AN ABSTRACT OF THE THESIS OF

Janet K. Rasmussen for the degree of Master of Science in Water Resource Science presented on May 22, 2009

Title: Reactive Polyphenols and Dissolved Nutrients in a Nitrogen-Limited Headwater Catchment, Western Cascades, Oregon, USA

Abstract approved:

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In undisturbed N-limited forested catchments, DON may represent over 90% of the total N lost in streams. Some ecologists have suggested that plant-derived reactive polyphenols may be responsible for DON binding and transport because polyphenol-protein complexes are known to precipitate out of solution, bind to mineral surfaces or organic matter in the soil, or be leached from the system. Sources of reactive polyphenols include tannins produced by plants, lignin degradation products, and fungal melanins. Therefore, polyphenols produced in natural ecosystems might have profound effects on the biogeochemistry and fertility of unpolluted forests. We investigated the hypothesis that reactive polyphenols are the mechanism behind the observation that pristine streams lose nitrogen primarily in the dissolved organic (DON) form compared to inorganic N (DIN).

More knowledge of natural C and N dynamics in unpolluted ecosystems is vital to improve our understanding of the real and potential effects of anthropogenic N pollution. Despite the interest in polyphenols as a mechanism of DON sequestration and transport, no study has investigated the flux of water-soluble phenols from the forest floor to the stream, or estimated the relative proportion of phenolic DOC in the ecosystem.

We examined the concentration and characteristics of phenols, DOC, and N in an unpolluted, N-limited second-order catchment in the Western Cascades of Oregon in order to estimate the relative proportions of each over sources and storm events. Samples were collected from soil lysimeters, from a physically isolated hillslope component, and from the stream.

We estimated that between 4.8 and 16.6% of the DOC was phenolic, while the aromatic DOC was between 27.5 and 65.8% phenolic. The proportion of reactive polyphenols was

approximately 74%, and highest in the organic horizon and a small tributary. Stream DON averaged 94.6% of the total N, but many of the total N and most of the inorganic N results were below detection limits.

We found positive correlations between DON and total phenols in some sources but not in others. While we found some support for the hypothesis that polyphenols are an important mechanism of DON transport and sequestration, future examination of phenols, DOC, and N, under controlled conditions with more sensitive N analysis might prove fruitful.

REACTIVE POLYPHENOLS AND DISSOLVED NUTRIENTS IN A NITROGEN-LIMITED HEADWATER CATCHMENT, WESTERN CASCADES, OREGON, USA

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Janet K. Rasmussen, Author

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Chapter 1:

General Introduction

Janet K. Rasmussen

N Limitation:

What factors influence the nitrogen (N) limitation of unpolluted forested ecosystems? In pristine ecosystems, N can be limited due to low atmospheric inputs in the absence of anthropogenic contributions, or by leaching of N to streams. Aggrading forests require N inputs in excess of outputs as well as conservation of existing N stores. Numerous studies have found that pristine forests lose significant dissolved organic N (DON) to streams, while the more labile inorganic forms of N, NO_3^- and NH_4^+, are conserved (Sollins et al. 1980, Hedin et al. 1995, Vitousek et al. 2002, Kraus et al. 2003, Vanderbilt et al. 2003). We investigated the hypothesis that reactive polyphenols are the mechanism behind the observation that pristine streams lose nitrogen primarily in the dissolved organic (DON) form compared to inorganic N (DIN).

Significant hydrologic loss of DON in these N-limited systems is puzzling (Hedin, 1995, Neff, 2003). DON can be lost when it binds to mineral or organic particles, is flushed out via streams, and/or becomes resistant to decomposition. In the organic horizon, DON derives primarily from polymerized amino groups (Yu et al. 2002), which can be easily decomposed by enzymes. Plant-derived polyphenols (PP), are capable of sequestering proteins (amino acids) into recalcitrant complexes, and thus may have a significant role in the N cycle (Hättenschwiler and Vitousek, 2000).

Plants preferentially use NO_3^- and NH_4^+ as their N source, although some plants have been shown to derive a portion of their N needs from amino acids and even polyphenol-protein complexes (PPC) (Bennett and Prescott 2004). Ericoid mycorrhizal fungi, symbionts of ericaceous species including rhododendron and salal, as well as some ectomycorrhyzal fungi, symbionts of conifers, have been shown to access N from polyphenol-protein complexes (Bending and Read, 1996).

Geographic variation in stream loss of N in the United States may be due to anthropogenic effects. In a review of N concentrations in forested streams in the United States, mean NO_3^- concentrations were 2.5 times as high in the Northeast as in the West, and mean NH_4^+ was 4.5 times higher (Binkley et al. 2004). Higher DIN in the Northeast streams is believed to be due to the much higher atmospheric N deposition secondary to

pollution. By contrast, mean DON concentration was only half as high in the Northeast as in the West. DON:DIN averaged 0.47 mg/L in the NE and 2.59 in the West.

Much of DON, as well as a portion of the soil organic N bound to minerals and organic particles is thought to be composed of polyphenol-protein complexes (Qualls et al. 1991, Yu et al. 2002, Berthrong and Finzi 2006). The polyphenols in these complexes are derived in part from higher plants as tannins. The co-evolution of tannin-rich plants in nutrient-poor soils suggests that tannins may influence nutrient availability (Kraus et al. 2003).

Reactive Polyphenols:

Polyphenols are substances that possess several or many hydroxyl (OH) substituents bonded onto an aromatic ring (Waterman and Mole, 1994). There are many methods available to quantify and characterize phenols, and one method is the capacity for polyphenols to precipitate proteins. Polyphenols that bind with proteins are *reactive* (RPP). Formation of polyphenol-protein complexes by tannins depends upon the chemistry, structure (chain length, conformation, substitution patterns) and concentration of both tannins and proteins. While tannins by definition will precipitate proteins, neither all tannins nor all polyphenolic compounds will precipitate proteins under all circumstances (Kraus et al, 2003).

Few studies investigating the effect of plant-derived reactive compounds with N cycling focus specifically on polyphenols. Studies involving tannins are numerous, as well as studies of “humic substances” which may include tannins and other polyphenolic compounds in their structure. Humic substances are operationally defined as a series of high-molecular weight yellow to black substances formed by secondary synthesis reactions and fractionated into humic or fulvic acids and humin according to solubility characteristics (Stevenson, 1994). In the past several decades, suggested structures have been proposed for humic and fulvic acids, all of which include prominent polyphenolic constituents (Burdon, 2001). Burdon suggests that chemical studies and NMR data indicate that humic substances are simply macromolecules composed of plant and microbial materials and their degradation products.

Polyphenolics are a common feature of tannins, “humic substances”, melanins, and lignin degradation products (Figure 1.1), and may be the primary constituent that makes these compounds important to N cycling.

The most well-studied plant polyphenols are a class of diverse molecules called tannins. Tannins have been found to reduce N availability by decreasing N mineralization in soils or binding N in polyphenol-protein complexes (Olson and Reiners 1983, Kraus et al. 2004, Kanerva et al. 2006, Talbot and Finzi 2008). Once in the soil, tannins may remain in solution, precipitate, adsorb to mineral surfaces, form polyphenol-protein complexes, or undergo microbial degradation.

Non-tannin sources of polyphenols which may react with N include lignin degradation products (Nierop and Filley 2007) and fungal melanins (Stevenson 1994, Butler and Day 1998, Caldwell 2005, Rillig et al. 2007). Nierop and Filley (2007) found that distinguishing the signatures of lignin and tannin-derived polyphenols was difficult in soils, so that determining the relative importance of each remains a problem. Fungal melanins were found to form complexes with proteins, complex metals, protect against UV radiation, and have antioxidant properties (Butler and Day 1998). Tan et al (2008) found evidence of humic acid-protein interactions under environmental conditions. Soil organic matter or any of the humic substances may potentially form polyphenol-protein complexes as a result of their polyphenolic constituents.

Tannins:

Tannins are large polyhydroxyl compounds chemically defined by a number of assays and functionally defined by their ability to bind proteins (Baldwin et al. 1983). They are estimated to be the fourth most abundant biochemical produced by plants, and may occur in all plant tissues. In rapidly cycling soft tissues, they may be more abundant than lignin. (Hernes and Hedges, 2000). They comprise a significant part of biomass C in land plants, in which the leaves, buds, seeds, fruit, bark, wood, and root tissues may contain up to 40% tannin by weight (Yu et al. 2003, Kraus et al. 2004). Tannins found in higher plants may be divided into two categories: condensed tannins (CT) and hydrolyzable tannins (HT). CT are polymers of three-ring flavanol structures,

of which there are at least a dozen variants. These may have varying linkage patterns and substituents, and may be produced by both monocots and dicots. HT are usually made up of gallic acid units linked to a central glucose; and are only produced by dicots. They are not found in gymnosperms.

Tannins were once thought to be metabolic waste products. Their ability to bind proteins has been known since ancient times as they have been used in the tanning of animal hides. It is their interaction with proteins (amide-N) that gives tannins the potential for varied influences in biogeochemical processes. While a large portion of tannin polyphenols enter the soil from the decomposition of the litter layer, roots are also an important source of tannins to the soil (Hättenschwiler et al. 2003, Meier et al. 2008).

Polyphenols, particularly condensed tannins, have been implicated in the sequestration of organic N into recalcitrant protein-polyphenol complexes. (Maie et al. 2003, (Smolander et al. 2005). In N-limited ecosystems, plants which are most tolerant of low nutrient conditions are able to compete with others which are not. These competitive plants, i.e., conifers and ericaceous shrubs, often produce larger amounts of tannins in their tissues than plants growing in N-rich systems (deMontigny et al. 1993, Northup et al. 1995, Hättenschwiler et al. 2003, Kraus et al. 2004).

Not all polyphenolic compounds will bind to amino-N. *Reactive* polyphenols, however, include tannins and other polyphenolic compounds which do bind to proteins as well as non-peptide-N, such as nucleic acids, polysaccharides, and lipids (Appel, 1993). These can be estimated by a number of protein precipitation assays (Baldwin et al. 1983, Waterman and Mole 1994, Hagerman and Butler, 1978), as well as the relatively new solid phase extraction (SPE) method (Dvorakova et al. 2007), in which polyphenols are removed from solution as they form hydrogen bonds with polyamide resin in syringe tubes. The reactants can then be eluted for further analysis.

Protein binding capacity may be affected by chain length and structural characteristics of the polyphenol molecule and by the relative concentration of polyphenols and proteins (Kraus et al. 2004, Kanerva et al. 2006, Nierop et al. 2006a, Talbot and Finzi 2008). The mechanisms by which polyphenols interact with DON

include the covalent bonding of amino N to phenolic rings (Stevenson 1994), by hydrophobic interactions with hydrophobic amino acid side chains, cross-linkages of the polyphenols (Charlton et al. 2002, Jobstl et al. 2004), and hydrogen bonding, in which many weak interactions combine to form a stronger bond (Table 1.1) (Appel, 1993). Hydrogen bonding is the dominant mechanism in condensed tannin binding, and hydrophobic interactions are dominant in hydrolyzable tannin binding (Hagerman et al. 1998a). The B ring of condensed tannins readily forms quinones in natural systems, and these are thought to bind with organic matter via condensation reactions (Maie et al. 2003).

A recent review of tannin effects on nutrients in forested ecosystems found that tannins can slow decomposition, complex proteins, be toxic to microbes and inhibit enzyme activities (Kraus et al. 2003). Other functions of tannins include herbivore and pathogen defense, metal complexation, and resistance to UV radiation, freezing, and drought. The presence of tannins in the ecosystem is generally associated with increased DON:DIN in litter extracts (Kraus et al. 2003). Nutrient losses are reduced due to the recalcitrance of the DON, particularly when it is complexed to polyphenols, bound to mineral surfaces or humic substances (Kraus et al. 2003, Tan et al, 2008).

Hydrolyzable tannins occur in some green algae, and in angiosperms. With some exceptions, woody plants such as conifers, and ericaceous shrubs, including rhododendron and salal produce more condensed tannin than other higher plants (Waterman and Mole 1994, Kraus et al, 2003). These plants predominate in the unpolluted forests of the Pacific Northwest as well as in boreal and tropical forests which are N-limited. Ecologists question whether these plants are especially adapted to a low N environment by their ability to uptake DON through the activity of mycorrhyzal fungi (Bennett and Prescott 2004, Jones et al. 2005), or if the plants have adapted a mechanism to perpetuate the low N environment by producing more reactive polyphenols in these environments (Schimel et al., 1998, Northup et al. 1995). Differences in tannin concentration have been found to vary not only with species, but within species, possibly increasing with decreasing soil fertility (Schimel et al., 1998, Northup et al. 1995). In contrast to the findings of Northup et al. (1995), however,

Hattenschwiler et al. (2003) found lower polyphenol concentrations in plant tissues growing on more N-limited sites.

Distribution of Polyphenols in Forested Ecosystems:

Polyphenols in forested ecosystems, other than those in living plant tissues, are mostly found in the litter layer and organic horizon of the soil. Most studies of polyphenols, tannins, and humic substances in the environment focus on plant litter and the upper horizons of the soil. Studies of humic substances or polyphenols may focus on surface or groundwaters as well. Polyphenols are often difficult to detect in the mineral soil horizons due to decomposition, precipitation, adsorption to mineral surfaces, or to the interfering effects of solvents used for extraction (deMontigny et al. 1993, Lorenz and Preston 2002, Kraus et al. 2003). Solvent extractions of polyphenols in the litter layer and soils include compounds that are not water-soluble and therefore will not be part of polyphenol concentrations in the streams.

In a study from an N-limited coastal environment, DON:DIN leaching from pine litter was strongly correlated ($r^2 = 0.90$) to the polyphenol content of the litter over a 3 week incubation period (Northup et al. 1995). Berthrong and Finzi (2006) found a strong correlation between aqueous methanol extractable phenols in soil and DON production in three forest soils. Yu et al. (2002) found a strong positive correlation ($r^2 = 0.94$) between phenols and amino-N compounds in organic horizon leachate.

When tannins have been added experimentally to soils, their negative effects on N mineralization may continue even though the tannins are no longer extractable by commonly used solvents within one to two weeks after addition (Nierop et al. 2006). This implies that insoluble polyphenols in the organic horizon and soil may still be influencing nutrient dynamics.

Griffiths and Caldwell (1992) showed that ectomycorrhyzal fungi in coniferous forests were able to utilize N from polyphenol-protein complexes, while Bending and Read (1996) demonstrated that ericoid mycorrhyzal fungi could access N from polyphenol-protein complexes. If tannin-rich conifers and ericaceous shrubs are thus able to utilize complexed DON, they would gain an advantage over other species which

cannot. Bennett and Prescott (2004), however, found that the ability of three tannin-producing species to use the bound N did not differ substantially based on the amount of tannins produced. The three species took up only 1-2% of the N from polyphenol-protein complexes after 20 days (Bennett and Prescott 2004).

Studies of environmental effects of polyphenol-protein complexes have depended upon complexes created *in vitro* with isotopic N. Polyphenol-protein complexes in nature, though presumed to exist, have not been unequivocally detected (Lorenz et al. 2000). Tannin-rich fractions isolated from salal humus in British Columbia, Canada, were actually found to be ^{15}N-depleted in NMR studies. When used in extraction of tannins, however, acetone will disrupt the hydrogen bonds between polyphenols and proteins, interfering with collection of intact polyphenol-protein complexes (Lorenz and Preston 2002).

Higher polyphenol concentrations are associated with higher DON and lower DIN concentrations in litter layer extracts from pristine forests (Northup et al. 1995, Yu et al. 2002, Berthrong and Finzi 2006). It has also been suggested that the DON in streams is linked to refractory organic compounds (Qualls et al. 1991, Hedin et al. 1995). Alternately, we propose that stream DON is primarily peptide-N made recalcitrant by association with reactive polyphenols, and/or colloidal clay particles with organic coatings composed in part of polyphenol-protein complexes. Could the association of polyphenols and DON in the litter layer be related to the increased DON flux in the stream? Despite this intriguing question, I have not found a study in which water-soluble phenols have been measured coterminously in litter layer, soils, and streams.

Analytical issues abound when measuring phenols. Techniques and standards vary considerably, making studies difficult to compare. One problem is the use of commercial standards which have inconsistent composition, and another is the use of standards which are dissimilar to the typical composition of tannin polyphenols in the environment (Kraus et al. 2003). Common colorometric assays produce an increase in absorbance for each phenolate ion that is oxidized. If the standard used has one, two, or three hydroxyl substituents, the capacity for reaction with the reagent varies

accordingly. When the nature of the phenolics in the environmental samples is unknown, the comparison with a standard is at best an educated guess (Waterman and Mole, 1994). Variation in species of polyphenols over space and time may also change results even when consistent standards are used.

Characterization of Dissolved Organic Carbon:

The characterization of dissolved organic carbon (DOC) gives clues to its source in the ecosystem. Phenols are aromatic carbon compounds, and aromatic C (i.e., lignins and tannins) is associated with plant sources, or in the case of melanins, with fungal sources. Aliphatic C (i.e., proteins, lipids, and carbohydrates) is more closely linked to microbial sources. The specific UV absorbance at 254 nm ($SUVA_{254}$) of DOC is a measure of the relative aromaticity (Chin et al. 1994, Weishaar et al. 2003, Hood et al. 2005). Many ecosystem studies have examined $SUVA_{254}$ in litter, soils, and surface waters (Van Verseveld 2007, Hood et al. 2006, Hood et al. 2005). Changes in $SUVA_{254}$ with soil depth, over seasons, and over storm cycles, indicate variation in DOC sources (Hood et al. 2006).

Phenols are found in the aromatic portion of DOC, but not all aromatic C is phenolic. Smemo et al, (2007), found that approximately 2.5 % of the DOC in soil at 75cm depth in a northern hardwood forest was phenolic. Gallet and Keller (1999) found that the DOC of the O horizon was 9.4% phenolic and the B horizon was 7.8% phenolic. If $SUVA_{254}$ is another estimate of phenols in DOC, then it could be used as to more rapidly estimate phenols in varying sources and seasons. If it is different in some sources or over seasons, then the distinct effect of phenols on N cycling might be elucidated.

Thesis Objectives and Hypotheses:

Tannin-rich plant communities dominate in the N-limited forested ecosystems of the Pacific Northwest, and DON prevails over DIN in stream export. This study is the first to our knowledge that traces and compares the concentration of phenols to that of DOC and N in soil and stream water as they change over the course of precipitation

events. We estimated water-soluble phenol concentrations from their source in the organic horizon, through the soils, and exiting the hillslope to the stream, as rapid changes in water flow and source materials occur over storm cycles. We estimated the reactive portion of total phenols (reactive polyphenols) in solution from various sources. Phenol, DOC and N concentrations in soil and stream may change by an order of magnitude over storm events and seasons, providing an opportunity to assess these relationships in a single ecosystem. Therefore, we investigate the hypothesis that reactive polyphenols are stabilizing otherwise labile DON for transport via streams.

DOC and DON accumulate in the organic horizon, with highest concentrations at the onset of a precipitation event following dry antecedent conditions (Vanderbilt et al. 2003, Van Verseveld 2007). Kuiters and Denneman (1987) found that phenols were positively correlated to DOC in forest soils. We chose to examine concentrations of phenols, DOC, and N fractions over an early fall storm event in order to quantify changes throughout the soil profile and stream simultaneously. The following spring, additional samples were obtained during saturated conditions of a small precipitation event with ongoing snowmelt. Because aromatic C ($SUVA_{254}$) has also been shown to be highest during an early fall storm, and phenols make up a portion of aromatic C, we expected that phenol concentration would correlate positively to DOC.

We sampled from the organic horizon, and three successive depths within the mineral soil, as well as from the bulk hillslope discharge and stream. Because polyphenols are capable of adsorption to mineral surfaces (Hättenschwiler and Vitousek 2000), and to precipitate out of solution, we expected phenol concentrations to be highest in the organic layer, and decrease with depth in the soil profile.

Streamwater $SUVA_{254}$ has been found to increase during storms compared to baseflow levels, suggesting increased derivation of DOC from riparian or organic horizon sources (Hood et al. 2006). $SUVA_{254}$ in soil solution has been found to decrease with depth, suggesting sorption of aromatic C compounds to mineral soil (Hood et al. 2006). $SUVA_{254}$ is a simple and rapid analysis frequently included in ecosystem water studies, whereas the quantification of phenols is less often done. We expected that changes in $SUVA_{254}$ and the proportion of phenols in DOC during storms and with soil

depth would be similar. If so, $SUVA_{254}$ data may be a proxy for phenol proportion in DOC.

Because Smemo et al. (2007) found that DOC in a northern hardwood forest soil was approximately 2.5% phenolic, we expected that our study site would have a higher proportion of phenolic DOC, owing to the high tannin production of dominant vegetation of conifers and ericaceous shrubs. We hypothesized that polyphenols make up a significant portion of DOC in an N-limited catchment, and therefore we predicted that phenols would be positively correlated to DOC and to aromatic DOC.

We hypothesized that polyphenols may be highly bound to DON in N-limited environments where DIN concentrations remain low due to high demand and impaired mineralization of sequestered DON. Evidence of this would include positive correlation between phenol concentration and DON:DIN in organic horizons of an N-limited forested catchment; and possibly in mineral soil and stream water.

