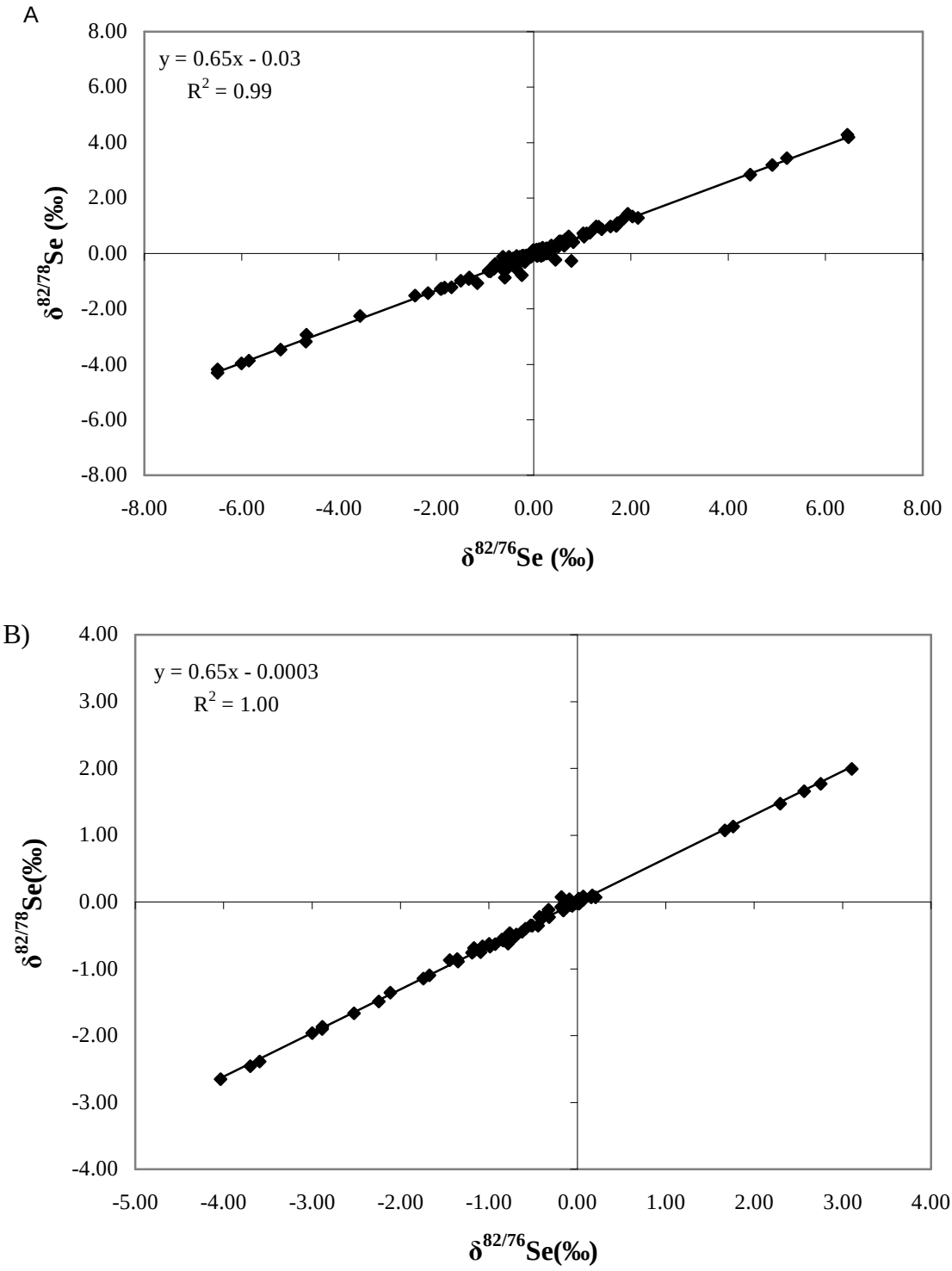

Figure A-6. Relationship between $\delta^{82/76}$Se and $\delta^{82/78}$Se values of microcosm samples and the NIST 3149 standard obtained in A) analytical runs of May 2009 and B) of March 2010.

Reference materials were chosen in order to cover a broad range of the environmental materials. The long-term reproducibility of $\delta^{82/76}$Se value of the SGR-1 (green river shale) standard was 0.2 ± s.d. 0.09‰ (n = 11) during 6 months.

4 Results and discussion

4.1 A method to quantitatively trap volatilized organoselenides for stable Se isotope analysis (Section B)

The first research objective was to develop an efficient method to collect Volatile methylselenides for Se isotope ratio analysis. To achieve this, I compared activated charcoal and alkaline peroxide trapping with respect to their suitability for the analysis of Se stable isotope ratios of organoselenides and tested the tightness of a microcosm system with the help of Se mass balances and the comparison of calculated mass-weighted mean $\delta^{82/76}$Se values in the whole microcosms with the $\delta^{82/76}$Se values of supplied Se oxyanions. It was demonstrated that the trap recovery was better for alkaline peroxide solution amounting to 95.6 ± standard deviation 5.4% (n = 3) than for activated carbon (<64%). The good performance of the alkaline peroxide trap was also confirmed in the microcosm experiments in which I recovered 96.6 ± 5.3 and 99.7 ± 12.1% (n =5) of the supplied Se(IV) and Se(VI), respectively. The mass balances of total Se in microcosm experiments run for 11-15 d with alkaline peroxide traps were 96 ± 15 and 102 ± 2.4% (n=3) for Se(IV) and Se(VI), respectively. The mass-weighted mean $\delta^{82/76}$Se values for the Se(IV) and Se(VI) batch experiments were -0.31 ± 0.05‰ (n = 3) and -0.76 ± 0.07‰ (n = 3) compared with -0.20 ± 0.1‰ and -0.69 ± 0.1‰ in the supplied Se oxyanions, respectively. The presented trapping method is reproducible and can be applied to quantitative studies of volatile methylselenides and their Se isotope ratios in the environment.

4.2 Isotope fractionation of selenium during fungal biomethylation by *Alternaria alternata* (Section C)

In Section B, my main objective was to quantify Se isotope fractionation during biomethylation of Se by the fungus species *Alternaria alternata* in microcosm experiments. The experiment was designed to understand the methylation processes using the stable isotopes of Se as tracers. My results show that there is a significant fractionation of Se isotopes during the methylation of Se and demonstrate that Se isotope fractionation among various inorganic Se species can provide new insights into the biogeochemical cycle of Se. Selenium isotopes were significantly fractionated during the methylation of Se(VI) ($\delta^{82/76}$Se of methylselenide = -3.97 to -3.25‰) compared to the initial $\delta^{82/76}$Se of Se (VI) of -0.69 ± 0.07‰ after incubation of 11-15 days. The incubation of Se(IV) for 3-5 days, showed a large Se isotope fractionation of at least -6‰. The accumulation and/or assimilation step of Se(VI) led to a small fractionation while the methylated Se was moderately fractionated. If *A. alternata* is capable of Se(VI) assimilation, I assume that Se(VI) is metabolized following the

sulfate reduction pathway because of the structural similarity of Se(VI) and sulfate. The transformation of Se(IV) usually is thought to follow almost the same reaction path as that of Se(VI). The greater isotope fractionation in the Se(IV) compared to that in Se(VI) experiments may be explained by the transport of the different inorganic Se species into the cells. The Se(IV) can be transported into the cell by distinct permeases whereas Se(VI) mainly follows the sulfate assimilation pathway. The isotope composition of Se in methylselenides provides information about the sources of methylselenides but is insensitive to different pH conditions in the surroundings of the methylating fungi.

4.3 Selenium partitioning and stable isotope ratios in urban topsoils (Section D)

The third section of my thesis treats the Se isotope analysis of ten soil samples to further our understanding of the biogeochemical cycle of Se in soil. Additional measurement of S concentrations and stable S isotopes helped in interpreting Se stable isotope ratios in soil. I determined Se partitioning among three operationally defined sequential extracts, the soluble, ligand-exchangeable, and plant protein-bound Se (Fraction 1), the Fe and organically bound Se (Fraction 2), and the residual Se (Fraction 3). The Se concentrations in the studied soils ranged between 0.094 and 0.52 mg kg^{-1} which were low to moderate Se concentrations. The largest contribution to total Se, extracted with NaOH, comprised up to 42% and thought to be associated with soil organic matter.

The $\delta^{82/76}$Se values of total Se in the topsoils were close to the bulk Earth composition with an average $\delta^{82/76}$Se value of -0.03 ± standard deviation 0.38‰ suggesting that there was no or little Se isotope fractionation in soil. The small isotope fractionation can be attributed to the low bioavailability of Se as a consequence of the presence of Fe oxides (adsorb the dominant Se(IV) forms strongly), organic matter, and SO_4^{2-} (prevents biouptake of the Se(IV) forms) in the study soils. Small Se isotope fractionations of -0.59 to -0.35‰ in mainly forest soils and of 0.26 to 0.45‰ in mainly alluvial soils were presumably caused by soil/plant-recycling and Se contamination by river water, respectively. In spite of the similarities in the assimilation of S and Se by organisms, the total S and Se isotope ratios in soil were not correlated. These results demonstrate that Se in urban soils developed from Se-poor substrates is little cycled through the biosphere. This is likely because of low bioavailability and competition with SO_4^{2-}.

4.4 Isotope fractionation of selenium by biomethylation in batch soil incubations with the fungus species *Alternaria alternata* (Section E)

The last section of my thesis offers a description of transformation processes of Se along the pathway from soil via microorganisms to volatile methylselenides. In this section, I first assessed the reduction in bioavailability of spiked Se oxyanions by sorption during three days, the time the samples were left to equilibrate after Se amendment before the incubation started. Then, closed microcosm experiments with soil spiked with Se(IV) or Se(VI), a growth medium, and the fungus species *Alternaria alternata* were conducted for 11 d. The equilibration of the spiked Se(IV) and Se(VI) for 3 days resulted in decreasing water-soluble Se (and thus bioavailable) concentrations by 32 to 44% and 8-14%, respectively, after three days associated with a little isotope fractionations of ε = -0.045 to -0.12‰ and -0.05 to -0.07‰, respectively. In two of the incubated soils – moderately acidic roadside and garden soils – between 9.1 and 30% of the supplied Se(IV) and 1.7% of the supplied Se(VI) were methylated, while in a strongly acidic forest soil no Se methylation occurred. The methylselenides derived from Se(IV) were strongly depleted in ^{82}Se ($\delta^{82/76}$Se = -3.3 to -4.5‰) compared with the soil (0.16 to 0.45‰) and the spiked standard (-0.20‰). The methylselenide yield of the incubations with Se(VI) was too small for isotope measurements. These results demonstrate that Se source species and soil properties influence the extent of Se biomethylation and that biomethylation results in Se isotopically light methylselenides.

4.5 Synthesis and conclusion

The present study was aimed at understanding the reactions involving Se and associated Se isotope fractionation in the environment. Reactions such as biomethylation and other transformation processes of Se change natural isotope ratios of Se. Accordingly, Se isotope ratios can be used as tracers for these reactions.

In the following, I summarize my answers to the research questions and provide a perspective of future investigations.

(1) Which procedure provides a quantitative trapping of organoselenides volatilized from microcosms in the laboratory to determine the stable Se isotope ratios with high precision?

The alkaline peroxide solution method was applied for the trapping of volatile methylselenides and the determination of their Se isotope composition. This method showed a

high recovery (95.6 ± 5.4%) and the mass-weighted mean $\delta^{82/76}$Se values for the Se(IV) and Se(VI) microcsom experiments (-0.31 ± 0.05‰ and -0.76 ± 0.07‰) were comparable with those of the supplied Se oxyanions (-0.2 ± 0.05‰ and -0.69 ± 0.07‰). The technique is therefore well suited for sampling of volatile organoselenides and measuring the Se isotope ratio in field studies.

(2) What are the controls on Se isotope fractionation during biomethylation of inorganic Se to methylselenides by the fungus species *Alternaria alternata*?

Selenium isotopes were fractionated during biomethylation by *Alternaria alternata* depending on the inorganic Se-species. The intensity of isotopic fractionation, expressed as ε, were as summarized in *Table A-3*.

Table A-3. Mean fractionation factors (and standard deviations) of the biomethylation of different Se source species at different pH values.

Source species	Experiment	Fractionation factor (‰)	pH
Se (VI)	**Section C**	-3.00 ± 0.26	4
Se (VI)	**Section C**	-2.72 ± 0.41	7
Se (IV)	**Section C**	> -6.00	7
Se (IV) +soil	**Section E**	> -3.3	/

The results of this part of my thesis illustrated that a considerable part of dissolved Se in the environment could be biomethylated associated with Se isotope fractionation. The variations in Se isotope ratios of volatile methylselenides may be used to infer Se source species and formation conditions of methylselenides in the environment.

(3) Does the Se isotope ratio vary in soils under different land use and is it possible to infer Se transformation processes from the Se isotope ratios?

Although Se isotope fractionation was small, it was possible to detect the effects of Se cycling by forest trees and Se contamination via river water. Selenium and S isotope ratios of topsoils under different (urban) land use were independent of each other in spite of their chemical similarity because different driving processes of isotope fractionation. In topsoils, Se isotope ratios are strongly influenced by preferred plant recycling of isotopically light Se via litterfall,

whereas S isotope ratios are mainly driven by mineralization of organic matter which contains a much larger reservoir of S than of Se relative to the S or Se fluxes. Consequently, the measurement of Se isotope ratios in the soil-plant-system provides further insight into the Se cycle. However, further studies under well controlled conditions are necessary to quantify the Se isotope fractionation associated with the complex mixture of Se transformation processes in the environment.

(4) How do different soil properties influence the extent of biomethylation of Se, which was spiked to the soil prior to incubation, by the fungus species *Alternaria alternata* and the Se isotope ratios ($\delta^{82/76}$Se) in methylselenides and which effect does the natural attenuation of the spiked Se have on the Se isotope ratios?

Selenium source species and soil properties influence the extent of Se biomethylation. Biomethylation generally yielded isotopically light methylselenides ($\delta^{82/76}$Se = -3.3 to -4.5‰) in line with the results of the incubation of *Alternaria alternata* with different Se source species in a growth medium without soil. The results of this part of my thesis be used to reveal pathways of biomethylation from different soils and source species. These results are also helpful in the context of bioremediation of Se-contaminated soils via biomethylation because they showed under which conditions biomethylation occurs to which extent. However, more soil incubations are needed before generalized conclusions can be drawn.

Several aspects of the isotope fractionation of Se in the environment require further study. Future research should be directed to solve the following scientific problems.

(1) Before the Se isotope signal in atmospheric methylselenides can be used as a tracer of Se source species and environmental conditions further possible Se-fractionation processes in the atmosphere need to be studied. Which effect do transformations of methylselenides in the atmosphere – particularly the sorption/desorption to aerosols – have on Se isotope ratios?

(2) Furthermore, as the Se isotope signal in bioavailable Se – which might be biomethylated – in soil differs, it is necessary to determine the variation in the Se isotope ratio among different soils under different land use and in different climate zones. The knowledge of the Se isotope signals in soil will advance our understanding of Se transformation processes in the environment and may even contribute to optimize bioremediation procedures of Se-contaminated soils.

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B A Method to Quantitatively Trap Volatilized Organoselenides for Stable Se Isotope Analysis

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1 Abstract

If volatile organoselenides are to be analyzed for their stable Se isotope composition to elucidate sources and formation processes, organoselenides need to be trapped quantitatively to avoid artificial Se isotope fractionation. We developed an efficient trap of organoselenides to be used in microcosms designed to determine the Se isotope fractionation by microbial transformation of inorganic Se to volatile organoselenides. The recoveries of volatilized dimethyldiselenide (DMDSe) from aqueous standard solutions by activated charcoal and alkaline peroxide solution with subsequent freeze-drying and purification via a cation exchange resin were tested. Microcosm experiments with the Se-methylating fungus *Alternaria alternata* in a growth medium were conducted, and tightness of the microcosm was assessed by comparing mass balances of total Se of the fungus, medium, and trapped organoselenides with the supplied Se mass. At the end of the experiment, we calculated $\delta^{82/76}$Se values of the whole microcosm and compared them with the $\delta^{82/76}$Se value of supplied Se(IV) and Se(VI). Our results demonstrated that activated charcoal cannot be used for quantitative trapping of organoselenides because generally <64% of the outgassed DMDSe were recovered. The mean recovery of Se volatilized from an aqueous DMDSe standard trapped in alkaline peroxide, in contrast, was 96 ± 11% (SD) after 2 h (n = 4). The mass balances of total Se in microcosm experiments with alkaline peroxide traps run for 11 to 15 d were 96 ± 15 and 102 ± 2.4% for Se(IV) and Se(VI) (n = 3), respectively. The mass weighted mean $\delta^{82/76}$Se values for the Se(IV) and Se(VI) batch experiments were -0.31 ± 0.05‰ (n = 3) and -0.76 ± 0.07‰ (n = 3), compared with -0.20 ± 0.05‰ and -0.69 ± 0.07‰ in the supplied Se oxyanions, respectively. We conclude that the alkaline peroxide trap can reliably be used to determine the Se isotope composition of organoselenides.

2 Introduction

Selenium is an essential trace nutrient for many organisms. However, Se is also toxic for most organisms at concentrations that are only slightly above the required levels. Selenium, like sulfur, can exist in the -II, 0, +IV, and +VI oxidation states. Inorganic Se(IV) species are up to 500 times more toxic than organoselenides (Chau et al., 1976). Methylation of Se is a widespread detoxifying process in the environment (McConnell and Portman, 1952). Organoselenides in the environment are likely to be generated by bioalkylation processes, similar to those known for Hg, Pb, As, and Sn (Craig and Maher, 2003). Microbial volatilization of Se as organoselenides was shown from soils (Karlson and Frankenberger, 1988a, 1989; Guo et al., 1999; Zhang et al., 1999), sediments (Calderone et al., 1990), waters (Chau et al., 1976; Cooke and Bruland, 1987), plants (Lewis et al., 1966; Zayed et al., 1998), and sewage sludges (Reamer and Zoller, 1980). The biotransformation of inorganic Se into volatile organoselenides depends on the Se source species (Doran, 1982), the availability and type of carbon sources (Karlson and Frankenberger, 1988b), temperature (Zieve and Peterson, 1981), and the presence of other trace elements, such as As or Cd (Diplock, 1979). Preliminary studies observed that organoselenides are a product of the metabolism of bacteria, fungi, plants, and humans (Challenger, 1945; Abuerrei et al., 1968; Thompsoneagle et al., 1989). Of the two most abundant organoselenides, the more volatile dimethylselenide (DMSe) diffuses more effectively from soil into the atmosphere, particularly at elevated temperature, than dimethyldiselenide (DMDSe) (Karlson et al., 1994; Guo et al., 2000). A variety of methods is applied for the trapping and determination of organoselenides. Several researchers used charcoal cartridges (Karlson and Frankenberger, 1988a; Martens and Suarez, 1999), glass wool (Jiang et al., 1989), a freezing procedure (Dejonghe et al., 1980), alkaline peroxide (Banuelos et al., 2005; Terry, Carlson et al., 1992), or concentrated nitric acid (Winkel et al., 2010; Zieve and Peterson, 1981). The nitric acid and alkaline peroxide as liquid traps have the advantage that no extraction of Se from a solid matrix is necessary (Jayaweera and Biggar, 1992). Previous studies showed recovery rates of organoselenides of 94 to 96% for different starting amounts of DMSe already in the first gas wash bottle of a series of bottles used to trap organoselenides (Terry et al., 1992). In three consecutive gas wash bottles containing different volumes of 0.05 mol L^{-1} NaOH and 30% H_2O_2, recoveries of, on average, 93, 7, and 0% were reported (Azaizeh et al., 1997; Azaizeh et al., 2003; Banuelos et al., 2005). In nitric acid and alkaline peroxide solutions, the two main organoselenide species DMSe and DMDSe are oxidized to Se(VI). In nitric acid, the

oxidation of DMSe and DMDSe results in dimethyl selenoxide and methylselenic acid, respectively, which can be separated by high-performance liquid chromatography–mass spectrometry or high-performance liquid chromatography hydride generation atomic fluorescence spectrometry (Winkel et al., 2010). In a nonreactive sorbent such as activated charcoal, DMSe and DMDSe are conserved. Because inorganic Se compounds, serving as sources for bioalkylation of Se, might differ in stable Se isotope ratios and because Se isotopes might be fractionated depending on the metabolic alkylation pathway (Johnson et al., 1999), the stable Se isotope ratios in organoselenides might offer an indication of different sources and formation pathways of organoselenides. The first Se isotope ratios were reported by Krouse and Thode (1962), who used gas source mass spectrometry. The development of thermal ionization mass spectrometry in the 1990s enabled the high-precision isotope analysis of heavier elements. The measurements of natural Se isotope ratios showed that Se isotope fractionation occurs during biotic (Johnson et al., 1999; Herbel et al., 2000; Ellis et al., 2003) and abiotic reactions (Johnson and Bullen, 2003). A significant fractionation of -4.0 to -9.0‰ relative to the reference material NIST SRM 3149, which has a Se isotope composition close to that of the Earth's crust (Carignan and Wen, 2007), was observed during dissimilatory reduction of Se oxyanions depending on bacteria species and the reduction process (Herbel et al., 2000). Ellis et al. (2003) reported a fractionation of the $^{80/76}$Se ratio of -2.6 to -3.1‰ during the reduction of Se(VI) to Se(IV) and of -5.5 to -5.7‰ during the transformation of Se(IV) to Se(0) in sediment slurry experiments with natural microbial communities. However, little or no fractionation was observed for Se(IV) sorption and oxidation of reduced Se in soils (Johnson et al., 1999). With the advent of the multicollector inductively coupled mass spectrometer coupled with a hydride generator, it became possible to analyze Se isotope ratios in natural samples with low Se concentrations (Rouxel et al., 2002). To make use of the information in stable Se isotope ratios of organoselenides with respect to source tracing and detection of formation pathways, it is important to develop a trapping method for Se isotope measurements of organoselenides with a recovery near 100%, which prevents potential isotope effects due to organoselenide loss because of incomplete trapping. Furthermore, the extraction of Se from the trap should be possible with simple purification steps and a low matrix effect. Our objective was to develop a procedure for the quantitative trapping of organoselenides volatilized from microcosms in the laboratory that allows for the determination of stable Se isotope ratios in organoselenides at high precision. To achieve this, we compared activated charcoal and alkaline peroxide trapping with respect to their suitability

for the analysis of stable Se isotope ratios of organoselenides and tested the tightness of a microcosm system with the help of Se mass balances and the comparison of weighted mean $\delta^{82/76}$Se values calculated according to the mass contributions of Se in the components of the microcosms (growth medium, fungus, and the organoselenide traps) in the whole microcosms with the $\delta^{82/76}$Se values of supplied Se oxyanions.

3 Materials and Methods

3.1 Material

Plastic and glassware were soaked in nitric acid and rinsed carefully with high-purity (>18 MW) deionized water before use. For the preparation of solutions, only high-purity reagents were used, and all deionized water was prepared by using a Milli-Q purification system (Milli-RO Plus30, Eschborn, Germany). Sodium selenate (Na_2SeO_4), Na_2SeO_3, and CH_3OH (analytical- reagent grade) were obtained from Merck (Darmstadt, Germany). Nitric acid, H_2O_2, DOWEX 50WX8 100- to 200-mesh cation-exchange resin, and the standards of DMSe [$(CH_3)_2Se$, ≥99.0% purity] and DMDSe [$(CH_3)_2Se_2$, 98% purity] were supplied by Sigma Aldrich (Steinheim, Germany). We prepared organoselenide stock solutions of 0.2 mg L^{-1} DMSe and 0.4 mg L^{-1} DMDSe. For the alkaline peroxide trap, we used a solution of 99.9% NaOH (Merck) and 30% *Trace*Select H_2O_2 (Sigma-Aldrich, Seelze, Germany). The packing material used for the activated charcoal trap included activated charcoal, glass wool (VWR, Darmstadt, Germany), and a Whatman no. 2 filter in a borosilicate cylinder (45 mm length; 22 mm diameter). Solutions of 1000 mg L^{-1} of ^{89}Y and ^{103}Rh in 0.5 mol L^{-1} nitric acid from CPI International (Amsterdam, The Netherlands) were used as internal standards. A stock solution of 3000 mg L^{-1} Se (SRM 3149; NIST, Gaithersburg, MD) was used to prepare Se standard solutions for the determination of Se concentrations with inductively coupled plasma mass spectrometry (ICP–MS).

3.2 Experimental procedure

We tested the trapping efficiencies of activated charcoal (in granulated form) for volatilization of DMSe and DMDSe from aqueous standard solutions. The trapping efficiency of the alkaline peroxide solution was tested by using an aqueous DMDSe standard. Because of the lower volatility of DMDSe (Henry constant: 0.218 kPa m^3 mol^{-1}) than DMSe (Henry constant: 0.144 kPa m^3 mol^{-1}) (Karlson et al., 1994), the risk that part of the compound remains in the stock solution used for the volatilization experiments is higher for DMDSe

than for DMSe. However, for DMSe there is a more pronounced risk of passing through the alkaline peroxide trap without reacting. We nevertheless considered it to be unlikely that DMSe passed all three consecutive gas wash bottles without reaction.

3.2.1 Activated charcoal

We modified a method of Karlson and Frankenberger (1988a), who determined the recovery of organoselenides labeled with the radioactive ^{75}Se isotope in an activated charcoal trap. We placed different amounts of granulated activated charcoal (three times each of approximately 0.2, 0.4, and 0.5 g, and one time each of approximately 0.3 and 0.6 g) in a borosilicate glass cylinder between a filter on the bottom and glass wool on the top. The activated charcoal–filled glass cylinder, acting as organoselenide trap, was attached to an outlet hole in the Teflon-impregnated butyl stopper used to close an Erlenmeyer flask containing 2.5 mL of the organoselenide stock solution (DMSe [n = 3] or DMDSe [n = 3]). The volatilizing DMDSe in the Erlenmeyer flask was allowed to equilibrate with the activated charcoal trap for 24 h. Thereafter, we pulled potentially remaining organoselenides into the activated charcoal trap by using a small vacuum pump (0.1 L min^{-1}) for 2 h. All volatilization experiments were repeated at least three times (see Table 1 for details). The trapped DMDSe and DMSe were eluted by soaking of the activated charcoal in 2 mL of methanol for 10 min according to the suggestion of Karlson and Frankenberger (1988a).

