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Investigation of bacterial hopanoid inputs to soils from Western Canada

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Abstract

Hopanoids have been widely used as characteristic biomarkers to study inputs of bacterial biomass to sediments because they are preserved in the geologic record. A limited number of studies have been performed on hopanoid biomarkers in soils. The present study examined the distribution and potential preservation of hopanoids in soils that are developed under different climatic conditions and varying vegetative inputs. Solvent extraction and sequential chemical degradation methods were employed to extract both "free" and "bound" hopanoids, from three grassland soils, a grassland–forest transition soil, and a forest soil from Western Canada. Identification and quantification of hopanoids in the soil samples were carried out by gas chromatography–mass spectrometry. Methylbishomohopanol, bishomohopanol and bishomohopanoic acid were detected in all solvent extracts. The base hydrolysis and ruthenium tetroxide extracts contained only bishomohopanoic acid at a concentration range of 0.8–8.8 μg/gC and 2.2–28.3 μg/gC, respectively. The acid hydrolysis procedure did not release detectable amounts of hopanoids. The solvent extraction yielded the greatest amounts of "free" hopanoids in two of the grassland soils (Dark Brown and Black Chernozems) and in the forest soil (Gray Luvisol). In contrast, the chemical degradation methods resulted in higher amounts of "bound" hopanoids in the third grassland soil (Brown Chernozem) and the transition soil (Dark Gray Chernozem), indicating that more hopanoids exist in the "bound" form in these soils. Overall, the forest and the transition soils contained more hopanoids than the grassland soils. This is hypothesized to be due to the greater degradation of hopanoids in the grassland soils and or sorption to clay minerals, as compared to the forest and transition soils.

1. Introduction

Soil organic matter (SOM) consists of plant and microbial residues at various stages of decay with different turnover times (Trumbore, 1997; Guggenberger et al., 1999; Kögel-Knabner, 2002; Certini et al., 2004; Karbozova-Saljnikov et al., 2004; Kölbl and Kógel-Knabner, 2004). Microbial biomass is estimated to comprise between 1% and 5% of SOM (Jenkinson and Ladd, 1981; Smith and Paul, 1990; Alef and Nannipieri, 1995; Glaser et al., 2004). It is necessary to estimate soil microbial biomass because

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it serves as a pool for the delivery of plant nutrients, plays a role in soil structure formation and stabilization, and acts as marker for soil fertility and sustainability (Smith and Paul, 1990; Alef and Nannipieri, 1995; Glaser et al., 2004; Williamson et al., 2005). It is also important to characterize and quantify microbial biomass in SOM, which is considered part of the fast-cycling pool that has a relatively shorter turnover time than the slow-cycling pool (Sadowsky and Schortemeyer, 1997; Fang et al., 2005). The fast-cycling pool has a dynamic role in regulating atmospheric CO_2 and it is important to understand its role in the global C cycle (Schlesinger and Andrews, 2000). Characteristic molecular markers (biomarkers) such as phospholipids, amino sugars and 3-hydroxy fatty acids are widely used to characterize and quantify bacterial inputs to SOM (Frostegård and Bååth, 1996; Keinänen et al., 2003; Glaser et al., 2004). Alternatively, hopanoids are widely used as characteristic biomarkers to examine bacterial inputs to sediments because they have the potential to provide information regarding bacterial biomass contributions to sedimentary organic matter (Innes et al., 1997; Farrimond et al., 2000; Talbot et al., 2003a). However, very little information on the preservation of hopanoids in soil environments exists (Winkler et al., 2001).

Bacteria of diverse taxonomic groups synthesize, as membrane lipids, the broad family of amphiphilic compounds known as biohopanoids (Ries-Kautt and Albrecht, 1989; Ourisson and Rohmer, 1992; Watson and Farrimond, 2000; Farrimond et al., 2003). As membrane components, the hopanoids are crucial for the life of the bacteria and act as surrogates for the membrane constituent cholesterol found in higher organisms (Ourisson and Rohmer, 1992; Farrimond et al., 2000; Talbot et al., 2001, 2003a). Identified bacterial hopanoids include simple C_{30} hopanoids, such as diplopterol and diploptene, and a range of bacteriohopanepolyols (BHPs), such as bacteriohopanetetrol and aminobacteriohopanetriol (Rohmer et al., 1992; Innes et al., 1997; Watson and Farrimond, 2000; Talbot et al., 2003a,b; Thiel et al., 2003). The composite BHPs are linked to polar moieties such as amino acids, sugar derivatives and nucleosides (Rohmer et al., 1992; Thiel et al., 2003; Talbot et al., 2003a). Diplopterol and diploptene are the biosynthetic precursors of BHPs and are found in low amounts in most hopanoid-producing bacteria as well as in some eukaryotes such as ferns and lichens, whereas the derivatives of C_{35} BHPs are only produced by prokaryotic bacteria (Talbot et al., 2003b). BHPs are synthesized by certain types of bacteria, such as some gram-negative bacteria, gram-positive bacteria, methanotrophs, cyanobacteria, acetic acid bacteria, N-fixers and purple non-sulphur bacteria (Talbot et al., 2003a,b).