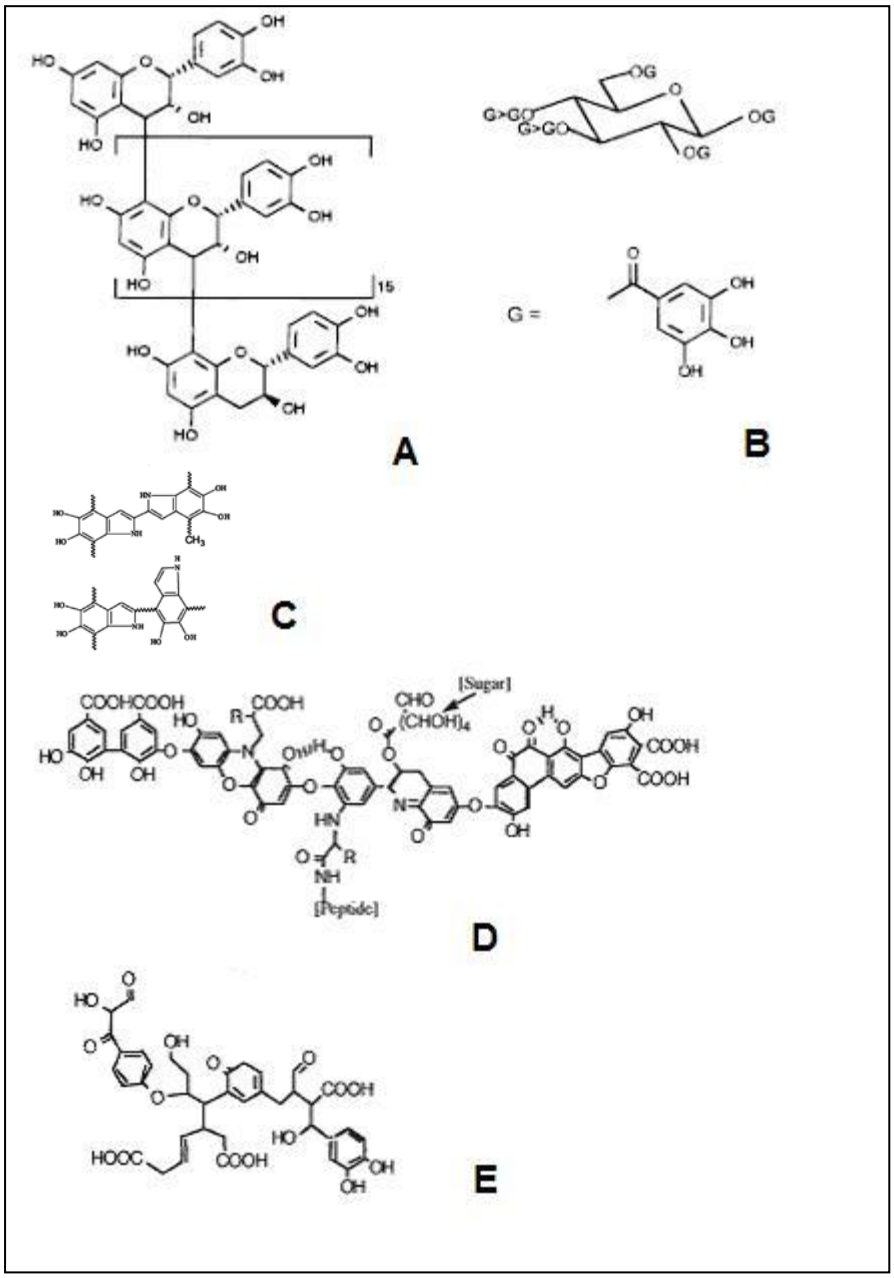

Figure 1.1: Structures of some polyphenolic substances. A) condensed tannin, B) hydrolyzable tannin, adapted from Hagerman, et al, 1998, C) fungal melanin, adapted from Zhong, et al, 2008; and D) suggested humic substance structure from Stevenson, 1982, and E) suggested humic substance structure from Stein, et al, 1997, both adapted from Burdon, 2001.

Table 1.1: Types of bonding between polyphenols and N, adapted from Appel, 1993.

Type of bond	Bond strength (kJ/mol)	Reversibility	pH of formation
Hydrophobic	< 4	reversible	any
Hydrogen	10-40	reversible	< 8
Ionic	100-1000	reversible	> 8
Covalent	100-1000	irreversible	> 8

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Chapter 2:

Reactive Polyphenols and Dissolved Nutrients in an N-Limited Headwater Catchment, Western Cascades, Oregon

Janet K. Rasmussen

Introduction:

What factors are responsible for N limitation in forested ecosystems? Previous studies have found that phenol concentrations are positively correlated to DON:DIN in organic horizons. Two possible implications of this are that reactive polyphenols bind DON into recalcitrant complexes, limiting the availability of N for mineralization; and/or that polyphenols interfere with enzymatic N mineralization processes. We investigated the hypothesis that reactive polyphenols are the mechanism behind the observation that pristine streams lose nitrogen primarily in the dissolved organic (DON) form compared to inorganic N (DIN).

Undisturbed forested ecosystems in regions with low atmospheric N deposition are generally N limited. N is lost via leaching to streams and to the atmosphere via denitrification. In these systems, leaching of DIN is low due to the high demand for this labile form, but loss of DON may be as high as in N polluted systems, such as in NE USA (Asano et al. 2006). When there is a great demand for N, plants may find ways to protect what little N they have (Vitousek et al. 2002).

Tannins, which are polyphenols produced by plants, have several mechanisms of N preservation. Many herbivores and pests are prevented from feeding on plants due to the unpalatability or noxious effect of tannins. During decomposition, polyphenols may inhibit N mineralization in the soil by binding with amide-N to form complexes which are resistant to microbial decomposition, and by inactivation of enzymes which break down proteins (deMontigny et al. 1993). The reactivity of tannins and their abundance suggest their importance in biogeochemical processes in the ecosystem (Hernes and Hedges 2004, Kraus et al. 2004).

Non-tannin sources of polyphenols which may react with N include lignin degradation products (Nierop and Filley 2007) and fungal melanins (Stevenson 1994, Butler and Day 1998, Caldwell 2005, Rillig et al. 2007). Nierop and Filley (2007) found that distinguishing the signatures of lignin- and tannin-derived polyphenols was difficult in soils, so that determining the relative effects of each remains a problem. Fungal melanins were found to form complexes with proteins (Butler and Day 1998).

Dissolved organic carbon (DOC) includes phenols, aromatic carbon compounds capable of complexation with DON. Concentrations of DOC and DON have been found to decrease with flowpath length as they move through the soil profile (Asano et al. 2006, deMontigny et al. 1993). Also, the aromaticity of DOC has been found, in some studies, to decrease with flowpath length. Dissolved organic matter binds readily to mineral surfaces as it moves downward through the soil profile, or is microbially decomposed, and may also precipitate out of solution as large aggregates (Sollins et al. 1996). For this reason, dissolved organic matter concentrations found in soil water generally decrease with depth, or with length of time in contact with mineral soil. Organic matter bound to mineral soil particles may have a long or relatively short residence time, depending on the type of bonding, and molecular structures involved.(Kleber et al. 2007).

We expect that polyphenols bind DON in the organic layer (Kuiters and Denneman 1987) and at least some of the resulting complexes are leached into streams without binding to soils. Therefore, tracing the concentrations and characteristics of C and N as they move from organic layer, through soil, from the isolated hillslope, and into the stream will help define a potential flowpath of phenols and DON.

The hydrology of a catchment is also a factor in the control of nutrient cycling (McKnight et al. 2001, Hood et al. 2005, Van Verseveld 2007). Steepness, aspect, soil characteristics, riparian and hyporrheic zone dynamics can affect the amount of time that solutes are in contact with soil, microbes, and plants. The hydrological processes can influence and explain nutrient chemistry, but nutrient chemistry can also help elucidate hydrological processes.

H. J. Andrews Experimental Forest (HJA) in the Western Cascades of Oregon is one of few well-studied unpolluted forested ecosystems in the world (Vanderbilt et al. 2003). Atmospheric deposition of N is extremely low, estimated 2kg N/ha/yr (Sollins et al. 1980). DON:DIN in stream water draining 3 old-growth watersheds in HJA ranged from 1.5 to 3.6 (Vanderbilt et al. 2003).

This study is the first to our knowledge that traces and compares the concentration of phenols with DOC, DON, and DIN in soil and stream water. We

measured concentrations of phenols, amide-binding polyphenolics, inorganic and total N, DOC, and $SUVA_{254}$ during a significant storm event in October 2007 and again during one of a series of storms in April 2008 in an experimental watershed. We compared two methods for fractionating the reactive portion of total phenols.

Tannins decrease in concentration with depth in the soil profile as they are degraded, bind to mineral surfaces, or precipitate out of solution (deMontigny et al. 1993). We expected that phenol concentrations would be highest in the organic layer, and decrease through the soil profile. Phenols have been estimated to compose 2.5% of DOC in deep soil solution (Smemo et al. 2007) and 7.8% of B horizon and 9.4% of O horizon soil solution (Gallet and Keller, 1999). We hypothesized that phenols make up a significant portion of the DOC in an N-limited catchment, therefore we predicted that phenol concentration would be positively correlated to DOC.

For the same reason, we expected that phenol concentration would correlate positively to UV absorption at 254nm, which approximates the aromatic DOC. Since $SUVA_{254}$, which can be used to calculate the proportion of aromatic DOC, is an easily and commonly measured parameter, it may serve as a proxy for phenol concentration.

Previous studies have shown a positive correlation between DON:DIN and phenol concentration in organic horizons (Northup et al. 1995, Berthrong and Finzi 2006), suggesting that high phenol concentrations were responsible for binding of DON; however these studies did not investigate that relationship between phenol concentration and DON:DIN in mineral soil and/or stream water. There are few other studies to corroborate this relationship. We hypothesized that phenol concentrations would correlate positively with DON and negatively with DIN in organic horizons and possibly in mineral soil and stream water.

Methods:

Site Description

The study site is in the H. J. Andrews Experimental Forest (HJA) in the Western Cascades of Oregon, USA, one of few well-studied unpolluted forested ecosystems in the world. While most studies of N cycling in North American forests have been

conducted in regions where atmospheric N deposition rates are significantly elevated, at HJA, N deposition has remained low, 1.6 to 2.0 kg N ha^{-1} yr^{-1} (Sollins et al. 1980), compared to inputs exceeding 8 kg N ha^{-1} yr^{-1} in many East Coast forests (Hedin et al. 1995). Additional N inputs from N-fixing epiphytes vary significantly, but may exceed N deposition (Holub and Lajtha, 2005). An estimated 1.5 kg N/ha/yr is lost via leaching to streams (Sollins et al. 1980).

Watershed 10 (WS10) of HJA (Figure 2.1) is a steep 10.2 ha catchment (44.2° N, 122.25° W) with elevation ranging from 470 – 680 m. It is a gauged catchment drained by a second order stream. The climate is Mediterranean, with average annual rainfall of 2220 mm (Van Verseveld 2007). The catchment was clearcut in 1975, and is now revegetated in mixed conifer (Douglas fir, western hemlock), with an understory including rhododendron and salal, all known to produce tannins (Kraus et al. 2003, Bennett and Prescott 2004, Halpern 2008). Other tannin-producing plants native to WS10 include Western redcedar, red alder, bigleaf maple, and huckleberry (Hernes and Hedges 2004).

Bedrock geology is Upper Oligocene to Lower Miocene volcanoclastic rocks, including mudflows, pyroclastic flows, tuffs, with basaltic and rhyolitic dikes (Swanson and James 1975). The soils are classified as coarse loamy mixed mesic Typic Hapludands (Yano et al. 2005).The depth is 1-6 meters to saprolite, with high subsurface flow rates. Surface soil conductivity was greater than 400 cm/hr, decreasing to as little as 10 cm/hr in some subsoils (Ranken 1974). There was no potential for overland flow due to the high conductivity, even during the heaviest precipitation.

Isotopic studies in WS10 indicate that baseflow water from the hillslope has a residence time of 1-2 years; and that during transitional or wet antecedent moisture conditions, “event water”, that is, water from the precipitation event, has a residence time of 10-30 hours. During the wet antecedent periods, there is an additional shallow reservoir of water with a residence time of 10-25 days (McGuire et al. 2005).

A south-facing hillslope within the catchment is instrumented with 25 Prenart lysimeters at various depths for collection of soil water, and 5 zero tension lysimeters which collect soil water passively from beneath the organic horizon. The lysimeters

were positioned at least 5 meters from the stream. A portion of this hillslope has been isolated at its base for a distance of 10 m with an impermeable barrier to bedrock, which collects all the water draining the hillslope above through a calibrated weir (Figure 2.2). The lower 60% of the stream (which includes the isolated hillslope area) has had its riparian and hyporheic zones effectively removed by several debris flows, the last in 1996, which have scoured it to bedrock (Van Verseveld 2007). Thus this is an ideal site for studying the distinct sources and pathways of nutrients and phenols as they pass through the soil profile, mix with groundwater, and move into the stream.

Sample collection

After a summer dry period, WS10 experienced several precipitation events. A significant storm, beginning 15 October, deposited over 190 mm of rainfall in 6 days (Figure 2.3). We define a storm as a period of precipitation greater than 10 mm, interrupted by less than 12 hours of no precipitation (Van Verseveld 2007). During and after this storm, stream and hillslope water was collected at 2 to 4 hour intervals using an ISCO sampler. Soil water was collected from the lysimeters two days before the storm began, twice daily for 3 days beginning 16 October, and then intermittently.

An unusually persistent snowpack prevented sampling during the winter months. As soon as WS10 was accessible in spring of 2008, another precipitation event was sampled. This storm event, from 27 April through 1 May, deposited over 40 mm of rain, and followed a series of more significant precipitation events. This storm coincided with ongoing snowmelt, so that the soils were thoroughly saturated.

During the spring storm, upper stream (above hillslope outlet), lower stream (at gauge) and hillslope samples were collected four times a day, at approximately 0600, 1000, 1400, and 1800. In addition, samples were collected from the first order left and right forks of the stream twice daily at 1000 and 1400. The left fork was characterized by a steeply incised, predominately bedrock, channel, and appeared to be the primary channel of the stream. The right fork, however, was a minor tributary and flowed through the litter layer and over soil. The hillslope over which it flowed faced northwest.

Precipitation amounts were obtained from the HJA database (McKee 2008) for Primet station, less than 1 km from WS10.

The catchment is gauged at the outlet of the second-order stream. Streamflow measurements were obtained from the HJA database (Rothacher 2007) for WS10 (Figure 2.4), and converted to units of ft^3/s using the formula provided by D. Henshaw, (2002), then to m^3/s. The hillslope catchment weir is 91 meters above the stream weir. A calibrated weir collects subsurface flow from the hillslope above. A capacitance water level recorder (TruTrack, Inc., model WT-HR) recorded stage at 10 minute intervals at 1 mm resolution, and these measurements were converted to cms units.

Samples from tension lysimeters were bulked according to depth and referred to as "shallow" (20 cm), "medium" (30-40 cm), and "deep" (70-110 cm). Samples from zero tension lysimeters were also bulked together.

Assays

All samples were filtered with pre-washed GF/F filters, kept cold, and frozen within 24 hours of collection. NANOpure water (Barnstead APS Ultra FF0501 0.22µ filtered) was used for blanks, rinsing, mixing reagents, and dilution of tannins. DOC and TN were measured on a Shimadzu TOC-V TSH Total organic carbon analyzer with attached TNM-1 total nitrogen measuring unit. The levels of detection were: DOC, 0.05 mg/L; TN, 0.05 mg/L. NH_4^+ and NO_3^- were measured on a Lachat QuikChem FIA+ 8000 Series analyzer, with detection limits of 0.01 and 0.002 mg/L, respectively. DON was calculated by subtracting NH_4^+ and NO_3^- from TN.

When calculating DON:DIN, we considered only those samples in which total N and at least one of the inorganic N results were greater than zero; and thus many data points were eliminated. There is some justification for substituting a smaller number for the zero or below detection limit results, using the assumption that there must be some N present in natural waters, and to avoid data bias against the more dilute samples (Hedin et al. 1995). However, in our study, 23% of the total N assays, 53% of the NO_3^- assays, and 69% of the NH_4^+ were below detection limits. The substitution of arbitrary values for these would result in uncharacteristic proportions of N species.

$SUVA_{254}$ was calculated by dividing the UV absorbance at 254 nm of a water sample, measured in inverse meters (m^{-1}), by the DOC concentrations, and is given in units of L mg-C^{-1} m^{-1}. Absorbance was measured using a Shimadzu UV 1201 spectrophotometer and 1 cm quartz cuvette. Duplicate samples were tested, and the difference between results averaged 0.002 absorbance units (n = 21).

Frequently tannins or phenols have been measured in litter extracts and soil solutions after extraction with solvents and/or fractionation of humic substances by solubility characteristics (Box 1983). Humic substances contain many polyphenolic groups, and are known to bind amide N. The fractionation process identifies humic substances as humic acids, bases, and insoluble humin (Suominen 2003). Tannins, however, depending upon their molecular weight and structure, may be found in any of the fractions. When phenols are extracted from litter and soils using solvents such as aqueous acetone, the hydrogen bonds between polyphenols and proteins may be disrupted (Lorenz et al. 2000). Large polyphenols may not be extracted when aqueous acetone is used (Nierop and Filley 2007)

The Folin-Ciocalteu assay can be used with unaltered environmental water samples to measure total phenolics in concentrations as low as 0.02 ppm. This colorimetric assay relies on the reduction of a phosphotungstic-phosphomolybdic complex to a blue chromophore that can be quantified using a spectrophotometer. This assay does not discriminate between tannin and non-tannin phenolics, but measures the total concentration of phenolic hydroxyl groups (Waterman and Mole 1994, Hättenschwiler et al. 2003, DeForest et al. 2005).

Total phenols were measured using the Folin-Ciocalteu assay (Waterman and Mole 1994, Ohno and First 1998, Suominen 2003, DeForest et al. 2005), with some modification. With samples at 25^{o}C, 2 mL undiluted sample at 25^{o}C was measured with a volumetric pipette into a 5 mL glass test tube, and 0.1 mL Folin Ciocalteu reagent (Sigma F9252) was added while vortexing. After 1-8 minutes, 0.3 mL buffer solution of 20% Na_2CO_3 (J. T. Baker 3604-05) was added while vortexing. Tubes were incubated at 25^{o}C for 1 hour, and absorbance read at 750 nm. Duplicate samples were tested, and the difference between results averaged 0.002 absorbance units (n = 25).

Reactive polyphenols were measured using two methods, casein precipitation and solid phase extraction (SPE), for comparison.

Casein was used for the protein precipitation method (Baldwin et al. 1983, Suominen 2003, Valachovic et al. 2004, Kuiters and Denneman 1987). Undiluted water samples, 6mL at 25^{o}C, were added to 200 mg casein powder (Sigma C5890), in 20 mL glass bottles with rubber stoppers, and shaken 3 hours in mechanical shaker. After centrifugation at 2700 rpm for 15 minutes, the supernatant was aspirated through a 5µ filter needle (Becton-Dickinson 305200), and re-assayed for phenols. The difference between the pre-casein and post-casein absorbance values was designated as the reactive polyphenol fraction (Kuiters and Denneman 1987, Suominen 2003, Smolander et al. 2005).

The casein precipitation method was not sufficiently sensitive to the low concentrations of phenols in some samples. Results could usually only be obtained in the most concentrated organic horizon samples. The background absorbance by the casein amino acids in the blanks was so large that it overcame the relatively small variance between the concentration of phenols before and after precipitation (DeForest et al. 2005).

For the Solid Phase Extraction (SPE) method, 5-10 mL of sample at 25^{o}C were filtered through polyamide tubes (Supelco DPA 6-S 6 mL tubes). The polyamide resin offers binding sites at amide and carbonyl ends. Prior to extraction, the tube was conditioned with 2 mL methanol (Fisher Scientific A412-1) followed by 2 mL water. Resin-bound polyphenols were eluted three times with 2 mL 70:30 acetone at approximately 2 mL/min. three times in succession (Supelco 1998, Liu et al. 2002). Eluent was re-assayed for phenols, and the difference between the pre-SPE and post-SPE absorbance values was designated as the reactive polyphenol fraction. Three blanks were prepared using NANOpure water.

A critical problem has been the choice of an appropriate standard for measuring tannins or phenols. Most researchers have depended upon commercially available standards (tannic acid, gallic acid), which are not typical of the phenolic compounds under study (Maie et al. 2003). Standards purified from the plant of interest offer a

significant improvement (Hagerman et al. 1998b, Kraus et al. 2004). Salal (*Gaultheria shallon*) is a common understory plant in WS10. Purified salal tannins prepared by Dr. Caroline Preston, Pacific Forestry Centre, Victoria, BC, Canada, were used as a phenol standard.

100 mg salal tannins were added to 1 L water, and filtered using Whatman GF/F filter with a nominal pore size of 7µm. Only 2.4% of the tannins were retained on the filter, thus 97.6% of the dry tannins were soluble in water at 25°C. Serial dilutions were prepared ranging from 0.0984 mg/L to 98.4 mg/L. A standard curve was prepared so that phenols in environmental samples could be measured in salal tannin equivalents (STE) (Appendix A), with the caveat that these are *water-soluble* salal tannins. Tannin standard solution was assayed for DOC.

Statistical Analysis

Calculations and linear regressions of relationships between samples, sources, and seasons were conducted using S Plus software and Excel. Correlations of phenol concentration, DOC, and UV_{254}, DON, and DIN were done using simple linear regression for each season and source.

Summaries of parameters measured over fall and spring, from different sources (Table 2.1) were made using flow-weighted averages of all samples collected over the season, including those outside of the isolated precipitation events studied.