3.2.2 Alkaline peroxide trap

The schematic diagram of the apparatus for the alkaline peroxide trapping procedure is shown in *Figure B-1*. Three gas wash bottles were combined via Teflon tubes (60 mm length, 10 mm diameter) to prevent adsorption of volatile Se compounds on the tubes. One of the gas wash bottles was connected to a N_2 reservoir via a gas flow-regulator (red-y smart controller, type GSC-A9SA-BB02; Vogtlin Instruments, Aesch, Switzerland). For each experiment, a new trap solution was prepared. The volatilizing DMDSe standard was drawn through three gas wash bottles filled with the trapping solution and connected to the serum bottle in which the DMDSe standard was filled. As trap solutions, 90 mL of 0.05 mol L^{-1} NaOH and 30 mL of 30% H_2O_2 were used. A constant N_2 stream of 0.75 L min^{-1} was passed through the serum and gas wash bottles for 2 h to remove any volatile Se compound. At the end of the experiment, the alkaline trap solution was heated for 2 h to eliminate the remaining H_2O_2 and to oxidize the trapped methylselenide completely to Se(VI). The recovery in alkaline peroxide

solution of organoselenides volatilized from aqueous standards was studied after different outgassing times of 60, 90, and 120 min.

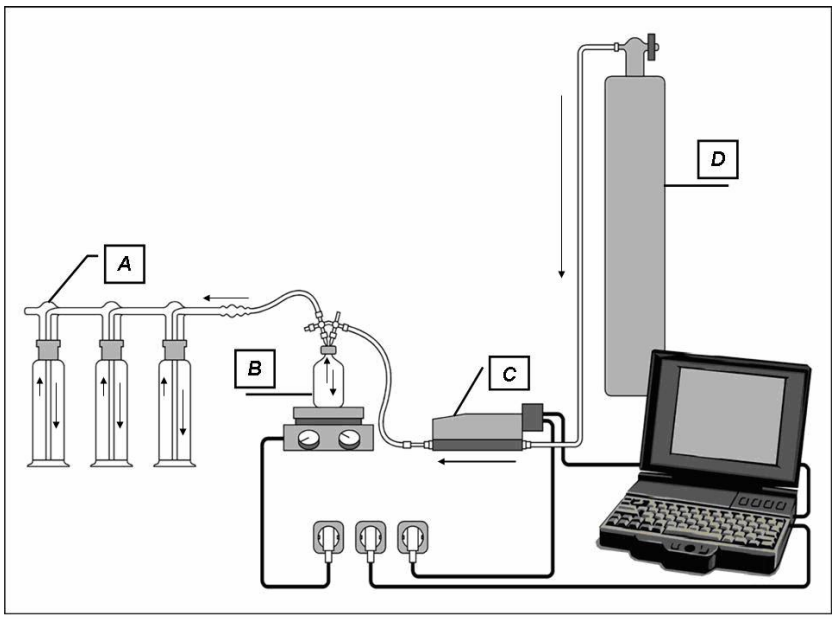


Figure B-1: Modified trapping method for volatile organoselenides after Terry et al. (1992). A) gas wash bottles each with 120 mL alkaline peroxide solution, B) microcosm with DMDSe standard or fungual culture, C) gas flow regulator, D) 99.9% N_2 gas. All parts of the apparatus are connected via Teflon tube lines and the arrows illustrate the direction of the gas flow.

The volume of the alkaline peroxide solutions containing the trapped organoselenides was reduced to 20 mL by freeze-drying. To eliminate Na from the trapping solution after the end of the experiment, chromatographic columns were used. Syringes (20 mL) were tipped with glass wool, and 2 g of DOWEX 50WX8 100- to 200-mesh cation-exchange resin were added covered with a second layer of glass wool. Before adding the Se(VI)-containing trap solution, the cation-exchange resin was conditioned with 4 mol L^{-1} HCl for 2 h, rinsed with deionized water, and dried at 40°C overnight. After loading the samples onto the columns, the Na^+ ions were replaced by H^+ ions, whereas the negatively charged Se(VI) passed through the columns. The removal of Na^+ ions was confirmed by pH measurements; pH values changed from strongly alkaline (pH ~12) to weakly acidic (pH ~5). To test the effects of freeze-drying and cation exchange on the recovery of organoselenides, we processed Se(VI) standards in the same way as the trapped organoselenides. The tests of the influence of freeze-drying and cation exchange on the recovery of Se were replicated three times at each Se concentration level of 1, 2.5, 5, 10, 25, 50, and 100 mg L^{-1} Se.

3.2.3 Microcosm experiments

3.2.3.1. Material

All solutions were prepared using high-purity (>18MΩ) deionized water. Stock standard solutions of 1000 mg L^{-1} Se as Na_2SeO_4 and as Na2SeO3 were purchased from Merck (Darmstadt, Germany). A strain of *Alternaria alternata* was obtained from the German Resource Center for Biological Materials (DSMZ, Braunschweig, Germany). To trap organoselenides, we used the alkaline peroxide trap as described above. All samples were filtered through 0.45-μm membrane filters (VWR, Darmstadt, Germany). The solution of 0.2% (w/v) $NaBH_4$ (Acros Organics, Fair Lawn, NJ) for the hydride generation was prepared daily in 0.2% (w/v) NaOH (Fisher Scientific, Fair Lawn, NJ). For the quality control and normalization of Se isotope measurements, we used SRM 3149 (National Institute of Standards and Technology, Gaitherburg, MD).

3.2.3.2. Procedure

In addition to the tests with aqueous solutions of DMDSe, the alkaline peroxide trap was used in microcosm treatments in which the known methylating fungus species *A. alternata* was incubated in a growth medium to which Se(IV) or Se(VI) was supplied. The microcosm in which we supplied Se(IV) and that in which we supplied Se(VI) were each replicated three times. The fungus was cultivated on agar plates at 30°C for 5 d and kept at 4°C before use. The liquid culture medium used was prepared as described by Thompsoneagle et al. (1989). An aerobic culture was grown in a 250-mL Erlenmeyer flask containing 80 mL of the medium at 30°C on a rotatory shaker for 7 d before the culture was used for incubation with inorganic Se species. Incubations were conducted in 80-mL serum bottles containing 20 mL cultured medium (10 g L^{-1} malt extract, 10 g L^{-1} glucose, and 0.5 g L^{-1} peptone), which was spiked with Se(IV) or Se(VI) to a final volume of 50 μg Se per bottle. At the end of the experiment after 11 to 15 d of incubation, we treated the alkaline peroxide trap solutions as described previously. One blank was prepared for each experimental batch to check possible Se contaminations. No Se was detected in any blank.

3.3. Analytical methods

3.3.1. Measurement of selenium concentrations

To determine the Se concentrations in the trap solutions, the isotopes ^{82}Se and ^{77}Se were measured relative to the internal standards with an ICP–MS HP4500 (Agilent, Waldbronn, Germany). The two Se isotopes were selected because they had the smallest signal-to-background ratio compared with the other stable Se isotopes. To calibrate the ICP–MS, we used the NIST SRM 3149 standard. The measurement of Se with ICP–MS had a detection limit of 2.7 mg L^{-1} Se, which was calculated as 3 SD of the mean Se concentrations in the blanks. The precision of the analysis by ICP–MS of three samples of 25 µg L^{-1} was 1.2%.

3.3.2. Measurement of selenium stable isotope ratios

Selenium isotope ratios were determined with multicollector ICP–MS (Nu Plasma; Nu Instruments, Wrexham, UK). The double-spike method was used to correct for instrumental mass bias, and Se was introduced into the instrument via a custombuilt hydride generator, following methods given by Clark and Johnson (2008). Here, a ^{74}Se + ^{77}Se double spike was used for all analyses (Zhu et al., 2008). We collected aliquots with a total Se mass of 100 ng from each sample because this is the optimum sample size for good counting statistics. An appropriate amount of double-spike solution was added so that the ratio of spiked ^{77}Se to ^{78}Se in the sample was close to 2:1. The samples were made up with 5 mol L^{-1} HCl (±0.2 mol L^{-1}) to a total of 5 mL. To convert Se(VI) to Se(IV), the samples were heated in a hot block held at 120°C for 1 h. After cooling at room temperature, the solutions were diluted to 2 mol L^{-1} HCl (±0.2 mol L^{-1}). Standard and blank solutions were prepared following the same procedure with the samples. The solutions were bubbled with N_2 for 20 min to remove volatile Br species, after which the glass tubes were equilibrated overnight with atmospheric Kr because the volatile Br species (^{81}BrH) and Kr (^{82}Kr) interfere with the measurement of ^{82}Se. The equilibration of the sample with the atmosphere ensures a stable background signal of Kr in all samples and blanks (in equilibrium with atmospheric Kr), which allows for the subtraction of the blank signal (on-mass zeros) of ^{84}Kr from the signal at mass 84 of all samples. Sample Se was introduced into the mass spectrometer as H_2Se generated via reaction with a 0.2% $NaBH_4$ solution in a continuous flow hydride generator similar to that described by Rouxel et al. (2002). Measurements of $^{74}Se/^{78}Se$, $^{76}Se/^{78}Se$, $^{77}Se/^{78}Se$, $^{80}Se/^{78}Se$, and $^{82}Se/^{78}Se$ were made with all Se isotopes measured simultaneously, with a minimum of 30 integrations of 5 s each. Interference from ^{74}Ge and ^{76}Ge was determined via measurement of ^{73}Ge on an ion

counting device and multiplication by mass bias–modified natural $^{74}Ge/^{73}Ge$ and $^{76}Ge/^{73}Ge$ ratios. Interferences by $ArAr^+$ at masses 76 and 78 were removed mostly by on-mass baseline measurements using high-purity HCl with a secondary correction using the mass 80 measurement. Intensity at mass 80 in excess of the expected 80Se signal was used to estimate the intensity of the residual $ArAr^+$ signal. Corresponding interferences at masses 76 and 78 were calculated using mass bias–modified natural Ar isotope ratios. Interferences of Kr^+ and $ArCl^+$ were corrected by on-mass baseline measurements. The HCl concentration of the blank solution matched that of the samples, and, like the samples, it was equilibrated with atmospheric Kr. The $^{82}Se/^{76}Se$ and $^{82}Se/^{78}Se$ ratios of each sample or standard were extracted from the interference-corrected Se isotope ratios measured on the sample-spike mixture via an iterative data reduction calculation that determines and corrects for mass bias, mathematically removes the spike from the mixture, and calculates the final results. This routine is described in earlier publications (Clark and Johnson, 2008; Johnson et al., 1999). All isotope data are reported by using the d notation relative to the standard NIST 3149 SRM:

$$\delta^{82/76}Se\ (‰) = [(^{82/76}Se_{sample})/(^{82/76}Se_{standard}) - 1]*1000 \qquad \text{(B-1)}$$

The $\delta^{82/78}Se$ results were also calculated and in all cases were consistent with the $\delta^{82/76}Se$ results. Normalized sample $\delta^{82/76}Se$ results were calculated by subtracting results of SRM-3149 analyses, which were done at least once for every five samples. Procedural standards (NIST3149) were also analyzed for Se isotope composition. The concentrations of Se in standards (unprocessed and processed) as well as a suite of samples were ~8 mg L^{-1}, which gave us a ^{78}Se signal intensity of 1.5 to 2.5 V. Furthermore, a processed standard (i.e., reduced to Se(IV) before injection to the hydride-generator coupled to the multiple-collector–inductively couple plasma/mass spectrometry) was analyzed every 10 samples. The overall mean $\delta^{82/76}Se$ value of all NIST 3149 measurements was -0.46 ± SD 0.09‰ (n = 13). The values are comparable with the $\delta^{82/76}Se$ value of the NIST 3149 (-0.44 ± SD 0.09‰; n = 6), which was processed in the same way for isotope analyses as the trapped organoselenides (i.e., volume-reduced by freeze-drying, poured through a cation-exchange column, bubbled with N_2, and equilibrated with atmospheric Kr). To test the tightness of the microcosms and the accuracy of our measurements, we established the mass balance of the microcosms:

$$m_{Se_{total}} = m_{Se_{medium}} + m_{Se_{fungus}} + m_{Se_{trap}} \quad \text{(B-2)}$$

where *m* is the mass of Se in the whole microcosm, the growth medium, the fungus and the trap at the end of the experiment. The mass-weighted mean $\delta^{82/76}$Se value was calculated as:

$$\delta^{82/76}Se_{microcosm} = \left(f_{medium} \times \delta^{82/76}Se_{medium}\right) + \left(f_{fungus} \times \delta^{82/76}Se_{fungus}\right) + \left(f_{trap} \times \delta^{82/76}Se_{trap}\right) \quad \text{(B-3)},$$

whereas f is represents the mass fractions of the medium, fungus, and trap, respectively.

4 Results and Discussion

4.1 Trapping efficiency of activated charcoal for organoselenides

Almost all DMSe volatilized from aqueous standards was recovered (*Table B-1*). The nearly complete recovery of DMSe is similar to findings of Karlson and Frankenberger (1988a,b), who reported recoveries of volatile organoselenides of 95.2% by using methanol as extractant and 1.08 g of activated charcoal in a study of Se volatilization from soils. In contrast, with activated charcoal only, a maximum of 64% of the DMDSe volatilized from aqueous standards was recovered (*Table B-1, Figure B-2*). This is consistent with findings of Jayaweera and Biggar (1992) and Haygarth et al. (1994), who attributed low recoveries of a maximum of 50% of volatilized DMDSe in activated charcoal traps by using a water–ethanol elution. The latter authors assumed that a revolatilization of the trapped organoselenides occurred during their experiments. However, if this were true, particularly the recovery of DMSe should be low because of its higher volatility compared with DMDSe, which was not the case. Instead, our recovery of trapped DMSe was even higher than that of DMDSe (*Table B-1*). Therefore, we checked whether the low recovery of DMDSe might be attributable to incomplete extraction of trapped organoselenides from the activated charcoal by plotting the recoveries of DMDSe versus mass of powdered activated charcoal (*Figure B-2*). The recovery of DMDSe decreased with increasing mass of activated charcoal, supporting our assumption of a strong sorption of DMDSe to activated charcoal that prevented its elution with 2 mL methanol. In contrast, the trapped DMSe was completely eluted with 2 mL methanol. The consistent low recovery of DMDSe from the activated charcoal trap with the previously published elution methods (Karlson and Frankenberger, 1988a,b; Jayaweera and Biggar,

1992; Haygarth et al., 1994) prevented the use of the activated charcoal trap in our microcosm experiments. An incomplete recovery might introduce artificial Se isotope fractionation.

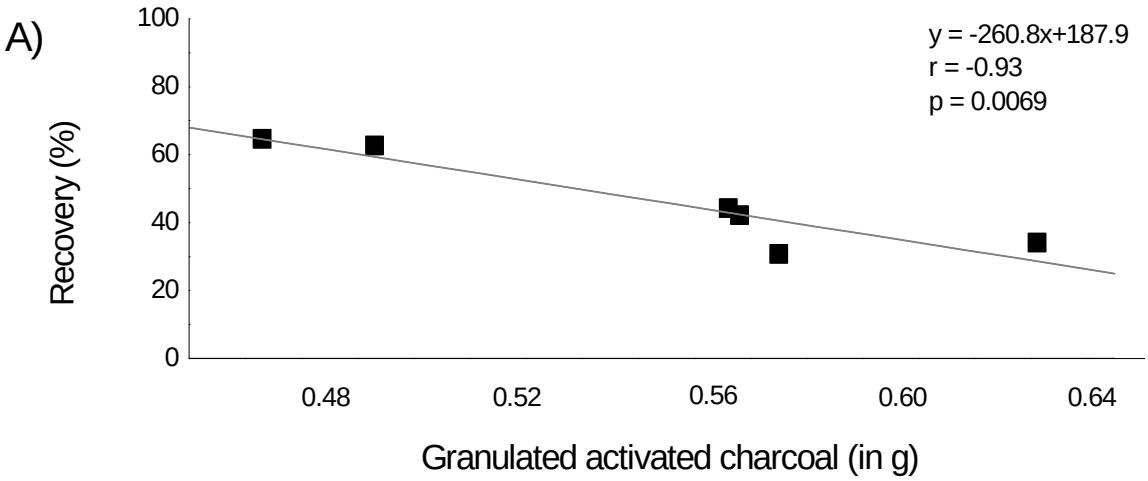


Figure B-2: Recovery of DMDSe by using different amounts of activated carbon.

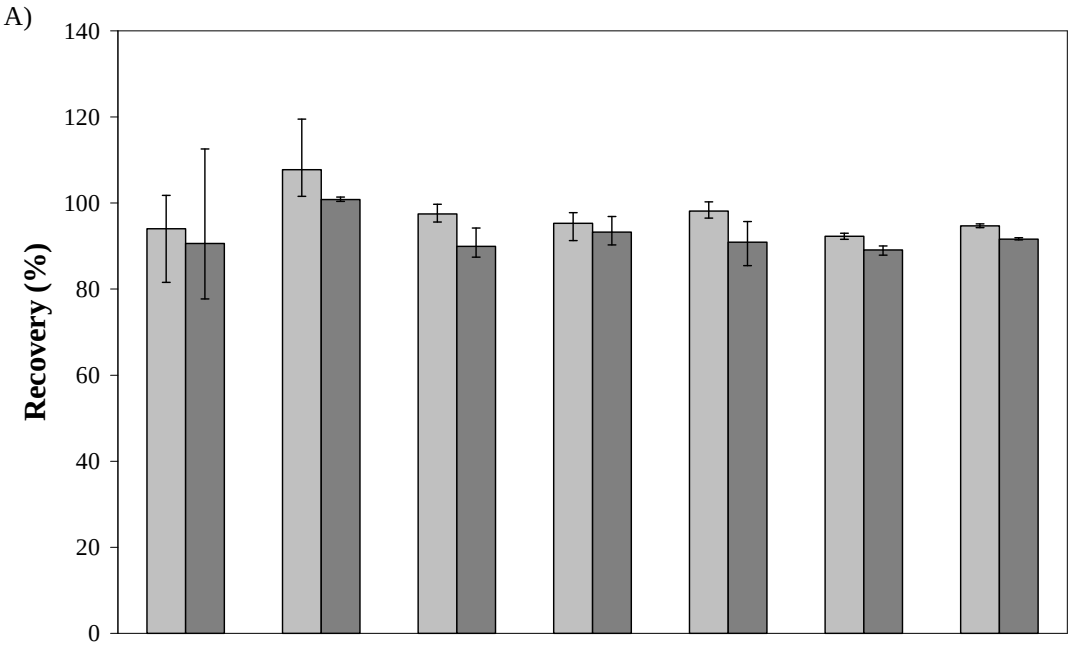
4.2 Trapping efficiency of the alkaline peroxide solution for organoselenides

The recovery of DMDSe in the trapping experiment with alkaline peroxide solution increased with increasing equilibration time with the highest yield near 100% after 120 min, illustrating that the minimum equilibration time should not be <120 min (*Table B-1*).

Table B-1. Mean recoveries of organoselenides volatilized from aqueous solutions or growth medium containing Alternaria alternata supplied with (hydro)selenite or selenate (microcosms only) by activated charcoal and alkaline peroxide solution as traps. Standard deviations are shown in brackets.

Type of analysis	No. of samples	Se species	Added Se	Recovered Se	Recovery of applied Se
			mg L^{-1}	mg L^{-1}	%
activated charcoal					
granular	3	DMSe	20	19 (0.9)	95 (4.4)
granular	3	DMDSe	40	25 (0.3)	64 (0.8)
granular	12	DMDSe	40	23 (6.3)	47 (13)
alkaline peroxide					
60min	6	DMDSe	8	4.9 (0.3)	65 (8.5)
90min	6	DMDSe	8	6.5 (0.5)	81 (6.1)
120min	4	DMDSe	4	4.8 (0.6)	96 (11)
microcosm	3	Se(IV)	5	4.8 (0.7)	96 (15)
microcosm	3	Se(VI)	5	5.2 (0.2)	102 (2.4)

In the tests with aqueous DMDSe solutions after 120 min, in the first, second, and third gas-wash bottle, on average 58.3 ± 0.9%, 23.5 ± 2.5%, and 18.9 ± 3.4% of the supplied Se were recovered, respectively (n = 3). For the test experiments with aqueous DMDSe solutions and all six microcosm experiments, the recovery was close to 100%. Our results agree well with recoveries reported by Banuelos et al. (2005) of 94 to 100% for field experiments with chambers using alkaline peroxide traps. We tested potential DMDSe losses because of the reduction of the volume of trap solutions to 20 mL by freeze drying and the removal of Na^+ via cation exchange. The mean recoveries of Se(VI) standards after freeze-drying were 97.1 ± 5.1% for ^{77}Se and 92.3 ± 4% for ^{82}Se (*Figure B-3A*). The recoveries were independent of the concentrations of Se between 1 and 100 mg L^{-1} for both tested Se isotopes. The mean recoveries of the whole sample preparation process (i.e., freeze-drying + cation-exchange resin) were 100.8 and 90.6% and for ^{77}Se and ^{82}Se, respectively (*Figure B-3B*). This illustrates that the processing of the alkaline peroxide traps did not reduce the Se recoveries.



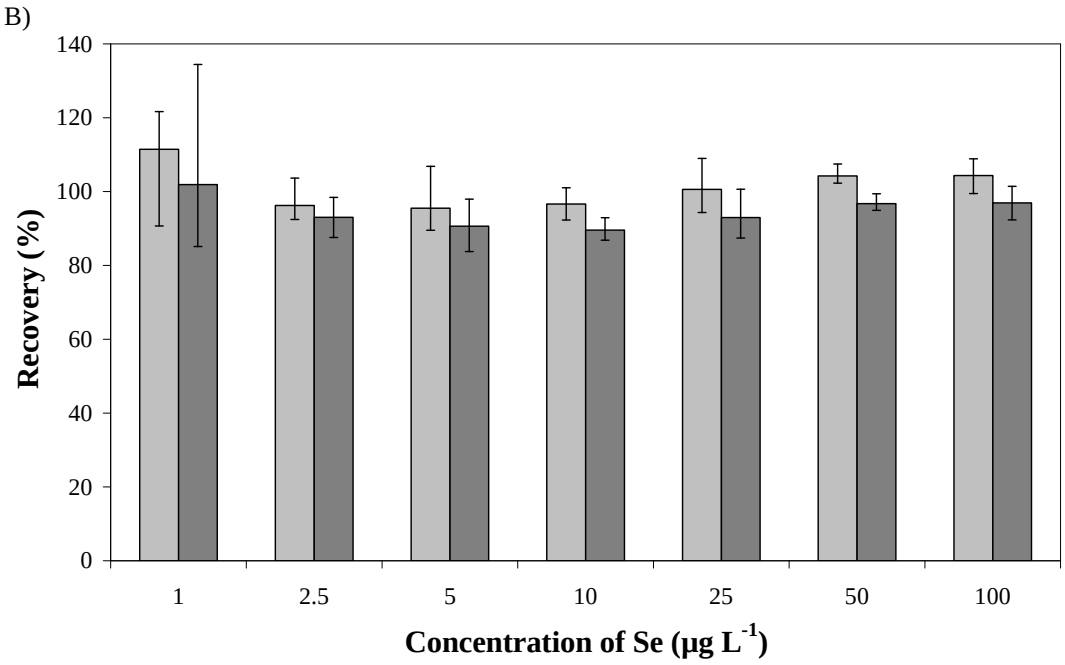


Figure B-3: Mean recovery of volatilized DMDSe in alkaline peroxide solutions after the A) freeze-drying step and B) freeze-drying and cation-exchange resin steps for varying initial Se concentrations. The columns show the concentrations of the Se isotopes ^{77}Se (light grey) and ^{82}Se (dark grey) measured with ICP-MS. Error bars represent standard deviations (n = 3).

4.3 Mass balances in microcosm experiments

After testing the performances of activated charcoal and alkaline peroxide traps with aqueous standards for equilibrium times of at maximum 2 h, it became clear that the activated charcoal trap cannot be used because of low recovery of organoselenides, which occurred likely as a consequence of too strong sorption to the activated charcoal. Therefore, the microcosm experiments were run with alkaline peroxide traps only, for which a satisfactory recovery of volatilized DMDSe was observed; the recovery was not significantly reduced by the subsequent freeze-drying and cation-exchange steps. Because the microcosm experiments were run for much longer time periods than the preliminary recovery tests, we assessed the performance of the microcosm by determining mass balances of Se and calculating mass-weighted mean $\delta^{82/76}$Se values for microcosm experiments in which Se(IV) and Se(VI) were provided as a source of organoselenides (*Table B-2*). We recovered nearly 100% of the supplied Se for the treatments with Se(IV) or Se(VI) as a source of organoselenides (*Table B-1*). The average mass-weighted mean $\delta^{82/76}$Se value of the three microcosms with Se(IV) as source was -0.31 ± 0.05‰ compared with -0.20 ± 0.10‰ in the supplied Se, and that of the $\delta^{82/78}$Se value was -0.20 ± 0.03‰ compared with -0.10 ± 0.10‰, illustrating a good match of

the calculated values with that of the supplied Se(VI). The same was true for the microcosm experiments with Se(VI), where the average massweighted mean $\delta^{82/76}$Se value of the whole microcosm was -0.75 ± 0.07‰, compared with -0.69 ± 0.10‰ in the supplied Se(VI), and the average mass-weighted mean $\delta^{82/76}$Se value of the whole microcosm was -0.52 ± 0.05‰, compared with -0.44 ± 0.10‰.These results illustrate that there were no significant losses of Se during the 11 to 15 d of incubation

Table B-2. Stable isotope ratios of Se ($\delta^{82/76}$Se and $\delta^{82/78}$Se) of the growth medium, the fungus (Alternaria alternata), the trapped organoselenides, supplied Se oxyanions, and calculated mass-weighted mean value of the microcosms. The standard deviation of the isotope measurements is 0.1‰ (determined by replicate measurement of NIST 3149) and the standard error of the calculated whole microcosm values is 0.17‰ using the Gaussian error propagation law.