Biohopanoids undergo a wide range of degradation processes such as loss or alteration of functional groups, structural modification and rearrangement, stereochemical changes and aromatization that lead to the formation of geohopanoids (Watson and Farrimond, 2000; Farrimond et al., 2003). Hopanoic acids, hopanols, C_{30} hopenes and hopanoidal aldehydes and ketones are types of geohopanoids that have been reported in the environment (Innes et al., 1997; Watson and Farrimond, 2000; Farrimond et al., 2003). Hopanoic acid, hopanols, and C_{30} hopenes are the most commonly observed geohopanoids in modern environments whereas hopanoidal aldehydes, ketones and hopanes comprise only minor amounts (Farrimond et al., 2002, 2003). Hopanoids are preserved in sediments owing to their pentacyclic C skeleton, which is relatively resistant to degradation, and end up as hopanes and other stable degradation products in the geological record (Innes et al., 1997; Talbot et al., 2001; Farrimond et al., 2002). Hopanoids have been found in sediments as old as 2.5 Ga and are classified as one of the most abundant natural products on the earth (Ourisson and Albrecht, 1992; Summons et al., 1999; Rohmer et al., 1992). Hopanoids provide information about the contribution of bacterial biomass to sediments as well as the paleoenvironmental conditions existing at the time they were deposited (Innes et al., 1997).

Many studies have been carried out to investigate hopanoids in sediments but only a limited number of studies have been performed on soils (Ries-Kautt and Albrecht, 1989; Jaffé et al., 1996; Bull et al., 1998; Winkler et al., 2001). Most studies focus on the solvent-extractable or "free" hopanoids but because hopanoids are highly functionalized, they are not directly amenable to analysis by conventional gas chromatography–mass spectrometry (GC–MS) methods (Innes et al., 1997). Therefore, solvent extractable ("free") functionalized hopanoids must be treated with periodic acid and sodium borohydride to yield simple primary alcohols with shortened side chains, which are more volatile and amenable to analysis by GC–MS (Innes et al., 1997). Hopanoids may also occur in a "bound" form, where they are linked to other organic molecules or macromolecular organic matter (Winkler

et al., 2001; Farrimond et al., 2003). Winkler et al. (2001) applied a sequential chemical degradation method to study the hopanoids that are bound through ether, ester, aliphatic and aromatic C bonds. The application of a sequential chemical degradation method was successful in identifying some of the bound hopanoids, demonstrating the value in employing both "free" and "bound" extraction methods when examining hopanoids in soil samples.

This study was carried out to analyze and quantify both "free" (solvent extractable) and "bound" hopanoids in selected grassland soils (Brown, Dark Brown and Black Chernozems), a grassland–forest transition soil (Dark Gray Chernozem), and a forest soil (Gray Luvisol) from Western Canada by GC–MS. The objective of this study was to compare the distribution of "free" and "bound" quantities of hopanoids in soils from different environments (i.e. grasslands, forest and grass–forest transition).

2. Material and methods

2.1. Soil samples

Samples were collected from grasslands, grassland–aspen forest transition zone, and an aspen forest (Table 1). Chernozemic (grassland) soils occur in semiarid climates and are developed under grassland vegetation (Janzen et al., 1998). These soils have surface horizons that have been darkened by the build-up of organic matter from decomposition of grasses (xerophytic or mesophytic) and forbs. The mean annual soil temperature (MAT) ranges from 1.7 °C in the Black Chernozemic soil zone to 5.0 °C in the Brown Chernozemic soil zone and the annual precipitation is 452 mm in the Black Chernozemic soil zone and 413 mm in the Brown Chernozemic soil zone (Campbell et al., 1990). Luvisolic soils are found in the Boreal Plain Ecozone (Janzen et al., 1998) just north of the Chernozemic soil zones. Luvisolic soils develop under forest vegetation, in subhumid to humid, mild to very cold climates (Soil Classification Working Group, 1998).

Soil samples were collected in October of 2003 from a transect in Alberta, Canada stemming from north of Edmonton to south of Lethbridge. The sites sampled were all well-drained, pristine areas that have not been used for agricultural production. The surface mineral horizons (Ah horizons) of Brown, Dark Brown, Black and Dark Gray of the Chernozemic sequence and one sample of an organic (O) horizon of forest soil (Gray Luvisol) were used in this study. Sample properties are listed in Table 1. All the soil samples were air-dried and sieved through 2 mm after sampling. The samples were stored at room temperature in glass jars prior to analysis.

2.2. Determination of carbon and nitrogen contents

Carbon and N contents of soils were determined on finely ground samples with an elemental Analyzer Vario EL III (Hanau, Germany) C, H, O, N, S. Soil samples were analyzed in duplicate. The C and N contents are reported in Table 1.

Table 1
Properties and sample details of soils analyzed in this study

Sample	Location	Soil sample depth (cm)[a]	pH[b]	Texture	Soil moisture regime[c]	Organic carbon content (%OC)	Nitrogen (%N)	Carbon to nitrogen ratio (C/N)
Grassland								
Brown Chernozem Ah	SE of Lethbridge	0–15	6.4	Loam	Subarid to semiarid	2.08	0.19	10.95
Dark Brown Chernozem Ah	Lethbridge	0–15	6.6	Silt loam	Semiarid	2.77	0.25	11.1
Black Chernozem Ah	Edmonton	0–15	6.75	Silt loam	Subhumid	4.41	0.36	12.3
Aspen-grassland								
Dark Gray Chernozem Ah	Tofield	0–10	6.1	Loam	Subhumid	5.00	0.33	15.2
Aspen forest								
Gray Luvisol O horizon	NW of Edmonton	0–15	5.3	n/a	Subhumid to humid	17.0	0.95	17.9

n/a, not applicable (this is an organic horizon).
[a] This is the depth sampled, not the depth of the horizon.
[b] Measured in deionized water.
[c] From Soil Classification Working Group (1998).

2.3. Extraction of hopanoid compounds from soil samples

The solvent extraction and sequential chemical degradation procedure used to isolate hopanoids from soil samples is illustrated in Fig. 1. Three major extraction procedures were applied: solvent extraction which isolates "free" hopanoids, acid and base hydrolyses to obtain hopanoids "bound" to SOM, and oxidation with ruthenium tetroxide (RuO_4) for further bond cleavage of "bound" hopanoids. All isolates were derivatized with diazomethane and *N*,*O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) prior to GC–MS analysis.