Flow weighted calculations were made as follows:

$$Ave_{fw} = \frac{\sum F \times C}{\sum F}$$

where F is the streamflow at the time of sample collection, and C is the concentration or parameter being averaged. The standard error for flow weighted averages was found as

$$SE_{fw} = \frac{SE_c}{\sqrt{\sum F^2}}$$

where SE_C is the standard error of the flow weighted concentration, calculated in S+.

Smoothed data for comparison of DOC concentration peaks in stream and hillslope, fall 2007, was calculated by averaging each data point with the previous and subsequent data point.

A common condensed tannin monomer (e.g., catechin), is approximately 60% C by weight, and the salal tannin standards that we tested contained 69.2% (SE = 5.2) DOC. Using the broad assumption that our phenols were derived from condensed tannins, we could approximate the proportion of phenolic DOC in our sources using our salal tannin standard.

$$\%\ phenolic\ DOC = \frac{0.60(PP(mg/L))}{DOC(mg/L)}$$

$SUVA_{254}$ has been found to correlate closely with percent aromaticity of DOC (Weishaar et al. 2003). By comparing $SUVA_{254}$ values with ^{13}C NMR determinations of percent aromaticity in well-characterized samples of natural humic substances, Weishaar et al., (2003) found % aromaticity significantly correlated with $SUVA_{254}$:

$$y = 6.52x + 3.63,\ R^2 = 0.97,\ n = 13$$

where y = % aromaticity and x = $SUVA_{254}$ The proportion of aromatic DOC that is phenolic was estimated as

$$\%\ polyphenolic\ aromatic\ DOC = \frac{\%\ polyphenolic\ DOC}{DOC} \times 100$$

Detailed protocols for phenol and reactive polyphenol assays, and standard preparation are found in Appendix A.

Results:

Correlation of phenols to DOC

Fall phenol concentrations were found to be correlated to DOC concentration in the organic horizon, hillslope, and stream (all p values < 0.001) (Table 2.2). In each of these, the relationship was positive and linear, with slopes between 0.23 and 0.33 (Figure 2.5). 94% of the phenol concentrations were explained by the DOC in the organic horizon, compared to 39% and 67% in the hillslope and lower stream. In the soils, there was no correlation between phenol concentration and DOC.

In the spring, phenol concentrations were positively correlated to DOC in the organic horizon, shallow and deep soils, and the right fork of the stream (all p values ≤ 0.05) (Table 2.2). The upper and lower stream data was also suggestive of a relationship

(p values ≤ 0.07). The slopes of regression lines of all these were between 0.069 and 0.250.

Using the assumption that our phenols were 60% DOC, and Weishaar's (2003) formula for converting $SUVA_{254}$ to % aromatic DOC, we estimated % phenolic DOC, % aromatic DOC, and % phenolic aromatic DOC based on average concentrations and values for each source over fall and spring (Table 2.3 and Figure 2.6). In fall, the highest phenol content (16.6 and 15.8%) of DOC was in the organic horizon and stream in the fall. The lowest was in the deep soil (4.8% phenolic DOC).

In spring, phenols made up an estimated 9.8% of DOC in the organic horizon and 8.6% in the stream. The highest proportion of phenolic DOC was in the right fork of the stream (12.1%), but overall, there was little difference by source.

Correlation of phenol concentration to A_{254}

$SUVA_{254}$ is absorbance at 254 nm normalized to DOC to find proportion of DOC that is aromatic. To discover if the ratio of phenols to DOC is correlated to $SUVA_{254}$, we eliminated DOC in the denominator, and compared absorbance at 750nm following the Folin Ciocalteu assay with the absorbance at 254 nm in each sample. We found strong evidence that phenol concentration in the fall is correlated to A_{254} in the organic horizon, hillslope and stream (all p values < 0.001) (Table 2.2). In each of these, the relationship was positive and linear, with slopes between 0.043 and 0.126 (Figure 2.7). In the soils, there was no correlation.

In the spring, phenol concentrations were highly correlated to absorbance at 254 nm in the organic horizon, soils, and all locations within the stream (all p values < 0.05). Slopes of regression of all these were between 0.065 and 0.134 (Figure 2.7).

Using Weishaar's (2003) formula, we estimated that DOC in the fall ranges from 15.7% aromatic in the deep soil to 26.1% aromatic in the organic horizon (Table 2.3 and Figure 2.6). We estimated that the phenolic portion of that aromatic DOC ranged from 43.3% in the shallow soil to 65.8% in the lower stream.

In spring, we found DOC was 19.6% aromatic in the organic horizon and 19.9% aromatic in the stream (Figure 2.6). We estimated that the phenolic portion of that aromatic DOC ranged from 27.5% in the left fork stream to 52.7% in the shallow soil.

Correlation of dissolved N to phenol concentration

We investigated the relationship between phenol concentration and DON:DIN. In the fall, we found no significant correlation between phenol concentration and DON:DIN in the organic horizon, soils, or hillslope (p values >0.10) (Table 2.4).In the lower stream, there was a positive correlation between DON:DIN and PP (n = 26, p = 0.0081, slope = 51). DOC predicted 26% of the DON:DIN in the lower stream (Figure 2.8).

We found that phenol concentrations were positively correlated to DON in the organic horizon, hillslope, and lower stream in the fall (p values < 0.05) (Table 2.4). There were only 4 data points for the deep soil samples, however, and the slope of the regression line for those was inconsistent with the others (Figure 2.9). For the lower stream, phenol concentration predicted only 7% of the DON (n = 62).

In the spring we found significant correlation between DON:DIN and phenol concentration in the right fork of the stream, (n = 9, p = 0.0016, R^2 = 0.78) (Table 2.4 and Figure 2.8). There were insufficient data to make a comparison in the shallow or deep soil.

In contrast to fall, we found no significant correlation between phenols and DON in the spring. There were insufficient data to make a comparison in the shallow or deep soil. The right fork of the stream was suggestive for a correlation, however (n = 9, p = 0.0651, R^2 = 0.41) (Figure 2.9).

Storm-related changes

In the fall, peaks of the hillslope hydrograph and the stream hydrograph were nearly synchronous, but the streamflow had a greater amplitude (Figure 2.10). Smoothed DOC peaks in both hillslope and stream are nearly synchronous with one another, and with hydrograph peaks (Figure 2.11). However, the DOC peaks in the hillslope precede those in the lower stream until the largest peak of the storm. Following that, the hillslope DOC concentration peaks lag behind those of the lower stream.

It is apparent that the DOC concentration in stream and hillslope over the peak of the hydrograph was similar, but lower in the hillslope before and after the peak (Figure 2.12). In the organic horizon and the soils, DOC clearly decreases with depth, and peaks were delayed by depth as well.

$SUVA_{254}$ decreased steadily during the fall storm in both lower stream and hillslope (Figure 2.13). Changes in $SUVA_{254}$ in the organic horizon and soils over the course of the storm are less evident.

Phenols in the stream are highest just after the beginning of the fall storm, and begin to decrease before the hydrograph peak (Figure 2.14). In the hillslope, concentrations are quite variable on the rising limb, then peak again on the receding limb before falling to baseflow values. On the receding limb, hillslope and stream concentrations are most similar. Phenols in the soil showed a sharp decrease from organic horizon, with delayed peaks, similar to the plot of DOC in soils. Phenols also decrease by depth.

The proportion of phenolic DOC changes over the course of the storm (Figure 2.15). The proportion decreases steadily in the stream, but rises and falls in the hillslope, coincident with the hydrograph peak. In the soils, the proportion of phenolic DOC is similar to the proportion of aromatic DOC ($SUVA_{254}$).

Over the course of the fall storm, DOC increased only 30% in the organic horizon over baseflow concentrations, but increased 5.3 times in the hillslope, and 4.3 times in the lower stream (Table 2.5). Peak phenol concentrations decreased by 20% in the organic horizon over baseflow values, while in the hillslope and stream, they increased 27.8 and 6.6 times, respectively. $SUVA_{254}$ did not increase in the organic horizon, but increased 3.9 times in the hillslope and 1.2 times in the stream. The $SUVA_{254}$ increases in the hillslope and stream, however, occurred after the peak of the hydrograph.

The spring precipitation event did not result in the same magnitude of hydrograph changes as the event sampled the previous fall (Figure 2.4). Still, DOC concentrations decreased over the duration of the storm in stream and hillslope and in the soils (Figure 2.16). DOC in the mineral soils dropped sharply from the organic

horizon, and was consistently lower in the soils in spring compared to fall. Flow-weighted average DOC in the right fork of the stream was 60% higher than at the lower stream weir (Table 2.1) The phenol concentrations peaked in the stream and hillslope, and fell to consistently low levels by the end of the storm (Figure 2.17). The phenol concentration in the right fork of the stream responded with a much higher increase over the peak of the hydrograph than at the other stream sampling sites. Phenols dropped sharply from the organic horizon to the mineral soil, but then remained at consistently low concentrations over the hydrograph.

In the fall, total N increased 3.5 times in the organic horizon from baseflow to peak. In the hillslope and stream, total N increased 1.7 and 2.1 times, respectively. NO_3^- and NH_4^+ increased by 4.1 and 7.5, respectively, in the organic horizon, but were not detected in baseflow samples of hillslope and stream (Table 2.5). DON decreased slightly over the course of the storm (Figure 2.18) in the stream and soils. Changes in the hillslope were not evident. DIN over the course of the storm showed no consistent pattern (Figure 2.19).

We had no baseflow samples from before the spring storm event, since it was preceded by frequent storms. We compared the initial measurements of DOC, PP, $SUVA_{254}$, and N at the beginning of the storm to peak values (Table 2.5). Over the course of the storm event, DOC increased only 10% in the organic horizon, did not increase in the hillslope and increased by 60% in the stream. Phenol concentrations did not increase in the organic horizon, but increased 5.6 times in the hillslope and 9.6 times in the stream. $SUVA_{254}$ did not increase in the organic horizon, but increased 2.3 times in the hillslope and 1.7 times in the stream. The $SUVA_{254}$ increase in the hillslope occurred after the peak of the hydrograph.

In the spring, total N did not increase in the organic horizon or hillslope. In the stream, total N increased by 20%. NO_3^- increased by 50% and 60%, respectively, in the organic horizon and hillslope and 2.4 times in the lower stream. NH_4^+ did not increase in any of the sources.

Concentrations and characteristics by depth

In the fall, flow-weighted average concentrations of DOC (Figure 2.20) and phenols (Figure 2.21) decreased sharply from the organic horizon to the mineral soil, and then continued to decrease with depth in the soil profile. Of these, the change was most noticeable and consistent in the phenol concentration, which decreased by over 70% from the organic horizon to the shallow soil (20 cm depth), and by nearly 95% in the deep soil (70-110 cm). For both DOC and phenols, the concentrations in the hillslope were most similar to the concentrations in the middle to deep soil. The stream concentrations were higher than the hillslope, however, most similar to those between the shallow and middle soil depths.

DOC was higher in the organic horizon at the beginning of the fall storm, but mean and flow-weighted values appear lower than in spring because fall data included a prolonged sampling of DOC-depleted organic horizon leachate at the end of the storm.

In the spring, flow-weighted average DOC and phenol concentrations were highest in the organic horizon and decreased sharply in mineral soil, hillslope and stream. (Figures 2.20 and 2.21). Stream DOC and phenols were lower than in the soils, except for the right fork.

In the fall, flow-weighted average $SUVA_{254}$ (Figure 2.22) decreased by depth from the organic horizon to the shallow and middle soils, but then increased from deep soil to hillslope to stream. Variation in $SUVA_{254}$ showed no pattern with respect to source or depth in the spring.

Flow-weighted average DON:DIN (Figure 2.23) decreased in the fall from the organic horizon to the mineral soil, but then increased through the soil profile. DON:DIN in the stream was similar to the organic horizon and hillslope DON:DIN was similar to shallow soil. All forms of N (Figure 2.24) decreased from the organic horizon to the shallow soil, but then remained relatively flat through the soil profile, decreasing again somewhat in the hillslope and stream.

In spring, variation by source of flow-weighted average DON:DIN showed no clear pattern. Standard errors were large over both seasons, and in shallow and deep soil, there were one data point each. Average values of all N species in the spring were

lower than those in the fall. Inorganic N , particularly NH_4^+, was much lower. As in the fall, the largest decrease in DON was from the organic horizon to the mineral soil. There was little variation with depth in the soil, and the hillslope and stream concentrations were somewhat lower than in the soils.

Samples from the left and right fork of the stream, taken only in the spring, showed significant differences in flow-weighted average values (Table 2.1), with the right fork more like the shallow, or even the organic layer, in having much higher concentrations of DOC and phenols and higher SUVA than in other stream sites. The left fork, however, was more like the hillslope water in most respects. We also sampled the stream just above the point at which the hillslope weir discharged into the stream, to see if any variation took place between that point and 91 m downstream at the weir. A consistent pattern emerged, indicating that higher values in the right fork water and lower in the left fork combined to some intermediate value at the level of hillslope discharge device, but decreased further downstream.

Assays for reactive polyphenols compared

Over 80% of the total phenols assayed in organic horizon and right fork of the stream were reactive polyphenols, compared with less than 60% in the hillslope samples (Figure 2.25). In other sources, the reactive polyphenols made up 60-80% of total polyphenols. The salal standards were 95-100% reactive polyphenols.

Of 111 casein precipitation assays (Table 2.6) 72 resulted in zero or negative %RPP values. These zero results were likely related to the background absorbance values of the casein overcoming the very slight absorbance values differences that would have been expected, especially for dilute samples. Of those results > 0, the mean was 67.8% RPP (SE = 3.12, n = 39), and the difference (in %RPP) between the 5 duplicate tests with results > 0 were 31.0 (SE = 9.13).

The SPE procedure, using polyamide resin filled tubes, was sensitive enough to produce reproducible results at lower phenol concentrations than the casein procedure. The primary difficulty with the SPE method was to regulate the suction pressure to pass the samples and solvents through the tubes at a consistently slow rate. The solvents tended to pass through more quickly than the water, so the suction was constantly in

need of adjustment; therefore, variation in the results may have occurred due to this mechanical problem.

Of a total of 228 SPE measurements, 22 resulted in zero or negative %RPP values. These zero results were found in the most dilute samples, for which differences in absorbance values before and after passing through the tubes were small. Of those results > 0, the mean was 74.1% RPP (SE = 1.43, n = 167), and the difference (in %RPP) between the 45 duplicate tests with results >0 were 17.9 (SE=2.57).

Comparison of %RPP results using both methods excluding zero values, indicated a mean difference of 11.5 %RPP (SE = 2.22, n = 35). Because the %RPP was found by subtraction of phenol concentration in eluted sample from original sample, more dilute samples with low absorbance values were skewed to lower results. A decrease of one unit from 2 units results in 50% reactivity, compared to a decrease of one unit from 100, which results in 99% reactivity. This may contribute to the lower reactivity of phenols in samples from sources in which the concentration of phenols was much lower, for example, the hillslope (Figure 2.26).

Discussion:

We expected to find that water-soluble phenols were reactive with amide-N, and that they would decrease from their highest concentration in the organic horizon with depth in the mineral soil. We expected that the phenolic proportion of DOC would be significant, and that phenols would be correlated to DOC. Because phenols are aromatic and derived from the terrestrial DOC pool, we expected that the proportion of phenols in DOC would be correlated to $SUVA_{254}$. We expected that phenol concentration would be positively correlated to DON:DIN or DON, in the organic horizon, or perhaps in the soils or stream.

Reactive polyphenols

Water-soluble phenols in WS10 were approximately 74% reactive with amide-N overall, indicating a potential for formation of polyphenol-protein complexes. Reactive polyphenols may conserve N by complexing it and binding to organo-mineral surfaces in the soils, but could also contribute to N loss via leaching from the system during

storms. Our investigation of the relative concentrations of phenols in WS10 soils and surface water provided clues to both processes. The organic horizon and right fork of the stream had a higher proportion of reactive polyphenols than sources in which the water had already been filtered through soils, perhaps removing preferentially the more reactive phenols.

Hydrologic processes

Hydrology can affect the nutrient dynamics and distribution in a catchment (McGlynn and McDonnell 2003), therefore a discussion of these processes is important before interpretation of the phenol, DOC, and N results. Many studies have focused on hydrological controls over C and N processes, emphasizing the importance of riparian and hyporrheic zones (Smith et al. 2008). We found that the distribution of phenols, DOC, and N through the soils and stream were also affected by antecedent wetness conditions, which were dry in fall, and wet in spring. The distribution changed over the course of the storms, particularly in the fall. Hydrology also affected the delivery of nutrients to the stream from the hillslope, again, more so in the fall. Riparian and hyporheic processes are less important in this steeply dissected bedrock channel stream, but as the stream rises during a storm, it may intersect with nearby organic horizon and shallow soils.

Kirchner's (2003) "Double Paradox" of catchment hydrology and geochemistry asks: 1.) if catchment water has a residence time measured in days to years, why do we see changes in the hydrograph nearly instantaneous with precipitation events? and 2.) if the water discharged is mostly "old", how does the chemistry of the water reflect chemistry of water inputs, i.e., high concentrations of nutrients from decaying vegetation in the litter layer?

We found that the hydrograph of the stream and hillslope were nearly synchronous with one another, and with precipitation in the fall. Fiori et al. (2007) describe a steep catchment with gradations of conductivity by depth, in which the soil conductivity is high. The stormflow rapidly enters the soil, and enhances the groundwater, developing a "groundwater mound" at the lower end of the hillslope. Streamflow development is rapid, as we saw in WS10.

What has puzzled hydrologists is the fact that although the concentrations of some conservative tracer elements are dampened in stream and deeper soil waters, other nutrients respond immediately to flow regimes (Kirchner 2003). The difference in nutrient chemistry between stream water and the more dilute baseflow water may be due to preferential flowpaths, riparian or hyporrheic processes, or a combination of these (McGlynn and McDonnell 2003, Hood et al. 2006, Van Verseveld 2007).

Change in aromaticity of DOC over a storm indicates a change in the source pool of nutrients (Hood et al. 2006, Van Verseveld, 2007). In the hillslope and stream, the proportion of phenols to total DOC changed over the course of the storm. While the proportion decreased steadily from an early peak in the stream, in the hillslope, it rose sharply with the peak of the hydrograph, then fell to below stream levels on the receding limb (Figure 2.15). In contrast, $SUVA_{254}$ decreased steadily in both the stream and hillslope (Figure 2.13).

We argue that phenols may be a particularly useful tracer of source pools. The delayed increase in phenolic DOC in the hillslope compared to the stream (Figure 2.15) may indicate that early in the storm, stream phenols are derived from different source waters, such near-stream or riparian. The removal of phenols by depleted fall soils may delay their delivery to the hillslope component on the rising limb. Within the hillslope, macropore flow during the peak of the storm may bypass the adsorption or metabolic processes to deliver higher concentrations of phenols to the hillslope discharge. Also, as the water table rises during peak precipitation, it may intersect increasingly with surface soils near the base of the hillslope, accessing nutrients and phenols.

In the spring, we collected samples from the upstream left and right fork of the stream, as well as just above the output of the hillslope collection device. By comparing concentrations of DOC, phenols, and N from these stream locations, it was evident that the left fork, a continuation of the main branch with a bedrock channel, contributed water that was more dilute in nutrients compared to downstream water and more similar to hillslope samples. The right fork was a very small but perennial stream, running over and through a thick organic layer. This small branch contributed a significantly higher nutrient load to the main stream, most likely because of its access to the organic

horizon, in contrast to the main branch which is steeply incised and has a bedrock channel. These observations were similar to those of Van Verseveld (2007), who examined C and N concentrations in WS10.

Antecedent wetness conditions were very important in explaining the differences we found between fall and spring phenol, DOC and N concentrations. In the fall, antecedent conditions were dry and baseflow prior to the storm was low and declining. In the spring, soils had been well-saturated by snowmelt and frequent storms. Baseflow was declining, but much higher than in the fall.

The decrease of flow-weighted DOC, phenols, and $SUVA_{254}$ by depth in the mineral soil was gradual in the fall, but abrupt in the spring. In the dry antecedent conditions of fall, abiotic processes may be more responsible for removal of DOC components. In the spring, microbial processes may cause the sharp drop in DOC and its constituents from organic horizon to shallow soil. In the fall, these concentrations continue to drop through the soil profile, but in spring, the concentrations remain essentially unchanged with depth in mineral soil.

Despite very different conditions in the fall and spring, the phenol concentration increased dramatically in both hillslope and stream at the onset of the storms. The phenol concentration was higher in the organic horizon in the fall than in the spring, since the accumulated litter had not been flushed by frequent precipitation events. The deciduous trees, including *Acer macrophyllum* and *Alnus rubra*, which grow mostly near the streams, were dropping their leaves in the fall. The leaves of these two trees have much smaller tannin content (0.63 to 0.96 %wt), than the needles of *Pseudotsuga menzeinsii* (5.61 %wt) (Hernes and Hedges, 2004) but the mass of leaves that are deposited on the forest floor in the fall may contribute significantly to the polyphenol stores.

In the spring, the organic horizon and soils had been nearly continuously flushed by repeated storms and snowmelt, still the phenol concentration in the organic horizon was high. Phenols dropped very steeply from the organic horizon to shallow soil, and then remained essentially unchanged in deeper soil profiles. Needles and leaves from the conifers and ericaceous shrubs, whose tissues have a much higher %wt of tannins

than deciduous species, continue to lose their leaves all year long. Snow cover may have slowed the decomposition of litter during the winter. With rapid melting and warmer temperatures, microbial processes as well as abiotic adsorption may account for this dramatic disappearance of phenolics in the soil.