Microcosm treatment		**Growth medium**			**Fungus**			**Traps**			**Supplied Se**		**Calculated whole microcosm**	
sample	Se source	$\delta^{82/76}$Se	$\delta^{82/78}$Se	Se (%)	$\delta^{82/76}$Se	$\delta^{82/78}$Se	Se (%)	$\delta^{82/76}$Se	$\delta^{82/78}$Se	Se(%)	$\delta^{82/76}$Se	$\delta^{82/78}$Se	$\delta^{82/76}$Se	$\delta^{82/78}$Se
		‰												
1	Se(IV)	3.3	2.19	15.4	-0.93	-0.63	63.4	-1.34	-0.77	21.2	-0.2	-0.1	-0.36	-0.23
2	Se(IV)	2.95	1.97	18	-1.17	-0.74	53.6	-0.78	-0.57	28.4	-0.2	-0.1	-0.32	-0.20
3	Se(IV)	1.96	1.33	12.9	-0.32	-0.23	60.1	-1.19	-0.76	27.1	-0.2	-0.1	-0.26	-0.17
1	Se(VI)	-0.59	-0.4	96.26	-1.36	-0.85	0.71	-3.29	-2.25	3.03	-0.69	-0.44	-0.68	-0.46
2	Se(VI)	-0.61	-0.38	85.17	-0.84	-0.57	7.46	-3.14	-2.39	7.37	-0.69	-0.44	-0.81	-0.54
3	Se(VI)	-0.63	-0.46	75.08	-0.95	-0.67	22.01	-3.14	-2.11	2.91	-0.69	-0.44	-0.77	-0.55

5 Conclusions

Of the two tested trapping methods for volatile organoselenides, only the alkaline peroxide trap showed a satisfactory recovery near 100%. The recovery by the alkaline peroxide trap was not deteriorated by the necessary preconcentration by freeze-drying and purification via cation exchange. We did not retrieve more than 64% of the volatilized DMDSe from the activated charcoal, likely because of incomplete desorption by soaking in methanol. This was true for the powdered and the granulated activated charcoal. The close negative relationship between mass of activated charcoal and Se recovery suggests strong sorption of DMDSe by the active charcoal as the reason for the failed elution. Consequently, only the alkaline peroxide trap can be used to study Se stable isotope fractionation processes in closed microcosm experiments, although we were not able to distinguish the Se isotope signals of DMDSe and DMSe with this approach. Our microcosm experiments run for 11 to 15 d demonstrated the good performance of the alkaline peroxide trap, which was illustrated by a near 100% recovery of the supplied Se in the components of the microcosm (growth medium, fungus, and trapped organoselenides) and a good match of the mass-weighted mean $\delta^{82/76}$Se and $\delta^{82/78}$Se values in the whole microcosms with those in the supplied Se salts. Thus, artificial Se stable isotope fractionation because of unaccounted losses during the microcosm experiment can be ruled out. Further method development (e.g., by improving the retention and elution of organoselenides on activated charcoal or the use of an alternative sorbent) is necessary if a separate consideration of the Se isotope signals in DMDSe and DMSe is intended.

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C Isotope Fractionation of Selenium During Fungal Biomethylation by *Alternaria alternata*

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1 Abstract

The natural abundance of stable Se isotopes may reflect sources and formation conditions of methylated Se. We aimed at (i) quantifying the degree of methylation of selenate [Se(VI)] and hydro-/selenite [Se(IV)] by the fungus *Alternaria alternata* at pH 4 and 7 and (ii) determining the effects of these different Se sources and pH values on $^{82}Se/^{76}Se$ ratios ($\delta^{82/76}Se$) in methylselenides. *Alternaria alternata* was incubated with Se(VI) and Se(IV) in closed microcosms for 11-15 d and additionally with Se(IV) for 3 to 5 d at 30°C. We determined Se concentrations and $\delta^{82/76}Se$ values in source Se(VI) and Se(IV), media, fungi, and trapped methylselenides. In Se(VI) incubations, methylselenide volatilization reached steady-state conditions before the 11^{th} d and the amounts of trapped methylselenide were not significantly different among the 11-15 d incubations. In 11-15 d, 2.9-11% of Se(VI) and 21-29% of Se(IV), and in 3 to 5 d, 3-5% of Se(IV) were methylated. The initial $\delta^{82/76}Se$ values of Se(VI) and Se(IV) were -0.69 ± s.d. 0.07‰, and -0.20 ± 0.05‰, respectively. The $\delta^{82/76}Se$ values of methylselenides differed significantly between Se(VI) (-3.97 to -3.25‰) and Se(IV) (-1.44 to -0.16‰) as sources after 11-15 d of incubation; pH had little influence on $\delta^{82/76}Se$ values. Thus, the $\delta^{82/76}Se$ values of methylselenide indicate the source species of methylselenides. The strong isotope fractionation of Se(VI) is probably attributable to the different reduction steps of Se(VI) to Se(-II) which were rate-limiting explaining the low methylation yields, but not to the methylation itself. The shorter incubation of Se(IV) for 3-5 d, showed a large Se isotope fractionation of at least -6‰ before steady state was reached. This initial Se isotope fractionation during methylation of Se(IV) is much larger than previously published.

2 Introduction

Selenium is an essential trace element for animals and humans with a narrow range of concentrations between sufficiency and toxicity (Maier and Knight, 1993) Selenium toxicity is mainly attributable to chemical similarity with S resulting in a nonspecific replacement of S by Se in proteins (Pilon-Smits et al. 2009) The concentrations of Se in soils range usually from 0.1 to 2 mg kg^{-1} (Ure and Berrow, 1982). Anthropogenic emissions of Se, mainly by changes in land use that release Se from naturally Se-rich soils developed on shales and certain other organic-rich sedimentary rocks, cause an increase of Se concentrations in the environment (Ure and Berrow, 1982). In ecosystems with elevated Se concentrations in soil such as those of the agricultural areas of the San Joaquin Valley in California or the Kendrick site in Wyoming, Se bioaccumulation can reach levels that adversely affect wildlife (Martens and Suarez, 1997, Naftz et al. 1993). Selenium occurs naturally in four oxidation states (-II; 0; IV; VI) which differ significantly in their nutritional and toxic relevance. The highly toxic oxidized forms of Se, selenate (Se[VI]) and (hydro)selenite (Se[IV]), are soluble in water but differ in mobility and bioavailability. Selenic acid [Se(VI)] is (similar to sulphuric acid) a strong acid with a pKa_2 value of 1.7 while selenous acid [Se(IV)] is weaker with a pKa_1 value of 8.3 and a pKa_2 value of 2.6. Accordingly, Se(VI) occurs mainly as SeO_4^{2-} (selenate) and Se(IV) as $HSeO_3^-$ (hydroselenite) and SeO_3^{2-} (selenite) in aqueous solutions depending on the pH, while the undissociated acids are only relevant in extremely acid solutions (Shriver et al. 1994) Selenate is more soluble than (hydro)selenite but nevertheless less bioavailable (Maier and Knight, 1993) Microorganisms play important roles in the environmental fate of toxic elements (Gadd, 2001) Selenium methylation is seen as a detoxifying response of some microorganisms to high Se concentrations. It is a widely distributed metabolic process and offers a potential remediation strategy in Se-contaminated areas (Frankenberger and Karlson, 1989). The first evidence of methylation of inorganic Se was reported by Challenger (1945) and was based on the finding of different methylation pathways as shown in *Figure C-1* (Chasteen and Bentley 2003; Reamer and Zoller, 1980). Inorganic Se species [Se(VI) and Se(IV)] and even some organoselenium compounds (selenomethionine, selenocysteine) are methylated to volatile dimethylselenide (DMSe) and, to a lesser extent, to dimethyldiselenide (DMDSe) (*Figure C-1*). The methylselenides compounds are 500-700 times less toxic than the inorganic Se species (McConnell and Portman, 1952) In general, formation of dimethylselenide is successive reduction and methylation of inorganic Se (Chasteen and Bentley, 2003) A number of microorganisms are known that are capable of Se methylation

including the bacterium species *Aeromonas veronii*, (Rael and Frankenberger, 1996) *Cornyebacterium sp*., (Doran and Alexander, 1977) *Pseudomonas fluorescens*, (Chasteen et al. 1990) and *Pseudomonas sp*., (Cau et al. 1976) and the fungus species *Alternaria alternata*, (Thompson-Eagle et al. 1989) and *Fusarium sp*. (Barkes and Fleming, 1974)

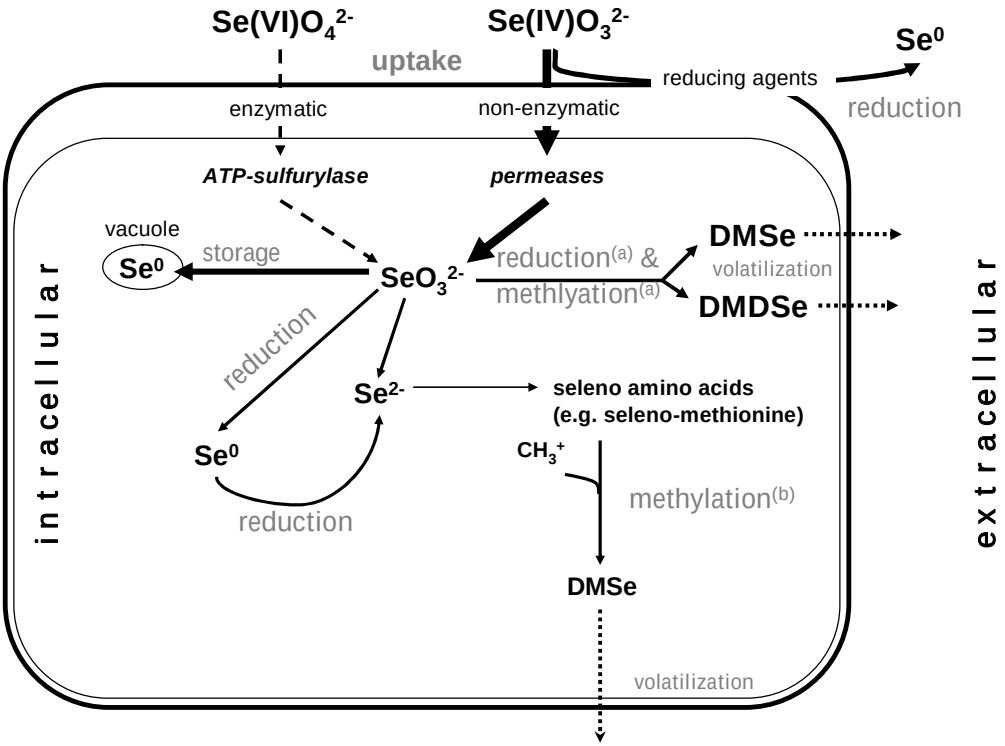

Figure C-1. Overview of the transformation paths of Se oxyanions to elemental Se(0) and to volatile methylselenides [DMSe (dimethyl selenide) and DMDSe (dimethyldiselenide)]. (a) Challenger mechanism of the formation of methylselenide (modified after Chasteen and Bentley 2003). (b) Methylation of selenoamino acids to volatile methylselenide (Sors et al. 2005)

It is also known that plants methylate Se (Zayed at al. 1998, 1994) The methylation pathway of Se is controlled by various methyl donors such as Sadenosylmethionine,16 N-methyltetrahydrofolate, and methylcobalamine (Fatoki, 1997) The fungal methylation is most likely controlled by the methyl donor S-adenosylmethionine (Fatoki, 1997) Because of numerous transformations of Se, Se isotopes are excellent tracers to investigate microbial and abiotic processes affecting Se (Rouxel et al. 2002) and to distinguish different Se sources in the environment (Ellis et al. 2003) Selenium has six stable isotopes: ^{74}Se (contribution to the total elemental mass: 0.89%), ^{76}Se (9.37%), ^{77}Se (7.64%), ^{78}Se (23.77), ^{80}Se (49.61%), and ^{82}Se (8.73%) (Wachsmann and Heumann, 1992) High-precision Se isotope measurements can

be performed with multicollector inductively coupled plasma mass spectrometry (MC-ICPMS) using a combination of high-efficiency Ar plasma ionization and a multicollector detector array (Rouxel et al 2002) Various Se isotope studies have measured different Se isotope ratios to express enrichments of heavier or lighter isotopes; in our study we measured $^{82}Se/^{76}Se$, and we thus express results of all earlier studies in terms of this ratio. Previous studies have demonstrated that Se isotope fractionation occurs as a consequence of reduction processes (Ellis et al. 2003, Herbel et al. 2000). A significant fractionation of $\delta^{82/76}Se$ of -6.00 to -13.50‰ was observed during dissimilatory reduction of Se oxyanions depending on bacteria species and the reduction process (Herbel et al. 2000) Ellis et al. (2003) reported separation factors ε of -3.9‰ to -4.7‰ during the reduction of Se(VI) to Se(IV) and of -8.3‰ to -8.6‰ during the further reduction of Se(IV) to Se(0) in microcosm experiments using sediment slurries with natural microorganism communities. *Alternaria alternata* is a widely distributed filamentous fungus species in the environment and has been isolated from almost all habitats (soils, rotten wood, plants). *A. alternata* was also isolated from Se-contaminated water ponds of Kesteron Reservoir in California (Thompson-Eagle et al. 1989). *A. alternata* is one of the most active terrestrial Semethylating organisms because it is capable of methylating a variety of Se-species as substrates and is resistant to high concentrations of Se (Thompson-Eagle et al. 1989). For bioremediation of Se-contaminated areas, it is important to understand the methylation processes using an environmentally relevant organism with high methylation activity and the stable isotopes of Se as tracers. The most oxidized species Se(VI) is the most abundant form of Se in contaminated regions, and methylation is an efficient detoxification process (Frankenberger and Karlson, 1989; Ure and Berrow, 1982). The objectives of this study were to (1) quantify the degree of methylation of Se(VI) and Se(IV) by the fungus *A. alternata* at pH 4 and 7 after the reaction has ended. (2) determine the effects of these different Se sources and pH values on $^{82}Se/^{76}Se$ ratios ($\delta^{82/76}Se$) in methylselenides, and (3) determine the initial Se isotope fractionation during methylation of Se(IV) before the reaction has ended.

3 Materials and Methods

3.1 Materials

All solutions were prepared using high purity (>18 MΩ) deionized water. Stock standard solutions of 1000 mg L^{-1} Se as Na_2SeO_4 and as Na_2SeO_3, respectively, were purchased from Merck (Darmstadt, Germany). To trap methylselenides, we used a solution of 99.9% NaOH (Merck, Darmstadt, Germany) and 30% TraceSelect H_2O_2 (Sigma-Aldrich, Seelze, Germany). All samples were filtered through 0.45 μm membrane filters (VWR, Darmstadt, Germany). For the quality control and normalization of Se isotope measurements, we used SRM 3149 (National Institute of Standards and Technology, Gaitherburg, MD). All plastic and glassware were soaked in 10% nitric acid and rinsed carefully with deionized water.

3.2 Incubations

We purchased a strain of *A. alternata* from the German Resource Center for Biological Materials (DSMZ, Braunschweig, Germany). The fungus was first cultivated on agar plates at 30°C for 5 days and then kept at 4°C prior to use. The liquid culture medium used was prepared as described in ref (Thompson-Eagle, 1989). An aerobic culture was grown in 250-mL Erlenmeyer flasks containing 80 mL of the medium at 30°C on a rotatory shaker for 7 days before the culture was used for incubation with inorganic Se species. Incubations were conducted in 80 mL serum bottles containing 20 mL cultured medium (10 g L^{-1} malt extract, 10 g L^{-1} glucose, and 0.5 g L^{-1} peptone) which were spiked with Se(IV) or Se(VI) to a final amount of 100 μg Se per bottle followed by inoculation of 1 mL of homogenized fungus suspension. The pH was adjusted with 1 M HCl or 1 M NaOH to 4 or 7. The serum bottles were plugged with sterile butylstoppers to create microaerobic conditions. During incubation the bottles were horizontally shaken at 100 rpm at 30°C. The serum bottles were covered with aluminum foil to suppress abiotic photochemical formation of methylselenides (Guo et al. 2003).

3.3 Experimental design

We set up four treatments, i.e., a combination of the supply of Se(VI) and Se(IV) with the two pH levels 4 and 7. The fungal cultures from each experiment were incubated for 11-15 days with Se(VI) and Se(IV), each at pH 4 and 7 and additionally for 3-5 days with Se(IV) at pH 7 only. The duration of the incubation of >10 days was chosen to allow the development of fungal activity and the production of measurable concentrations of methylselenides. Peitzsch

(2008) showed that the trapped concentrations of methylselenides in a similar incubation with *Alternaria alternata* were constant after incubation times of 10-21 days. We collected trapped methylselenides on days 11-15 (on each day a separate bottle was opened) to make sure that the biomethylation had come to an end after 10 days in our experiment. If the latter was the case, the five sampling dates (on days 11-15) could be considered as replicates of our treatments. For the additional experiment with Se(IV) we chose an incubation time of 3-5 days at pH 7 to determine separation factors ε of kinetic isotope fractionation of Se isotopes for the methylation of Se(IV). Each treatment of the 3-5-day-lasting Se(IV) experiment was duplicated. Uninoculated serum bottles were used as a control.

3.4 Trapping of methylselenides

We used the trapping method modified after Terry et al. (1992) to collect methylselenides. Gas wash bottles were connected to each other via Teflon tubes. Each gas wash bottle contained 120 ml of alkaline peroxide solution (90 mL of 0.05 M NaOH and 30 mL of 30% H_2O_2). For each trap the alkaline peroxide solutions were freshly prepared. The methylselenides were driven out of the sampling flask into the trapping solution by a N_2 stream of 0.75 L min^{-1} for 2 h which was controlled via a gas flow regulator. After collection of volatile Se, growth media and fungi were separated by filtration through 0.45 µm membrane filter, freeze-dried, and digested by 2 mL of HNO_3 at 140°C to determine the Se remaining in the media and accumulated in the fungi.

3.5 Element and isotope analyses

Selenium concentrations were measured with ICP-MS HP4500 (Agilent, Waldbronn, Germany) and/or ELEMENT 2 (Thermo Scientific, Waltham, MA). The ICP-MS measurements had a detection limit of 0.27 µg L^{-1} Se. Se isotope ratios were determined with MC-ICPMS (Nu Plasma, Nu Instruments; Wrexham, U.K.). The double spike method was used to correct for instrumental mass bias (Johnson and Bullen, 2004; Johnson et al. 1999) and Se was introduced into the instrument via a custom-built hydride generator, following methods given in a recent publication (Johnson et al. 1999). Here, a ^{74}Se + ^{77}Se double spike was used for all analyses. We collected aliquots with a total Se mass of 100 ng from each sample. Then, an appropriate amount of double spike solution was added so that the ratio of spiked ^{77}Se to ^{78}Se in the sample was close to 2:1. The samples were made up with 5M HCl (±0.2 M) to a total of 5 mL. To convert Se(VI) to Se(IV), the sample was heated in a hot block held at 120°C for 1 h. After cooling at room temperature the solutions were diluted to

2M HCl (±0.2M). Standard and blank solutions were prepared following the same procedure together with the samples. Measures to eliminate interferences of $^{81}BrH^{+}$ and ^{82}Kr are described in the Supporting Information. Sample Se was introduced into the mass spectrometer asH_2Se generated via reaction with a 0.2% $NaBH_4$ solution in a continuous flow hydride generator similar to that described by Rouxel et al. (2002) Measurements of $^{74}Se/^{78}Se$, $^{76}Se/^{78}Se$, $^{77}Se/^{78}Se$, and $^{82}Se/^{78}Se$ isotope ratios were made with all Se isotopes measured simultaneously, with a minimum of 30 integrations of 5s each. The treatment of interferences by Ge and $ArAr^{+}$ are described in the Supporting Information. The $^{82}Se/^{76}Se$ and $^{82}Se/^{78}Se$ ratios of each sample or standard were extracted from the interference-corrected Se isotope ratios measured on the sample-spike mixture via an iterative data reduction calculation that determines and corrects for mass bias, mathematically removes the spike from the mixture, and calculates the final results. This routine is described in earlier publications (Clark and Johnson, 2008; Johnson et al. 1999)

All isotope data are reported by using the δ notation of $^{82}Se/^{76}Se$ relative to the standard NIST 3149 SRM (Eq C-1):

$$\delta^{82/76}Se\ (‰) = [(^{82/76}Se_{sample})/(^{82/76}Se_{standard}) - 1]*1000 \qquad \text{(C-1)}$$

Accuracy and precision of our isotope ratio measurements are described in the Supporting Information.

The kinetic isotope effect is described by the isotope fractionation factor α, which is defined as in Equation C-2.

$$\alpha = R_{Product} / R_{Reactant} \qquad \text{(C-2)}$$

where $R_{Product}$ and $R_{Reactant}$ refer to the isotope ratio of the product and the reactant, respectively.

For convenience, α is expressed in terms of ε, which is defined by Equation C-3

$$\varepsilon = (\alpha - 1)x1000‰ \qquad \text{(C-3)}$$

and which is closely approximated by Equation C-4.

$$\varepsilon = \delta_{Product} - \delta_{Reactant} \qquad \text{(C-4)}$$

4 Results and Discussion

4.1 Controls and replicates

In the controls, we did not detect Se in the methylselenide traps, indicating that no methylation occurred without presence of the fungus. This was true for both Se(VI) and Se(IV). Furthermore, the growth medium was free of Se as shown by the analysis of two replicate aliquots of the medium. The mass balances of total Se inmicrocosm experiments run for 11-15 days with alkaline peroxide traps were 96.6 ± 5.3% and 99.7 ± 12.1% for Se(IV) and Se(VI), respectively (n = 5).

4.2 Extent of methylation

In all treatments, detectable amounts of methylselenides were produced by *Alternata alternaria* from inorganic Se. *Figure C-2* illustrates that steady-state conditions of methylation were reached for both, incubations with Se(VI) and with Se(IV) before the 11th d of the incubation because methylation yields did not change anymore between days 11 and 15. Therefore, we considered the incubations of 11-15 d as replicates of steady-state methylation experiments.

Markedly less Se (2.9-11%) was methylated if Se(VI) was provided, compared to Se(IV) (21-29%) in the incubations of 11-15 days. In the 3-5 day incubations, 3-5% of the supplied Se was methylated, i.e., much less than in the 11-15 day incubations confirming that the biomethylation reaction was still incomplete after 5 days allowing for the assessment of kinetic isotope fractionation during Se(IV) methylation. The results were in line with previous studies (Dungan and Frankenberger, 2001; Zhang and Chasteen, 1994; Thomspon-Eagle et al. 1989; Chau et al .1976, Barkes and Fleming, 1974) reflecting that the formation of methylated compounds occurs for all Se species; however, it is most rapid for Se(IV). If Se(VI) was provided, there was a close and significant correlation between Se accumulated in the fungus and the dry weight of the fungus (r = 0.98).

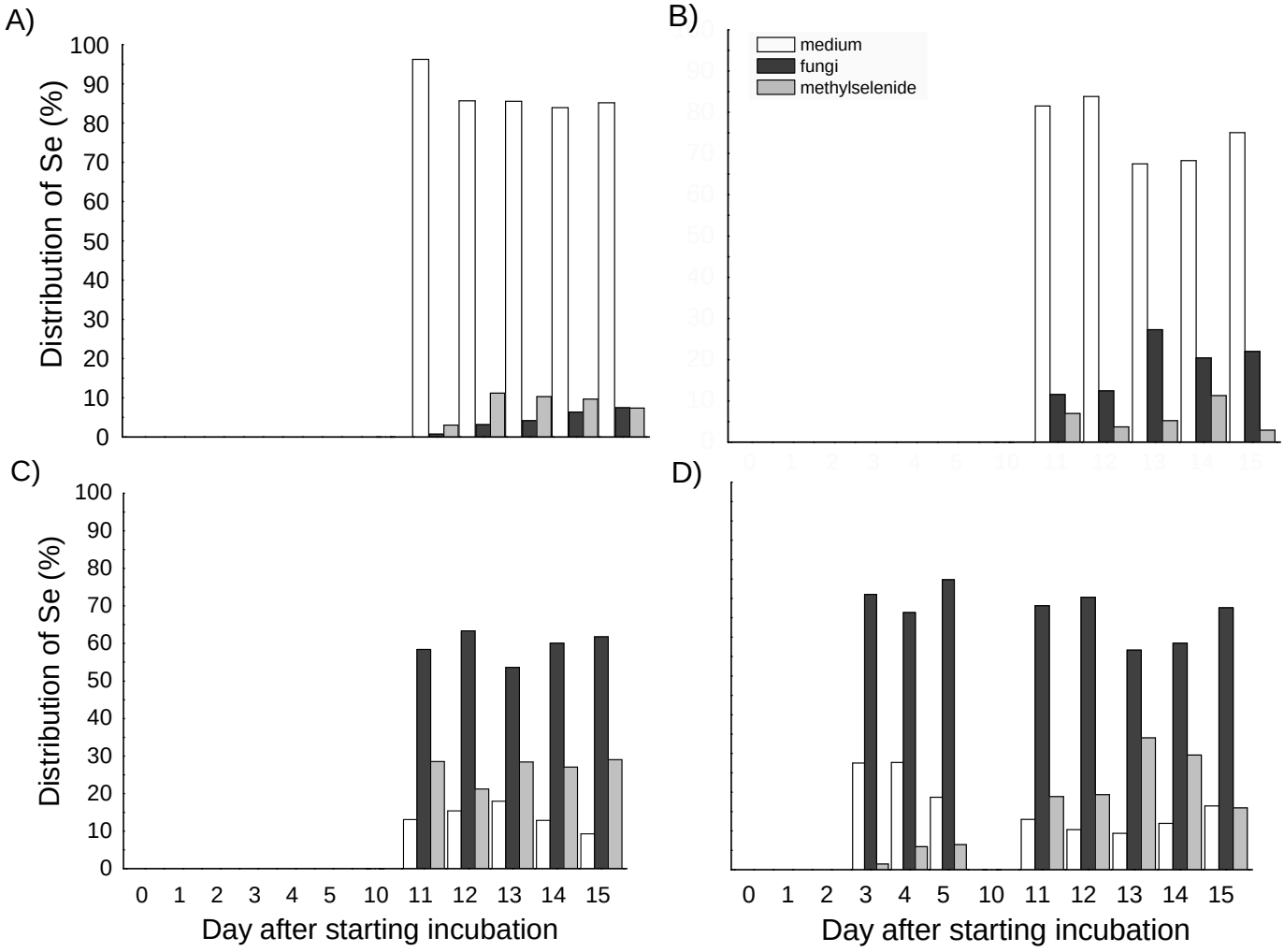

Figure C-2: Distribution of Se among media, fungi, and trapped methylselenides in % of the supplied A) Se(VI) at pH 4, B) Se(VI) at pH 7, C) Se(IV) at pH 4, and D) Se(IV) at pH 7 after incubation of 3-5 and 11 to 15 d.