2.3.1. Solvent extraction of soil samples

The solvent extractable or "free" hopanoids were extracted from soil samples according to Winkler et al. (2001) with minor modifications. Approximately 30 g of each mineral (Ah) soil sample (Brown, Dark Brown, Black and Dark Gray Chernozems) and approximately 10 g of the O horizon (Gray

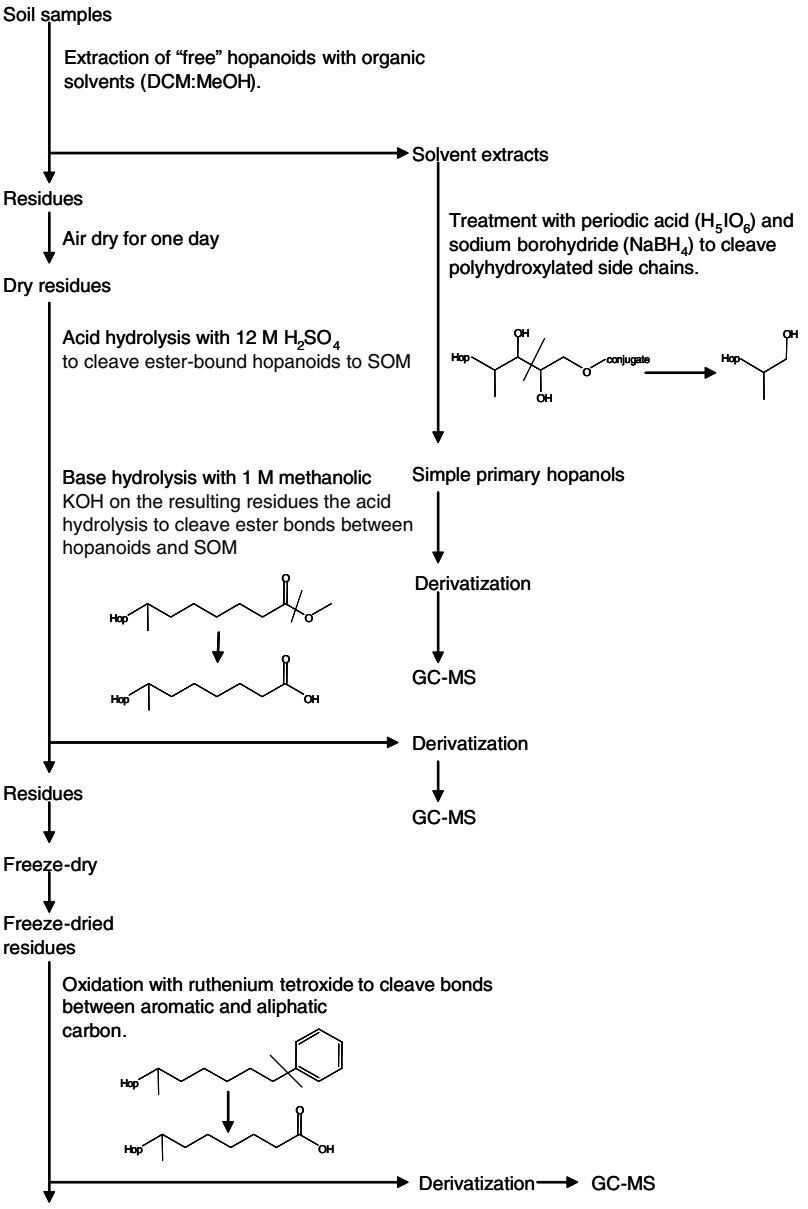

Fig. 1. Solvent extraction and sequential chemical degradation methods to extract hopanoids from soil samples (modified from Winkler et al., 2001).

Luvisol) was each extracted 3 times with a mixture of 80 mL of methanol and dichloromethane (1:1, v/v) by sonication for 15 min (Fig. 1). The extracts were filtered through pre-extracted cellulose filters (Fisher brand P8 filters) and then through glass fiber filters (Fisher brand GF8 filters) to remove smaller soil particles from the extracts. The organic solvents were concentrated by rotary evaporation, transferred into 2.0 mL vials and dried under a stream of N_2. The residues from the solvent extraction were air dried and kept at −20 °C until further analysis.

The extracts were treated with periodic acid (H_5IO_6) and sodium borohydride ($NaBH_4$) to convert polyhydroxylated hopanols to simple primary hopanols according to Innes et al. (1997). H_5IO_5 (300 mg) and a 3 mL mixture of tetrahydrofuran and milliQ water (8:1, v/v) were added to the extracts and the mixtures were stirred for 1 h at room temperature to oxidize 1,2-diols to aldehyde products. Then 10 mL of milliQ water was added and the mixtures were extracted 3 times with 20 mL of chloroform. The combined chloroform extracts were concentrated by rotary evaporation and then completely dried in 2.0 mL glass vials under a stream of N_2 gas. The dry extracts were then stirred with 100 mg $NaBH_4$ in 3 mL ethanol for 1 h at room temperature to reduce the aldehyde products to alkoxide intermediates (RCO^-). Fifteen milliliters of 0.1 M potassium dihydrogen phosphate was added to the mixtures then extracted 3 times with 20 mL of chloroform. Adding the potassium dihydrogen phosphate solution after the hydride-addition step protonates the alkoxide intermediates to produce simple alcohol products. The combined solvents were concentrated by rotary evaporation and evaporated to dryness under a stream of N_2 in 2.0 mL vials.