Our results were similar to Van Verseveld (2007) in that during the fall transition period, DOC and DON concentrations were higher in the stream than in the hillslope, but during the spring wet period, the DOC, DON, and NO_3^- concentrations were similar in the stream and hillslope. We also found that $SUVA_{254}$ and phenol concentration were higher in stream than the hillslope in fall, but similar in the spring.

During precipitation events, the "flushing response" causes DOC concentrations to peak prior to the hydrograph peak. A clockwise hysteresis pattern of DOC versus flow indicate that during storm events, flow volume does not predict concentration (Hood et al. 2003, Hood et al. 2006). DOC and particularly the phenol concentrations increased rapidly during the rising limb in the hillslope runoff. The rapid increase in hillslope DOC and phenols may be accounted for by mixing of near surface water with baseflow. The deeper soil profiles showed a delay in increased DOC and phenol concentration over the fall storm (Figure 2.12), therefore the increased DOC and phenol concentration in the hillslope discharge did not result from immediate downward percolation of nutrients at the surface, but from mixing of surface and deeper water, perhaps at the base of the hillslope or via preferential flowpaths. Near-stream runoff and leaching from shallow soil and litter layers during high flows may also be responsible for nutrients in the stream.

Correlation of phenols to DOC, aromatic C and N

Water-soluble phenols were highly correlated to DOC in the fall, except in the soil layers (Table 2.2), where much of the phenolic DOC may have been preferentially removed from solution by adsorption or metabolized. This is consistent with the steep gradient in DOC, aromatic DOC, and phenolic DOC between the organic horizon and mineral soil, which occurred both in fall and spring (Figure 2.6).

Fall phenolic DOC averaged 11.6% (st.dev. = 4.3%) in all sources. In the spring, when antecedent conditions were saturated, phenols made up less of the DOC, but the

proportions were more consistent (mean = 8.9%, st.dev. = 1.7%) between organic horizon, soils, hillslope and stream sources. This suggests that an equilibrium had been reached in the soils, so that productions of phenols was keeping pace with adsorption and metabolic processes. While Smemo et al (2007) estimated that approximately 2.5% of DOC was phenolic in northern forest soil control plots at 75 cm depth, we estimated phenolic DOC content of deep soil at 4.8% in fall and 9.3% in the spring. Smemo et al. (2007) used a 0.45µ filter, which may have removed larger polyphenol complexes that passed through our 7µ filters. We found that phenols and $SUVA_{254}$ were higher in deep soils in the spring than in the fall, as did Smemo et al. (2007). The conifers and ericaceous shrubs of WS10 do produce more tannins than deciduous trees of northern hardwood forests, but the difference could also have been due to the variation in tannin standards used.

Phenols were highly correlated to UV_{254} except in soil layers during the fall, where the phenolic aromatic DOC had likely been preferentially removed from solution. Phenols accounted for over half of aromatic DOC in the organic horizon and stream in the fall. In the spring, phenols were highly correlated to UV_{254} in all sources. The saturated soils in spring had likely reached an equilibrium with respect to phenols and aromatic C.

The aromaticity of DOC was similar in both the organic horizon and in the stream during the fall, and lower in the mineral soils and hillslope. Phenols accounted for an estimated 63.6% of the aromatic DOC in the organic horizon leachate, and 65.8% in the stream during fall. We expected that $SUVA_{254}$ could be used as an approximation of phenols in DOC, but the significant differences between phenolic DOC (Figure 2.15) and aromatic DOC (Figure 2.13) during the fall storm argues against this. Also, the proportion of phenolic C to aromatic C differs by a factor of 2 between sources in the same season.

Northup et al. (1995) found that the DON:DIN was positively correlated to condensed tannins (as catechin) and total phenolics (as tannic acid) in their study, but also that litter phenolic content was positively correlated to DON and negatively correlated to DIN. We found significant correlation between DON:DIN and phenol

concentrations only in the fall lower stream, and spring right fork of the stream. Still, we cannot rule out the hypothesis that phenols may be important factors in DON sequestration and transport.

When we examined the regression of DON on phenols (Table 2.4 and Figure 2.9), we found significant correlation in the organic horizon, deep soil, and lower stream in the fall, and suggestive evidence of positive correlation in the spring right fork of the stream. The highest proportion of reactive polyphenols were also found in the organic horizon and right fork of the stream.

The correlation between DON and phenols in deep soil, however, may have been an artifact, as there were only 4 data points, the slope is inconsistent with others, and there is no reason to expect a correlation between DON and phenols in deep soil. The correlation in lower stream in the fall is also somewhat questionable, as the phenol concentration predicted only 7% of the DON concentration. Our study was hampered by exceedingly low N concentrations, reducing the numbers of paired data for evaluation. The nitrogen concentrations may have been so low that polyphenol-protein binding was impaired. The binding of polyphenols and proteins depends on the relative concentration of each (Hagerman et al. 1998a). Also, we restricted our study to the water-soluble phenols, while methanol and acetone-extracted phenols in soil are likely important in the sequestration of DON (Nierop et al. 2006).

NH_4^+ levels were much higher in the fall than in the spring. Inorganic N has been seen to increase after fall rains at Cascade Head, Oregon, as it did at HJA. (Asano et al. 2006). The decreased NH_4^+ in all sources in spring may be related to immobilization and mineralization processes. NH_4^+ averaged 0.020 mg/L in the fall (n = 11), and 0.003 mg/L in the spring (n = 7), however, in most samples, NH_4^+ was not detected).

NO_3^- concentrations in streams are low and seasonal in aggrading systems (Vitousek and Reiners 1975). In a long term study, NO_3^- concentrations in three mature or old-growth forest streams in HJA averaged 0.001 to 0.004 mg/L and NH_4^+ concentrations averaged 0.007 to 0.009 mg L^{-1} (Vanderbilt et al. 2003). In our study,

NO_3^- concentrations averaged 0.005 mg/L in the spring (n = 18) and 0.007 mg/L in the fall (n = 26).

Interestingly, the decrease in flow-weighted phenol concentration through the soil profile is accompanied by an increase in DON:DIN (Figures 2.21 and 2.23): a relationship that is quite the opposite of our expectations. Some of the increased DON:DIN is related to the sharp decrease in DIN through the soil profile that is evident in the fall. Also, the positive correlation we expected to see between phenols and DON:DIN would likely take place in the organic horizon (binding) and stream (transport). In the soils, phenols bound to mineral surfaces may not be detectable by our assay, though still may be actively binding DON.

Assays for reactive polyphenols

We found that the SPE method was superior to the casein precipitation method for separating the reactive portion of phenols in dilute environmental samples. Both methods gave results within ~10% for the same samples. The variation between assays for the same sample was less than the variation between duplicate samples using the same test (17.9% for SPE and 31.0% for casein). However, the casein precipitation method gave values of zero for the majority of the assays, particularly for more dilute samples. The primary difficulty with the SPE method was maintaining a consistent suction, since solvents tended to pass the tube more quickly than the samples due to differences in viscosity.

Conclusion:

Changes in DOC and phenols in their pathways from the organic horizon to the stream suggested mechanisms controlling nutrient flux in this N-limited headwater catchment. Most importantly, the change in proportion of phenolic DOC over the course of a fall storm in the hillslope and stream suggested changes in source pools.

We had expected that hillslope water would not represent a significant source of nutrient supply to the stream because of its primary “old water” composition. However, hillslope DOC and phenol concentrations were strongly correlated to stream concentrations, and higher than expected. We found that a small tributary stream

provided a significant portion of nutrients to the main channel, and suspect near-stream sources may provide the remainder of the differences in organic matter content seen between stream and hillslope values. In this headwater catchment, hyporheic and riparian processes played much less of a role in nutrient dynamics (except in the right fork of the stream) than we would expect in most catchments, because of the bedrock channel.

We estimated that the average proportion of phenolic DOC is 15.8% in the fall stream, 16.6% in the organic horizon leachate, but only 4.8% in the deep soil. In our spring samples, the proportions varied less: from 6.3% in the left fork of the stream to 12.1% in the right fork, and were more consistent with depth. Phenol concentrations in the organic horizon were higher prior to the fall storm than at the onset of the spring storm, but were much lower in the soil profile and in the stream over both seasons. The ratio of phenols to DOC was positively correlated to $SUVA_{254}$ (as evidenced by phenols to UV_{254}) in all sources over both seasons, except in the soils during fall. Where correlations existed ($p \leq 0.05$), the slopes were consistent and linear. The use of $SUVA_{254}$ as a proxy for phenols, however, is not supported, because the phenolic proportion of aromatic DOC varied more than $SUVA_{254}$ between sources and over time.

We found no evidence supporting our hypothesis that DON:DIN would be positively correlated to phenols in the organic horizon, as Northup et al. (1995) found. However, we found positive correlations between DON:DIN and phenols in the fall stream, and suggestive evidence of positive correlation in the right fork of the stream in spring. An important consideration is that we only examined water-soluble phenols, which may be more important in N flux in streams than in organic horizon sequestration processes. We found positive correlations between phenols and DON in the organic horizon and lower stream in the fall, however, and also suggestive evidence of a correlation in the right fork in the spring. The increase in DON:DIN in the Northup et al. (2006) study is driven more by the decrease in inorganic N than the increase in DON. We found that DIN in WS 10 changed very little in the spring and inconsistently in the fall, so that DON:DIN was less informative. While the average %DON was nearly the same in the stream over fall and spring (93.7% and 94.4%, respectively), it

was generally lower in fall soils and higher in spring soils, due to antecedent moisture conditions and biogeochemical processes.

The positive correlations between DON:DIN and DON and phenols in stream samples supports our hypothesis that reactive polyphenols are a mechanism of transport of DON in N-limited headwater catchments.

We found a new method for fractionating reactive polyphenols that performed more reliably than casein precipitation for dilute environmental samples, using Solid Phase Extraction with polyamide filter tubes. Fractionation of water samples with SPE may provide more detailed characterization of the polyphenols if large enough samples are used so that the eluted portion is sufficient for elemental analysis. We estimated that the water-soluble phenols were approximately 74% reactive with amide-N.

We did not have sufficient sample for analysis of the eluent fraction, but in future, such analysis may help determine the composition of reactive polyphenols, and their N content.

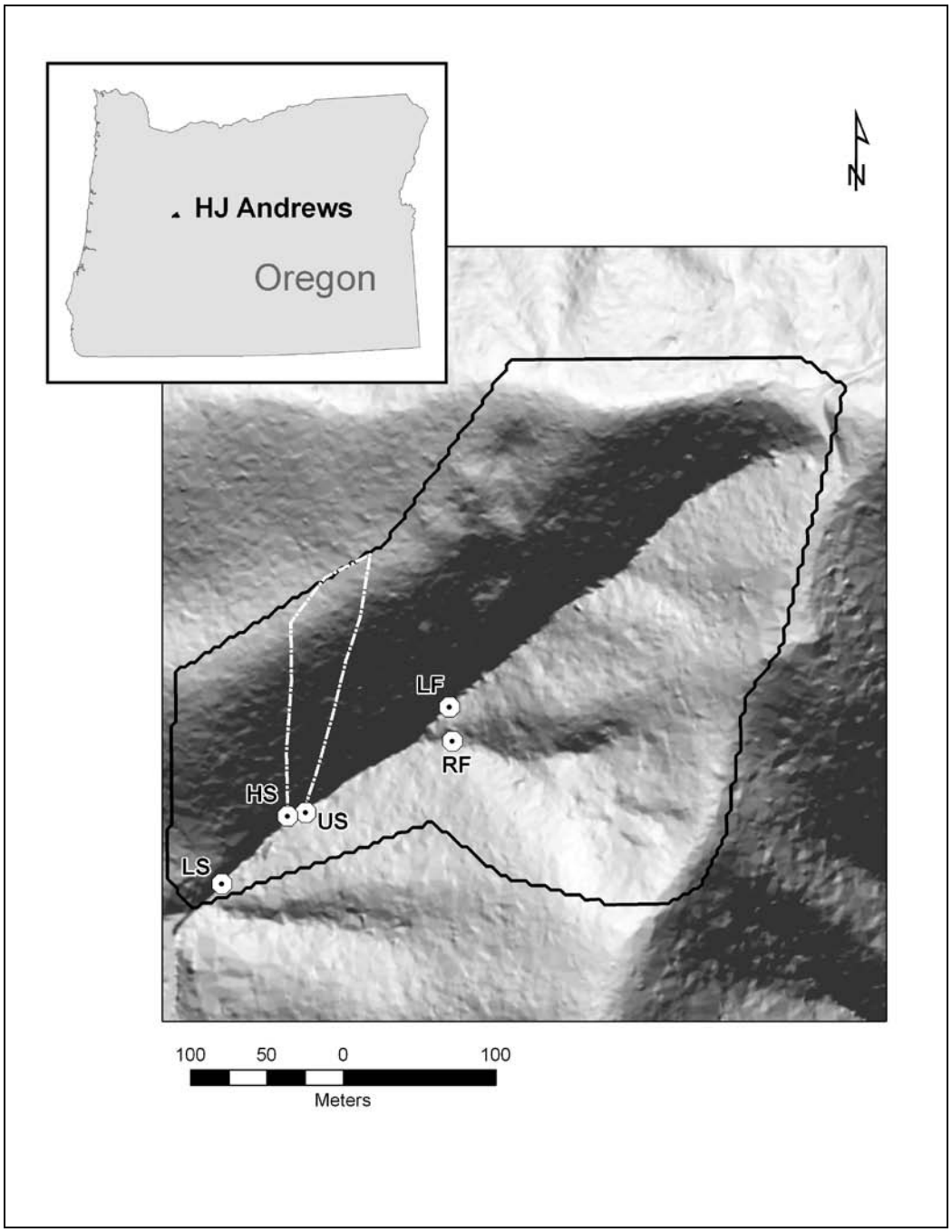


Figure 2.1: Study site: Watershed 10 in H. J. Andrews Experimental Forest, Western Cascades, Oregon. Sample collection sites are left fork (LF), right fork (RF), upper stream just above hillslope outflow (US), hillslope discharge (HS), and lower stream at gauging station (LS). The area enclosed by dashed line is the hillslope collection area, in lower half of which the lysimeters are positioned.

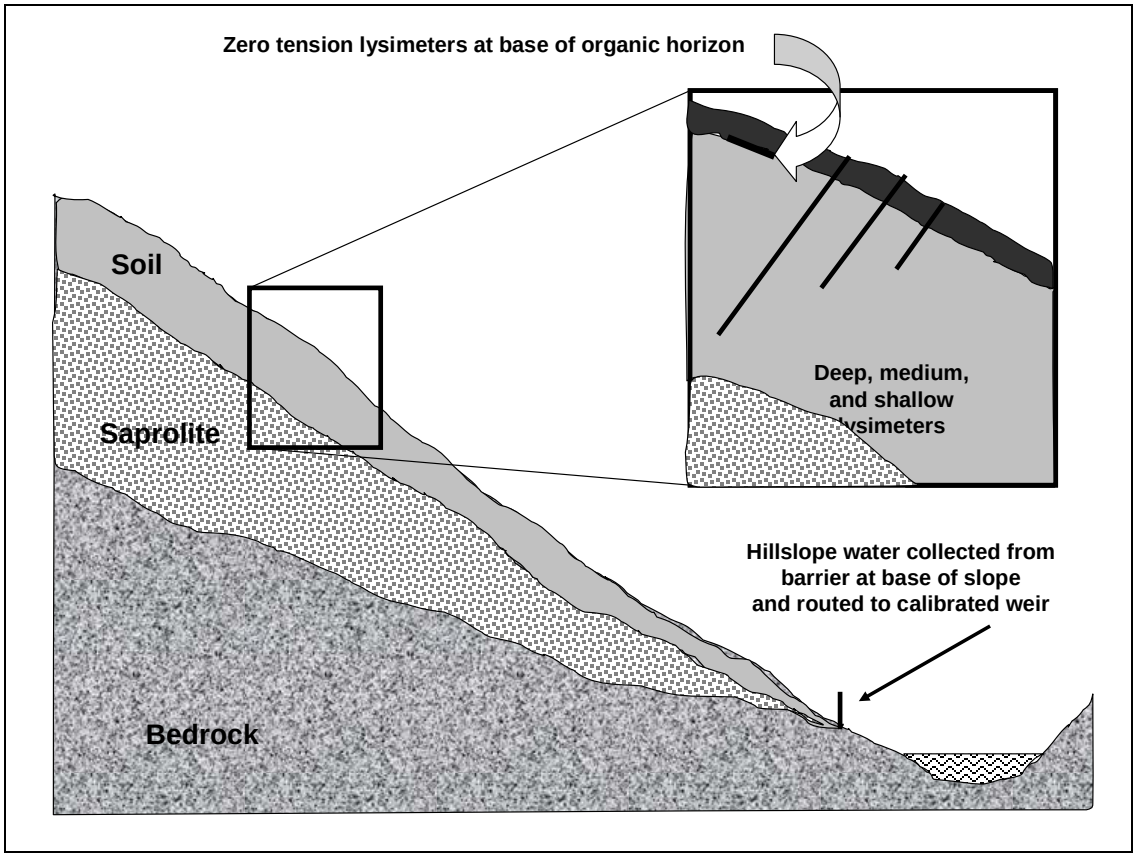


Figure 2.2: Diagram of hillslope soil water collection. Zero tension lysimeters at the base of organic horizon, tension lysimeters at three depths within the soil. Impermeable barrier at base of slope collects all water draining hillslope into a calibrated weir, from which sample is collected.

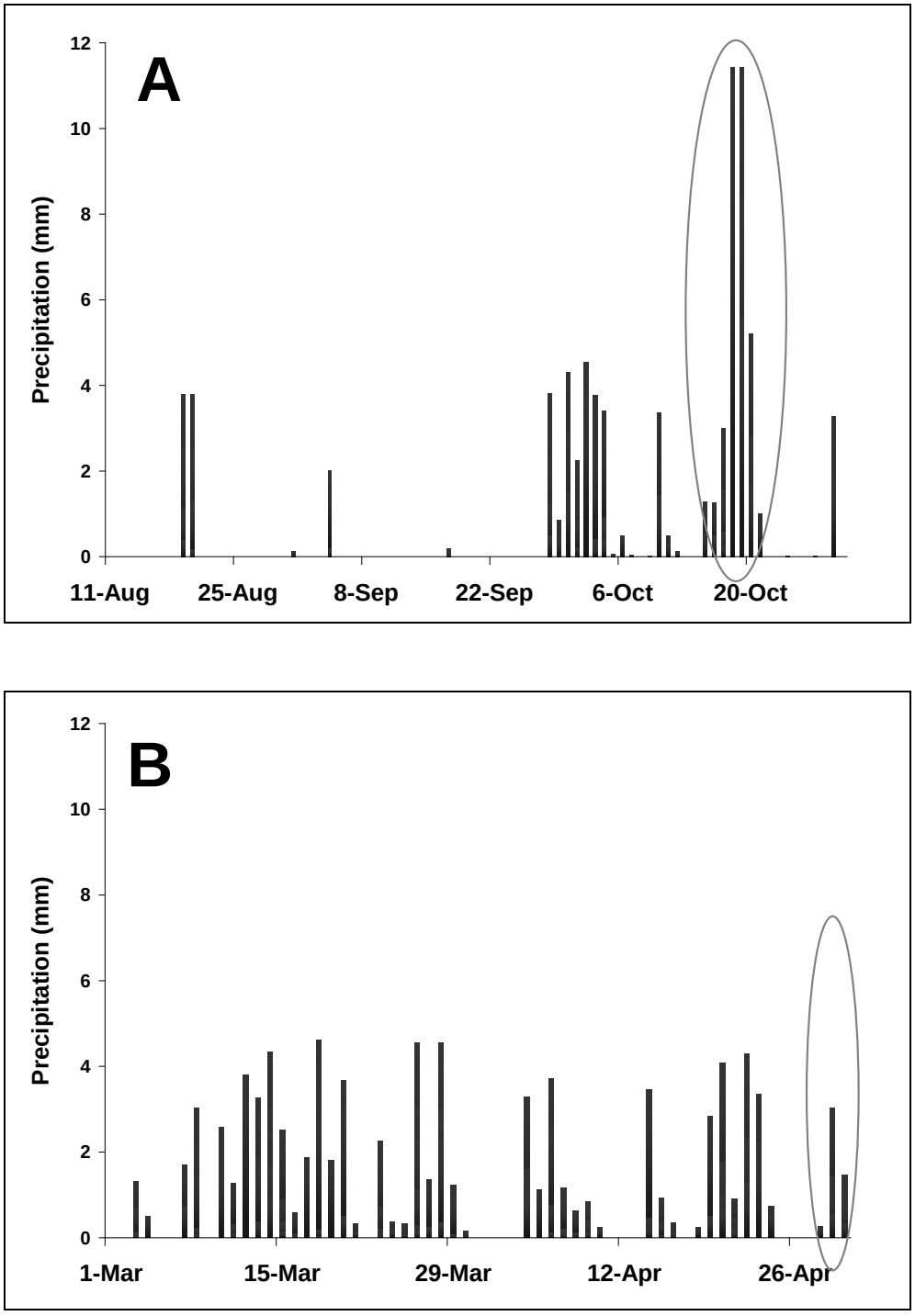


Figure 2.3: Precipitation records from Primet weather station near WS10. Study periods circled.
A) from 11 August through 31 October, 2007 B) from 1 March through 30 April, 2008.