This suggests that the methylation rate of Se(VI) resulting in the removal of Se from the organisms was not as rapid as other Se conversion processes, e.g., reduction of Se(VI) to Se(IV) and subsequent reduction to Se(0) by the fungi. As Se(IV) can be easily reduced to Se(0) by many reductants produced by the cells of *A. alternata*, both intra- and extracellular Se(0) might occur in fungi and media. In the Se(VI) experiment, there was no Se(0) visible, but we can assume that a small amount of supplied Se(VI) was reduced toSe(0) (*Figure C-1*). Under neutral conditions, more Se from Se(VI) was accumulated in *A. alternata* at the end of the incubation than under acid conditions (*Figure C-2*). This accumulation went along with a higher final dry mass of fungi (31.8 ± 6.5 mg) under neutral than under acidic conditions (25.2 ± 4.1 mg) illustrating that the fungus grew better under the neutral pH conditions.

However, at pH 4, the Se concentration in the fungi (based on dry mass) was higher than at pH 7, suggesting that Se(VI) was more bioavailable at pH 4 than 7 which is in line with findings of Riedel and Sanders (1996).

4.3 Selenium isotope fractionation during methylation in the Se(VI) experiments

If Se(VI) was provided, the Se in methylselenides was substantially depleted in the heavier isotopes relative to the dissolved Se(VI) source (*Table C-1*). Some samples could not be isotopically analyzed because of their low Se concentrations yielding <100 ng Se, the minimum mass needed for reliable Se isotope ratio determination. The small contribution of Se in methylselenides relative to total Se in the microcosms with Se(VI) (Figure 2A,B) explains why the $\delta^{82/76}$Se values of the Se remaining in the media and the fungal Se did not change substantially despite the preferential methylation of lighter Se isotopes. At both tested pH levels, fungal Se was slightly (-1.03 ± 0.19‰) depleted in the heavier isotopes relative to the supplied Se and the remaining Se in the media (*Figure C-3A,B*). The Se(VI) experiments had small amounts of volatilization and incorporation into fungi, and under these conditions, the difference in $\delta^{82/76}$Se between the trapped methylselenides and the Se remaining in the medium is very close to ε. The average ε values in the Se(VI) treatments were similar at pH 4 (ε = -3.00 ± 0.26‰) and pH 7 (ε = -2.72 ± 0.41‰).

Our results demonstrate that (1) the accumulation and/or assimilation step of Se(VI) leads possibly to a small fractionation while (2) the methylated Se is likely moderately fractionated. If *A. alternata* is capable of Se(VI) assimilation, we assume that Se(VI) is metabolized following the sulfate reduction pathway because of the structural similarity between Se(VI) and sulfate. Roy and Trudinger (1970) proposed that all green plants, fungi, and most bacteria can reduce sulfate to sulfide which is subsequently incorporated into sulfur-containing organic molecules. In this case, Se(VI) was presumably activated by adenosinetriphosphate (ATP)-sulfurylase to adenosine-phosphoselenate (APSe) (Lauchli, 1993) and then reduced in different steps to finally methylselenide. The enzymatic pathway limits the assimilation because ATP-sulfurylase is an enzyme catalyzing the primary step of intracellular sulfate activation (Ullrich et al. 2001) and, in this case, of Se(VI) activation to APSe. The uptake of sulfate and selenate is unidirectional and occurs together with a reductive step in the cytoplasm for which ATP is required (Roy and Trudinger, 1970; Lauchli, 1993) This step may produce a small fractionation as observed in the accumulated Se in the fungus (*Figure C-3A,B*). However, the reaction of sulfate with ATP sulfurylase to form APS is reported to cause no fractionation of S isotopes (Chambers and Trudinger, 1978) The next metabolic step is the

reduction of APSe to the Se(IV)-form which can occur enzymatically by ATPS-reductase or nonenzymatically by glutathione. It is well-known that the S isotope fractionation during assimilatory sulfate reduction is small with an isotope fractionation ($\delta^{34}S$) of +0.5‰ to -4‰ in organic sulfur compounds compared to the initial sulphate (Chamber and Trudinger, 1978). A similarly small isotope fractionation was observed for Se(VI), if the Se isotope ratios of the fungus are compared with those in the source and media (*Figure C-3A,B*). The final reduction of Se(IV) leads to the formation of Se(-II) which can be metabolized into selenoamino acids (selenomethionine, selenocysteine).40 As described by Sors et al. (2005) the selenoamino acids are methylated and converted to methylselenides. Since the Se in methylselenides is moderately fractionationed at pH 4 (ε around -3‰ at both tested pH levels, *Figure 3A,B*), the methylation of assimilated Se must have undergone a fractionation of approximately-2.5‰ to -3.0‰ in favor of lighter isotopes.

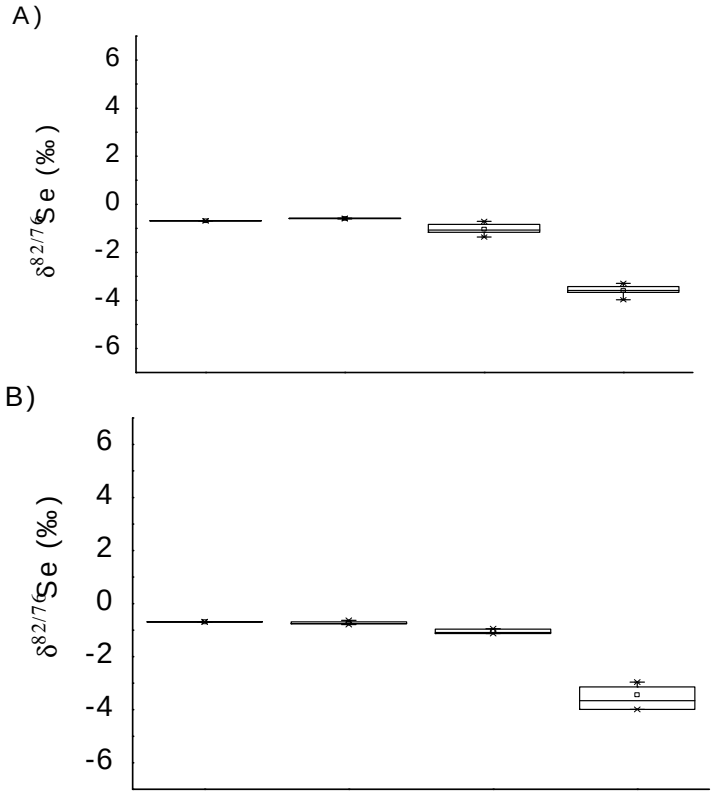

Figure C-4 Box plots of the $\delta^{82/76}$Se in the supplied Se and in media, fungi, and methylselenides at the end of the incubation of treatments with A) at pH 4, and B) Se(VI) at pH 7 after incubation of 11 to 15 days. The central lines of the boxes illustrate the mean, the upper and lower limits of the boxes the 25 and 75 percentiles of the variance and the whiskers the maximum and minimum values

4.4 Selenium isotope fractionation during methylation in the Se(IV) experiments

After incubation of Se(IV) for 11-15 days, the Se in the medium was almost completely consumed, and consequently, only a small Se isotope fractionation effect was observed ($\delta^{82/76}$Se values in methylselenides ranged -1.44‰ to -0.16‰ similar to the $\delta^{82/76}$Se value of -0.20‰ (SD 0.05‰ of the source Se(IV), irrespective of the pH values of 4 or 7). Future speciation of Se in medium and fungus and determination of the Se isotope ratios of these species can give us information about colloidal or suspended Se(0) in the mediumor fungus. But in this manuscript we do not further discuss the Se isotope ratios of the 11-15 day incubation of Se(IV) but focus on the incubation with a shorter duration of only 3-5 days to assess possible kinetic Se isotope fractionation before steady-state conditions were reached. Indeed, after 3-5 days, more of the Se(IV) remained in solution (*Figure C-2D*), and we were able to arrive at a useful estimate of the magnitude of kinetic isotope fractionation by methylation.

The $\delta^{82/76}$Se values of the trapped methylselenides, fungal Se, and the remaining Se in the media could not be explained by a simple Rayleigh distillation model (Herbel et al. 2000; Johnson et al. 1999) because of the complexity of Se pools and transfer and transformation processes. However, minimum values for the total fractionation induced by methylation and transformation of extra- and intracellular Se species by *A. alternata* could be reasonably calculated from the results of the short-term Se(IV) incubations. Although the evolution of $\delta^{82/76}$Se values of the medium during the first three days was not determined, our results indicate that these values were greater than 0‰, since on the third day the $\delta^{82/76}$Se value of the medium was +6.3‰ (*Table C-1*). The apparent shift of $\delta^{82/76}$Se of the medium to lower values after 3-5 days of incubation

(*Figure C-4*) may be a consequence of mixing of isotopically lighter colloidal Se(0) or some other form of Se produced by the fungus that passed through the filtration step during sample preparation for isotope measurement. The produced Se(0) might be formed in the cell and extracellularly43 (*Figure C-1*), and some extracelluar Se(0) may have passed through the filters as a very fine precipitate and mixed with the medium. As the Se concentration in the medium decreased from day 3 to day 5 during the incubation, this component would be expected to increasingly shift the mixture to lower $\delta^{82/76}$Se values, as is observed. The measured $\delta^{82/76}$Se value of the trapped methylselenide on the third day was -6.18‰ (*Figure C-4*).

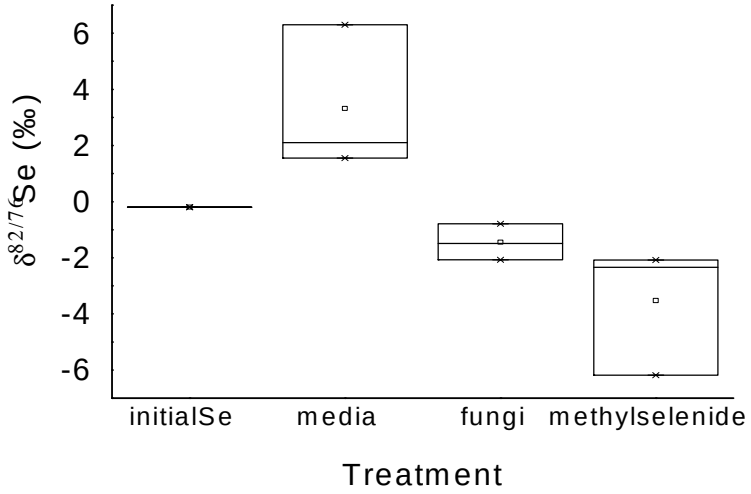

Figure C-3: Box plots of the $\delta^{82/76}$Se in the supplied Se and in media, fungi, and methylselenides at the end of the incubation of treatments with Se(IV) after incubation of 3 to 5 days. The central lines of the boxes illustrate the mean, the upper and lower limits of the boxes the 25 and 75 percentiles of the variance and the whiskers the maximum and minimum values

Therefore, the ε value for the whole methylselenide formation from selenite must have been more negative than -6‰. Since the $\delta^{82/76}$Se value of the fungus is not very negative, Se in the medium before the third day of incubation could not have been much heavier than the measured $\delta^{82/76}$Se for the medium on the third day (+6.3‰).

This suggests ε must have been less negative than -12.5‰ constraining the separation factor to a value between roughly -6‰ and -12.5‰, perhaps around -9‰. Our results demonstrate that the methylselenides are significantly enriched in ^{76}Se compared to the supplied Se(IV) because of kinetic Se isotope fractionation before biomethylation came to an end. However, because we did not determine concentrations and Se isotope ratios in intermediate metabolites, our estimate must be considered with great care. For the accumulation of Se in the fungus, ε is estimated as -4‰ to -5‰ from the difference between a rough estimate for the average $\delta^{82/76}$Se value of the mediumduring the experiments and the measured $\delta^{82/76}$Se value for the fungal Se (*Table C-1*). If it is assumed that the Se in the fungus is mainly Se(0) and a smaller part of isotopically heavier organic Se compounds (mainly Se-containing amino acids), we suggest an ε for the formation of Se(0) around -6‰. The by ca. 1‰ more pronounced difference in the Se isotope ratios between the Se in the fungus and the methylselenides in our short-term Se(IV) than in the longer Se(VI) incubations (*Table C-1*) suggests a simple rate-limiting step control of the apparent fractionation. The greater isotope

fractionation in the short term Se(IV) than that in longer Se(VI) experiments may be explained by the transport of the different inorganic Se species into the cells. Selenite can be transported into the cell by distinct permeases whereas Se(VI) mainly follows the sulfate assimilation pathway during the uptake (Ullrich et al. 2001) (*Figure C-1*). Algal methylation resulted in a smaller Se isotope fractionation between source Se(VI) and methlyselenide ($\varepsilon < 1.1‰$) (Johnson et al. 1999) than in our experiments with Se(IV) (estimated $\varepsilon \sim -9‰$). These differences suggest different methylation pathways for different organisms. In the literature, a mechanism that involves four steps in which the Se is reduced to form methylselenide is described (Challenger, 1945). Other studies proposed that the methylation of inorganic Se may first involve reduction of Se(IV) to Se(0) and then reduction to the selenide form, which is subsequently methylated (Doran and Alexander, 1977) The reduction of oxidized Se to Se(0) and the subsequent methylation processes are likely to have different ε values. Relative to Se(VI), Se(IV) is more reactive because of its more polar character which resulted in the comparatively high Se accumulation by *A. alternata* (*Figure C-2C,D*). The main process was the reduction of Se(IV) to Se(0) (both intra- and extracellularly) which gives rise to a macroscopically visible reddish color of the fungi indicating the presence of elemental Se(0). This supports our assumption that the fungal Se is likely mainly Se(0), probably together with small amounts of organic Se bound to amino acids. The estimated ε value for the formation of Se(0) of ~-6‰ to -8‰ is comparable with values from previous studies which observed Se isotope fractionation for the reduction of Se(IV) to Se(0) with ε values of -1.1 to -8.4‰ (Herbel et al. 2000).

Previous studies showed that, in addition to methylation, fungi such as *Fusarium sp.* (Ramadan et al. 1988) and *Aspergillus parasiticus* (Moss et al. 1987) were capable of reducing Se(IV) to elemental Se(0). Gharieb et al. (1995) found an enhanced reduction of Se(IV) by incubation of the fungus *Trichoderma reesii* in malt extract medium (same as in our study) because the synthesis of selenite-reducing enzymes was greater in a complex medium. But it is not yet well understood if selenitereducing enzymes are produced as response to high Se(IV) concentrations or as a part of the general metabolism. Previous studies (Dungan and Frankenberger, 2000, 1999; Doran and Alexander, 1977) reported that the formation of Se(0) and the production of dimethylselenide occurred simultaneously in bacteria. Therefore, the reduction of Se(IV) is a multistep process involving the uptake into the cell and reduction of Se(IV) to Se(-II) or both extracellular and intracellular reduction to Se(0).

We conclude that the isotope composition of Se in methylselenides provides information about the sources of methylselenides but is not very sensitive to different pH conditions between 4 and 7 in the surroundings of the methylating fungi. During incubation of Se(IV), two parallel main pathways were observed simultaneously, one leading to Se(0) and another leading to methylselenides. The difference in $\delta^{82/76}$Se values between the fungal Se and the methylselenides may reflect a stronger fractionation for the methylation pathway than the reduction to Se(0).

Our results demonstrate that Se isotope fractionation between various inorganic Se species can provide new insights into the biogeochemical cycle of Se. The knowledge of the Se isotope fractionation during fungal biomethylation helps in determining source species of methylselenides and formation processes. This knowledge is necessary to assess risks arising from Se contaminations and design appropriate remediation measures. Future investigation will be required to determine if different isotope fractionations of Se occur by various organisms depending on their methylation pathways.

C Isotope fractionation of selenium during fungal biomethylation of *Alternaria alternata*

Table C-1. $\delta^{82/76}$Se values in Se(+IV) and Se(+IV) standards, media, fungi, and methylselenides of the treatments with Se(+IV) and Se(+VI) at each of the pH values of 4 and 7.

Sample	Se species	Day 3	Day 4	Day 5	Day 11	Day 12	Day 13	Day 14	Day 15
					‰				
Standard	+VI				-0.69± standard deviation 0.07 (n=2)				
Standard	+IV				-0.20± 0.05 (n=2)				
					pH 4				
Medium	+VI				-0.59	-0.59	-0.58	-0.58	-0.61
Fungus	+VI				-1.36	-1.17	-1.07	-0.71	-0.84
Methylselenides	+VI				-3.29	-3.42	-3.97	-3.67	-3.59
					pH 7				
Medium	+IV	6.3	2.1	1.55					
Fungus	+IV	-2.07	-1.49	not determined					
Methylselenides	+IV	-6.18	-5.81	-2.80					
Medium	+VI				-0.69	-0.76	-0.79	-0.76	-0.63
Fungus	+VI				-1.13	-1.09	not determined	-0.96	-0.95
Methylselenides	+VI				-2.96	-3.66	-3.99	not determined	-3.14

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D Selenium partitioning and stable isotope ratios in urban topsoils

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1 Abstract

A combination of sequential extraction with stable isotope ratio measurements of Se might offer new insights into biogeochemical processes governing Se turnover in soils. Therefore, we determined the Se partitioning among three operationally defined sequential extracts (0.1 M K_2HPO_4-KH_2PO_4 at pH 7, 0.05 M NaOH, conc. HNO_3) and the stable isotope ratios of total Se ($\delta^{82/76}$Se values) in ten topsoils under five different land uses (alluvial grasslands, forests, house gardens, parks, and roadside grassland) from the city of Bayreuth (ca. 73,000 inhabitants) in Germany. Furthermore, we determined S and SO_4^{2-} concentrations and stable isotope ratios of total S (δ^{34}S values) to support our interpretation of the Se concentrations and isotope ratios because of the chemical similarity of Se and S. All topsoils had low total Se concentrations (0.09-0.52 mg kg^{-1}). The largest contribution to total Se was extracted with NaOH comprising up to 42% thought to be associated with organic matter and metal oxides. The $\delta^{82/76}$Se values of total Se in the topsoils were close to the bulk Earth composition with an average $\delta^{82/76}$Se value of -0.03± SD 0.38‰ suggesting that there was no or little Se isotope fractionation in soil. We attribute the small isotope fractionation to the low bioavailability of Se as a consequences of the presence of Fe oxides (adsorb the dominating Se(IV) forms strongly), organic matter, and SO_4^{2-} (prevents biouptake of the Se(IV) forms) in the study soils. Small Se isotope fractionations of -0.59 to -0.35‰ in mainly forest soils and of 0.26 to 0.45‰ in mainly alluvial soils were presumably caused by soil/plant-recycling and Se contamination by river water, respectively. In spite of the similarities in the assimilation of S and Se by organisms, the total S and Se isotope ratios in soil were not correlated. Our results demonstrate that Se in urban soils developed from Se-poor substrates is minimally cycled through the biosphere likely because of low bioavailability and competition with SO_4^{2-}.

2 Introduction

Selenium is a trace element which is essential in small amounts for microorganisms, animals, and humans but toxic at high concentrations. Low concentrations of Se in food grown on Se-poor soils could e.g., lead to the Keshan disease, a human heart disease (Chen et al., 1980), whereas high Se concentrations in food grown on Se-rich soils could cause e.g., the "alkali disease", i.e. Se poisoning of animals and humans (Wilber, 1980). The primary source of Se in food is usually the soil Se pool. As a result of the generally low Se concentrations in soils of Europe, Se deficiency is widespread in Europe (Ure and Berrow, 1982). Therefore, understanding of the Se cycling in the environment is important with respect to the prevention of animal and human deseases as a consequence of Se deficiency.

The concentrations of Se in soils usually range from 0.1-2 mg kg^{-1} with a worldwide mean concentration of 0.4 mg kg^{-1} Se but some soils contain up to 8000 mg kg^{-1} Se (Berrow and Ure, 1989). Selenium concentrations in soil depend on type of parent materials, presence of fine particles, distance to the oceans, and organic materials in soils (McNeal and Balistrieri, 1989). Soils derived from igneous rocks contain low Se concentrations whereas the weathering of sedimentary rocks from the Creataceous and Paleocene results in seleniferous soils with concentrations of up to 200 mg kg^{-1} Se (Girling, 1984). The Se concentration of German soils varies from 0.02 to 2 mg kg^{-1} mainly related to the degree of anthropogenic influence (Hartfiel and Bahners, 1988). Consequently, grassland and arable soils in Germany show a north-south gradient with decreasing Se concentrations towards the south (Hartfiel and Bahners, 1988; Hartfiel and Schulte, 1988). The anthropogenic input of Se to soils originating mainly from the combustion of fossil fuels, pyrometallurgical activities and the agriculture on naturally Se-rich soils developed on shales and certain other organic-rich sedimentary rocks redistributes Se in the environment (Pyrzynska, 2002).

Insufficiency or toxicity of Se depends on both, Se concentration in soils, water and food, and the chemical form of Se determining bioavailability and adsorption. The occurrence of different chemical Se species in soil is related to pH, redox potential, and other soil solution properties including ionic strength and concentration of dissolved organic matter controlled by soil mineralogy and soil microbial activities. The water-soluble fractions of Se in soils play an important role in the biogeochemical cycling of Se in ecosystems and are the best indicators of Se deficiency or excess in soils (Sun et al., 2009). The oxidized forms of Se, selenate Se(VI) and selenite/hydroselenite Se(IV), are water soluble and hence more mobile and bioavailable. However, both Se(VI) and Se(IV) can be

adsorbed on Fe oxides (Balistrieri and Chao, 1987), clay minerals (BarYosef and Meek, 1987), Mn and Al oxides (Foster et al., 2003), and organic matter (Abrams et al., 1990). Usually Se(IV) is more strongly sorbed than Se(VI) onto clay minerals and oxides (Balistrieri and Chao, 1987). The sorption of Se(IV) resembles that of phosphate while Se(VI) sorption is more similar to sulfate, which in soils is usually less retained than phosphate (Barrow and Whelan, 1989). The sorption of Se is more pronounced under reducing conditions but also occurs in aerated soils (Haudin et al., 2007) and is favored under acidic conditions because of a higher positive surface charge (Neal et al., 1987). In well-drained neutral soils, Se(IV) species dominate, while Se(VI) species occur mainly in well-drained alkaline soils whereas well aerated acidic soils and soils with high organic matter contain more organic Se(-II) forms (Kabata-Pendias, 2004). Gustafsson and Johnson (1992) reported that increasing soil organic matter concentrations result in lower plant availability of Se. The reduction of Se(IV) and Se(VI) results in the formation of insoluble, immobile elemental Se [Se(0)] in the soil matrix where the re-oxidation to soluble Se-species proceeds only slowly (Zawislanski and Zavarin, 1996).

To identify and quantify different chemical forms of Se, sequential extraction is an established method (Chao and Sanzolone, 1989; Martens and Suarez, 1997; Bujdos et al., 2000; Hagarova et al., 2005) although no generally accepted procedure exists. In all methods published to date, a phosphate buffer is used to extract soluble and ligand-exchangeable Se because phosphate competes with Se(IV) and Se(VI) for the same binding sites in soils (Barrow and Whelan, 1989b). To extract Se associated with metal oxides and organic matter, various extractants are used. The metal oxide-bound and organic Se pool was either assessed by extraction with NaOH (Hagarova et al., 2005; Keskinen et al., 2009; Nakamura et al., 2005), concentrated HCl (Chao and Sanzolone, 1989) or $K_2O_8S_2$ (Martens and Suarez, 1997).

It is well known that S and Se have similar chemical properties (Terry et al., 2000) and both elements follow similar metabolic pathways in plants and animals (Terry et al., 2000; Sors et al., 2005). The plant availability of Se(VI) and Se(IV) in solutions can be influenced by the presence of competing anions such as sulfate and phosphate (Hopper and Parker, 1999). Particularly, Se(VI) uptake is inhibited by sulfate (Bell et al., 1992) and uptake of Se(VI) occurs as an active process into the roots via sulfate transporters and the sulfate assimilation pathway. The uptake of Se(IV), in contrast, occurs passively and can also be inhibited by competition with sulfate for anion exchange sites at the surface of plant roots

(Asher et al., 1977). An increasing sulfate/selenate ratio in soil solution generally leads to lower Se concentrations in plants (Sors et al., 2005).

The natural abundance of stable Se isotopes can be used to investigate microbial and abiotic processes and to distinguish different Se sources in the environment (Rouxel et al., 2002). Selenium has six stable isotopes: ^{74}Se (contribution to the total elemental mass: 0.89%); ^{76}Se (9.37%); ^{77}Se (7.64%); ^{78}Se (23.77); ^{80}Se (49.61%), and ^{82}Se (8.73%) (Wachsmann and Heumann, 1992). The first precise isotope measurements were carried out using gas-source mass spectrometry by Krouse and Thode (1962). A thermal ionization mass spectrometry (TIMS) method was developed by Johnson et al. (1999). In TIMS analyses, $^{80/76}$Se ratios were determined. More recent high-precision Se isotope measurements ($^{82/76}$Se ratios) have used multicollector inductively-coupled plasma mass spectrometry,(MC-ICP/MS) using a combination of high-efficiency Ar plasma and multicollectors (Rouxel et al., 2002). The interference of $ArAr^+$ on mass 80 generated in the plasma, however rendered the use of ^{80}Se as target isotope impossible. Therefore, $^{82/76}$Se ratios are usually determined in measurements with MC-ICP/MS (Zhu et al., 2008).