2.3.2. Acid and base hydrolysis on the solvent-extracted residues

Acid and base hydrolyses were performed to cleave ester-bound hopanoids to SOM (Winkler et al., 2001). The dry extraction residues were stirred in 40 mL of 12 M H_2SO_4 for 16 h at room temperature (Fig. 1). After dilution to 2.5 M H_2SO_4 with milliQ water, the mixtures were hydrolyzed under reflux for 5 h. The suspensions were vacuum filtered through glass fiber filters (Fisher brand GF8 filters) and the residues were kept for base hydrolysis. The filtrates were extracted 3 times with 100 mL chloroform. The solvents were dried over anhydrous Na_2SO_4, concentrated by rotary evaporation, transferred to 2.0 mL vials and dried under N_2.

The residues from the acid hydrolysis were hydrolyzed under reflux with 40 mL of 1 M methanolic KOH for 3 h (Fig. 1). After adding 40 mL formic acid and chloroform (1:4, v/v) the mixtures were stirred for a few minutes. The mixtures were then vacuum filtered through glass fiber filters (Fisher brand GF8 filters) and the residues were washed with 80 mL of chloroform. The filtrates were evaporated to near dryness. After the addition of 20 mL of milliQ water, the mixtures were extracted 3 times with 20 mL of chloroform. The solvents were dried over anhydrous Na_2SO_4, concentrated by rotary evaporation, transferred to 2.0 mL vials and dried under N_2.

2.3.3. Oxidation with ruthenium tetroxide

Soil samples were oxidized with RuO_4 to cleave C—C SOM-bound hopanoids. The resulting residues from base hydrolysis were freeze-dried prior to being subjected to oxidation with RuO_4. The residues were stirred with 10 g Na periodate, 25 mg ruthenium (lll) chloride hydrate in 42 mL acetonitrile, 30 mL water and 30 mL dichloromethane for 24 h at room temperature (Fig. 1) (Winkler et al., 2001). After adding 40 mL of formic acid in chloroform (1:4 v/v) to the mixtures, they were stirred for 3 min. The mixtures were vacuum filtered through glass fiber filters (Fisher brand GF8 filters) and the residues were washed with 50 mL of chloroform. Sufficient Na_2SO_3 was added to reduce I and then the organic layer was separated from the aqueous layer. The aqueous phase was extracted with 3 portions of 20 mL of chloroform. The combined organic solvents were dried over anhydrous Na_2SO_4, then concentrated by rotary evaporation, and evaporated to dryness under a stream of nitrogen in 2.0 mL vials.

2.4. Derivatization of hopanoid compounds

All extracts (from solvent extraction, acid and base hydrolyses and oxidation with RuO_4) were first methylated by reaction with diazomethane in ether for 1 h at 30 °C. After removal of the ether in a stream of N_2, the methylated samples were reacted with *N*,*O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) and anhydrous pyridine for 3 h at 70 °C to form trimethylsilyl (TMS) derivatives of alcohols. The samples were analyzed as methyl esters and TMS ethers by gas chromatography–mass spectrometry as described in Section 2.5.

2.5. Gas chromatography–mass spectrometry (GC–MS) parameters

The derivatized extracts were analyzed with an Agilent model 6890 gas chromatograph (GC) equipped with an Agilent model 5973 quadrupole mass selective detector (MSD), and an Agilent 7683 auto-sampler. Separation was performed on an HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) with the following temperature program: the column temperature was held at 65 °C for 2 min, and then the temperature was increased from 65° to 300 °C at a rate of 6 °C/min, with a final isothermal hold at 300 °C for 20 min. The injection volume was set at 1 μL (splitless mode) and the injector temperature was set at 280 °C. Helium was used as the carrier gas. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV ionization energy and in full scan mode from m/z 50–650. Data were obtained and processed with Agilent Chemstation G1701DA software. Individual compounds were identified by comparison of mass spectra with the literature (Ourisson and Albrecht, 1992; Winkler et al., 2001). Cholestane was used by Winkler et al. (2001) as an external standard and was also used in this study for comparisons with published literature values. Quantification of individual hopanoid compounds was achieved by comparison of peak areas of hopanoids with standard components in the total ion current (TIC).

3. Results and discussion

3.1. Carbon and nitrogen contents

The C and N contents as well as the C/N ratios are listed in Table 1 for the grassland, the grassland–forest transition and the forest soils. The grassland soil Ah horizons contained between 2.08% and 4.41% C, the transition soil Ah horizon and the forest soil O horizon contained 5.00% and 17.0% C, respectively. The C content increased in the grassland climosequence from the Brown Chernozem to the Black Chernozem. Higher N concentrations were observed in the forest soil than in the grassland soils (Brown, Dark Brown and Black Chernozems) and the transition soil (Dark Gray Chernozem). The C/N ratio ranged from 11.1 to 17.9, with the highest C/N found in the Gray Luvisol. The low C content and low C/N ratio in the Brown Chernozemic soil indicates that the SOM is in a more advanced stage of decomposition than in the other grassland soils and the forest soils from this region.

3.2. Hopanoid distribution in soil samples

3.2.1. Solvent extractable (“free”) hopanoids in soils

Methylbishomohopanol, bishomohopanol, and bishomohopanoic acid were detected in all solvent extracts. The concentrations of total solvent extracts ranged between 7.4–25.4 μg/gC (Table 2).