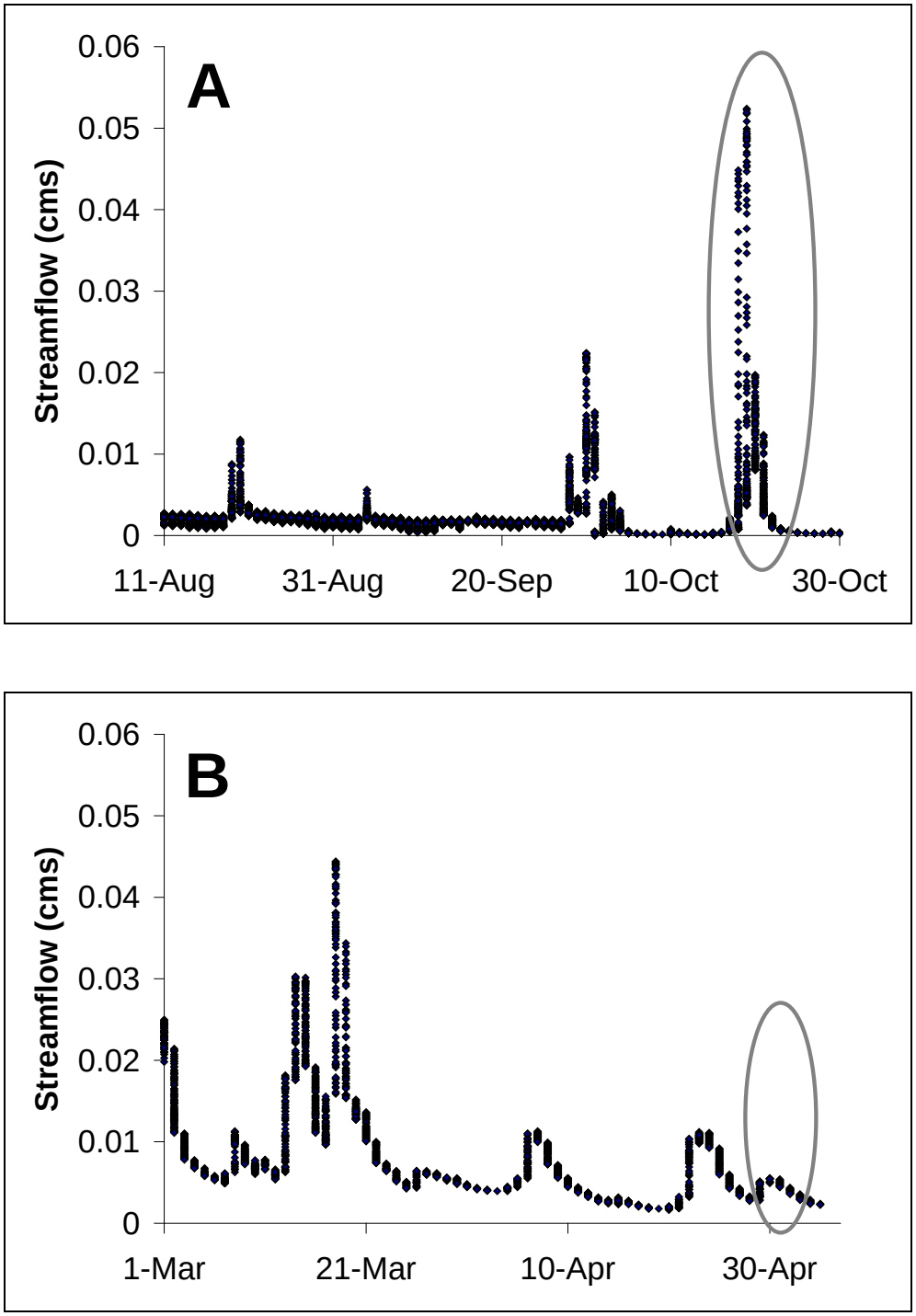


Figure 2.4: Hydrographs of WS10 with study periods circled. A) late summer and fall, 2007. B) spring 2008. During the spring, snowmelt is also occurring.

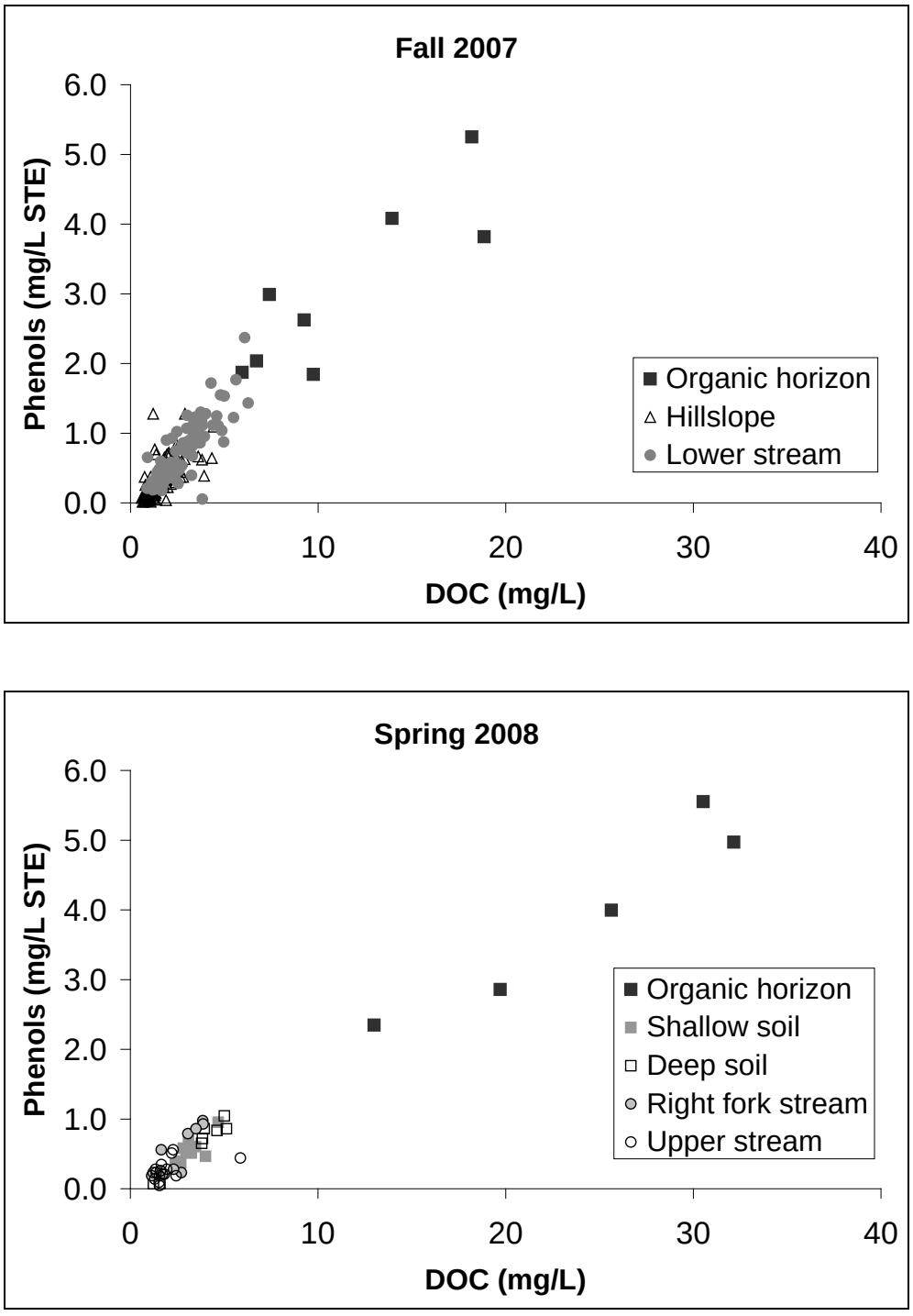


Figure 2.5: Relationship of phenols to DOC for fall, 2007 and spring, 2008. Only sources with significant correlations are shown (p value < 0.05). Refer to Table 2.2 for linear regression results.

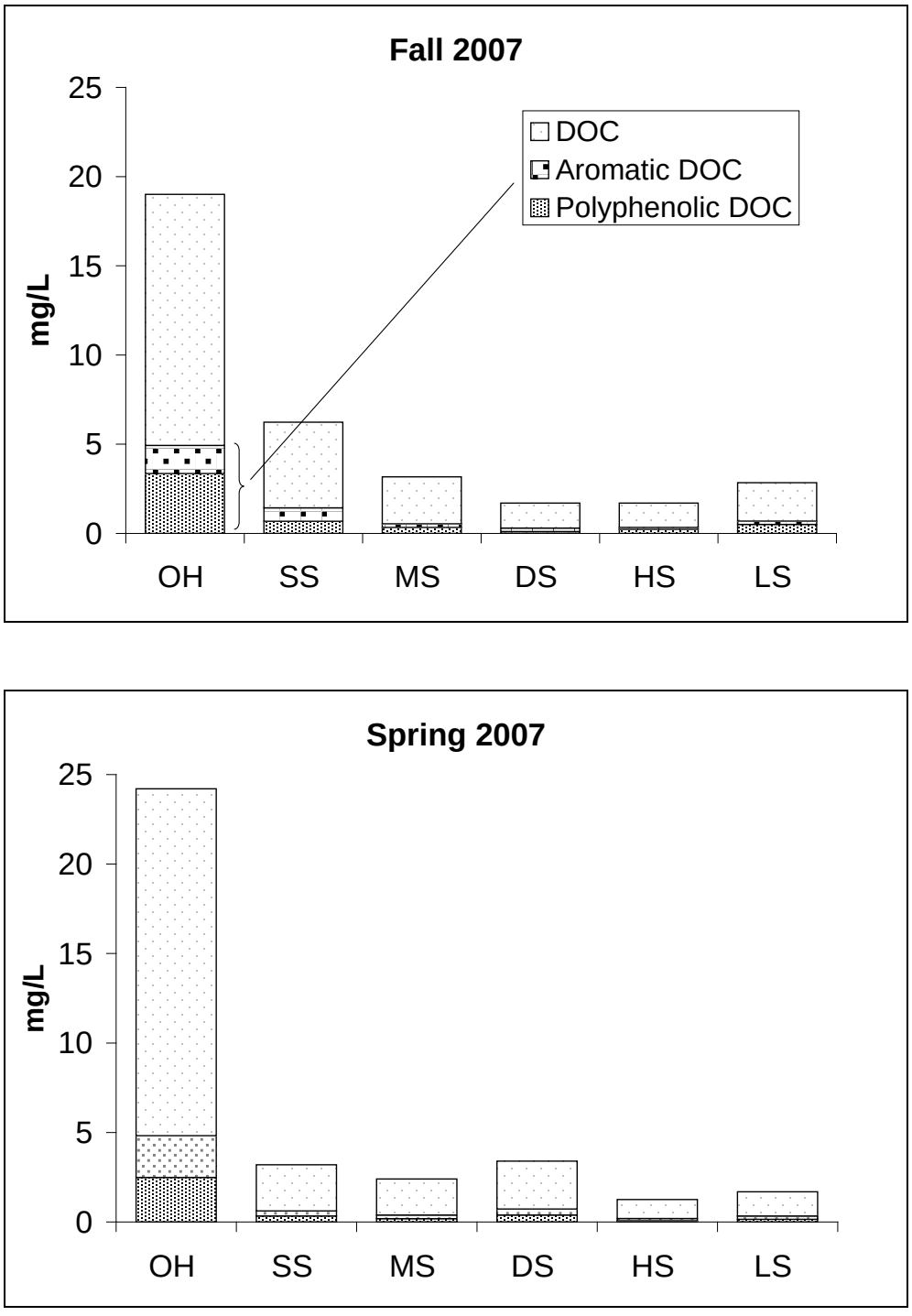
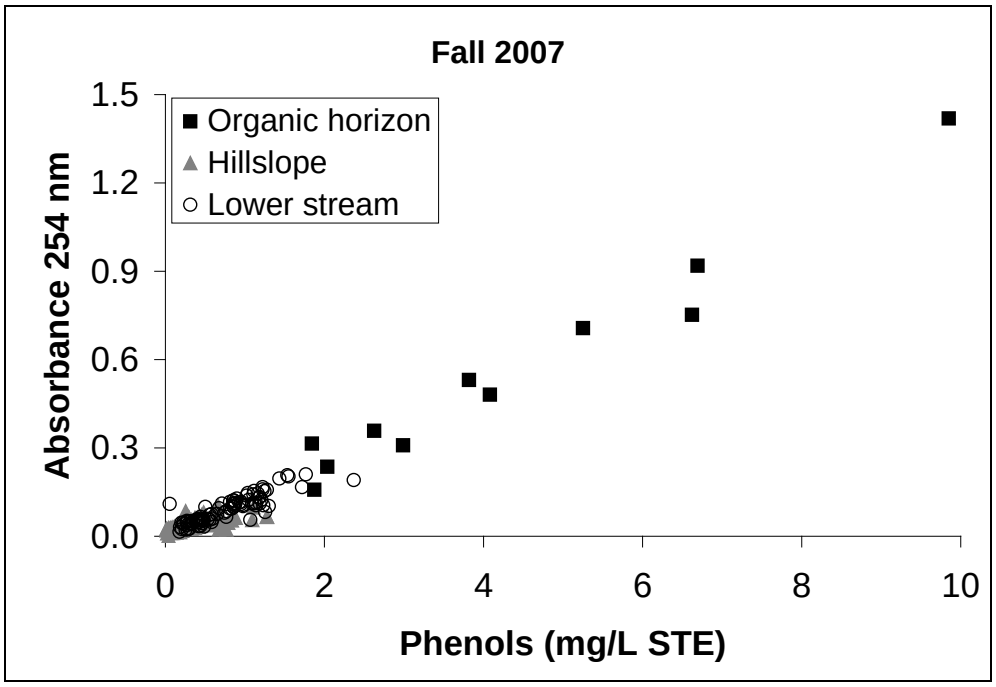


Figure 2.6: Comparison of mean DOC, with estimated aromatic DOC and phenolic DOC by season and source, in organic horizon (OH), shallow soil (SS), middle soil (MS), deep soil (DS), hillslope (HS), lower stream (LS). Phenolic DOC is a subset of aromatic DOC.



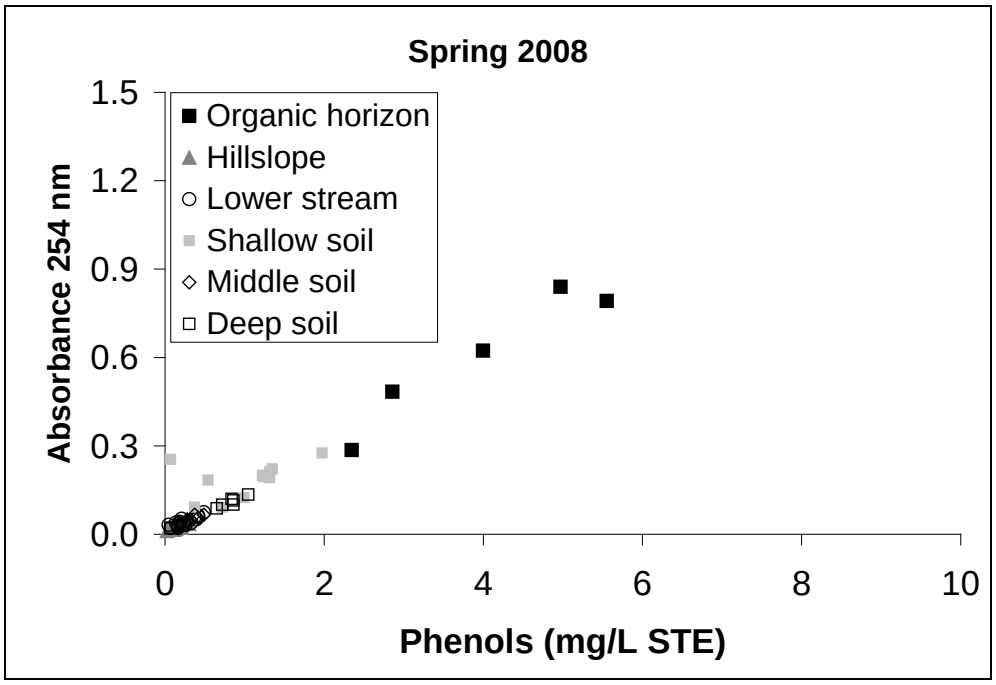
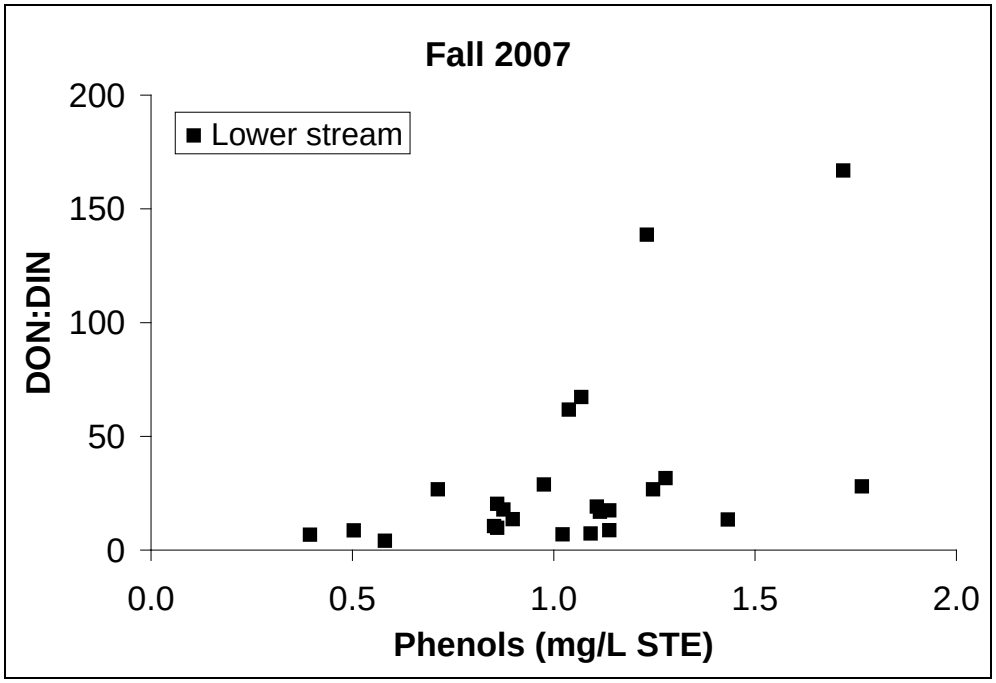


Figure 2.7: Relationship of absorbance at 254 nm to phenols for fall, 2007 and spring, 2008. Only sources with significant correlations (p value < 0.05) are shown for fall, 2007. All sources in spring were significantly correlated, but three other sampling sites on the stream are not shown. Refer to Table 2.2 for linear regression results.



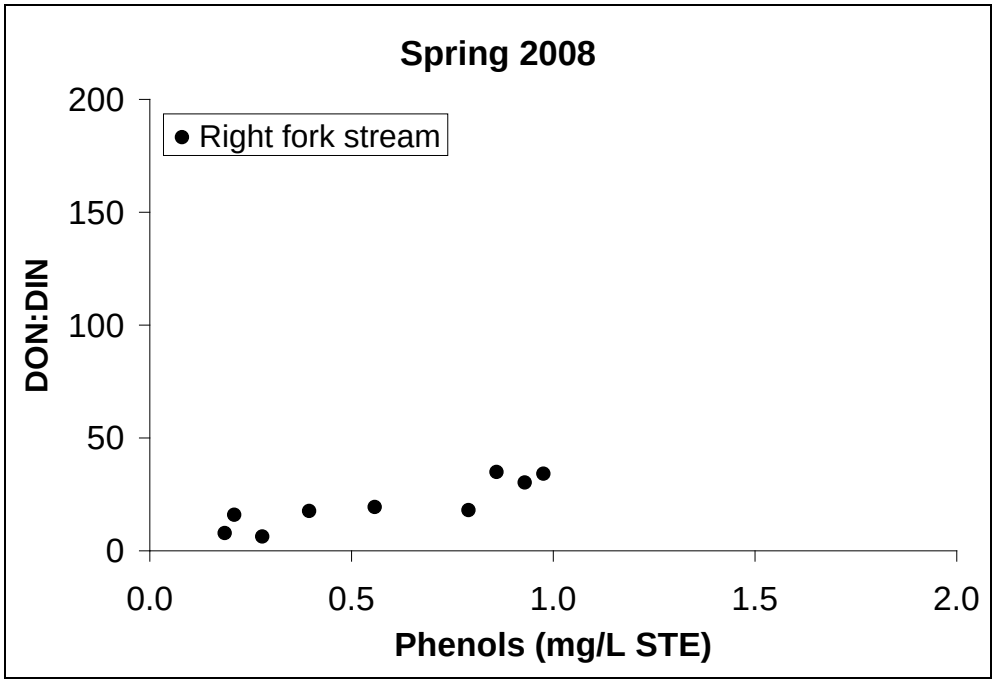


Figure 2.8: Relationship of DON:DIN to phenol concentration for fall, 2007, and spring, 2008. Only sources with significant correlations (p value < 0.05) are shown. Refer to Table 2.4 for linear regression results.