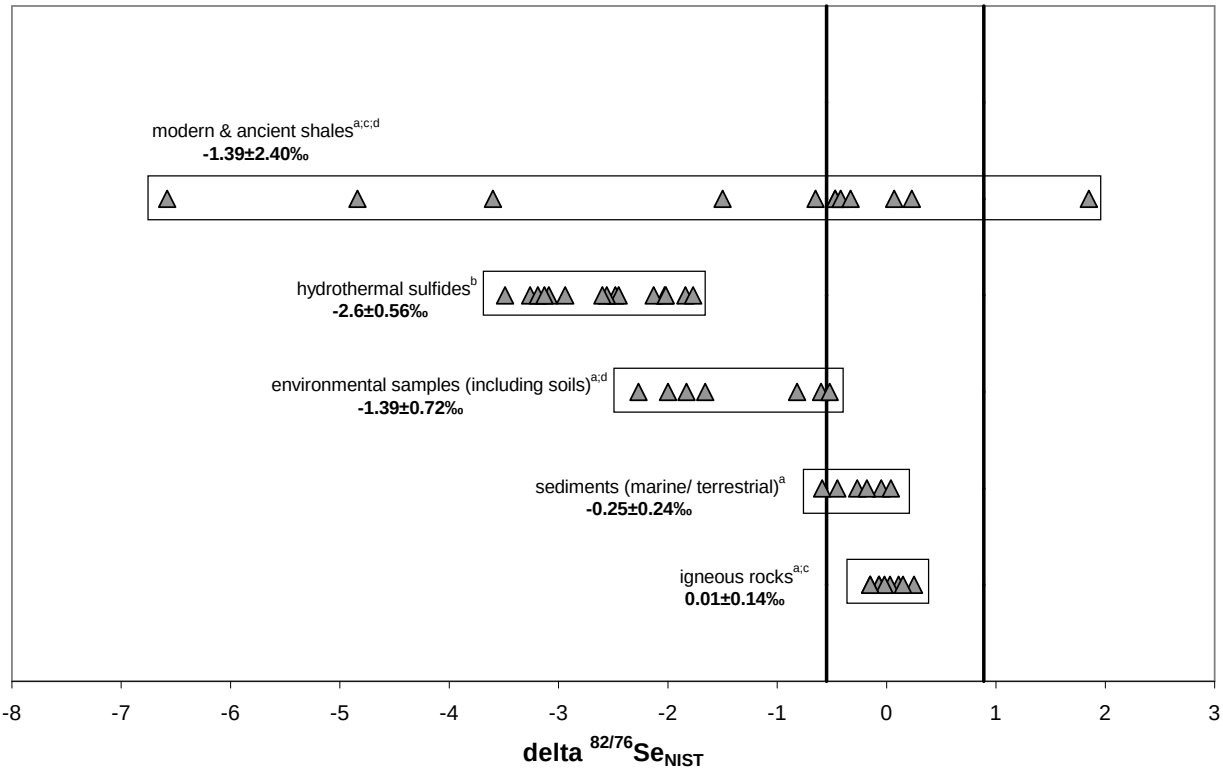


Figure D-1: Reported $\delta^{82/76}Se_{NIST}$ values in different natural samples. We used data of Rouxel et al. (2002), Layton-Matthews et al. (2006), and Wen et al. (2007) and recalculated the $\delta^{82/76}Se_{NIST}$ value if $\delta^{82/76}$Se values relative to other standards were reported.

The largest Se isotope variations were observed in shales (*Figure D-1*) while in meteorites and igneous rocks Se isotope variations are limited to a small range of <0.5‰ (Rouxel et al., 2002), suggesting that crystallization of high temperature melts results in a negligibly small isotope fractionation, i.e. shift in the $^{82/76}$Se ratio between melt and crystallized mineral. The combination of a sequential extraction procedure with the determination of stable Se isotope ratios in soils is a particularly promising approach to gain new insights into the Se cycle.

As Se concentrations in soils of urban areas are particularly important both with respect to possible toxicity of excess concentrations as well as for food production in gardening or peri-urban agriculture, we combined a well established sequential extraction approach to determine Se concentrations, bioavailability, and biogeochemical transformation in soils with stable Se isotope analysis. Furthermore, we determined total S and SO_4^{2-} concentrations and total S stable isotope ratios to assess the interaction of Se and S in our study soils. In particular we tested the following hypotheses:

(i) Like elsewhere in central Europe, total Se concentrations in urban soils of the German city of Bayreuth are low and mainly strongly bound in little bioavailable pools resulting in possible Se scarcity of plants grown in the urban area,

(ii) the natural abundance of stable Se isotopes can be used to infer processes such as plant uptake and recycling via litterfall, changing redox conditions or anthropogenic contamination governing the Se concentrations and cycling in the soil-plant system, and

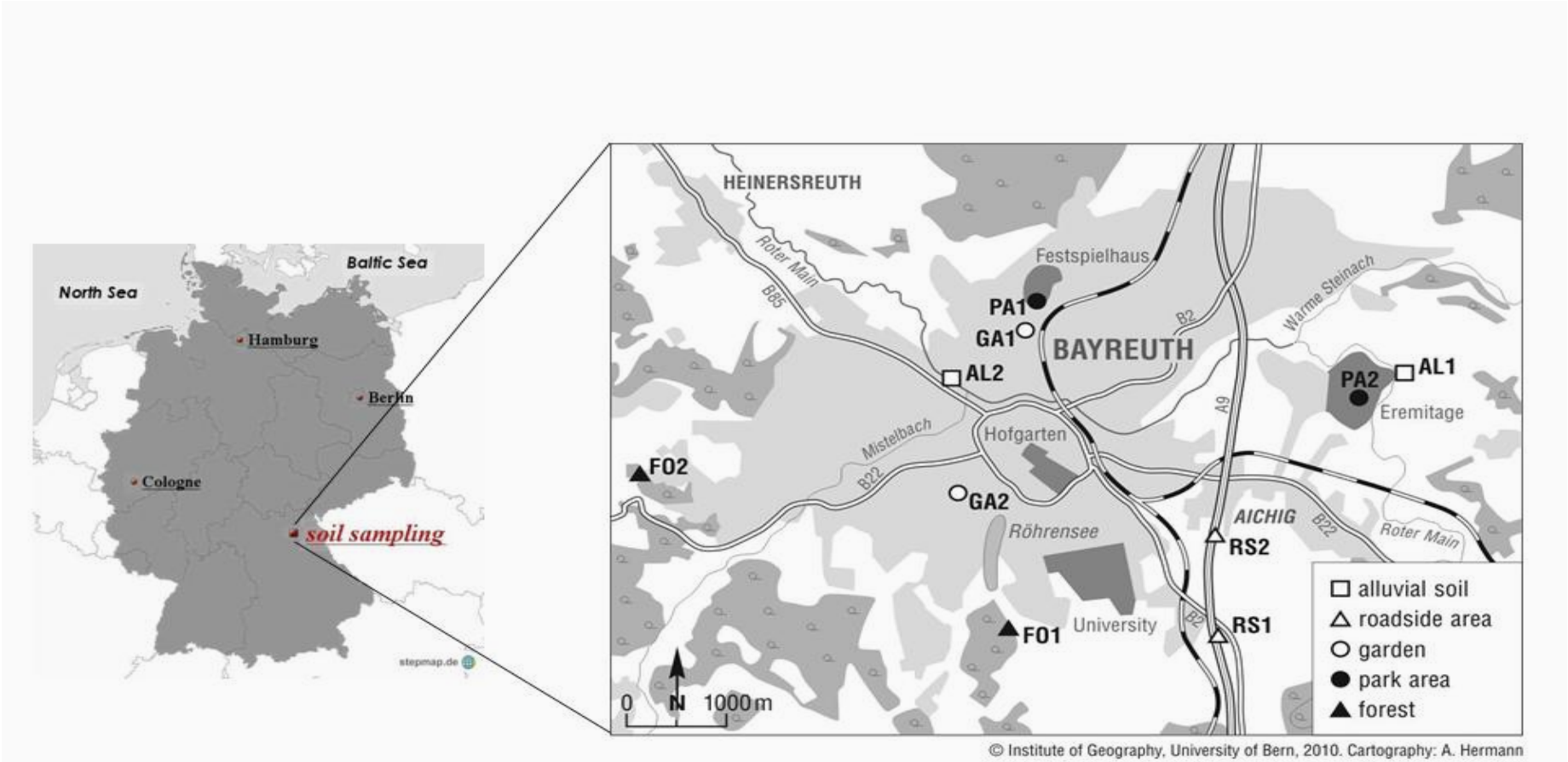
(iii) the analytically more easily accessible S concentrations and isotope ratios can be used to draw conclusions on the Se cycling because of the chemical similarity of S and Se.

3. Material and methods

3.1 Soil sampling

The study soils were collected from the city of Bayreuth in Bavaria, SE Germany (49°94´ N, 11°57´ E, ca. 73,000 inhabitants, *Figure D-2*). We collected topsoils (0–5 cm) from ten sites with different land use as described by Krauss and Wilcke (2003). The sampled land-use types were alluvial grasslands (AL1 and AL2), forests (FO1 and FO2), house gardens (GA1 and GA2), parks (PA1 and PA2), and grass-covered roadsides (RS1 and RS2). All samples were air-dried and sieved to <2 mm and stored in closed Al containers.

Figure D-2: Location of the study sites in the city of Bayreuth, Germany. AL, alluvial soil; RS, grass-covered roadside soil; GA, home garden soil; PA, urban park soil; FO, forest soil.



3.2 Soil characterization

Texture was determined by the pipet method. The pH was measured in a soil:water suspension (1:2.5 m/m) with a potentiometric glass electrode (Thermo Fisher Scientific, Orion U402-S7, Waltham, MA, USA). The concentrations of C, N, and S were determined with an elemental analyzer before and after removal of carbonates with concentrated HCl until effervescence ceased (Elementar Vario EL, Elementar Analysensysteme, Hanau, Germany). Carbonate-C was calculated by difference between C determinations before and after applying HCl. Effective cation-exchange capacity (ECEC) was determined as the sum of the charge equivalents of Na, K, Ca, Mg, and Al extracted with 1 M NH_4NO_3. The base saturation (BS) is the contribution of the sum of the charge equivalents of the base metals K, Na, Ca, and Mg to the ECEC. Oxalate-extractable Fe, Al, and Mn concentrations (Fe_o, Al_o, Mn_o) were determined with the method of Schwertmann (1964) and dithionite-citrate-extractable Fe concentrations (Fe_d) with that of Mehra and Jackson (1960). Sulfate was extracted with deionized water. The metal concentrations were measured by atomic absorption spectrometry using Varian AA 400 (Varian Inc., Mulgrave, Australia). Inorganic SO_4^{2-} concentrations were determined with ion chromatography (IC 761, Metrohm, Zofingen, Switzerland).

For total digests, samples were ground by an agate mortar, 1.0 g of each sample was weighed in 120-mL teflon vessels and 10 mL of concentrated HNO_3 was added. The vessels were closed, placed in a microwave system (MARS5Xpress, CEM Corp., Matthews, NC, USA), and digested by the following program: 20 min rising temperature time; 200°C digesting temperature; 3000kPa (435psi) high pressure and 60 min holding temperature time.

We modified the sequential extraction method of Martens and Suarez (1997) by omitting the deionized water extraction step because we expected very low water-soluble Se concentrations given the low total Se concentrations in the study soils. Similar extracts were used by Herbel et al. (2002) to determine stable Se isotope ratios in various sediment pools. Soil samples (1.0 g) were placed into 50-mL centrifuge tubes. For Extract 1, 25 mL of 0.1 M K_2HPO_4-KH_2PO_4 buffer (pH 7) (Roth, Karlsruhe, Germany) was added and shaken on a horizontal shaker at room temperature for 1 h. Thereafter, the samples were centrifuged at 10.000 rpm (=11.400g) (Beckman J2-MC, Beckman Coulter, Brea, CA, USA) for 30 min and the supernatant was removed. Extract 1 of the sequential extraction contains soluble, ligand-exchangeable, and plant protein-bound Se forms which are all readily bioavailable. The remaining sample was treated with 25 mL of 0.05 M NaOH for Extract 2 which was prepared from 99.99% NaOH (Merck, Darmstadt, Germany). The solutions were heated in a water bath

at 90°C for 2 h, then centrifuged, and the supernatant removed as described above. Extract 2 is thought to contain mainly metal oxide- and organically bound Se (but not Se occluded in Fe oxides). For the last extract (Extract 3), the soil aliquots were treated with 5 mL of concentrated nitric acid (*Trace*Select; Sigma-Aldrich, Seelze, Germany) and heated at 90°C for 30 min to remove the residual, strongly bound Se, mainly associated with silicates and crystalline oxides. After cooling to room temperature, 20 mL of deionized water were added and the solutions were heated again for 90 min. The supernatants from all extraction steps were filtered through 0.45 µm membrane filters (VWR, Darmstadt, Germany). The sample volumes of Extracts 1 and 2 were reduced to 10 mL by freeze-drying to preconcentrate Se. The samples of the Extract 3 were evaporated at 60°C to a volume of 6 mL. To remove organic compounds from all samples, 1.5 mL of 30% H_2O_2 (suprapur, Merck, Darmstadt, Germany) was added (Johnson et al., 1999) and reacted at 70°C for 45 min. Selenium concentrations in the extracts were determined with inductively-coupled plasma-mass spectrometry (ICP-MS, ELEMENT 2, Thermo Scientific, Waltham, MA) by monitoring masses 77, 78 and 82.

For all preparation steps, we used chemicals with the purest grade and all plastic and glassware were soaked in 10% nitric acid and rinsed carefully with high purity deionized water (>18 MΩ cm^{-1}).

3.3 Stable isotope analyses

Before the Se isotope ratios were determined, two-step purification was carried out by (1) hydride generation and (2) anion exchange (AG1-X8 resin, Eichrom, Lisle, IL USA) using procedures described by Clark and Johnson (2008). The generation of Se hydrides served to eliminate most matrix effects. Sodium borohydride solution (0.2%) for hydride generation was prepared daily by dissolving 1.0 g of $NaBH_4$ and 1.0 g of NaOH in 500 mL H_2O. The generated H_2Se was trapped in 6 mL alkaline peroxide solution and then heated for complete conversion to Se(VI) and elimination of remaining H_2O_2. Then, the Se was separated from interfering elements (mainly Ge and As) via ion exchange, using 1 mL bed volume columns of BioRad AG1-X8 anion exchange resin. Selenium(VI) was eluted with 5 mL of 6 M HCl into glass tubes followed by conversion to Se(IV) at 100°C for 1 h and diluted to 2 M HCl (±0.2 M). Prior to purification steps and analysis with multicollector ICP/MS, the samples were spiked with the a ^{74}Se + ^{77}Se double-spike solution in the Se(IV) form to correct instrumental mass bias of the mass spectrometer (Clark and Johnson, 2008).

Standard and blank solutions were prepared following the same procedure together with the samples. The Se isotope ratios were determined using MC-ICP-MS (Nu Plasma, Nu Instruments; Wrexham, UK) coupled with a hydride generator (GILSON Miniplus3; Middleton, WI). All isotope data are reported using the $\delta^{82/76}$Se notation relative to the certified standard NIST 3149 SRM:

$$\delta^{82/76}\text{Se (‰)} = [(^{82/76}\text{Se}_{\text{sample}})/(^{82/76}\text{Se}_{\text{standard}}) - 1]*1000 \qquad \text{(D-1)}$$

We were able to determine Se isotope ratios in the total digests of our samples, but not in the other digests because of the low Se concentrations. The only exception was Fraction 2 of one alluvial soil sample (AL2, *Figure D-2*) which contained sufficient Se for isotope analysis.

To ensure the accuracy of our Se concentration and isotope ratio measurements, we used the certified reference material Green river shale, SGR-1 (3.5 mg of Se kg^{-1}) from U.S. Geological Survey (USGS, Reston, VA, USA). For precision, samples were run in duplicate. Furthermore, several procedural blanks (n = 4) were run in which on average 8.5 ng of total Se was found compared to at least 120 ng Se in our samples.

The measured total Se concentration of the reference material SGR-1 (Green River Shale) was 3.27±0.03 mg kg^{-1} Se close to its certified value of 3.5 mg kg^{-1} Se. We further analyzed the isotope composition of SGR-1 (Green River Shale) and found a $\delta^{82/76}$Se value of 0.2±0.1‰ (n = 7) which similar to the value of 0.42‰ (n= 1, recalculated relative to NIST SRM 3149) reported by Rouxel et al. (2003). The external precision of two standard deviations of the $\delta^{82/76}$Se value for unprocessed NIST SRM 3149 was 0.03‰ (n=16) and -0.15‰ for the processed standards (n = 5).

The isotope composition of total S was determined with the Eschka method (2:1 mixture of MgO and Na_2CO_3, Fluka Analytical, Seelze, Germany). Twenty g of homogenized soil sample was mixed with at least 40 g of Eschka`s mixture and placed in a porcelain crucible with an additional layer of Eschka`s mixture on the top. The crucible was heated at 850°C for 2 h in a muffle furnace, in order to oxidize all S to the SO_4^{2-} form. A 0.5 M $BaCl_2$-solution was prepared from $BaCl_2$ x $2H_2O$ (Merck, Darmstadt, Germany) to precipitate dissolved SO_4^{2-} as $BaSO_4$. The precipitate was collected on a 0.45 µm cellulose acetate filter (Sartorius Stedim Biotech GmbH; Göttingen, Germany). Samples in form of precipitated $BaSO_4$ were prepared for S isotope analysis. Stable S isotope ratios were determined with the IRMS Delta S 7193 mass spectrometer (Thermo Finnigan MAT GmbH, Dreieich, Germany) coupled with an elemental analyzer (Elementar Analyzer EA 1108, Fision Instruments S.p.A, Rodano,

Italy). The S isotope ratio was expressed in the common delta notation relative to the Canyon Diablo Troilite (CDT) standard:

$$\delta^{34}S\ (‰) = \left(\frac{\left(\frac{^{34}S}{^{32}S}\right)_{sample} - \left(\frac{^{34}S}{^{32}S}\right)_{CDT}}{\left(\frac{^{34}S}{^{32}S}\right)_{CDT}} \right) * 1000 \qquad \text{(D-2)}$$

The precision of our S isotope analysis was 0.4‰.

4 Result and discussion

4.1 Total selenium concentrations

The Se concentrations of the studied urban soils varied between 0.094 and 0.52 mg kg^{-1} (*Figure D-3*). The highest Se concentrations were observed in the northnorthwest of the sampling area which includes samples AL2, GA1, and PA1. In contrast, the lowest Se concentrations are not tied to a particular region of the sampling area, but were detected in the forest soils FO1 (0.097 mg kg^{-1} Se) and FO2 (0.094 mg kg^{-1} Se).

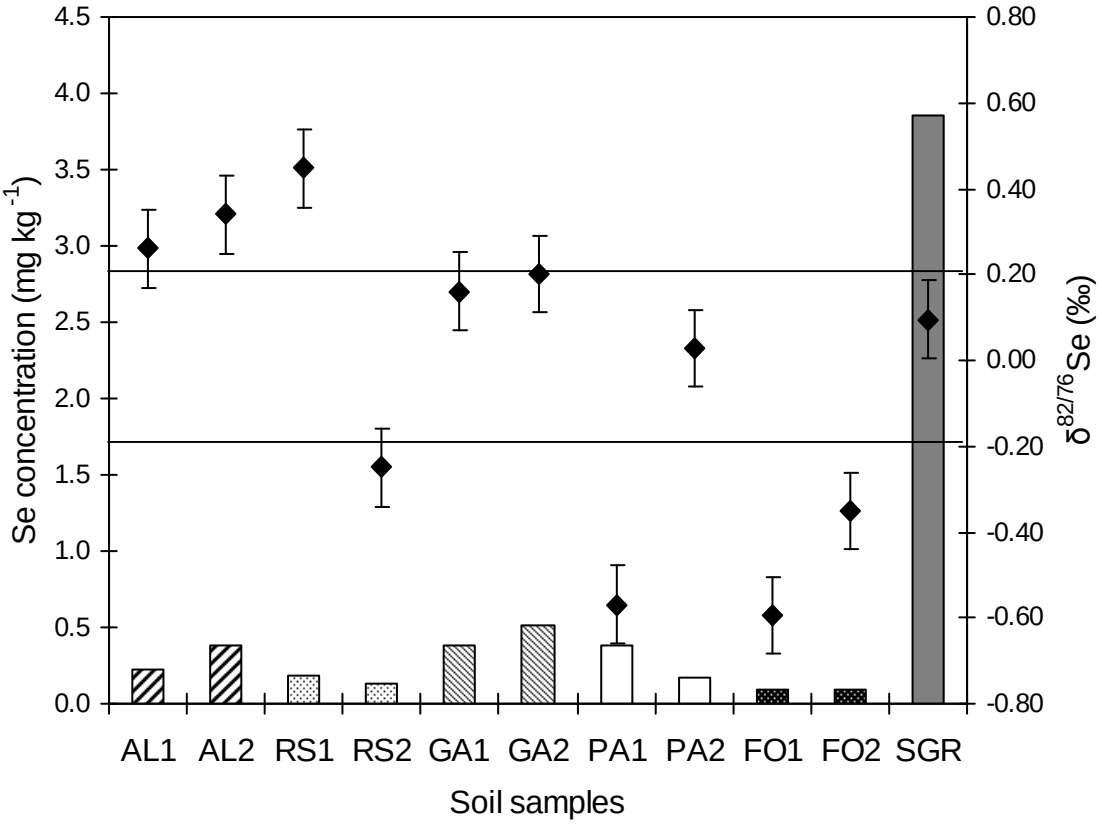


Figure D-5: Total Se concentrations and $\delta^{82/76}$Se values of topsoils from the city of Bayreuth, Germany and in the reference material SGR-1 (green river shale). Vertical error bars indicate maximum and minimum values (n=4).

According to the classification of Tan et al. (1994) the examined soils show Se deficiency (i.e. <0.15 mg kg^{-1} Se) or moderate Se concentration levels (0.175 to 0.4 mg kg^{-1} Se). The observed total Se concentrations are comparable with other parts of central and northern Europe, such as Austria (with a mean Se concentration of 0.35 mg kg^{-1} (Danneberg, 1989), Scotland (0.2 mg kg^{-1}, Ure and Berrow, 1982), Serbia (0.2 mg kg^{-1}, Maksimovic et al., 1991), and Finland (0.2 mg kg^{-1}, Ylärantka, 1983) all ranging in the low to moderate level of the classification of Tan et al. (1994). Given the low to moderate Se concentrations in our samples, it can be assumed that the anthropogenic input of Se is small and thus the likely main source of Se in soils is the parent rock. This assumption is corroborated by the fact that the soils of the study area are formed from sedimentary rocks of the Triassic and Jurassic which have usually low Se concentrations (Ebens and Shacklette, 1982).

The pH and ECEC (r = 0.75) showed significant positive correlations with total Se concentrations of the study soils (P <0.05, *Figure D-4*). We attribute the positive correlation

of pH with total Se concentrations mainly to increasing concentrations of lime with increasing pH. In calcite, Se oxyanions may substitute CO_3^{2-} (House and Donaldson, 1986) and both Se(IV) and Se(VI) can be adsorbed on $CaCO_3$ (Singh et al., 1981). This conclusion is confirmed by a significant correlation of total Se with $CaCO_3$-C concentrations (r = 0.78) in our soils. The correlation between ECEC and total Se concentration may be explained by the fact that the ECEC mainly reflects the clay and organic matter concentrations. It is known that trace element concentrations including Se in clay-rich soils are generally elevated (Hamdy and Gissel-Nielsen, 1977). However, pH and ECEC are also closely positively correlated (r = 0.74) because of the increasing negative charge of the organic matter with increasing pH. Therefore, again the variation in pH might be the dominating reason for the variation in total Se concentrations.