Table 2
Amounts of hopanoids in the soil samples from solvent extraction and sequential chemical degradation methods (μg/gC)

Compounds	Molecular weight	Molecular formula	Brown Chernozem (Ah horizon)	Dark Brown Chernozem (Ah horizon)	Black Chernozem (Ah horizon)	Dark Gray Chernozem (Ah horizon)	Gray Luvisol (O horizon)
Solvent extraction							
Methylbishomohopanol	470	$C_{33}H_{58}O$	1.2	0.6	0.6	1.4	2.5
Bishomohopanol	456	$C_{32}H_{56}O$	5.1	10.0	6.5	5.2	20.9
Bishomohopanoic acid	470	$C_{32}H_{54}O_2$	1.1	0.5	1.8	2.2	2.0
Total “free” hopanoids			7.4	11.1	8.9	8.8	25.4
Base hydrolysis							
Bishomohopanoic acid	470	$C_{32}H_{54}O_2$	8.8	3.5	3.3	4.3	0.8
Ruthenium oxidation							
Bishomohopanoic acid	470	$C_{32}H_{54}O_2$	bdl	5.2	2.2	28.3	8.4
Total “bound” hopanoids			8.8	8.7	5.5	32.6	9.2
Total (“free” + “bound”) hopanoids			16.2	19.8	14.4	41.4	34.6

bdl, below detectable limits.

Concentrations of individual hopanoid compounds varied between 0.6 and 2.5 μg/gC for methylbishomohopanol, 5.1–20.9 μg/gC for bishomohopanol and 0.5–2.2 μg/gC for bishomohopanoic acid. The highest amount of methylbishomohopanol was detected in the O horizon of the forest soil (Gray Luvisol) and the lowest amount was observed in the grassland soil (Dark Brown Chernozem). The Gray Luvisolic soil also contained the highest concentration of bishomohopanol, whereas the highest concentration of bishomohopanoic acid was detected in the transition soil (Dark Gray Chernozem). The highest total concentration of hopanoids was observed in the forest soil (Gray Luvisol) and the lowest was found in the grassland soil from the most arid climate (Brown Chernozem). In all the solvent extracts, bishomohopanol was the most abundant hopanoid detected.

The detected bishomohopanoic acid, methylbishomohopanol and bishomohopanol in the analyzed soil samples are reported to arise from highly functionalized biohopanoids (Innes et al., 1997; Winkler et al., 2001). Hopanoic acids are hypothesized to form from the oxidation of biohopanoids and are believed to be degradation products of BHPs (Innes et al., 1997). However, methylbishomohopanol and bishomohopanol have been reported to be the major degradation products of tetrafunctionalized biohopanoids, bacteriohopanetetrol, and aminobacteriohopanetriol and are believed to be formed by oxidative cleavage and then by reduction of the highly functionalized biohopanoids in environmental samples (Innes et al., 1997). Alternatively, they can be generated from the periodic acid and sodium borohydride treatment of the highly functionalized hopanoids, where this treatment cleaves off polyhydroxylated side chains and produces primary alcohols (Innes et al., 1997; Winkler et al., 2001).

3.2.2. Ester-bound hopanoids in soils after acid and base hydrolysis

Acid and base hydrolysis is used to cleave the ester bonds between hopanoids and SOM and release ester-bound hopanoids. Hopanoids were not detected in the acid hydrolysis extracts. This suggests that acid hydrolysis did not release ester-bound hopanoids from soils or below-detectable amounts of hopanoids in the analyzed soil samples. Winkler et al. (2001) also did not detect hopanoids in the acid hydrolysis extracts of soils in their study. Quirk et al. (1984) also observed that acid hydrolysis of peat produced low amounts of hopanoids. Further research should examine the efficacy of acid hydrolysis to extract soil-bound hopanoids.

Bishomohopanoic acid with 32 C atoms was the only hopanoid observed after base hydrolysis. The base hydrolysis released between 0.8 and 8.8 μg/gC bishomohopanoic acid (Table 2) with the highest concentration in the grassland soil (Brown Chernozem), and the lowest amount in the forest soil (Gray Luvisol). The base hydrolysis yielded 7–8 times more of bishomohopanoic acid in the grassland soils (Brown, Dark Brown, and Black Chernozem) and twice as much in the transition soil (Dark Gray Chernozem) in comparison to solvent extraction. However, less bishomohopanoic acid was measured in the forest soil (Gray Luvisol) after base hydrolysis than that detected after solvent extraction. This observation suggests that more bishomohopanoic acid is linked to SOM via ester bonds and/or sorption is reducing bishomohopanoic acid in the grassland and grassland–forest transition soil samples in comparison to the forest soil. The C/N ratios (Table 1) indicate that the forest soil is the least "humified" and implies that less bishomohopanoic acid is incorporated into the SOM matrix. However, the employed methodology can not discriminate between "bound" and "sorbed" hopanoids and thus, both mechanisms are plausible.

3.2.3. "Bound" hopanoids in soils after oxidation with ruthenium tetroxide

Oxidation with RuO_4 was used to cleave bound hopanoids from SOM. RuO_4 cleaves aliphatic–aromatic C bonds between hopanoids and SOM, and once the bond is cleaved, the aromatic ring from the organic matter is replaced by a carboxyl group (Richnow et al., 1992; Winkler et al., 2001). Binding of hopanoids to the aromatic structures is suggested to be a Friedel–Craft type of reaction (Richnow et al., 1992). However, RuO_4 can also cleave ester and ether bonds (Richnow et al., 1992; Winkler et al., 2001). Therefore, the detected bishomohopanoic acid in the RuO_4 extracts could also stem from the cleavage of ether groups and unreacted ester groups that survived the base hydrolysis procedure.