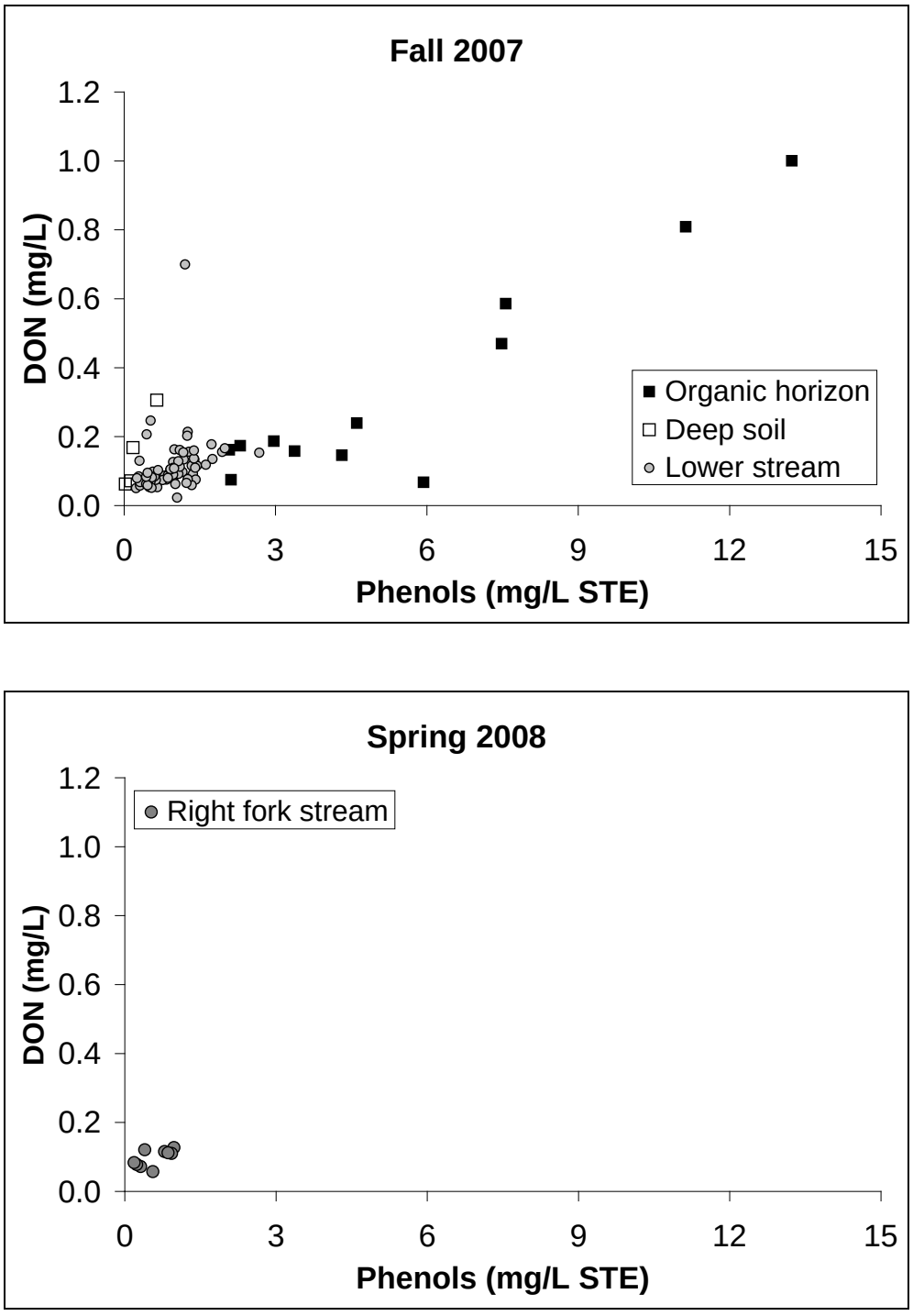


Figure 2.9: Relationship of DON to phenol concentration for fall, 2007, and spring, 2008. Fall sources with significant correlations (p value < 0.05) are shown. In spring, only the regression of DON on phenol concentration in the right fork of the stream was suggestive of correlation (p value = 0.0651), and is shown on the same scale as in the fall for comparison. Refer to Table 2.4 for linear regression results.

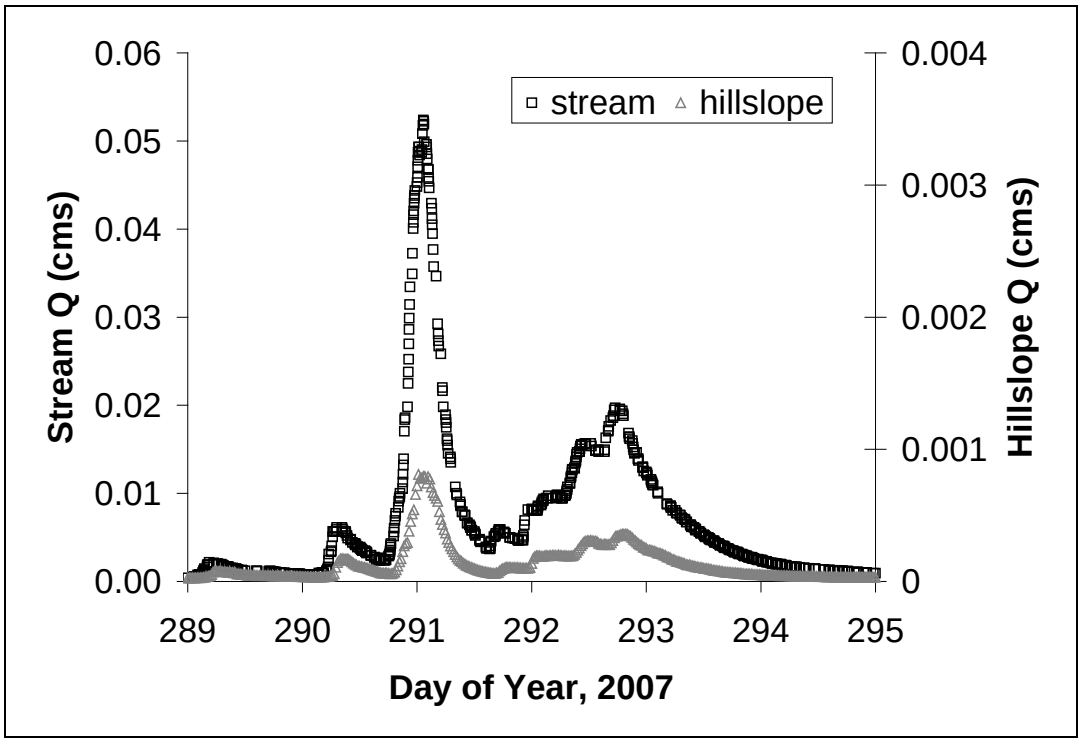


Figure 2.10: Stream and hillslope hydrographs.

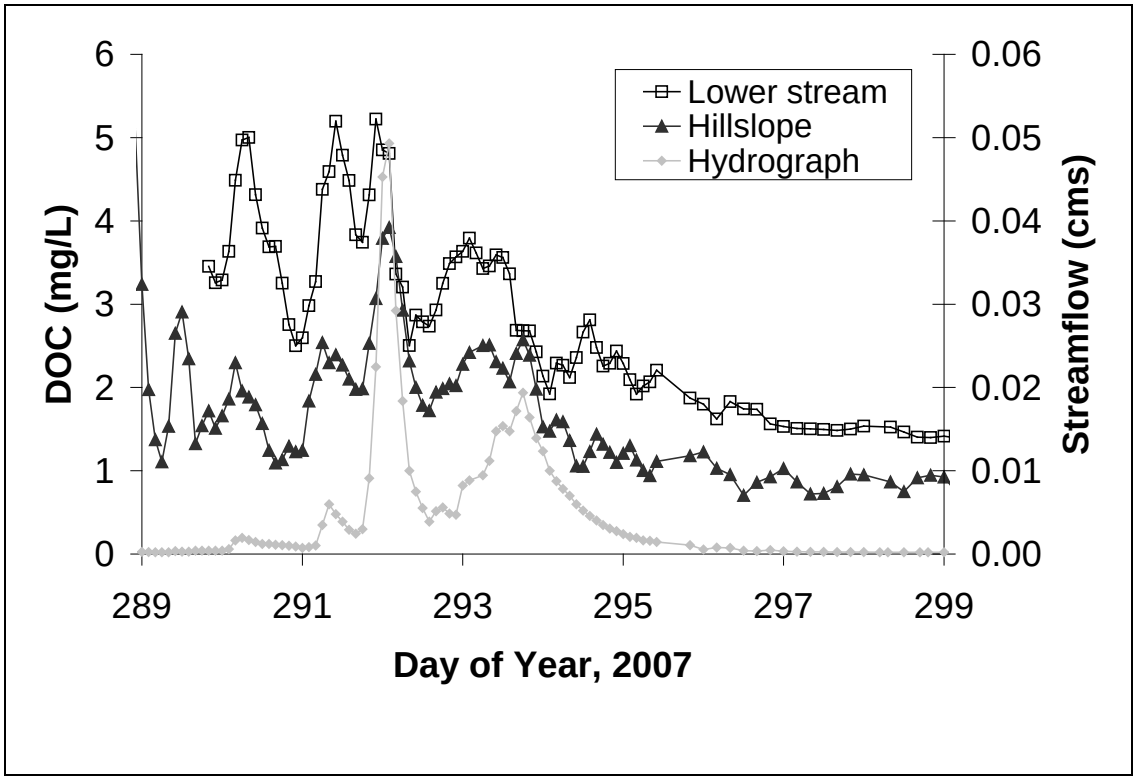


Figure 2.11: Comparison of DOC peaks (smoothed) in stream and hillslope water over fall 2007 storm. Data was smoothed by averaging each data point with the preceding and following point.

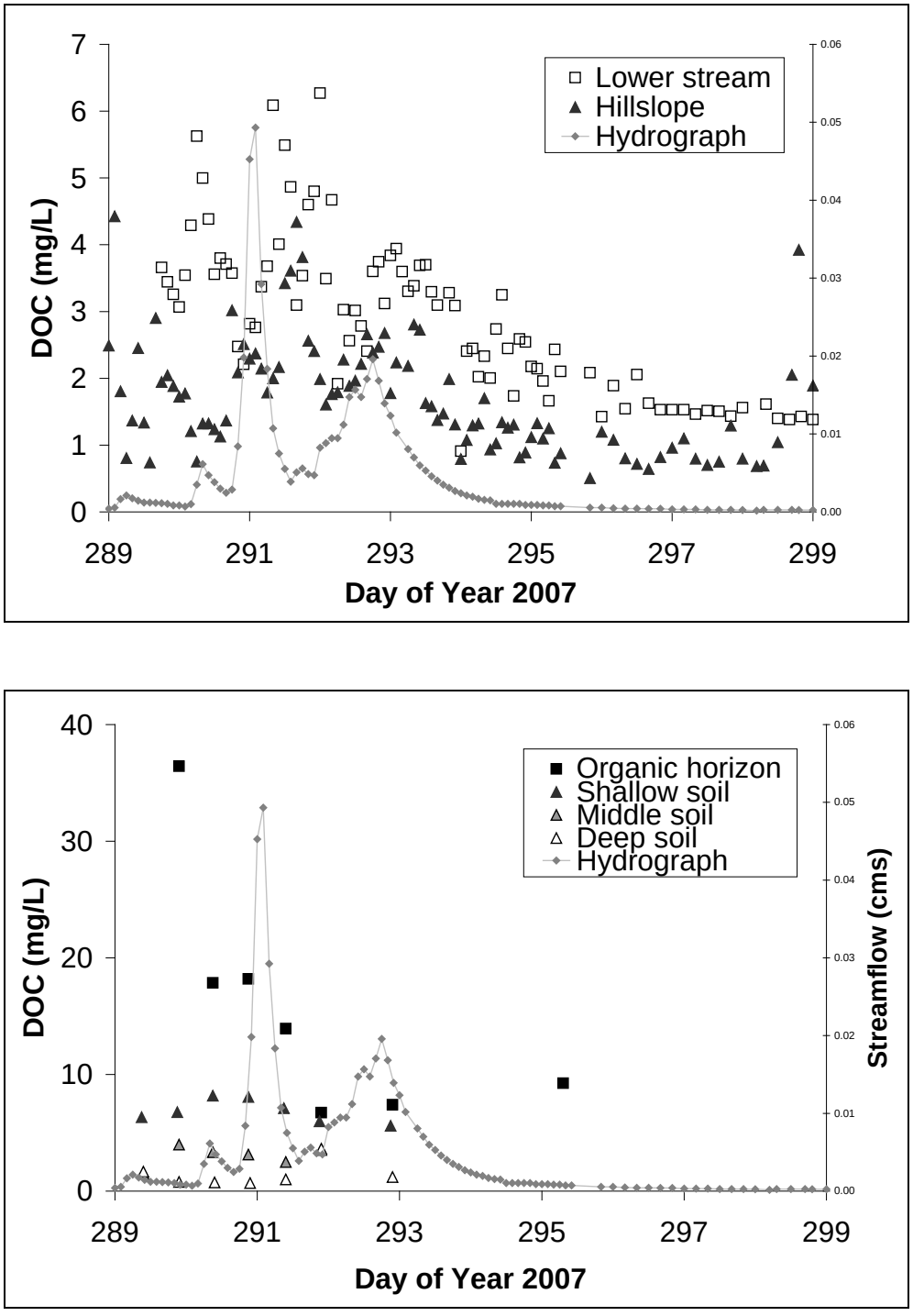
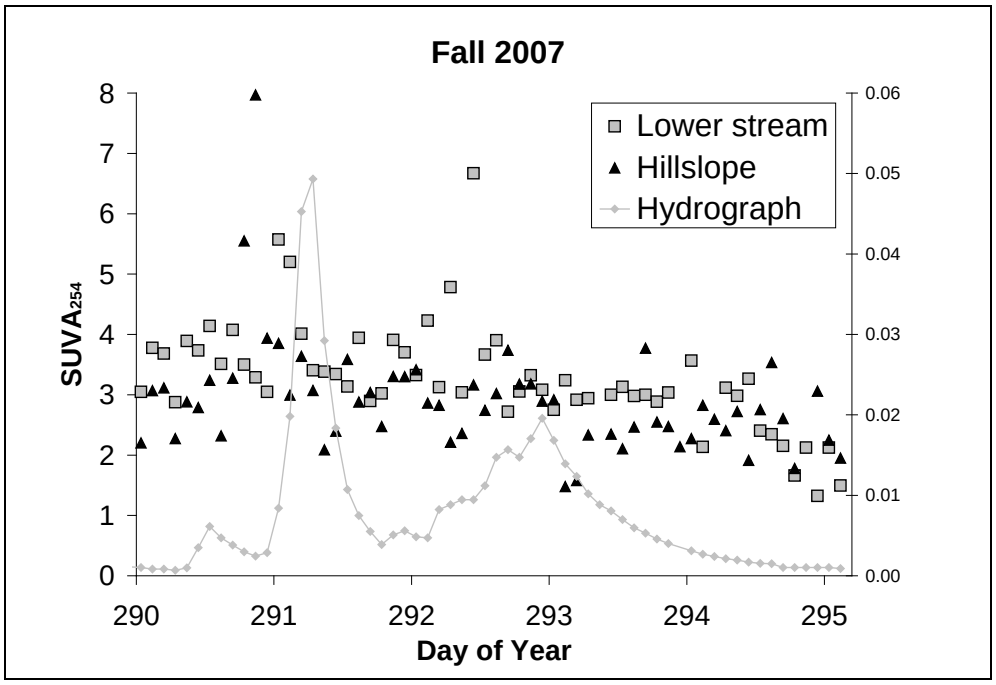


Figure 2.12: DOC in stream and hillslope; and in soils, over fall 2007 storm. Note different scale on DOC axis on second plot.



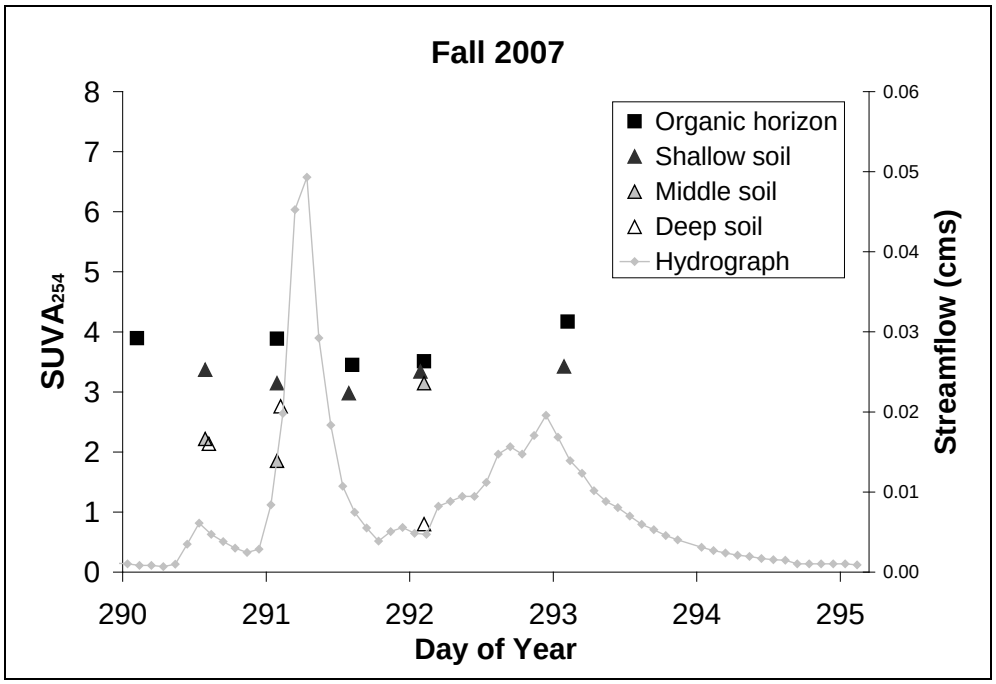


Figure 2.13: $SUVA_{254}$ over fall 2007 storm in stream and hillslope, and in organic horizon and soils.

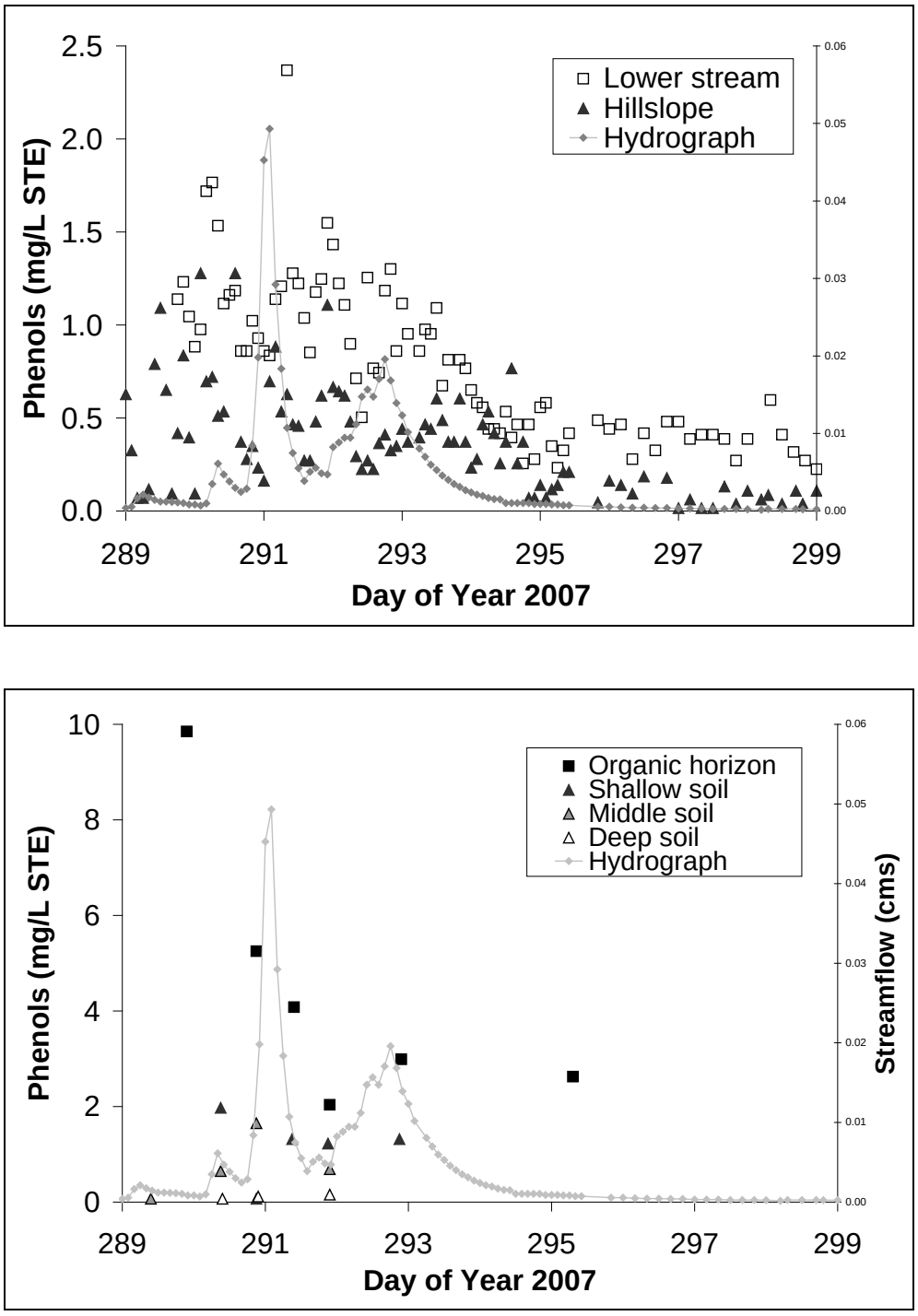


Figure 2.14: Phenol concentrations over fall 2007 storm, in stream and hillslope, and in soils. Note different scale on phenols axis on second plot.