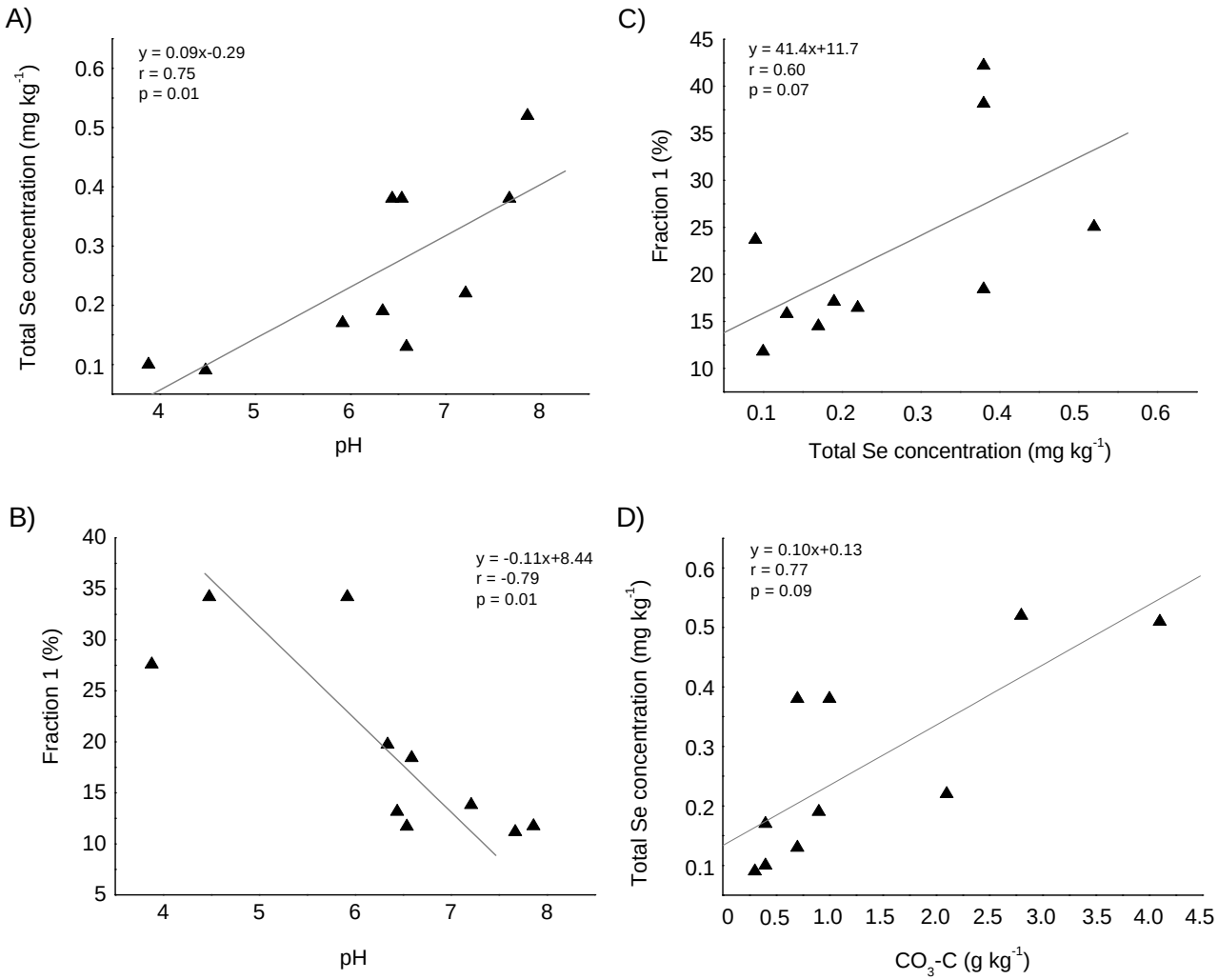


Figure D-4: Relationship between A) pH and total Se concentration in soils, B) pH and contribution of Se in Fraction 1 (water-soluble and ligand exchangeable Se) to the total Se concentration, C) total Se concentration and contribution of Se in Fraction 1 to the total Se concentration, and D) CO_3-C and total Se concentrations

4.2 Selenium partitioning

The sum of Se concentrations of the sequential extraction was close to the total Se concentrations determined by microwave digestion with concentrated HNO_3 (slope of the plot of Se in total digests vs. sum of Se concentrations in the three steps of the sequential extraction m = 0.78, r = 0.96). The Se concentration in Extract 1 (K_2HPO_4-KH_2PO_4 buffer) ranged between 12% (alluvial soils) and ~35% (forest soils) of total Se. The small contribution of Se in Extract 1 to total Se is a consequence of the fast transformation of bioavailable Se to strongly adsorbed and organically complexed Se(VI) or Se(IV) only extractable with Extract 2 (Bujdos et al., 2000). Furthermore, the Se forms removed by Extract 1 can be quickly leached to greater soil depth and taken up by plants and soil organisms. Only in Se-rich ("seleniferous") soils, water-soluble and ligand-exchangeable Se may form a similarly large pool as the organic matter-associated Se (Tokunaga et al., 1991).

The contribution of Extract 1 to total Se concentrations correlated negatively with increasing pH ($P < 0.05$, *Figure D-4B*). This may be explained by the increasing bioavailability of Se with increasing pH because of the less positively charged surface area and thus reduced Se sorption in soil. The positive correlation between total Se concentrations and the contribution of Extract 1 to total Se (*Figure D-4C*) can be attributed to the fact that Se reaching the soil from external sources (e.g., via river water) is less strongly bound than native Se, as was observed for other elements (Chlopecka et al., 1996; Wilcke and Kaupenjohann, 1998).

Extract 2 (0.05 M NaOH) contained the largest fraction of total Se in all study soils (42-77%; *Figure D-5*). This finding is similar as reported by Strawn et al**.** (2002) who suggested that Se is preferentially associated with Fe oxides in Luvisols in the Panoche Hills, California, in which – likely similar to our soils – Se(IV) was the predominant Se species. High concentrations of organically bound Se, which is also thought to be removed by Extract 2, were detected in Podzols in Sweden (Gustafsson and Johnson, 1992) and various agricultural soils of Japan (Yamada et al., 2009). Furthermore, fractions containing Se associated with organic matter were shown to be the largest Se pools in soils of Spain (Rodriguez et al., 2005), Slovakia (Hagarova et al., 2005), and Finland (Keskinen et al., 2009). Schweder et al. (1996) observed that there was a close correlation between the concentrations of Se and soil organic matter suggesting that Se was mainly bound to organic matter similar to S. In topsoils in central Spain, Se also occurred predominatly in fractions associated with humified organic matter (Rodriguez et al., 2005). It is assumed that the Se in Extract 2 of the sequential

extraction is unavailable to plant uptake because of the sorption of Se(IV) by Fe minerals and complexation by organic substances (Gustafsson and Johnson, 1992).

A similar portion of total Se as with Extract 1 is extracted with Extract 3 (HNO_3), which contains Se not available for the biosphere and dissolved transport in soil. In our samples, the Se extracted with Extract 3 contributed ~12-15% (alluvial and forest soils) to the total Se concentrations and with ~35% a substantially higher portion at the sites GA1 and PA1 (*Figure D-5*). Both, GA1 and PA1 are in the north of the sampling area where we observed the lowest Se concentrations. This might be attributable to either low native Se concentrations in the substrates from which these soils developed or to their location upwind from possible anthropogenic Se sources in the urban area.

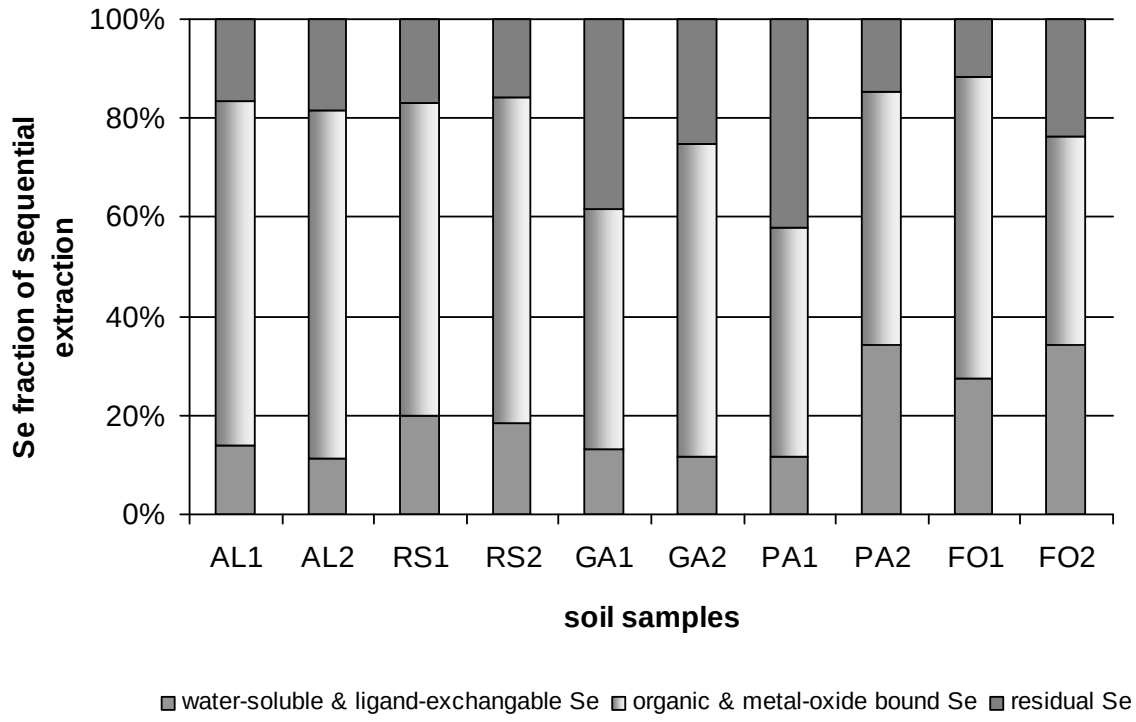


Figure D-5: Partitioning of Se among the fractions of the sequential extraction procedure. Black/uppermost: phosphate fraction (Fraction 1): soluble, ligand-exchangeable and plant protein-bound Se; light grey/central: NaOH fraction (Fraction 2): Fe and organically bound Se fraction; grey/lowermost: HNO_3 fraction (Fraction 3): residual Se.

4.3 Stable selenium isotope ratios

The $\delta^{82/76}$Se values of the studied soil samples were close to the Earth bulk composition of Se with the average $\delta^{82/76}$Se value of -0.03±0.38‰ (*Figures D-1* and *D-3*). The Se isotope ratios in our study soils were similar to those of marine and terrestrial sediments (Fig. 1,

Rouxel et al., 2002) which can be attributed to the fact that the study soils mainly derived from Triassic (Keuper) and Jurassic sediments. However, three soil samples (AL1, AL2, RS1) had clearly lower δ^{82}Se values of -0.59 to -0.35‰ and three samples had clearly higher $\delta^{82/76}$Se values of 0.26 to 0.34‰ (*Figure D-3*) than the 0 ±0.20‰ considered as the Earth bulk signal.

The group of soils with isotopically heavier Se includes two alluvial soils from the alluvial plain of the river Roter Main (AL1 and AL2). In these soils, an anthropogenic input of heavy Se via the river water is possible which might be immobilized because of adsorption, reduction processes, and biological transformation into organic matter. Johnson et al. (2000) reported $\delta^{82/76}$Se values for water of the San Joaquin River of 4.96±2.51‰ (recalculated to $NIST_{3149}$ and $^{82/76}$Se ratio) and Sacramento River in California of 5.96±2.81‰ (recalculated to $NIST_{3149}$ and $^{82/76}$Se ratio) which are affected by Se-rich waste water from oil refining Alternatively, the temporarily low redox potential during waterlogging of these soils might have favored Se methylation which releases light Se and leaves the remaining soil heavier (Schilling et al. 2011). Interestingly, the $\delta^{82/76}$Se value of Se in Extract 2 of the sequential extraction of the alluvial soil AL2, the only Se isotope ratio which we were able to determine in any of the sequential extracts, was -0.57‰, i.e. isotopically lighter than that of the bulk soil ($\delta^{82/76}$Se = 0.34‰). Consequently, one or both of Extracts 1 and 3 needed to be Se isotopically heavier than the bulk soil to balance the isotope budget. As both, introduced dissolved Se via the river water and methylated Se would affect the Se isotope signal of particularly the soil pool extracted with Extract 1, a heavier $\delta^{82/76}$Se value in Extract 1 would be in line with our above interpretations.

The group of soils with isotopically lighter Se includes two forest soils. We offer two possible explanations for this finding. For several elements it was shown that plants favor the uptake of the light isotopes (Trust and Fry, 1992; Kiczka et al. 2010). If this was also the case for Se at the studied forest sites, one can expect an isotopically light Se signal in the topsoil which contains recycled plant material. Herbel et al. (2002) observed that the assimilation of Se by plants fractionated the Se isotope ratio towards the lighter Se isotopes with a separation factor ε ($\varepsilon \approx \delta_{reactant} - \delta_{product}$) of -1 to 0‰. The further reduction of Se to Se(-II) in the plants does not fractionate Se isotopes. In line with this observation, Johnson et al. (1999) reported ε values of -1 to -2.5‰ for the whole path from uptake from soil via transport in plant to foliage and litterfall. Alternatively, the light Se isotope in the forest soils might be partly faster reduced which fractionates Se towards a lighter isotope signal in solution (Johnson 2004). The dissolved reduced Se-species, particularly (hydro-)selenite can be rapidly incorporated into

organic matter under acidic conditions (pH <5, Gustafsson and Johnsson, 1992). The main mechanism for Se retention in surface horizon of forest soils includes the microbial reduction and the subsequent incorporation into humic substances (Gustafsson and Johnsson, 1994).

For the remaining four soils, we propose no or little Se isotope fractionation because the analyzed topsoils are well-oxidized while it is known that large Se isotope fractionations only occur via oxyanion reduction that requires reducing conditions (Rashid and Krouse, 1985; Johnson, 2004). Under reducing conditions, conversion of Se(VI) to Se(IV) causes Se isotope fractionations of 4.5 to 10.5‰ and reduction of Se(IV) to Se(0) of 9 to 13.5‰ (Johnson, 2004). Given the small Se isotope fractionation because of Se(IV) adsorption on Fe minerals ($\delta^{82/76}Se_{adsorbed\text{-}dissolved}$ < 0.75‰, Johnson et al., 1999), it can be assumed that the adsorption of Se on iron minerals results in little or no Se isotope fractionation in our studied topsoils. Furthermore, the lack of a significant isotope variation in the topsoils of the four soils with $\delta^{82/76}Se$ values close to the Earth bulk signal is attributable to uniform Se sources. Even if the native Se in the parent substrates of the study soils was in reduced form, an abiotic oxidation of Se(-II) in parent rocks or Se(0) in soils to the oxidation state 0, +IV, +VI would not be associated with changed Se isotope ratios (Johnson, 2004).

4.4 Total sulfur and sulfate concentrations and stable S isotope ratios

The contribution of SO_4^{2-}-S to total S ranged from 1.2 (AL2) to 14% (FO1) illustrating that most S was organically bound in all samples (*Table D-1*). In agricultural topsoils of Iowa, SO_4^{2-}-S concentrations ranged between 1 and 26 mg kg^{-1} S (Tabatabai and Bremner, 1972). Thus, some of our study soils had elevated SO_4^{2-}-S concentrations (*Table D-1*). This becomes particularly evident if soils in the urban area of Bayreuth are compared with the forest soils at the outskirts of the city (*Table D-1*). The elevated SO_4^{2-} concentrations in some of our urban study soils are likely the consequence of the proximity to many combustion activities like house firing, traffic, and industry (Charlson et al., 1992). All study soils had 50 to 250 times higher SO_4^{2-}-S concentrations than total Se concentrations; the abundant SO_4^{2-} likely interfered with the uptake of Se. Mackowiak and Amacher (2008) reported in an experiment with seleniferous soils that sulfur as soil amendment decreased the Se uptake by forage plant species significantly. This would be particularly true for the likely minor Se(VI) which is taken up by soil organisms and plant roots via the same assimilatory pathway as SO_4^{2-}. But also Se(IV) which usually dominates in aerated soils (Kabata-Pendias, 2004) competes with anion exchange sites at the surface of plant roots (Asher, 1977).

The $\delta^{34}S$ values of total S ranged from -2 to +3.3‰ (Figure 5). In eight of the ten studied soils, the $\delta^{34}S$ values were positive (*Figure D-6*) which was also reported for many soils from Russia (Chukhrov et al., 1978) and forest soils in the Czech Republic (Novak et al., 1996). Similar to Se, S may be derived mainly from parent rocks which tend to have $\delta^{34}S$ values near the Canyon Diablo Troilite (CDT) standard (0‰ per definition) as revealed by studies of basic sills (Shima et al., 1963) and carbonatites (Mitchell and Krouse, 1975). The positive shift of the $\delta^{34}S$ values of most studied soils can partly be explained by the addition of S via deposition to soils (Alewell and Gehre, 1999). Sulfate formed by atmospheric oxidation of anthropogenic SO_2 is slightly enriched in ^{34}S (Nriagu and Coker, 1978). Furthermore, S isotopes are additionally fractionated during transformation and transport processes in the soil-plant system (Mayer et al., 1995). The main transformation processes causing S isotope fractionation in soil are mineralization and assimilation of S by plants (Kaplan and Rittenberg, 1964; Krouse and Tabatabai, 1986; Trust and Fry, 1992). Reductive incorporation of SO_4^{2-} into soil organic matter and assimilatory reduction in plants would also explain the positive shift in $\delta^{34}S$ values of most studied soils. Previous studies showed that the S isotope fractionations for assimilatory SO_4^{2-} reduction are small, with ε values in a range of -0.9 to -2.8‰ for different microorganisms (Kaplan and Rittenberg, 1964). Furthermore, Krouse and Tabatabai (1986) and Trust and Fry (1992) reported that higher plants were depleted in ^{34}S by 1.5‰ relative to the SO_4^{2-} sources. Consequently, the remaining S in soil usually becomes isotopically heavier. Novak et al. (1996) furthermore reported that $\delta^{34}S$ of bulk soils increase with increasing depth because of advancing mineralization of organic S implying that the dissolved S released by mineralization is isotopically lighter than the solid phase S. Therefore, the two negative $\delta^{34}S$ values in the alluvial soils AL1 and AL2 might be attributable to the input of S via river water (*Figure D-6*, Novak et al., 1996).

As *Figure D-6* clearly reveals, $\delta^{34}S$ and $\delta^{82/76}Se$ values of bulk soil were independent from each other in spite of the chemical similarity of S and Se. A particularly striking difference is the fact that topsoils had mostly positive $\delta^{34}S$ values (except the two alluvial soils AL1 and AL2) but close to zero or negative $\delta^{82/76}Se$ values (except three soils including again the two alluvial soils) in spite of the fact that plant uptake favors both the light S and Se isotopes and leaves the solid soil isotopically heavier. For S, microbial mineralization fractionating towards a heavier S isotope ratio in the remaining soil solid phase is obviously more important than the recycling of isotopically light S via plant litter. For Se, in contrast the reverse is true possibly because light Se is taken up from a larger soil volume and

accumulated in the surface organic layer. Therefore, the ratio of element flux with litterfall to element storage in soil is much larger for Se than for S. Another important difference is that in alluvial soils S introduced by the river water shifted the $\delta^{34}S$ to more negative values while Se introduced by the river water did the reverse. Alternatively, reduction processes in the alluvial soils may have resulted in reverse directions of isotope fractionation of S and Se.

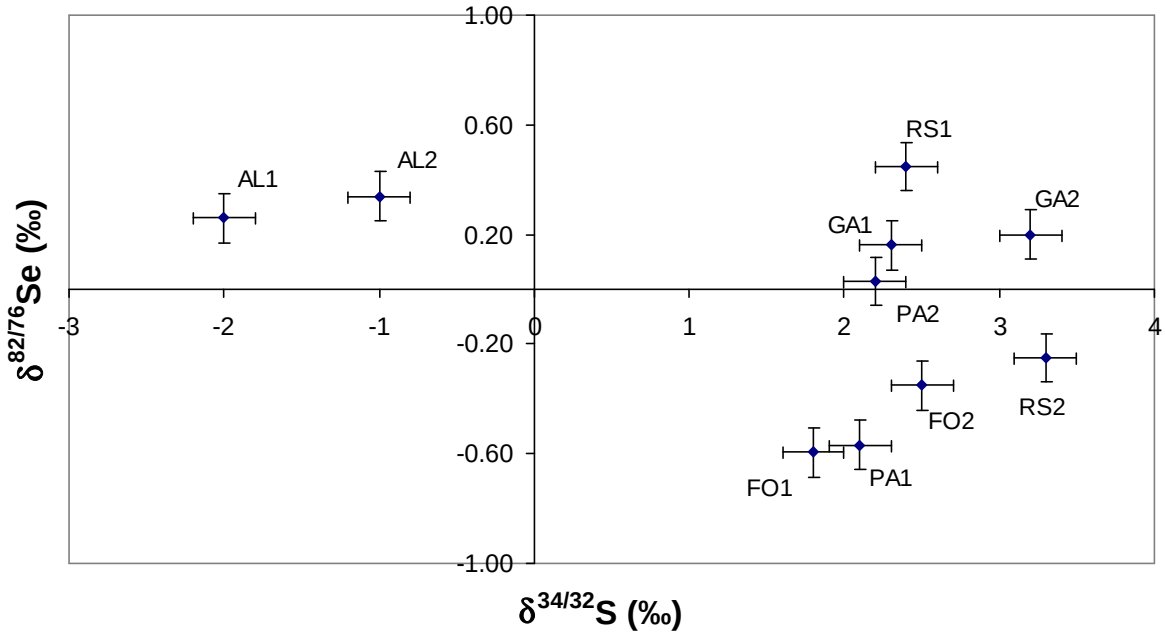


Figure D-6: Relationship between $\delta^{34/32}S_{CDT}$ and $\delta^{82/76}Se_{NIST}$ values of topsoils from the city of Bayreuth, Germany.

Table D-1. Selected physico-chemical properties of soil samples.

Sample	**Soil type** (IUSS Working Group WRB, 2006)	**Texture†**	**$pH_{(H2O)}$**	**OC‡**	**CO_3-C§**	**Total N**	**C/N¶**	**ECEC§**	**BS#**	**Al_o ††**	**Mn_o ††**	**Fe_o ††**	**Fe_d ‡‡**	**Total S**	**SO_4^{2-}-S**
				g kg^{-1}	g kg^{-1}	g kg^{-1}		mmolc kg^{-1}	%	g kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	mg kg^{-1}
Alluvial soils															
AL1	Eutric Fluvisol	silt loam	7.21	22.9	2.1	1.8	12.4	124	100	0.8	489	4.2	7.9	0.45	16.3
AL2	Eutric Fluvisol	silt loam	7.67	31.4	4.1	2.5	12.7	93	100	1.1	670	4.1	8.0	0.51	42.3
Roadside soils															
RS1	Ubric Anthrosol	sandy loam	6.34	18.9	0.9	1.9	9.8	81	99	1.0	744	2.9	5.9	0.34	14.9
RS2	Urbic Anthrosol	clay	6.59	41.1	0.7	3.7	11.1	136	99	0.8	569	1.5	7.6	0.64	9.6
Garden soils															
GA1	Stagnic Luvisol	sandy loam	6.44	23.5	0.7	2.0	12.0	144	99	0.9	421	2.8	6.7	0.42	12.2
GA2	Haplic Luvisol	sandy loam	7.86	33.4	2.8	2.2	15.3	195	100	1.0	413	2.0	5.1	0.56	10.7
Park area soils															
PA1	Eutric Gleysol	sandy loam	6.54	50.5	1.0	4.2	12.1	213	98	1.2	946	3.8	8.9	0.80	12.3
PA2	Eutric Gleysol	sandy loam	5.92	21.1	0.4	1.8	11.5	56	97	1.0	880	1.9	6.5	0.34	7.8
Forest soils															
FO1	Dystric Gleysol	sandy loam	3.88	23.7	0.4	1.2	19.1	14	87	1.1	70	2.0	3.8	0.24	1.7
FO7	Eutric Cambisol	loam	4.48	14.8	0.3	0.7	20.6	21	96	0.2	0	0.5	1.7		6.3

† According to Soil Survey Staff (2010).
§ Concentration ratio of OC and soil N.
 # Base saturation.
‡ Soil organic carbon.
¶ Effective cation exchange capacity.
†† Oxalate-extractable Al, Mn, and Fe
.‡‡ Dithionite-citrate-extractable Fe.

5. Conclusion

Our results demonstrate that like elsewhere in central Europe, total Se concentrations in urban soils of the German city of Bayreuth were low and bioavailable portions small. The soils therefore likely tended to cause Se deficiency in animals and humans consuming food produced on these soils, in line with the general trend in central Europe.

The Se isotope ratios showed a small variation around zero (i.e., the Se isotope ratio of the international reference material). Alluvial soils had isotopically heavier Se which we mainly attribute to the input of heavy Se via the river water. In contrast, forest soils had isotopically lighter Se, probably because of the preferential uptake of light Se by plants and recyling to the soil via litterfall. The reason for the small isotope fractionation of Se may be related to the low bioavailability of Se as revealed by our sequential extraction and to the competition of the Se oxyanions with the high SO_4^{2-} concentrations in the study soils for uptake in the biosphere.

In spite of the chemical similarity and similar isotope fractionation during plant uptake of Se and S, bulk isotope ratios of Se and S in soil were unrelated in our study soils with low Se concentrations. We suggest that the various turnover processes of Se in soils with low Se concentrations differ in importance from those of S. While the Se isotope ratio in bulk soil is influenced by plant recycling of light Se via litter fall, the S isotope ratio in bulk soil is mainly driven by mineralization of organic matter leaving the solid soil S isotopically lighter.

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E Isotope Fractionation of Selenium by Biomethylation in Batch Soil Incubations with the Fungus Species *Alternaria alternata*

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1 Abstract

The natural abundance of stable Se isotopes reflects sources and formation conditions of methylated Se. We tested the effects of (i) different inorganic Se species [Se(IV) and Se(VI)] and (ii) different soil samples on the extent of fungal biomethylation of Se and the Se isotope ratios ($\delta^{82/76}$Se) in methylselenides. Furthermore, we assessed the reduction in bioavailability of spiked Se oxyanions by natural attenuation during three days, the time the samples were left to equilibrate after Se amendment before the incubation started. We conducted closed microcosm experiments with soil spiked with Se(IV) or Se(VI), a growth medium, and the fungus species *Alternaria alternata* for 11 d. The concentrations and isotope ratios of Se were determined in all components of the microcosm with multicollector ICP-MS. The equilibration of the spiked Se(IV) and Se(VI) for 3 d resulted in decreasing water-soluble Se (and thus bioavailable) concentrations by 32 to 44% and 8-14%, respectively, after three days associated with a small isotope fractionations of ε = -0.045 to -0.12‰ and -0.05 to -0.07‰, respectively. In two of the incubated soils – moderately acid roadside and garden soils - between 9.1 and 30% of the supplied Se(IV) and 1.7% of the supplied Se(VI) were methylated while in a strongly acid forest soil no Se methylation occurred. The methylselenides derived from Se(IV) were strongly depleted in ^{82}Se ($\delta^{82/76}$Se = -3.3 to -4.5‰) compared with the soil (0.16-0.45‰) and the spiked standard (0.20‰). The methylselenide yield of the incubations with Se(VI) was too small for isotope measurements. Our results demonstrate that Se source species and soil properties influence the extent of Se biomethylation and that biomethylation results in Se isotopically light methylselenides.

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2 Introduction

Selenium is an essential trace nutrient for many organisms. However, Se is also toxic for most organisms at concentrations which are only slightly above the required levels. Its toxicity is mainly attributable to chemical similarity with S resulting in a nonspecific replacement of S by Se in proteins.

The concentrations of Se in soils usually range from 0.1-2 mg kg^{-1} with a worldwide mean concentration of 0.4 mg kg^{-1} Se (Berrow and Ure, 1989) but some seleniferous soils contain as much as 38 mg kg^{-1} Se. Selenium concentrations in soil depend on type of parent materials, presence of fine particles, distance to the oceans and organic matter concentration and composition in soils (McNeal and Balistrieri, 1989). The Se concentration of German soils varies from 0.02 to 2 mg kg^{-1} (Hartfiel and Bahners, 1988; Hartfiel and Schulte, 1988). Like in most Europe, German soils are natively poor in Se. In urban topsoils of the city of Bayreuth in Germany, Se concentrations of 0.09-0.52 mg kg^{-1} were found (**Section D**, p.63). Since the 1980s, nutritional problem resulting from particularly Se-poor soils such as in some areas of Finland have even been counteracted by fertilizing with Na_2SeO_4 (Keskinen et al. 2009).