Bishomohopanoic acid with 32 C atoms was the only hopanoid detected in the RuO_4 extracts. The concentration of bishomohopanoic acid ranged between 2.2–28.3 μg/gC (Table 2) with the highest concentration of bishomohopanoic acid observed in the transition soil (Dark Gray Chernozem). The RuO_4 oxidation yielded higher amounts of bisho-

mohopanoic acid than the solvent extraction in most samples. For instance, approximately 10 times more bishomohopanoic acid was detected in the Dark Brown Chernozem, about 13 times more in the forest–grassland transition soil (Dark Gray Chernozem), and about 4 times more in the forest soil (Gray Luvisol); indicating that more of the bishomohopanoic acid exists "bound" to the SOM through aliphatic–aromatic C and/or O linkages rather than in a "free" form. In samples such as the Gray Luvisol, Dark Gray and Dark Brown Chernozems, the RuO_4 method also released more bishomohopanoic acid than the base hydrolysis procedure. This suggests that these soils contain more bishomohopanoic acid bound via aliphatic–aromatic and/or ether bonds than in other samples (Brown and Black Chernozems). Bishomohopanoic acids that are bound to SOM via ester bonds and were not cleaved by base hydrolysis (i.e. survived the base hydrolysis procedure) may have been subsequently cleaved by RuO_4 and detected after this part of the sequential chemical extraction.

3.3. Occurrence of the detected hopanoids in bacteria

The detected bishomohopanol can arise from the C_{32}, C_{33}, C_{34}, or C_{35} tetrafunctionalized hopanoids bacteriohopanetetrol and aminobacteriohopanetriol or a range of composite hopanoids (Innes et al., 1997). Bacteriohopanetetrol is produced by a number of organisms such as cyanobacteria, purple non-sulfur bacteria, methylotrophs and some gram-positive and gram-negative bacteria (Talbot et al., 2003b). Aminobacteriohopanetrol is mostly synthesized by type ll methanotrophs but it has been synthesized by other bacteria such as N-fixing bacteria and purple non-sulfur bacteria (Talbot et al., 2003b). The detected methyl bishomohopanol could be either derived from 2- or 3- methyl BHPs. In ring A of the triterpenoid pentacyclic ring system, a methyl group can occur at the position C-2 or C-3 (Fig. 2). GC–MS analysis cannot be used to determine if the methyl group is attached to the C-2 or C-3 position in ring A. The methyl group at the C-2 position is recognized as a structure that occurs widely in bacteria, but the methyl group at the C-3 position is also an important characteristic because 3-methylbiohopanoids occur in acetic acid bacteria and in certain methanotrophs (Talbot et al., 2003b). 2-methyl biohopanoids are synthesized by cyanobacteria, methylotroph *Methylobacterium organophilum*, N-fixing bacteria and pink-pigmented facultative methylotrophs (Talbot et al., 2003b). However, it has been reported that quantitatively significant amounts of these compounds can only be synthesized by cyanobacteria (Summons et al., 1999). Some studies have observed that different bacterial groups can produce distinct and identifiable BHPs and have suggested that BHPs can be used as potential biomarkers in environmental samples (Farrimond et al., 2000; Talbot et al., 2003b). However, Talbot et al. (2003a,b) relied on data from intact BHPs (analyzed by liquid chromatography–MS) that are more informative for delineating BHP sources. Although the detection of hopanoids in soils demonstrates promise for analyzing bacterial community structures, further studies need to be performed to assess the applicability to soil environments.

3.4. Distribution of hopanoids in different soil types

The concentration of the total hopanoids varied among the soils analyzed in this study (Table 2). The grassland soils contain similar amounts of "free" and "bound" hopanoids (Table 2; Fig. 3).

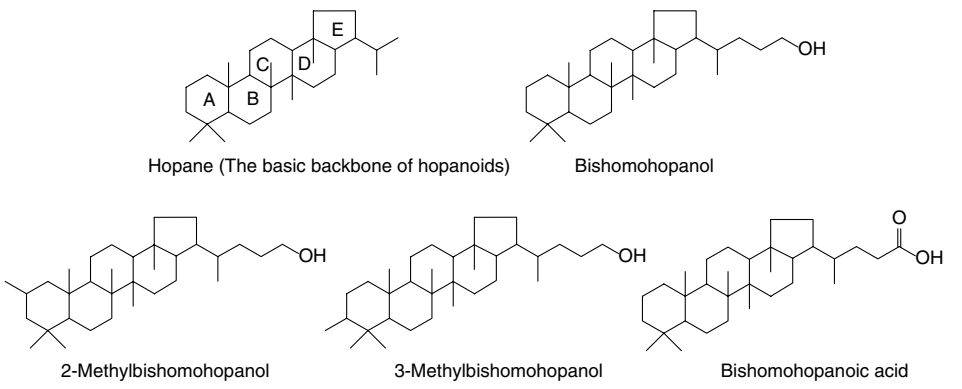

Fig. 2. Chemical structure of hopanoid markers identified in soil extracts.

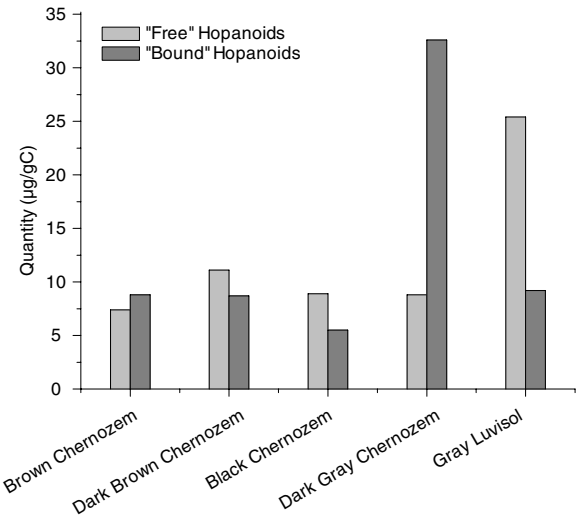

Fig. 3. The total distribution of "free" and "bound" hopanoids in the analyzed soil samples.