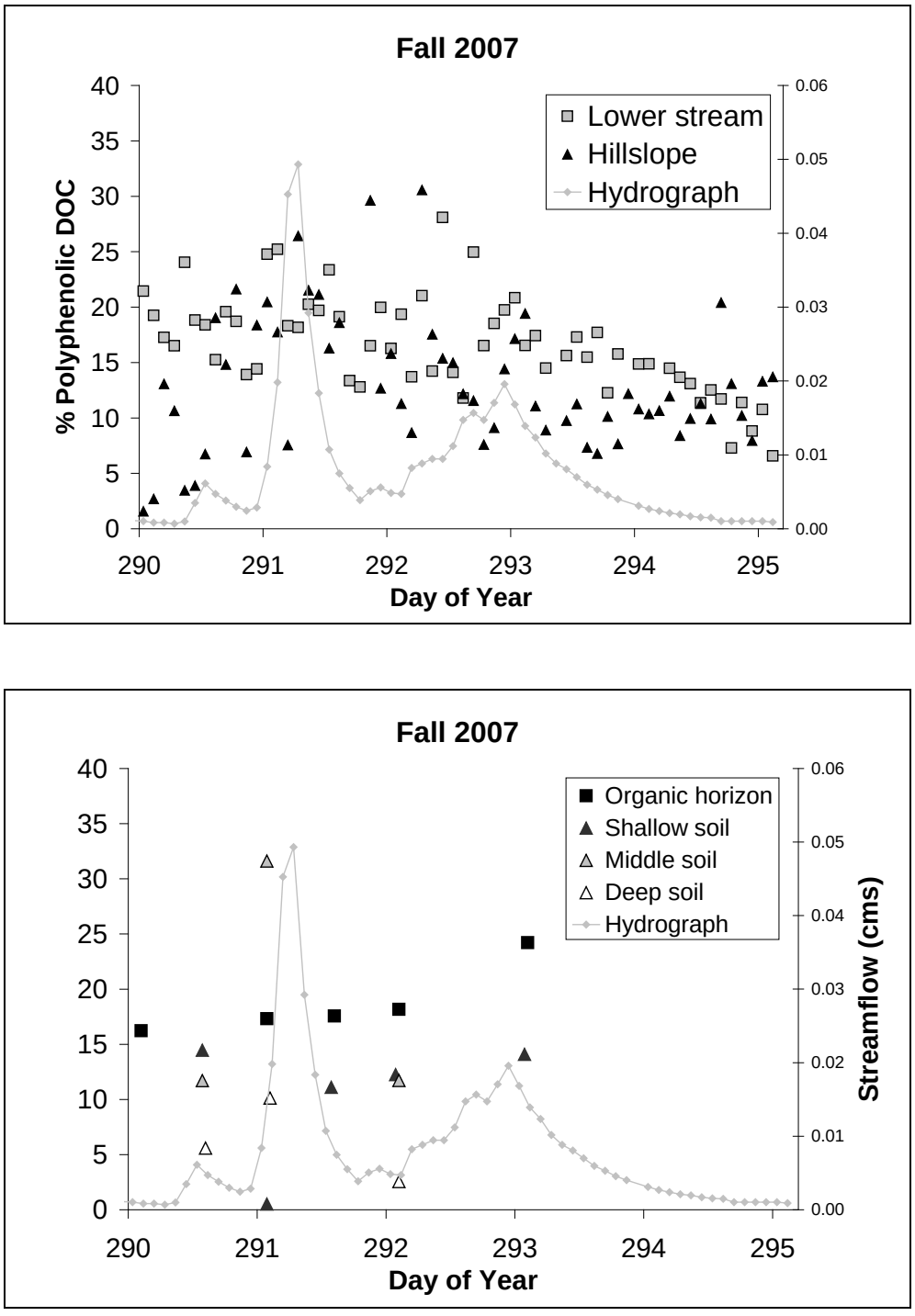


Figure 2.15: % Phenolic DOC over fall 2007 storm in stream, hillslope, and soils.

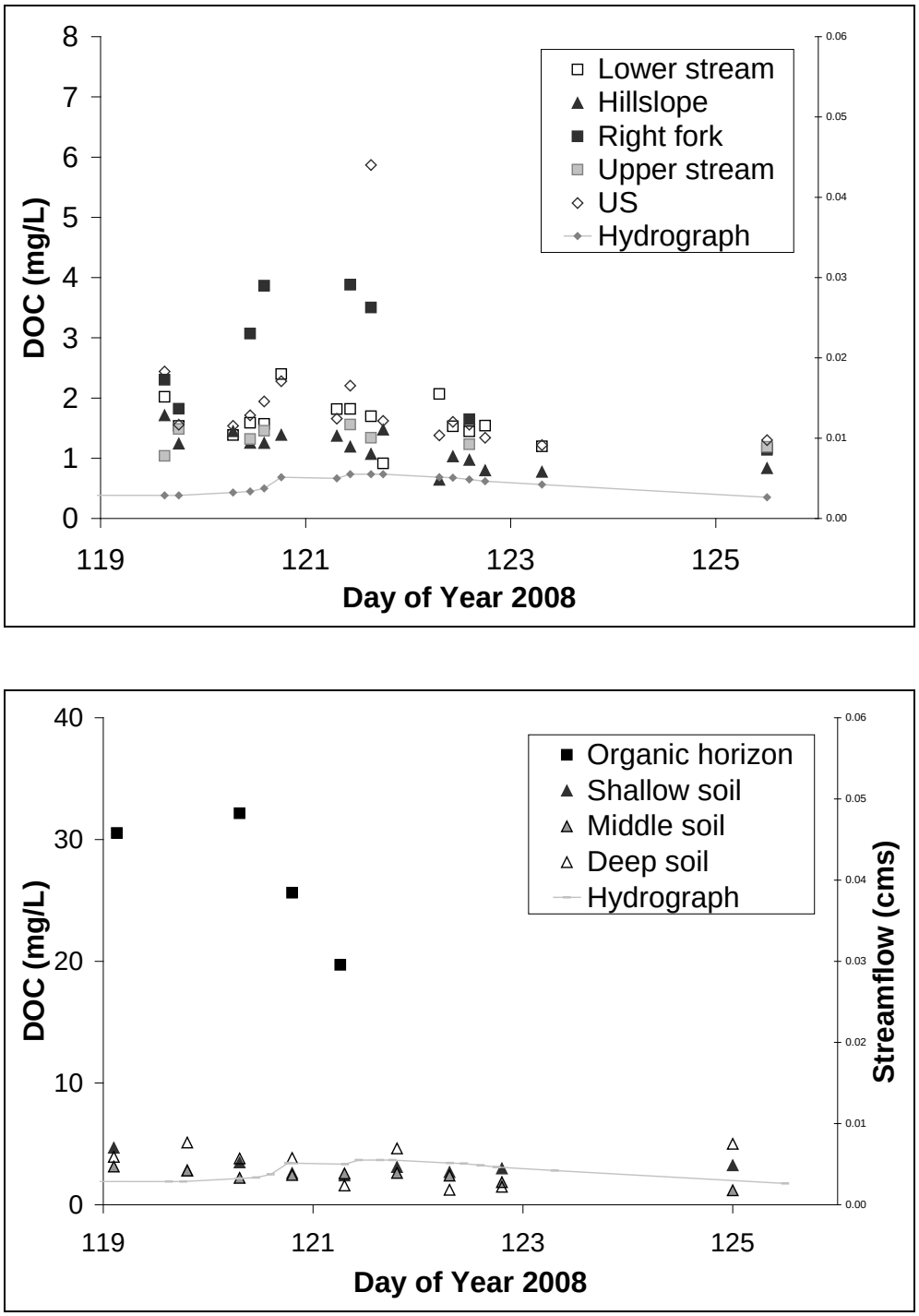


Figure 2.16: DOC over spring 2008 storm in stream and hillslope, and in soils. Note different scale on DOC axis on second plot.

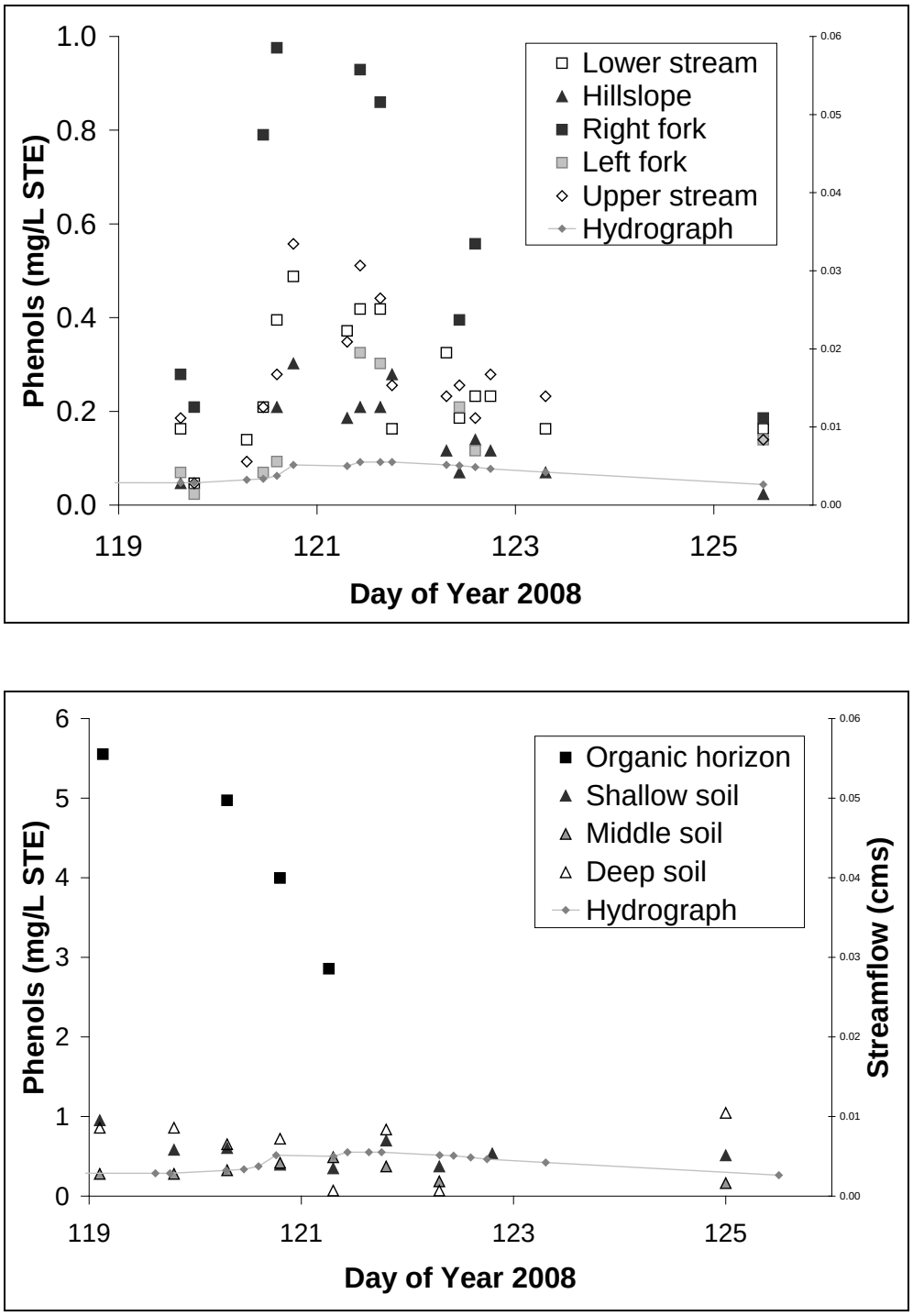


Figure 2.17: Phenol concentrations over spring 2008 storm, in stream and hillslope, and in soils. Note different scale on phenols axis on second plot.

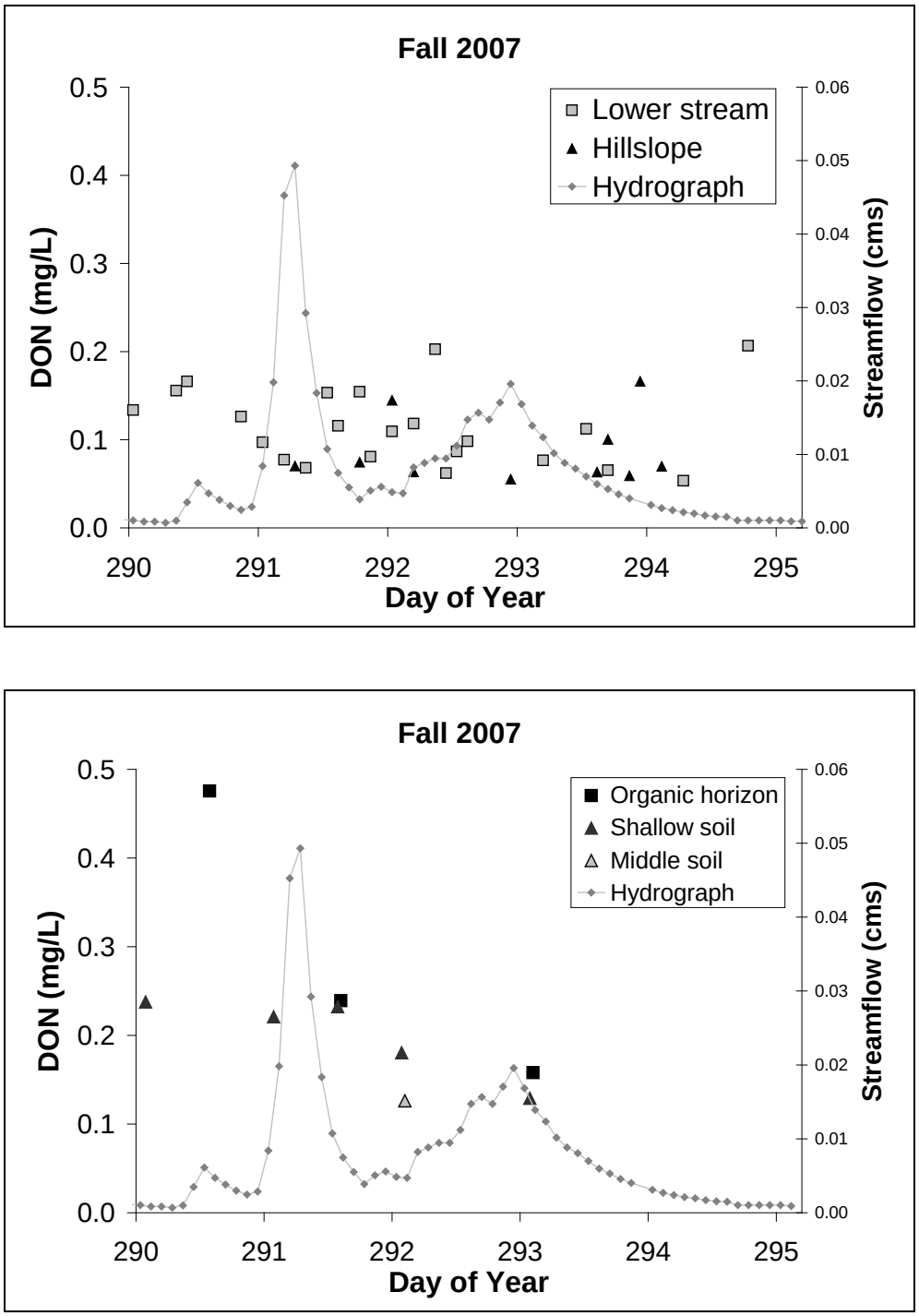


Figure 2.18: DON concentration over fall 2007 storm event in stream, hillslope, and soils.

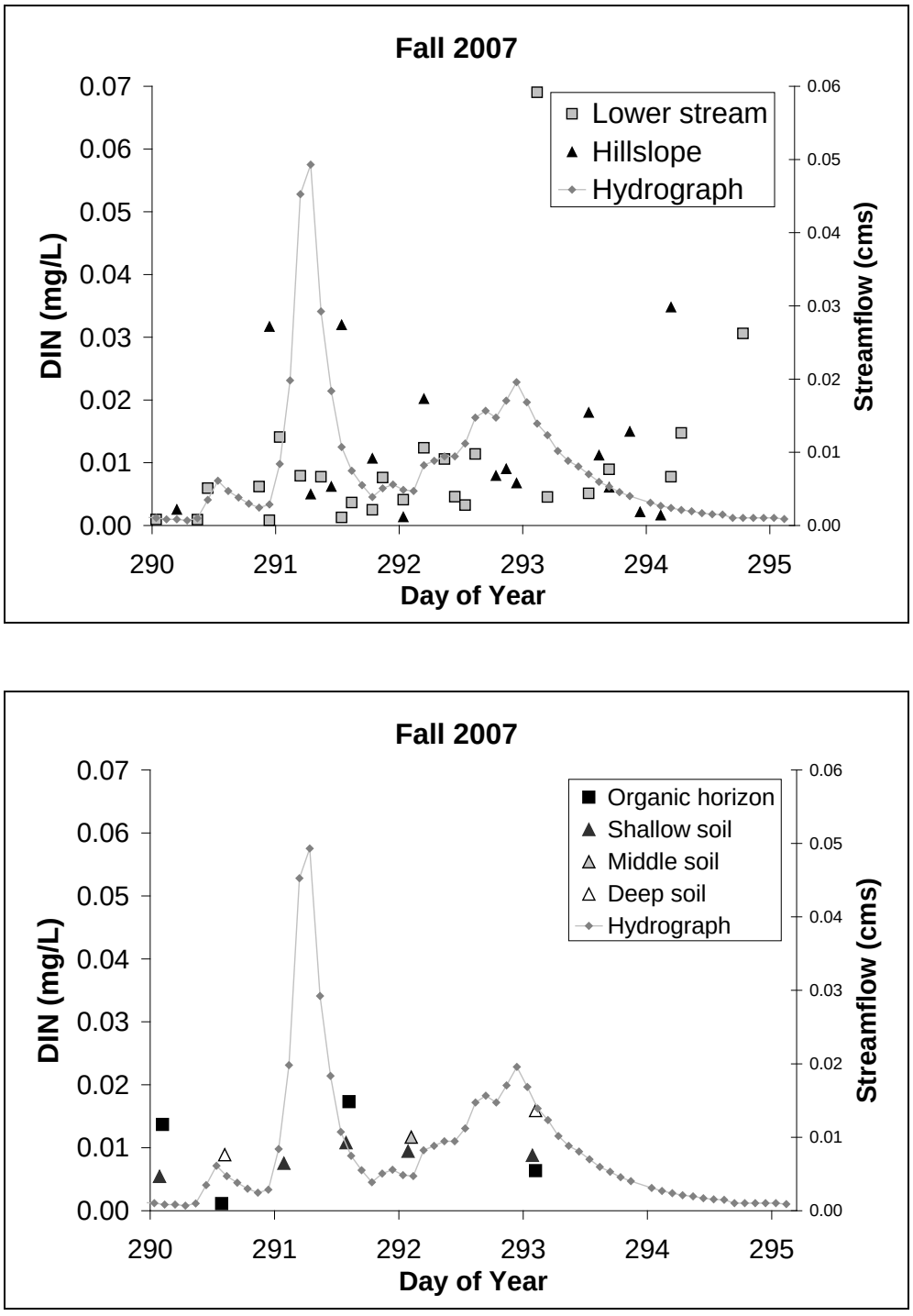


Figure 2.19: DIN concentration over fall 2007 storm event in stream, hillslope, and soils.

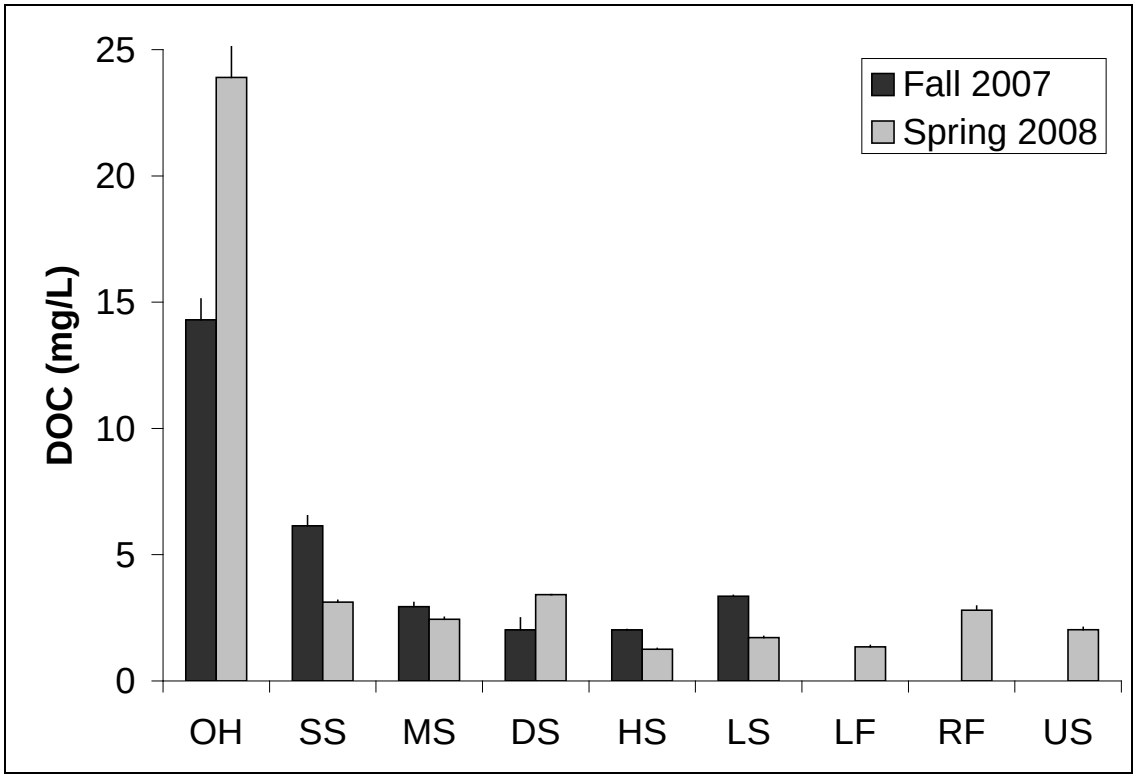


Figure 2.20: Flow-weighted mean DOC by source. Error bars represent one standard error of the mean. Organic horizon (OH), shallow soil (SS), middle soil (MS), deep soil (DS), hillslope (HS), lower stream (LS) (91 m below hillslope weir), left fork (LF), right fork (RF), upper stream (US) (just above hillslope weir).