Selenium occurs naturally in four oxidation states (-II; 0; +IV; +VI) which differ significantly in their nutritional and toxic relevance. The oxidized forms of Se, selenate ($Se[+VI]O_4^{2-}$), selenite ($Se[+IV]O_3^{2-}$), and hydroselenite ($HSe[+IV]O_3^{-}$), are soluble in water and hence show a high mobility, bioavailability, and toxicity. However, Se oxyanions can be adsorbed on different minerals and organic matter in soils and sediments reducing their bioavailability. Both Se(VI) and Se(IV) can be adsorbed on Fe oxides (Balistrieri and Chao, 1987), clay minerals (BarYosef and Meek, 1987), Mn and Al oxides (Foster et al., 2003), and organic matter (Abrams et al. 1990). Usually Se(IV) is more strongly sorbed than Se(VI) on clay minerals and oxides (Balistrieri and Chao, 1987). The strength of sorption of Se(IV) resembles that of phosphate while Se(VI) is sorbed about as strongly as sulfate, which in soils is usually less retained than phosphate (Barrow and Whelan, 1989). It was reported that Se(IV) adsorption on iron (hydr)oxides occurs via the formation of outer-sphere and inner-sphere surface complexes (Parida et al. 1997). The sorption of Se is more pronounced under reducing conditions but also occurs in aerated soils (Haudin et al. 2007) and is favored under acidic conditions because of a higher positive surface charge (Neal et al. 1987). Sorption together with other dissipation processes including dispersion, dilution, precipitation, and volatilization are described as natural attenuation (Wiedemeier et al. 1999). Immobilization by

natural attenuation in soils has an initial fast reaction (minutes or hours), which is followed by a slow reaction (days or years, Buekers et al. 2007).

Microorganisms play important roles in the environmental fate of toxic elements (Gadd, 2001). Biomethylation of Se is seen as detoxifying response of some microorganisms to high Se concentrations. It is a widely distributed metabolic process and offers a potential remediation strategy in Se-contaminated areas (Frankenberger and Karlson, 1989). Inorganic Se species [Se(+VI) and Se(+IV)] and even some organoselenium compounds (selenomethionine, selenocysteine) are methylated to volatile dimethylselenide (DMSe) and, to a lesser extent, to dimethyldiselenide (DMDSe). The organoselenium compounds are 500 to 700 times less toxic than the inorganic Se species (McConnell and Portman, 1952). Especially fungi, which are a major and often dominant component of the microbiota in soils contribute to biomethylation of Se (Gadd, 2001). Field studies showed that the methylation of Se depends on physical, chemical, and biological soil properties (Frankenberger and Karlson, 1989; Jayaweera and Biggar, 1992) with higher volatilization rate at high organic carbon concentrations, temperature (Calderone et al. 1990), and soil water contents (Frankenberger and Karlson, 1994; Terry et al. 2000).

The natural abundance of stable Se isotopes can be used to investigate into microbial and abiotic processes and to distinguish different Se sources in the environment (Rouxel et al., 2002). Various Se isotope studies have measured different ratios of the Se isotopes. In our study we measured $^{82}Se/^{76}Se$ and we thus express results of all earlier studies in terms of this ratio. The Se isotope ratios of environmental samples vary widely depending on transformation processes. The $^{82/76}Se$ ratios of igneous rocks have a homogeneous Se isotope composition and therefore are used as proxy for the bulk earth composition (Rouxel et al. 2002). In contrast, hydrothermal sulfides, terrestrial and marine sediments and soils vary in $^{82/76}Se$ values by up to -12.8‰ (Hagiwara, 2000; Rouxel et al. 2002; Wen et al. 2007; **Section D** p. 78-79).

Previous studies have demonstrated that Se isotope fractionation occurs as a consequence of dissmilatory reduction processes (Herbel et al. 2000; Ellis et al. 2003). A significant decrease in $\delta^{82/76}Se$ values of -6.0 to -13.5‰ was observed during dissimilatory reduction of Se oxyanions depending on bacteria species and the type of reduction process (Herbel et al., 2000). Ellis et al. (2003) reported fractionation factors ε of -2.6 to -3.1‰ during the reduction of Se(VI) to Se(IV) and of -5.5 to -5.7‰ during the further reduction of Se(IV) to Se(0) in microcosm experiments using sediment slurries with natural microorganism communities.

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Studies of isotope fractionation during biomethylation reported fractionation factors of -1.65‰ for cyanobacterial mats (Johnson et al. 1999), -3.25 to -4‰ and up to -6‰ by the fungus *Alternaria alternata* depending on the Se source species (**Section C** p. 50-58).

Our objectives were to test the effects of (i) different inorganic Se species [Se(IV) and Se(VI)] and (ii) different soil samples on the extent of biomethylation by the fungus species *Alternaria alternata* of Se and the Se isotope ratios ($\delta^{82/76}$Se) in methylselenides in closed microcosm incubations. Furthermore, (iii) we determined the reduction in bioavailability of spiked Se oxyanions and the associated Se isotope fractionation by natural attenuation during three days and, the time the samples were left to equilibrate after Se amendment before the microcosm incubation started.

3 Material and method

3.1 Soils

The soils were collected from the city of Bayreuth in Bavaria, SE Germany (49° 94´ N, 11° 57´ E, ca. 73,000 inhabitants). We collected topsoils (0–5 cm) from three sites with different land use as described by Krauss and Wilcke (2003). The sampled land-use types were a house garden (GA1), a grass-covered roadside (RS2), and a forest soil (FO1). The characterization of the soil properties were described previously (Krauss and Wilcke, 2003 and **Section D**, p. 71). Texture was determined by the pipet method. The pH was measured in a soil : water suspension (1:2.5 m/m) with a potentiometric glass electrode (Thermo Fisher Scientific, Orion U402-S7, Waltham, MA, USA). The concentrations of C, N, and S were determined with an elemental analyzer after destruction of CO_3^{2-} with concentrated HCl until effervescence ceased (Elementar Vario EL, Elementar Analysensysteme, Hanau, Germany). Carbonate-C was calculated by difference between C determinations before and after applying HCl. Effective cation-exchange capacity (ECEC) was determined as the sum of the charge equivalents of Na, K, Ca, Mg, and Al extracted with 1 M NH_4NO_3. The base saturation (BS) is the contribution of the sum of the charge equivalents of the base metals K, Na, Ca, and Mg to the ECEC. Oxalate-extractable Fe, Al, and Mn concentrations (Fe_o, Al_o, Mn_o) were determined with the method of Schwertmann (1964) and dithionite-citrate-extractable Fe concentrations (Fe_d) with that of Mehra and Jackson (1960). Sulfate was extracted with deionized water. The metal concentrations were measured by atomic absorption spectrometry

using Varian AA 400 (Varian Inc., Mulgrave, Australia). Inorganic SO_4^{2-} concentrations were determined with ion chromatography (IC 761, Metrohm, Zofingen/Switzerland).

The soils had total Se concentrations of 0.38 ± sd 0.03 (GA1), 0.19 ± 0.02 (RS2), and 0.1 ± 0.002 mg kg^{-1} (FO1). Most Se was not bioavailable, as it was bound to metal oxides and organic matter which is not bioavailable.The readily bioavailable water-soluble, ligand-exchangeable, and plant protein-bound Se forms accounted for 13.2 to 27.6% of the total Se concentrations (**Section D**, p. 74-83).

Table E-1. Selected physico-chemical properties of soil samples.

Sample	Soil type (IUSS Working Group WRB, 2006)	Texture†	$pH_{(H2O)}$	OC‡	CO_3-C§	Total N	C/N¶	ECEC§	BS#	Al_o ††	Mn_o ††	Fe_o ††	Fe_d ‡‡	Total S	SO_4^{2-}-S
				g kg^{-1}	g kg^{-1}	g kg^{-1}		$mmol_c$ kg^{-1}	%	g kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}	g kg-^{1}	mg kg^{-1}
GA1	Stagnic Luvisol	sandy loam	6.44	23.5	0.7	2.0	12.0	144	99	0.9	421	2.8	6.7	0.42	12.2
RS2	Urbic Anthrosol	clay	6.59	41.1	0.7	3.7	11.1	136	99	0.8	569	1.5	7.6	0.64	9.6
FO1	Dystric Gleysol	sandy loam	3.88	23.7	0.4	1.2	19.1	14	87	1.1	70	2.0	3.8	0.24	1.7

† According to Soil Survey Staff (2010).
‡ Soil organic carbon.
§ Concentration ratio of OC and soil N.
¶ Effective cation exchange capacity.
 # Base saturation.
†† Oxalate-extractable Al, Mn, and Fe.
‡‡ Dithionite-citrate-extractable Fe.

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3.2 Materials

All solutions were prepared using high purity (>18MΩ) deionized water. Stock standard solutions of 1000 mg L^{-1} Se as Na_2SeO_4 and as Na_2SeO_3, respectively, were purchased from Merck (Darmstadt, Germany). To trap methylselenides, we prepared a solution from 99.9% NaOH (Merck, Darmstadt, Germany) and 30% *Trace*Select H_2O_2 (Sigma-Aldrich, Seelze, Germany). All samples were filtered through 0.45 µm membrane filters (VWR, Darmstadt, Germany). For the quality control and normalization of Se isotope measurements we used SRM 3149 (National Institute of Standards and Technology, Gaitherburg, MD, USA). All plastic and glassware were soaked in 10% nitric acid and rinsed carefully with deionized water.

3.3 Equilibrating of soil with Se oxyanions/natural attenuation experiments

To 80-mL serum bottles filled with 2.0 g of soil, a 0.01 mol L^{-1} $NaNO_3$ background solution containing 10 mg L^{-1} Se as either Na_2SeO_3 or Na_2SeO_4 was added. Control samples contained either 10 mg L^{-1} Se or only the buffer solution (0.01 mol L^{-1} $NaNO_3$) in the same ionic background. The filled serum bottles were shaken for 1 to 3 d at 100 rpm. To assess the natural attenuation kinetics, an aliquot of the supernatant was sampled 30, 60, and 120 min, and 24, 48 and 72 h after starting the equilibration. Blank controls with no soil but the same concentration of Se were set up to check losses or contaminations. Blanks as well as soil equilibrations were realized in duplicates. Concentrations of Se were determined with ICP-MS (ELEMENT 2, Thermo Scientific, Waltham, MA).

3.4 Microcosm experiments

We purchased a strain of *A. alternata* from the German Resource Center for Biological Materials (DSMZ, Braunschweig, Germany). The fungus was first cultivated on agar plates at 30°C for 5 d and then kept at 4°C prior to use. The liquid culture medium used was prepared as described in Thompsoneagle et al. (1989). An aerobic culture was grown in 250-mL Erlenmeyer flask containing 80 mL of the medium at 30°C on a rotatory shaker for seven days before the culture was used for incubation with soil spiked with different inorganic Se species.

Microcosm experiments were conducted in 80-mL serum bottles containing 20 mL culture medium (10 g L^{-1} malt extract, 10 g L^{-1} glucose, and 0.5 g L^{-1} peptone) which was amended with 2 g of soil spiked either with 50µg Se(IV) or Se(VI) and equilibrated for three days by inoculation of 1 mL of homogenized fungus suspension. The serum bottles were plugged with sterile butyl-stoppers to create microaerobic conditions. The serum bottles were covered with aluminum foil to suppress abiotic photochemical formation of methylselenides (Guo et al. 2003). During incubation the bottles were horizontally shaken at 100 rpm at 30°C. The incubations were run for 11 d. The duration of the incubation of >10 d was chosen to allow the development of fungal activity and the production of measurable concentrations of methylselenides. Peitzsch (2008) showed that the trapped concentrations of methylselenides in a similar incubation with *Alternaria alternata* were constant after incubation times of 10 to 21 days illustrating that steady-state conditions were reached after 10 d. Uninoculated serum bottles were used as a control. At the end of the experiment, we collected the soil, the medium, the fungus, and the trapped methylselenides separately to determine Se concentrations and isotope ratios.

3.5 Trapping of methylselenides

We used the trapping method modified after Terry et al. (1992) to collect methylselenides. Gas wash bottles were connected to each other via Teflon tubes. Each gas wash bottle contained 120 ml of alkaline peroxide solution (90 mL of 0.05 M NaOH and 30 mL of 30% H_2O_2). For each trap the alkaline peroxide solutions were freshly prepared. The methylselenides were driven out of the sampling flask into the trapping solution by a N_2 stream of 0.75 L min^{-1} for 2 h which was controlled via a gas flow regulator. After collection of volatile Se, growth media and fungi were separated by filtration through 0.45 µm

membrane filter, freeze-dried, and digested by 2 mL of HNO_3 at 140°C to determine the Se remaining in the media and accumulated in the fungi.

The microcosm setup and performance was rigorously tested (**Section B** p. 33-38). We reported that the mass balances of total Se in these microcosm experiments run with alkaline peroxide trap were 96 ± 15 and 102 ± 2.4% (n=3) for Se(IV) and Se(VI), respectively. The average mass-weighted mean $\delta^{82/76}$Se value of the these microcosms with Se(IV) as source was -0.31 ± 0.05‰ compared to -0.20 ± 0.05‰ in the supplied Se and that of the $\delta^{82/78}$Se value -0.20 ± 0.03‰ compared to -0.10 ± 0.10‰ illustrating a good match of the calculated mass-weighted mean $\delta^{82/76}$Se values of the whole microcosm at the end of the incubation with that of the supplied Se(VI).

3.6 Selenium isotope analysis

Se isotope ratios were determined with MC-ICP-MS (Nu Plasma, Nu Instruments; Wrexham, UK). The double spike method was used to correct for instrumental mass bias (Johnson et al. 1999), and Se was introduced into the instrument via a custom built hydride generator, following methods given in a recent publication (Clark and Johnson, 2008). Here, a ^{74}Se + ^{77}Se double spike was used for all analyses. We collected aliquots with a total Se mass of 100 ng from each sample. Then, an appropriate amount of double spike solution was added so that the ratio of spiked ^{77}Se to ^{78}Se in the sample was close to 2:1. The samples were made up with 5 M HCl (±0.2 M) to a total of 5 mL. To convert Se(VI) to Se(IV), the sample was heated in a hot block held at 120°C for one hour. After cooling at room temperature the solutions were diluted to 2 M HCl (±0.2 M).Standard and blank solutions were prepared following the same procedure together with the samples. The Se isotope ratios were determined using MC-ICP-MS (Nu Plasma, Nu Instruments; Wrexham, UK) coupled with a hydride generator (GILSON Miniplus3; Middleton, WI). All isotope data are reported using the $\delta^{82/76}$Se notation relative to the certified standard NIST 3149 SRM (Eq. E-1).

$$\delta^{82/76}\text{Se}\ (‰) = [(^{82/76}\text{Se}_{\text{sample}})/(^{82/76}\text{Se}_{\text{standard}}) - 1]*1000 \qquad \text{(E-1)}$$

Our natural attenuation experiments were closed systems and unidirectional, so that the Rayleigh distillation model, which is often used to describe the process of kinetic isotope

fractionation can be applied. Therefore, we calculated the Se isotope fractionation from experimental data between educt and product with the Rayleigh distillation model (Eq. E-2).

$$\delta_{p_{(t)}} = \delta_{r(0)} - \varepsilon_{pr} \frac{f\ln(f)}{1-f} \qquad \text{(E-2)}$$

where $\delta_{p(t)}$ expresses the $\delta^{82/76}$Se of Se remaining in solution, $\delta_{r(o)}$ is the initial $\delta^{82/76}$Se of the educt at time 0, *f* is the fraction of educt remaining after time (t) and ε describes the isotope fractionation factor given by Eq. E-3.

$$\varepsilon = 1000 \times (\alpha - 1) \text{ (in ‰)} \qquad \text{(E-3)}$$

where the isotopic fractionation factor α is described in Eq. E-4.

$$\alpha = \frac{R_{\mathrm{Product}}}{R_{\mathrm{Reactant}}} \qquad \text{(E-4)}$$

as the isotope ratio R in two different Se species.

To test the tightness of the microcosm experiment and the accuracy of our measurements, we established the mass balance of the microcosms (Eq. E-5).

$$m_{Se_{total}} = m_{Se_{medium}} + m_{Se_{fungus}} + m_{Se_{trap}} + m_{Se_{soil}} \qquad \text{(E-5)}$$

where *m* is the mass of Se in the whole microcosm, the growth medium, the fungus, the trap and the soil at the end of the experiment. The mass-weighted mean $\delta^{82/76}$Se value was calculated with Eq. E-6.

$$\delta^{82/76}Se_{microcosm} = \left(f_{medium} \times \delta^{82/76}Se_{medium}\right) + \left(f_{fungus} \times \delta^{82/76}Se_{fungus}\right) + \left(f_{trap} \times \delta^{82/76}Se_{trap}\right) + \left(f_{soil} \times \delta^{82/76}Se_{soil}\right) \qquad \text{(E-6)}$$

where f represents the mass fractions of the medium, fungus, trap and soil respectively. The error of the mass balance was calculated with the Gaussian error propagation law and amounted to 0.25‰ for all treatments.

4 Results and Discussion

4.1 Natural attenuation

In the blank equilibrations (Se solution without soil) no Se losses occurred and in the blanks without Se no Se contamination occurred. During equilibration of the added Se oxyanions, the Se concentration in solution continuously decreased during three days in contact with all three soils (*Figure E-1*, *Table E-2*). The dissolved concentration of Se(IV) was consistently more reduced than that of Se(VI) in all soils. After three days, only 32-44% of the added Se(IV) was still dissolved. In contrast, between 86 and 92% of the added Se(VI) remained in solution with little change between days one and three illustrating that the small sorption occurred during the first 24 h. The stronger sorption of Se(IV) than of Se(VI) causes the generally lower mobility of Se(IV) in soils (Mayland et al. 1991). Balistrieri and Chao (1987) observed that goethite sorbed less Se(VI) than Se(IV) at the same pH. The lower Se(VI) adsorption can be explained by the competition with the sulfate in the study soils (*Table E-1*) as it was also reported by Merrill et al. (1986). Furthermore, the different soils showed different extents of Se(IV) sorption with the roadside soil exhibiting the least and the forest soil the most pronounced sorption. We assume that the Se sorption in the three study soils is mainly controlled by pH. Gho and Lim (2004) showed that the adsorption of Se(IV) to tropical soils decreased from 83% at pH 3 to 59% at pH 7 and that of Se(VI) from 46% to 15%, respectively. Similar observations were reported for arable soils in Finland (Vouri et al. 1989). Consequently, the most acid soil (FO2) sorbed Se(IV) most strongly and the moderately acid soils GA 1 and RS2 much less (*Table E-2*).

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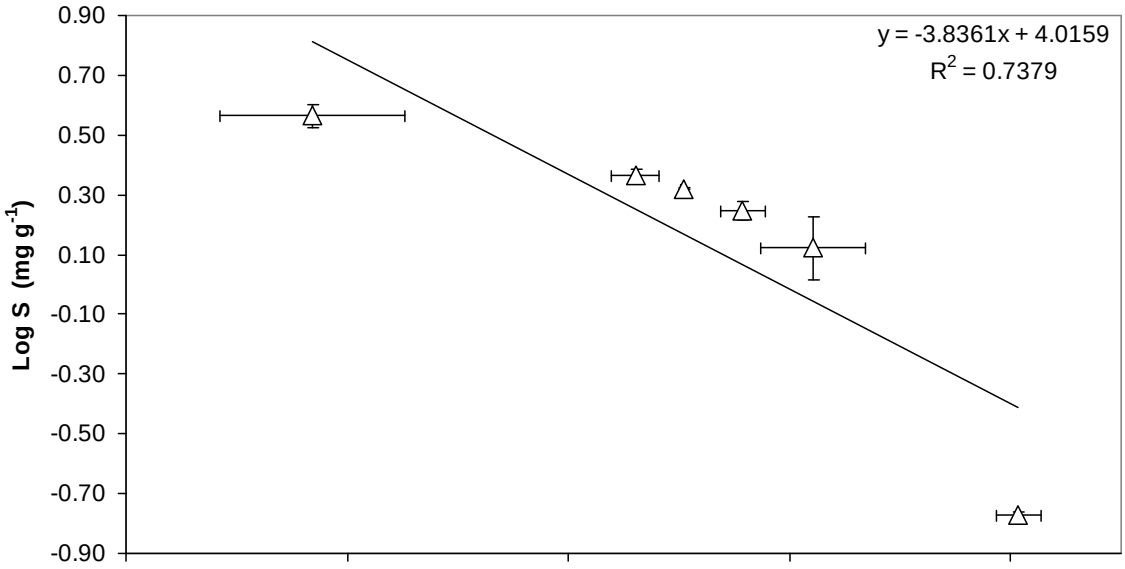
Table E-2. Reduction of water-dissolved Se by natural attenuation during equilibration of Se(IV) and Se(VI) for three days and associated $^{82/76}$Se fractionation factors.

Sample	Day of incubation	Natural attentuation Fraction of Se in solution (%)		Fractionation factor ε ± s.d.(‰)	
		Se(IV)	Se(VI)	Se(IV)	Se(VI)
Garden (GA1)					
	1	49.7	91.8		
	2	35.5	91.1		
	3	31.9	90.8		
				-0.12±0.1	-0.07±0.1
Roadside area (RS1)					
	1	55.6	91.0		
	2	46.9	90.0		
	3	44.0	89.5		
				-0.12±0.1	-0.06±0.1
Forest (FO1)					
	1	35.4	87.9		
	2	32.0	87.0		
	3	31.6	86.3		
				-0.045±0.1	-0.05±0.1

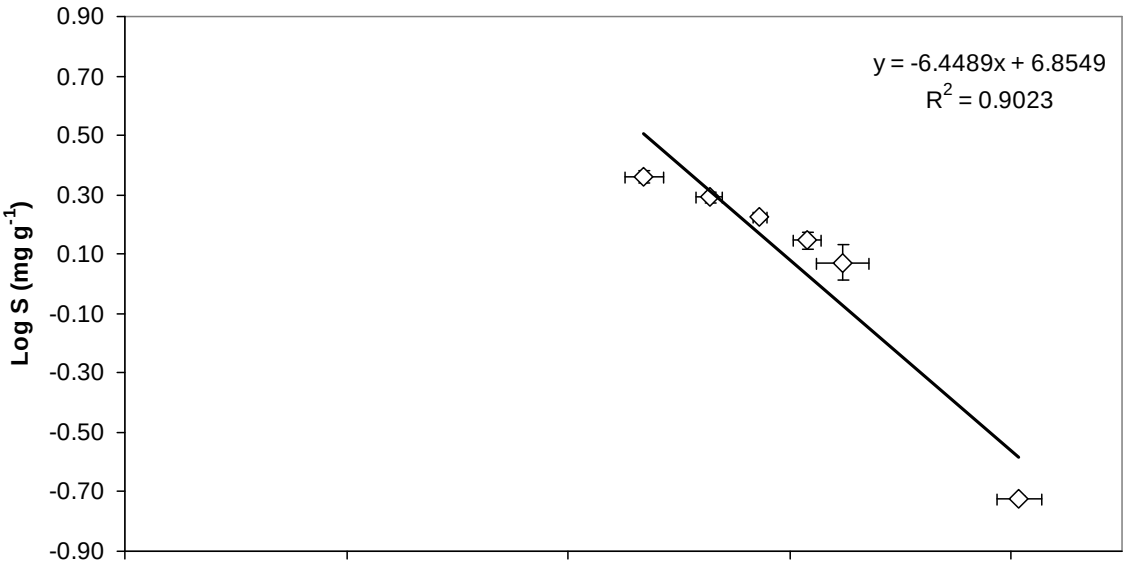
To assess the kinetics of the natural attenuation process, we assumed an exponential dissipation law and plotted the concentration in solution after 30 min to 72 h versus the concentration in solid phase in a double logarithmic way to derive the rate constant from the slope of the regression lines (*Figure E-1*). In line with the final sorption percentage of the added Se, the rate constants were similar for the roadside and garden soils (-3.8 and -4.0 mg g^{-1}) and higher for the forest soil (-6.5). Dimirkou et al. (2009) observed a similar rate constant for the sorption of arsenate to goethite while the rate constant for the sorption of chromate to urban surface soils in central Scotland at pH 2 was with 2.3 mg g^{-1} (pH 2) somewhat lower (Markiewicz-Patkowska et al. 2005).

E Isotope fractionation of selenium by biomethylation in microcosm incubations of soil with the fungus species *Alternaria alternata*

A)



B)



C)

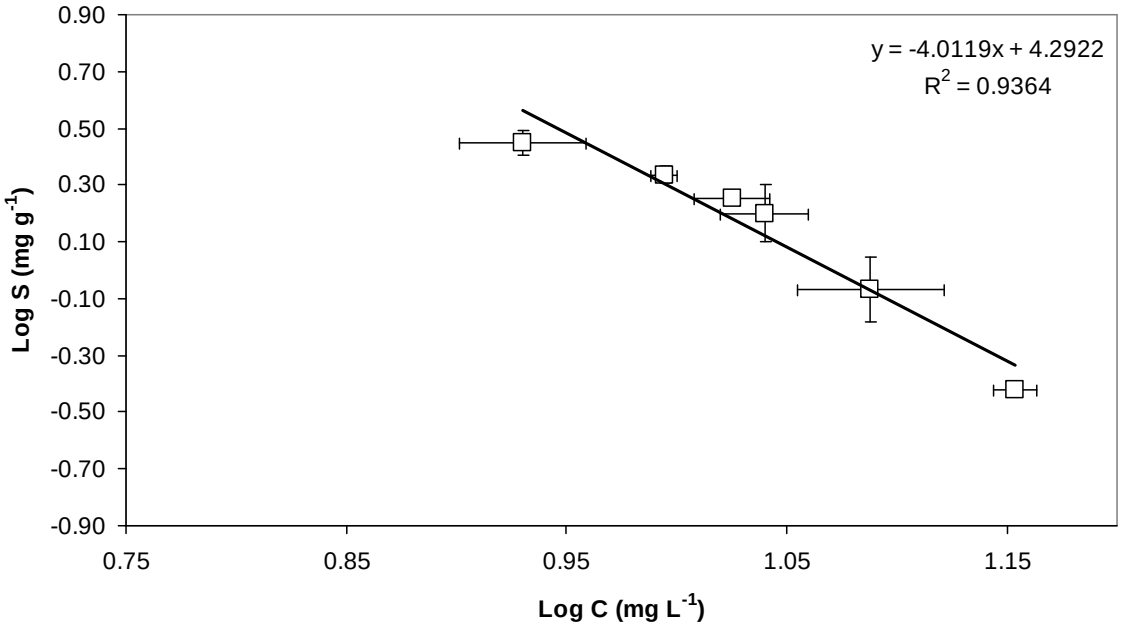


Figure E-1: Relationship between the concentration in solution (C) and in solid phase after equilibration for 30 min to 72 h for A) a garden soil (GA1), B) a roadside soil (RS2), and C) a forest soil (FO1). The error bars represent the standard deviations of duplicates.