However, the amounts of "free" and "bound" hopanoids vary in the transition and forest soils. The transition soil contained more "bound" hopanoids than "free" hopanoids and the opposite trend was observed for the forest soil, which contains higher amounts of "free" hopanoids. This suggests that more of the hopanoic acids in the Dark Gray (Ah horizon) Chernozem are incorporated into the organic matter and/or physically protected from biodegradation by the soil matrix (Baldock and Skjemstad, 2000). In the Gray Luvisol (O horizon) sample, the incorporation of hopanoic acids did not occur in great abundance, therefore less "bound" hopanoids were observed. The C/N ratio of the mineral horizons (Ah) suggests that SOM is more degraded than the O horizon implying that the amount of "bound" hopanoic acids is related to the degree of SOM alteration. The mineral horizons (Ah), which are reported to contain smectite minerals (Salloum et al., 2000), may also sorb hopanoid biomarkers and thus reduce their extractability with solvents.

The total amount of extracted hopanoids from soil samples are lower than those reported by Winkler et al. (2001). However, Bull et al. (1998) detected low concentrations (2.239 µg/g) of solvent-extractable bishomohopanoic acid in soils from Rothamsted (England) that were treated with farm yard manure. This indicates that varying amounts of hopanoids in soils are a function of bacterial activity which is controlled by environmental factors such as pH, temperature, moisture and nutrients. Different concentrations of hopanoids among soil types may also be due to the fact that hopanoids are biodegraded under conditions favourable to microbial activity (Winkler et al., 2001) and SOM turnover.

3.5. Fate of hopanoids in Western Canadian soils

Hopanoid degradation products (i.e. hopanes, extended hopanes, and hopenes) were detected in the soil samples, suggesting that hopanoids are likely undergoing degradation rather than preservation. Hopenes, hopanes, homohopene, and homohopanes were detected in the Ah horizons whereas only hopene was detected in the O horizon of the forest soil (Fig. 4). Hopanes and homohopanes in the environment originate from hopanoids after reductive alteration to unsaturated and saturated hydrocarbons (Peters and Moldowan, 1991). For example, bacteriohopanetetrol can be oxidized to a C_{32} acid in the presence of free O_2 which is then altered to C_{31}-homohopanes after decarboxylation (Peters and Moldowan, 1991).

Detection of norhopane in the grassland soils and the forest–grassland transition soil indicates that microbes have biodegraded hopanes to norhopanes which are reported as products of hopane biodegradation (Moldowan and McCaffrey, 1995; Tritz et al., 1999; Bost et al., 2001). Biodegradation of norhopanes, hopanes and homohopanes by bacteria has also been observed in crude oil (Bost et al., 2001; Frontera-Suau et al., 2002). Moldowan and McCaffrey (1995) proposed two mechanisms for homohopane degradation by bacteria. In one mechanism, the constituent (methyl group) of the cyclic C is removed where bacteria induce the demethylation of hopanes. In the second mechanism, bacteria oxidize the hydrocarbon side chain of homohopane molecules. Therefore, biodegradation of hopanes and homohopane in soils is plausible and may explain the low concentrations detected in this study. In addition, norhopanes could be degraded further by bacteria in soils as observed in crude oil. In the grassland and transition soils, hop-(17)-21-ene was detected, indicating that bacteria introduced a double bond between C-17 and C-21 (Fig. 5; Tritz et al., 1999). Then through abiotic oxidation, the hop-(17)-21-ene could be oxidized into different products in the soils as indicated by Tritz et al. (1999); (Fig. 5). Detection of 17α(H),21β(H)-homohopane in the grassland and transition soils also suggests that bacteria introduced a double

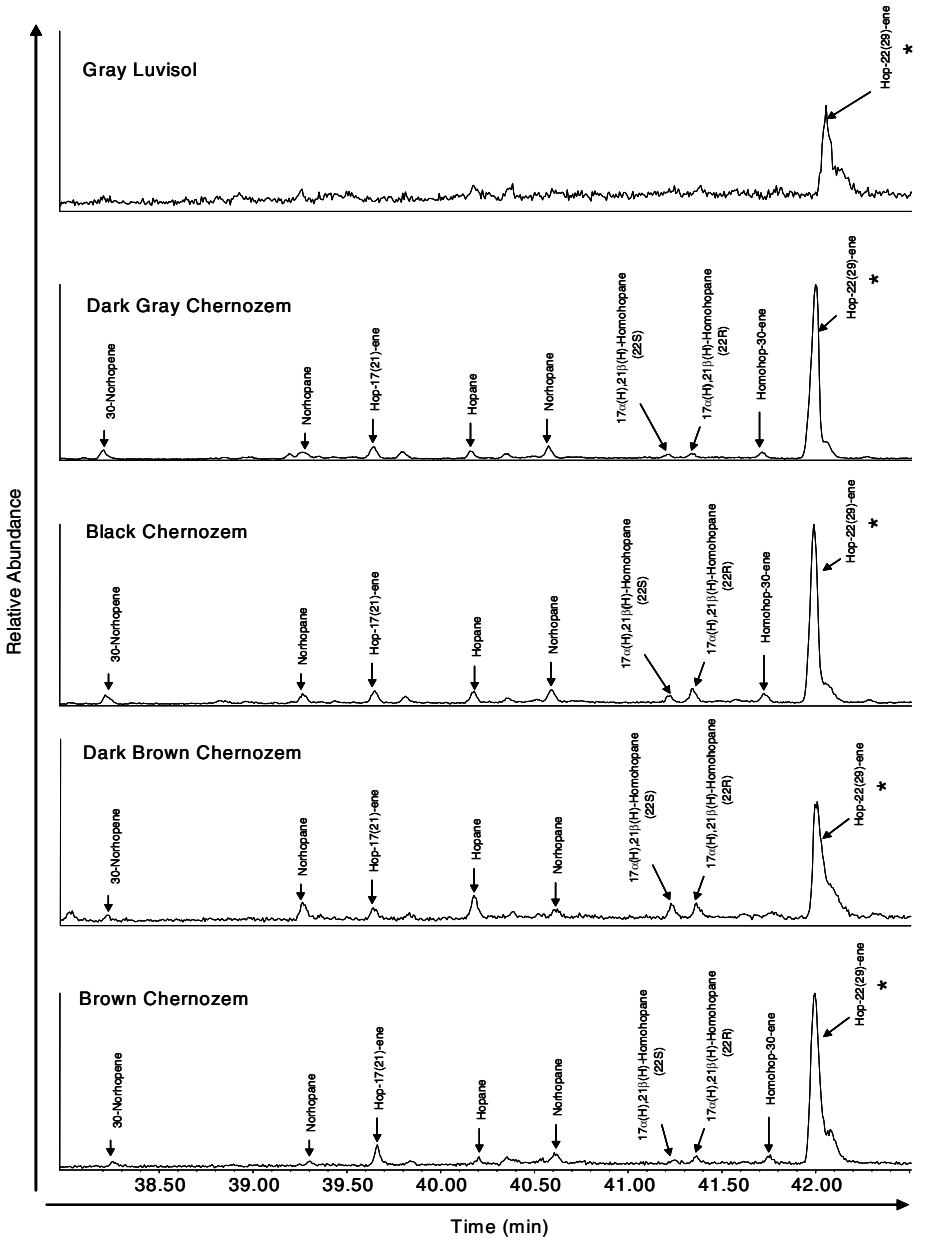

Fig. 4. Total ion chromatogram (TIC) with selected molecular ion of m/z 191 of hopane degradation products in the soil samples (Philp, 1985). (*Note*: *The position of the double bond is not known).

bond between C-17 and C-21 in the homohopane skeleton which serves as the biologically originated intermediate for converting ββ-homohopane into αβ-homohopanes (Tritz et al., 1999). The authors hypothesize that the variability in soil conditions (pH, moisture, soil temperature and soil moisture) are responsible for the observed varying amounts of hopanoids in the transition (Ah horizon) and the forest (O horizon) soils compared to those found in the grassland soils (Ah horizons).

4. Conclusions

The solvent extract method yielded the highest concentrations of hopanoids in two of the grassland soils (Dark Brown and Black Chernozems) and in the forest soil (Gray Luvisol) in comparison to the sequential chemical degradation method, which released higher concentrations in the Brown Chernozem and in the grassland–forest transition soil. More hopanoids exist in the ''bound'' form in the Dark

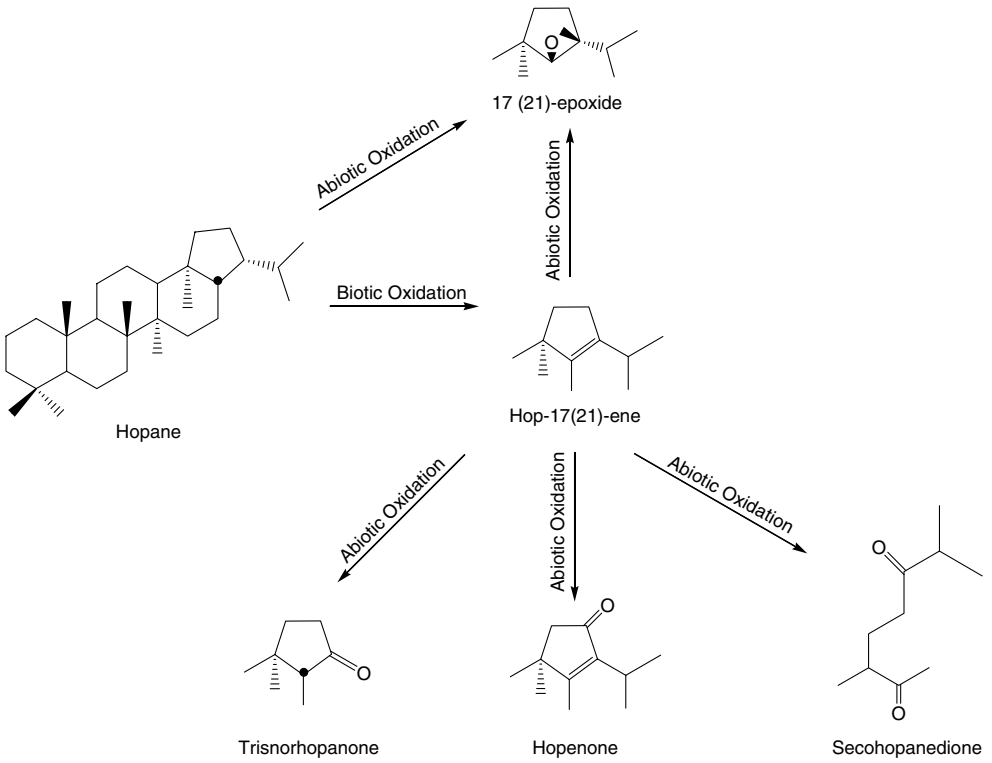

Fig. 5. Oxidation processes of hopanes by abiotic and biotic mechanisms (adapted from Tritz et al., 1999).

Gray Chernozem than in the other soils, where more hopanoids are found to be in the free form. In the grassland soils and the grassland–forest transition soil, degradation products of hopanoids were detected (hopanes, hopenes, homohopanes and homohopene). However, only hopene was detected in the forest soil. The detection of hopanes and hopenes indicates that hopanoids are undergoing degradation in soils and explains why low concentrations are detected in these samples relative to those reported for sediments. Furthermore, we hypothesize that the slower rate of degradation in the grassland–forest and forest soils results in greater accumulation/preservation of hopanoids. Further research is needed to assess the mechanisms of hopanoid incorporation and/or sorption and their potential use as proxies of biogeochemical processes in soil.

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