We observed a minor Se isotope fractionation as a consequence of the natural attenuation. The sorbed Se was negligible isotopically lighter than the Se remaining in solution. To determine enrichment factors ε, we used a Rayleigh model which could be readily applied to the closed reactors in which the soil equilibration with Se took place. In *Figure E-2*, the modelled courses of the remaining (i.e., dissolved Se) and product (i.e., sorbed Se) are shown together with our measurement results for the garden soil GA1 as an example.

E Isotope fractionation of selenium by biomethylation in microcosm incubations of soil with the fungus species *Alternaria alternata*

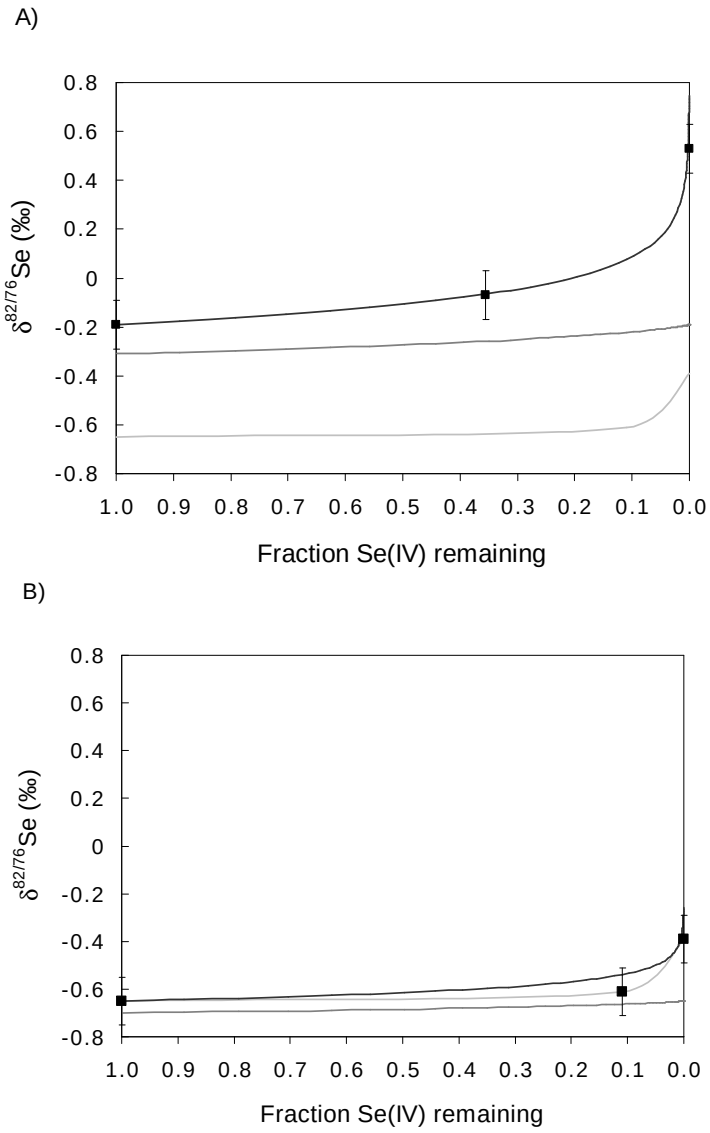


Figure E-2: Selenium isotope fractionation factor (ε) during natural attenuation of a garden soil (GA1) during three days; A) incubated with Se(IV) and B) incubated with Se(VI). The lines represent the modelled courses of $\delta^{82/76}$Se values of the remaining Se (black), educt (light grey) and accumulated product (dark grey) using a Rayleigh fractionation model. The error bars represent the standard deviation of repeated measurements of the certified standard NIST 3149.

The derived ε values for the natural attenuation of Se(IV) were identical for the garden and roadside and garden soils (GA1 and RS2) and smaller for the forest soil, for which a larger extent of sorption was observed (*Table E-2*). The ε values for the natural attenuation of Se(VI) were consistently smaller than those of Se(IV) and similar in all three studied soils. This is in line with findings of Johnson et al. (1999) of a small Se isotope fractionation of

ε = -0.53‰ for the adsorption of Se(IV) on hydrous ferric oxides. Similarly, ε values of the sorption of other oxyanions such as chromate and sulfate were < 0.1‰ and < 2‰, respectively (Ellis et al. 2004; Van Stempvoort et al. 1990). Our results demonstrate that the isotope fractionation because of the equilibration of the study soils with Se prior to incubation in the growth medium with *Alternaria alternata* is small.

4.2 Methylation

In the controls with Se(IV) and Se(VI), we did not detect Se in the methylselenide traps, indicating that no methylation occurred without presence of the fungus. The Se budgets of all microcosms (i.e, recovery of added Se via the incubated spiked soil) averaged 100.6 ± 8.1% illustrating that our recovery of Se was almost complete.

In the incubations of the garden (GA1) and roadside soils (RS2), methylation of both Se(IV) and Se(VI) occurred, while in the incubation of the forest soil (FO1), neither for Se(IV) nor Se(VI) methylation was observed (*Figure E-3*). In GA1 and RS2, 9-30% of the total Se in the microcosm microcosm was recovered in the methylselenide trap, whereas if Se(VI) was supplied we found < 2% of the supplied Se in the trap.

This is consistent with the results of previous microcosm incubations with the same fungus species and the same growth medium but without soil where Se(IV) was also to a much larger degree biomethylated by *Alternaria alternata* than Se(VI) (**Section C** p. 51-59).

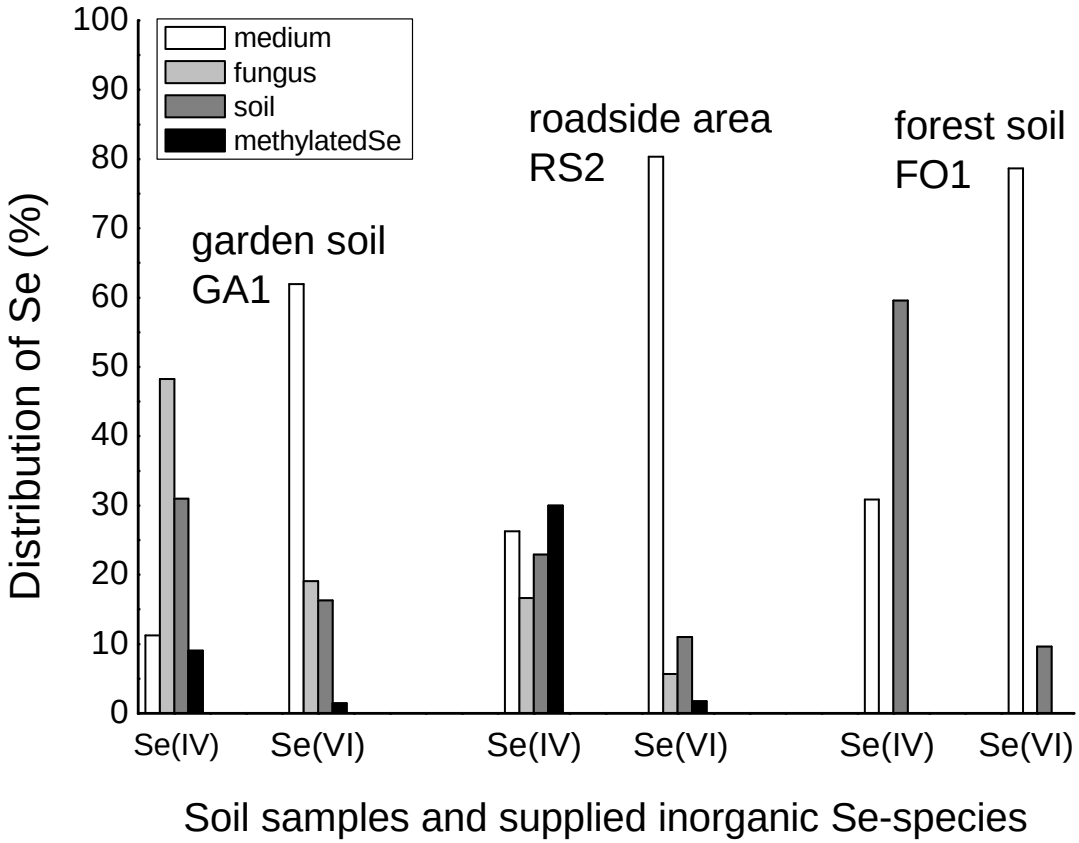


Figure E-3. Distribution of Se among soil, aqueous solution, fungi, and trapped methylselenides in % of the spiked Se(IV) and Se(VI) for the incubations with a garden (GA1), a roadside (RS2), and a forest soil (FO1) after incubation of 11 days.

The results confirmed previous studies by Barkes and Fleming (1974) that the formation of methylated compounds occur for all Se species, but is most rapid for Se(IV). The reason for the lower degree of methylation of Se(VI) is attributable to the fact that the methylation of Se(VI) by microorganisms requires more energy than that of Se(IV) (Terry et al. 2000). The reduction of Se(VI) to Se(IV) seems to be a rate-limiting step for biomethylation. We assume that Se(VI) is metabolized following the sulfate reduction pathway because of the structural similarity between Se(VI) and sulfate. If this was true, Se(VI) is presumably activated by adenosinetriphosphate (ATP)-sulfurylase to adenosine-phosphoselenate (APSe) (Lauchli, 1993) and then reduced in different steps to finally methylselenide (Challenger, 1945). On contrast, Se(IV) is mainly transported into the cell by distinct permeases (Bryant and

Laishley, 1988). Doran and Alexander (1977) proposed that the methylation of Se(IV) first involves reduction Se(0) and then reduction to the selenide form, which is subsequently methylated (Doran and Alexander, 1977).

One possible reason for differences in the degree of methylation between the RS2 and GA1 soils might be the availability of a C source in soils which can enhance the production of DMSe (Frankenberger and Karlson, 1989). However, the total C concentration was higher in RS2 than in GA1 (*Table E-1*). Karlson and Frankenberger (1989) reported that salinity of soils decrease the rate of methylation because of the low tolerance of soil microbes to Na^+ and Cl^-. Therefore, the roadside soil might show reduced Se methylation because it is more impacted by salt application for deicing in winter than the garden soil. Another driver of biomethylation is the pH which was similar in GA1 and RS2 but much lower in FO1. The optimal production of DMSe by *Alternaria alternata* occurs at pH 6.5 (Thompsoneagle et al. 1989). The low pH in FO1 might therefore explain why we did not observe any growth of the fungus and consequently also no biomethylation.

The mass-weighted mean $\delta^{82/76}$Se value of the whole microcosm at the end of the incubation (i.e. after 11 d) was -0.02 to 0.3‰. for the experiments with Se(IV) and -0.10 to -0.35‰ for the experiments with Se(VI) with a total standard deviation of ± 0.25‰ (calculated according to the Gaussian error propagation law) compared to the initial values of the spiked soil of -0.2± 0.05 and -0.69 ± 0.07‰, respectively. Different to the natural attenuation experiments, the $\delta^{82/76}$Se values of the trapped methylselenides, accumulated Se in fungi, Se of the soil, and the remaining Se in the media could not be explained by a simple Rayleigh distillation model because of the complexity of Se pools, transfer, and transformation processes.

If Se(IV) was provided, the Se in methylselenides was substantially depleted in the heavy isotope relative to the dissolved Se(IV) source (*Table E-2*). The negative $\delta^{82/76}$Se values are consistent with the finding that the biomethylation by *Alternaria alternata* grown in a medium with Se(IV) resulted in a strong Se isotope fractionation ($\varepsilon > -6‰$) after an incubation of 3 to 5 days (**Section C**, p. 54) (Schilling et al. 2011). Algal methylation ($\varepsilon < -1.65‰$) and the methylation observed during incubation of two soil samples ($\varepsilon \sim -0.9‰$) produced a smaller Se isotope fractionation than in our experiment (Johnson et al. 1999). The Se isotope fractionation during biomethylation of Se(IV) in our experiment is also in line with the findings of Herbel et al. (2002) that the reduction of Se(IV) to Se(0) was associated with ε

values of -1.1 to -8.4‰ (Herbel et al. 2002). The latter was also observed in an own previous study where the formation of Se(0) by *Alternaria alternata* showed an ε value of -6‰ (**Section C** p. 54) (Schilling et al. 2011).

Table E-3. $\delta^{82/76}$Se values of the unspiked soil and the components of the microcosms after 11 days of incubation.

Sample	Supplied Se	$\delta^{82/76}$Se values				
		Aqueous solution (medium)	Soil	Fungus	methylated Se	Unspiked soil
		(‰)				
Standard	Se(IV)	-0.20± s.d. 0.05 (n=2)				
Standard	Se(VI)	-0.69± 0.07 (n=2)				
GA1	Se(IV)	1.70	0.35	-0.87	-3.56	0.16
GA1	Se(VI)	0.29	-0.81	-1.83	-	0.16
RS2	Se(IV)	1.11	-1.34	0.33	-4.67	0.45
RS2	Se(VI)	0.98	-0.61	-7.53	-	0.45
FO1	Se(IV)	0.51	0.33	-	-	-0.59
FO1	Se(VI)	-0.34	-0.30	-	-	-0.59

The accumulated Se in the fungus had a wide range of $\delta^{82/76}$Se values of -7.53 to 0.33‰ (*Table E-3*) and was consistently Se isotopically lighter than the supplied Se and the remaining Se in the media. The two lightest $\delta^{82/76}$Se values in fungi occurred in the incubations with Se(VI) in which little methylation was observed. We attribute these negative $\delta^{82/76}$Se values to the effect of Se uptake by the fungus. The different $\delta^{82/76}$Se values in the fungi grown in the presence of different soils might be related to different $\delta^{82/76}$Se values of the bioavailable Se portion in the two studied soils. In the incubations with Se(IV), the fractionation towards lighter $\delta^{82/76}$Se values was less pronounced because the subsequent methylation process which used more of the assimilated Se than in the incubations with Se(VI) again preferred the light Se isotope.

The soil-bound Se tended to become isotopically lighter than at the beginning of the experiment in GA1 and RS2 except for GA1 incubated with Se(IV) but isotopically heavier in FO1. While the fractionation of the $\delta^{82/76}$Se values towards heavier isotope ratios is consistent with the preferred uptake and methylation of the light Se isotope, the shift of the Se isotope signal towards lighter signals is unexpected. We can only attribute this to the possibility that we did not fully separate the fungus from the soil in these microcosms. Furthermore, the analytical uncertainty is probably highest for the soil extractions where the risk of matrix effects during Se isotope measurement is highest.

All growth media at the end of the incubations were Se isotopically heavier than the supplied Se because all processes occurring in the microcosms (natural attenuation, uptake by fungi, biomethylation) prefer the lighter ^{76}Se (Johnson et al. 1999; Herbel et al. 2000; Schilling et al. 2011; **Section C** p. 50-58).

In our approach where we added the fungus to the Se-spiked soil, the fungus mainly grew on the surface of the soil and presumably used Se which was dissolved in soil solution and had a slightly more negative $\delta^{82/76}$Se value as a consequence of the precedent equilibration and associated natural attenuation. This is different to natural situations where the methylating microorganisms might live inside soil aggregates. The latter was described by Ellis et al. (2003) who reported that microbes lived in particle interiors with a heavier Se isotope pool than in exterior solution. Consequently, the reduced Se products were isotopically heavier than expected if exterior solution was the main source of Se (Ellis et al. 2003).

5 Conclusions

In our incubations, Se(IV) was more strongly biomethylated than Se(VI) consistent with previous similar incubations but without soil. Biomethylation only occurred in two soil samples with pH values (in water) of 6.4 to 6.6 but not in an acid forest soil (pH 3.9). Equilibration of the soil samples with the spiked Se for three days prior to starting microcosm incubations with fungi inoculate resulted in decreasing water-soluble and thus bioavailable Se concentrations in solution. This was particularly pronounced for Se(IV) of which the dissolved concentrations decreased to about one third of the initial value while for Se(VI) only about 10-15% of the dissolved Se was sorbed. The sorption was the more pronounced with more acid the soil, presumably because of the higher positive surface charge.

E Isotope fractionation of selenium by biomethylation in microcosm incubations of soil with the fungus species *Alternaria alternata*

While the natural attenuation during equilibration of the soil for three days with the spiked Se resulted in minor Se isotope fractionation ($\varepsilon < 0.12‰$), the biomethylation of Se(IV) caused strong isotope fractionations with a fractionation factor $\varepsilon > 9‰$. The biomethylation of Se(VI) produced too little methylselenide for an Se isotope analysis in our experiments.

We conclude that the extent of biomethylation of Se and associated Se isotope fraction depends on type of Se source species and soil properties such as pH. In turn, Se sources and formation processes can potentially be inferred by measuring the Se stable isotope ratio in methylselenides.

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F Appendix

Table F-1. Measured. $\delta^{82/76}Se$ and $\delta^{82/78}Se$ values of the standard NIST SRM3149 (solution) (**Sections B-E**)

Table F-2. Measured selenium-concentrations of the microcosm-expermiments (medium, fungus and methylselenide) and their calculated sum (**Section C**)

Table F-3. Measured total Se concentration of different soil samples (**Sections D-E**)

Table F-4. Measured values from the extraction of selenium sepcies from soil samples with KH_2PO_4-K_2HPO_4 (fraction 1), NaOH (fraction 2) and HNO_3 (fraction 3) (**Sections D-E**)

Table F-5. Measured. $\delta^{82/76}Se$ and $\delta^{82/78}Se$ values of the reference material SGR-1 (green river shale) (**Section D**)

Table F-6. Selenium recoveries after purification (anion exchange chemistry): Test samples-number 1-6 NIST SRM3149 and number 7-12 Se(IV)-standard (**Section D**)

Table F-1. Measured. $\delta^{82/76}Se$ and $\delta^{82/78}Se$ values of the standard NIST SRM3149 (solution) (Section B-E)

Standard number	Isotope measurement $\delta^{82/76}Se$ (‰)	$\delta^{82/78}Se$ (‰)	Standard number	Isotope measurement $\delta^{82/76}Se$ (‰)	$\delta^{82/78}Se$ (‰)
1	0	0	28	-0.03	-0.02
2	-0.02	-0.02	29	0.01	-0.01
3	0.05	-0.07	30	0.01	0.02
4	0.06	-0.04	31	0.01	0.03
5	-0.13	0.09	32	0.02	0.05
6	0.04	-0.02	33	-0.05	0.01
7	0.09	-0.07	34	0.07	0.05
8	0	0	35	0	0.01
9	-0.08	-0.02	36	-0.02	-0.03
10	-0.02	-0.01	37	-0.06	-0.03
11	0.04	-0.02	38	0.02	0.01
12	0.12	0.07	39	-0.08	-0.04
13	-0.03	-0.03	40	0.05	0.03
14	0.02	0.02	41	0	0
15	0.07	0.02	42	-0.02	-0.01
16	0.09	0.04	43	-0.11	-0.03
17	0.05	0	44	0.01	0.01
18	0.06	0	45	-0.09	-0.02
19	-0.04	0	46	0.06	0.02
20	0.06	0.08	47	0.02	-0.01
21	0	0.06	48	0	0.01
22	0.07	0.08	49	-0.06	-0.04
23	-0.05	-0.02	50	-0.06	-0.07
24	-0.08	-0.04	51	0.02	-0.01
25	-0.04	-0.01	52	0	0.03
26	-0.04	-0.03	53	0.08	0.06
27	0	-0.02	54	0.01	0.03
average		**0.002**			
standard deviation		**0.037**			

Table F-2: Measured selenium-concentrations of the microcosm-expermiments (medium, fungus and methylselenide) and their calculated sum

Sample	Se species	pH	Time after incubation						
			Day 3	Day 4	Day 5	Day 11	Day 12	Day 13	Day 14	Day 15
			Se (in µg)			Se (in µg)				
medium	IV	4				7.01	7.32	8.48	5.89	4.40
fungus	IV	4				31.23	30.11	25.31	27.48	29.38
methylselenide	IV	4				15.25	10.10	13.40	12.37	13.80
Σ	IV					53.50	47.53	47.19	45.74	47.59
medium	VI	4				44.69	48.56	40.47	55.49	51.95
fungus	VI	4				1.82	1.79	3.92	4.20	4.55
methylselenide	VI	4				7.73	6.34	9.70	6.40	4.50
Σ	VI					54.25	56.69	54.09	66.10	61.00
medium	IV	7	13.19	12.16	9.33	6.53	5.52	4.54	6.07	6.83
fungus	IV	7	34.02	35.84	37.58	34.24	37.61	27.47	29.60	28.04
methylselenide	IV	7	1.40	3.88	3.26	9.49	10.35	16.50	14.99	6.61
Σ	IV		48.61	51.88	50.17	50.27	53.49	48.51	50.66	41.48
medium	VI	7				44.97	44.77	45.72	44.55	35.60
fungus	VI	7				6.37	13.32	18.47	13.34	10.44
methylselenide	VI	7				3.83	3.95	3.53	7.35	1.38
Σ	VI					55.17	62.05	67.72	65.24	47.42
standard	IV	DS-corrected				48	48	48	48	48
standard	VI	DS-corrected				58	58	58	58	58

Table F-2: Measured total Se concentration of different soil samples

Soil type	**Sample**	**Total Se**					**Total Se**	
							average	**standard deviation**
		mg kg^{-1}					**mg kg^{-1}**	
alluvial soil	AL1	0.20	0.21				0.21	0.001
alluvial soil	AL2	0.40	0.34				0.37	0.034
roadside area	RS1	0.18	0.20				0.19	0.009
roadside area	RS2	0.30	0.34				0.32	0.021
garden soil	GA1	0.53	0.49				0.51	0.019
garden soil	GA2	0.34	0.38				0.36	0.021
park area	PA1	0.13	0.15				0.14	0.007
park area	PA2	0.10	0.10				0.10	0.001
forest soil	FO1	0.07	0.09				0.10	0.002
forrest soil	FO2	0.09	0.09				0.09	0.021
green river shale	SGR	3.26	3.32	3.25	3.24	3.28	3.27	0.037
blank		0.003	0.002	0.001			0.002	0.001

Table F-3: Measured values from the extraction of selenium sepcies from soil samples with KH_2PO_4-K_2HPO_4 (fraction 1), NaOH (fraction 2) and HNO_3 (fraction 3)

Soil type	Sample	Sequential extraction			Sequential extraction (average)			
		Fraction 1	Fraction 2	Fraction 3	Fraction 1	Fraction 2	Fraction 3	Σ
		mg kg^{-1}			mg kg^{-1}			
alluvial soil	AL1	0.03	0.15	0.03	0.03	0.15	0.04	0.22
		0.03	0.16	0.04				
alluvial soil	AL2	0.04	0.28	0.04	0.04	0.29	0.04	0.37
		0.04	0.30	0.04				
roadside area	RS1	0.02	0.08	0.02	0.03	0.08	0.02	0.13
		0.03	0.08	0.02				
roadside area	RS2	0.03	0.11	0.02	0.03	0.11	0.02	0.16
		0.03	0.10	0.03				
garden soil	GA1	0.05	0.16	0.14	0.05	0.18	0.14	0.37
		0.05	0.20	0.14				
garden soil	GA2	0.07	0.33	0.13	0.06	0.33	0.13	0.52
		0.06	0.33	0.13				
park area	PA1	0.07	0.19	0.15	0.08	0.19	0.15	0.42
		0.08	0.19	0.15				
park area	PA2	0.07	0.11	0.03	0.07	0.11	0.03	0.21
		0.07	0.11	0.03				
forest soil	FO1	0.04	0.08	0.01	0.04	0.08	0.02	0.13
		0.04	0.07	0.02				
forrest soil	FO2	0.03	0.04	0.02	0.04	0.04	0.02	0.10
		0.04	0.04	0.03				

Table F-4. Measured. $\delta^{82/76}Se$ and $\delta^{82/78}Se$ values of the reference material SGR-1 (green river shale)

Reference material (SGR-1) number	**Isotope measurement**		**Signal intensity**
	$\delta^{82/76}Se$	**$\delta^{82/78}Se$**	**78lnt (in V)**
1	0	0.02	2.20
2	0.2	0.22	1.23
3	0.35	0.29	0.98
4	0.27	0.19	1.42
5	0.15	0.12	0.61
6	0.22	0.14	0.86
7	0.22	0.34	0.83
8	0.17	0.07	1.39
9	0.22	0.11	2.38
10	0.28	0	1.22
11	0.11	-0.02	1.47

Table F-5. Selenium recoveries after purification (anion exchange chemistry): test samples-number 1-6 NIST SRM3149 and number 7-12 Se(IV)standard (Section D)

Test sample number	**Recovery (in %)**	**Isotope measurement**	
		$\delta^{82/76}$ Se	**$\delta^{82/78}$ Se**
1	87.12	0.08	0.08
2	80.69	0.18	0.14
3	65.37	-0.06	-0.07
4	79.48	n.d.	n.d.
5	85.25	-0.12	-0.17
6	87.38	n.d.	n.d.
7	85.10	-0.50	-0.35
8	88.51	-0.48	-0.31
9	78.61	-0.68	-0.44
10	77.79	-0.62	-0.39
11	90.90	-0.53	-0.36
12	93.43	-0.51	-0.34
unproc. Se(IV)	100	-0.44	-0.31
SRM 3149	100	-0.01	-0.02