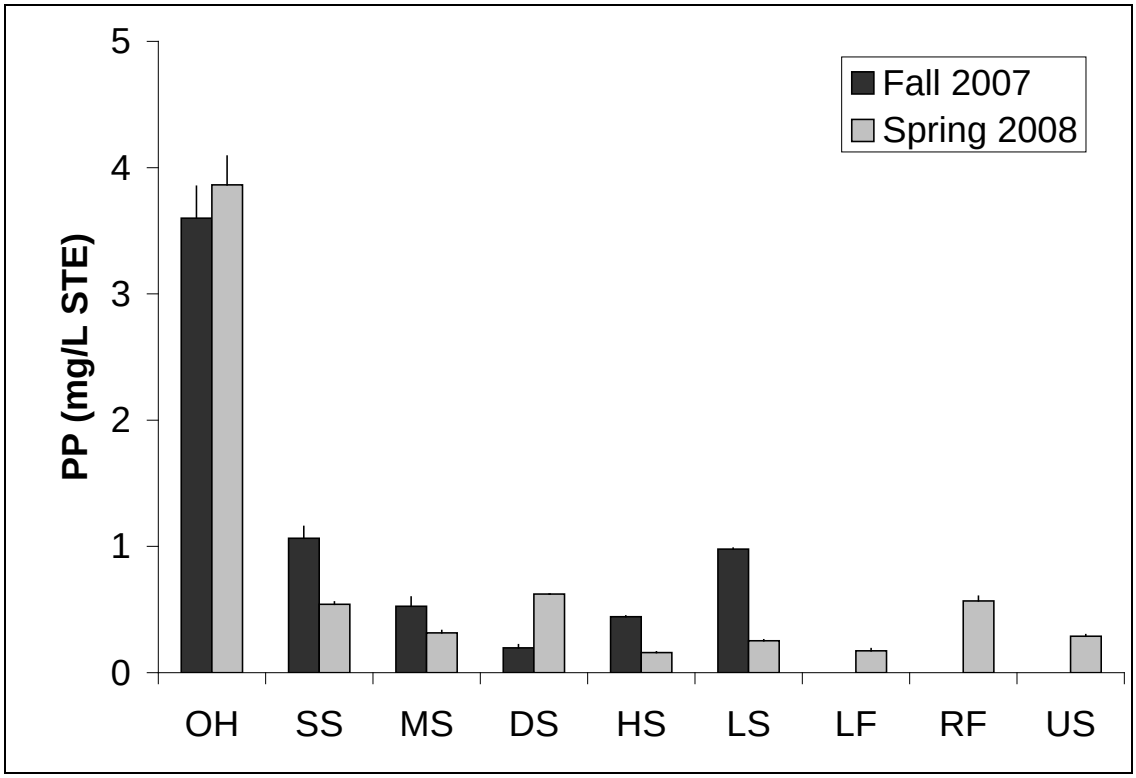


Figure 2.21: Flow-weighted mean phenol concentration by source. Error bars represent one standard error of the mean. Organic horizon (OH), shallow soil (SS), middle soil (MS), deep soil (DS), hillslope (HS), lower stream (LS) (91 m below hillslope weir), left fork (LF), right fork (RF), upper stream (US) (just above hillslope weir).

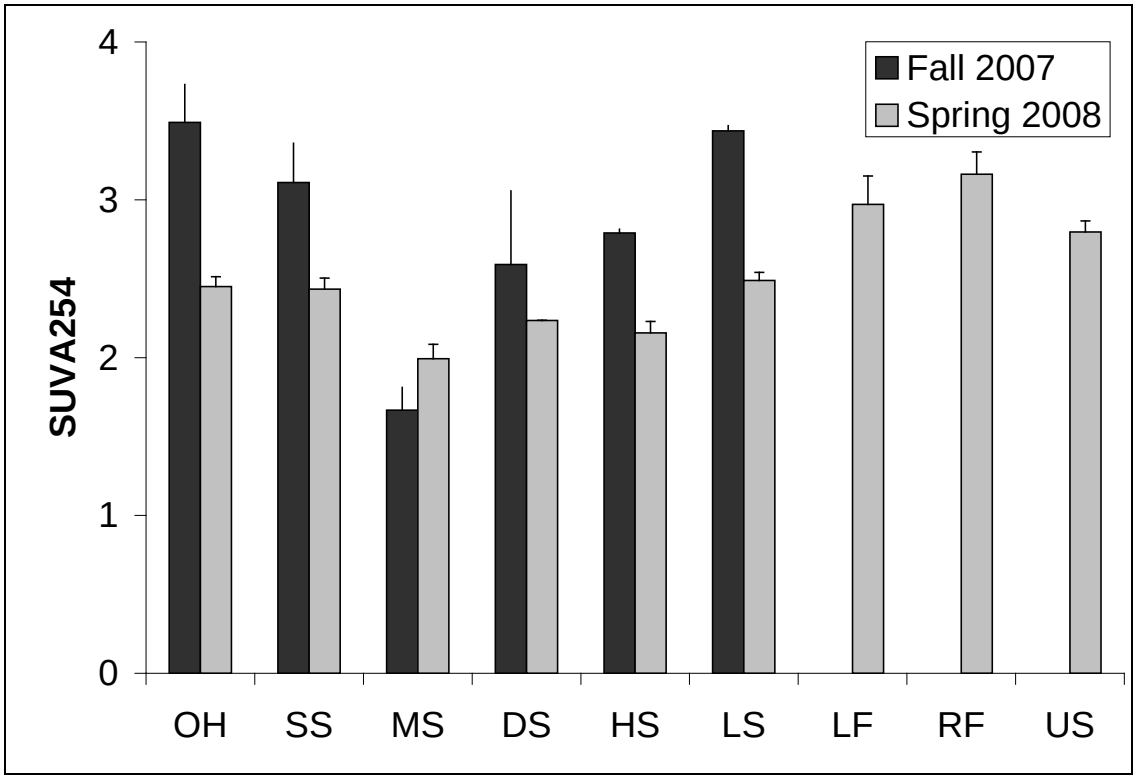


Figure 2.22: Flow-weighted mean $SUVA_{254}$ by source. Error bars represent one standard error of the mean. Organic horizon (OH), shallow soil (SS), middle soil (MS), deep soil (DS), hillslope (HS), lower stream (LS) (91 m below hillslope weir), left fork (LF), right fork (RF), upper stream (US) (just above hillslope weir).

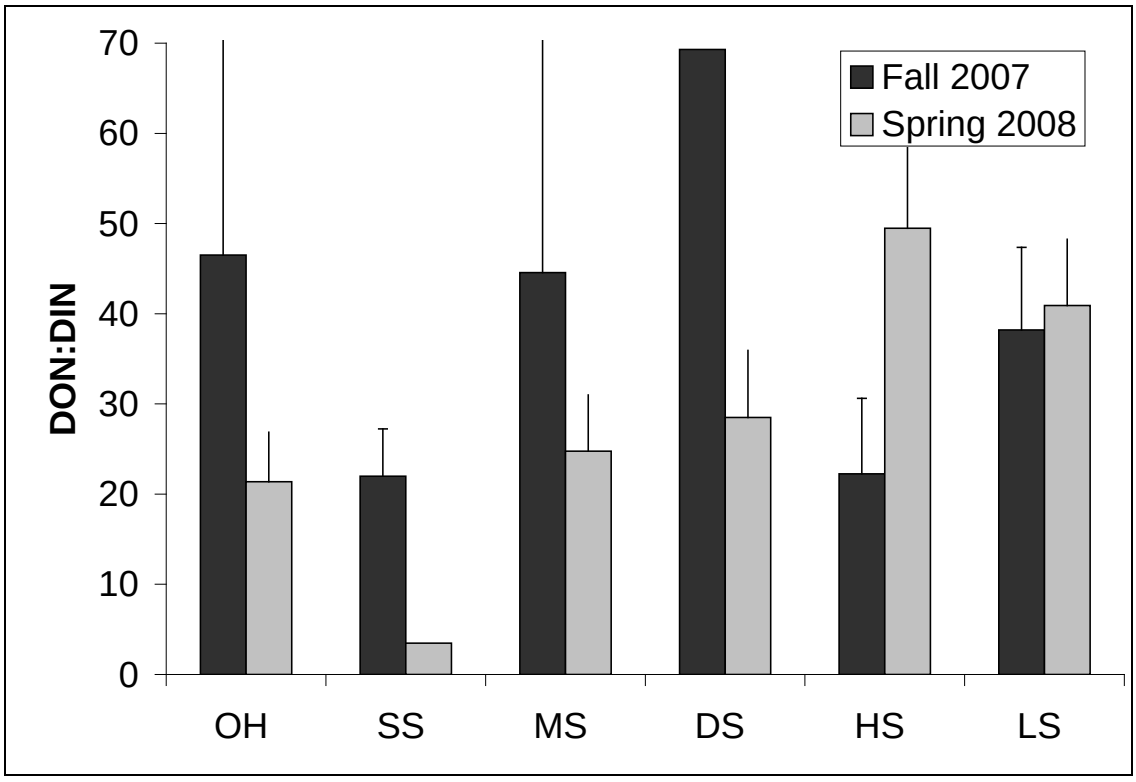


Figure 2.23: Flow-weighted mean DON:DIN by source. Error bars represent one standard error of the mean. Where there is no error bar, data set contained only one point. Organic horizon (OH), shallow soil (SS), middle soil (MS), deep soil (DS), hillslope (HS), lower stream (LS).

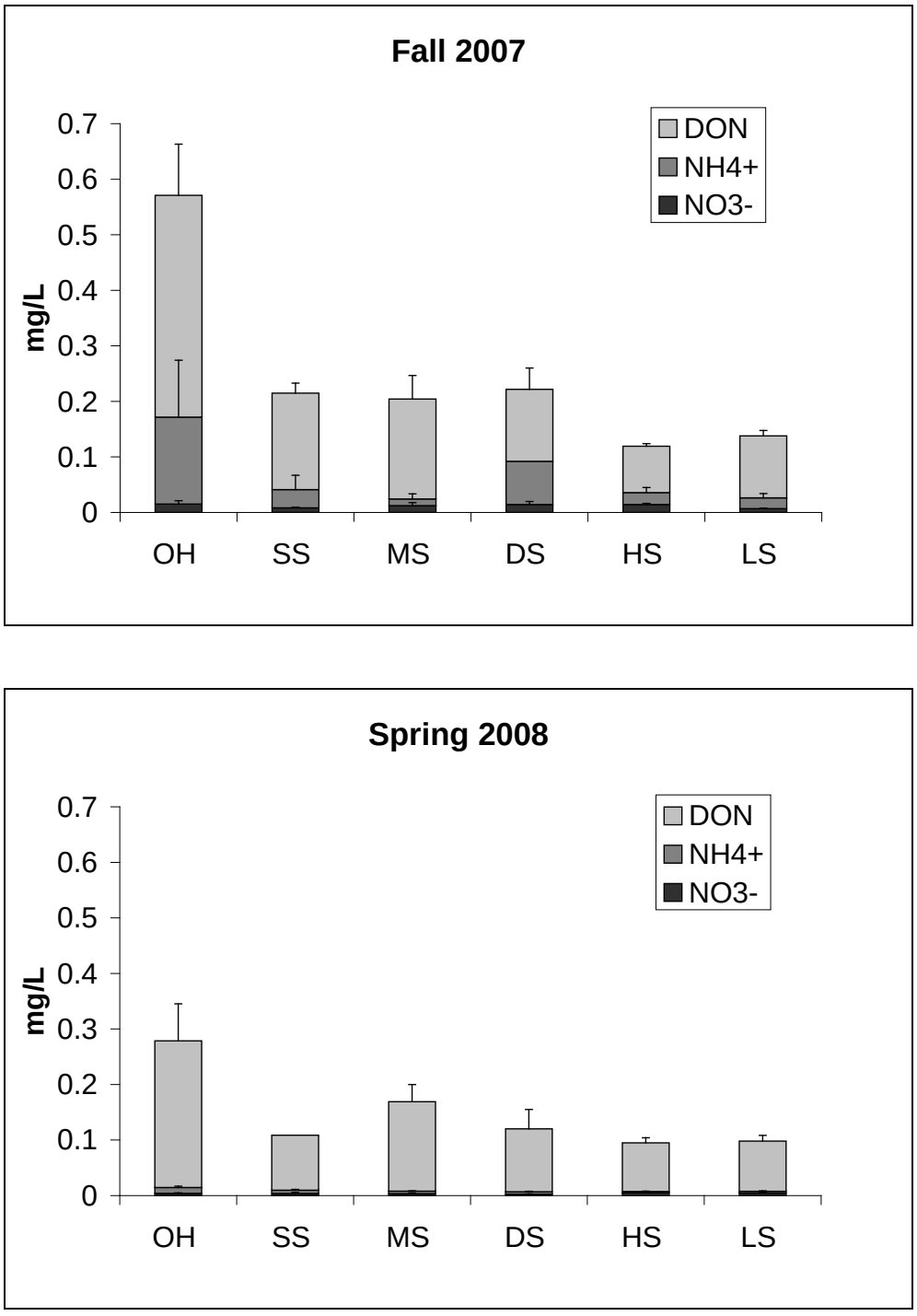


Figure 2.24: Mean DON and DIN concentrations by source. Error bars represent one standard error of the mean. Organic horizon (OH), shallow soil (SS), middle soil (MS), deep soil (DS), hillslope (HS), lower stream (LS)

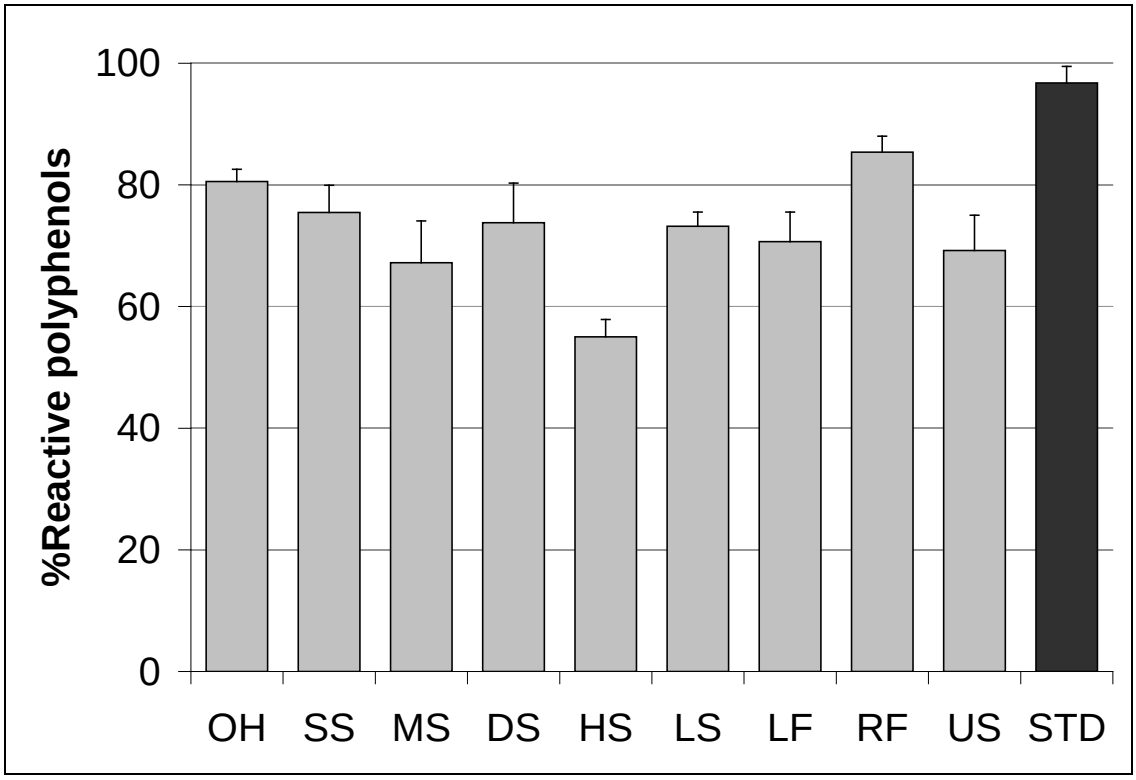


Figure 2.25: % Reactive polyphenols by source. Organic horizon (OH), shallow soil (SS), middle soil (MS), deep soil (DS), hillslope (HS), lower stream (LS), left fork (LF), right fork (RF), upper stream (US), salal standards (STD).

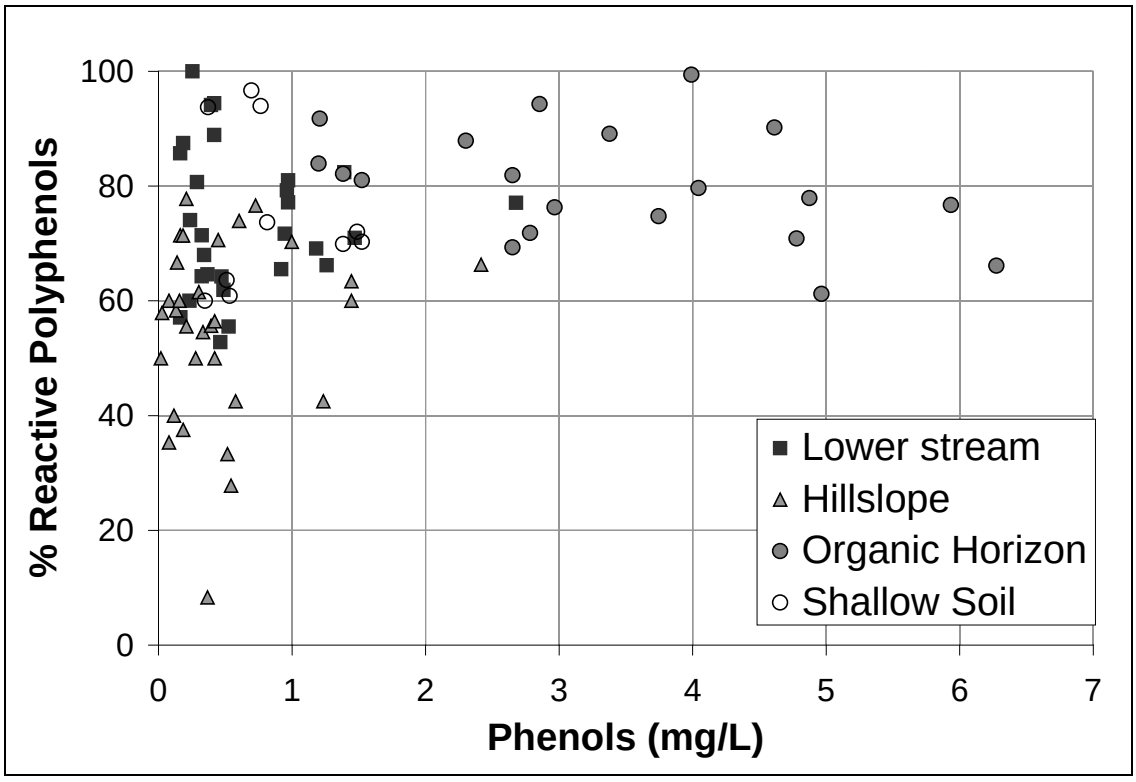


Figure 2.26: % Reactive polyphenols versus total phenol concentration of samples by source.

Table 2.1: Flow weighted averages of parameters by source and season. Standard errors in parentheses.

Source	Season	n	DOC (mg/L)	n	N (mg/L)	n	NH4+ (mg/L)	n	NO3- (mg/L)	n	DON: DIN	n	DON (mg/L)	n	$SUVA_{254}$	n	PP(mg/L STE)
Organic horizon	**F**	16	14.3 (0.828)	16	0.286 (0.016)	11	0.0534 (0.006)	7	0.0157 (0.0018)	11	14.0 (1.69)	12	0.3015 (0.0161)	12	3.49 (0.24)	12	3.60 (0.25)
Organic horizon	**S**	5	23.9 (1.55)	4	0.267 (0.028)	4	0.010 (0.009)	4	0.0040 (0.0005)	3	27.2 (8.64)	4	0.2550 (0.0280)	5	2.45 (0.062)	5	3.863 (0.229)
Shallow soil	**F**	13	6.14 (0.400)	13	0.147 (0.009)	3	0.0368 (0.017)	7	0.00878 (0.0010)	8	17.8 (2.16)	4	0.1270 (0.0120)	11	3.11 (0.248)	11	1.07 (0.093)
Shallow soil	**S**	10	3.12 (0.070)	1	0.128 (na)	10	0.0056 (0.0003)	8	0.0034 (0.0006)	1	3.48 (na)	1	0.0991 (na)	10	2.43 (0.070)	10	0.542 (0.020)
Middle soil	**F**	14	2.94 (0.165)	11	0.272 (0.033)	4	0.0116 (0.004)	5	0.0180 (0.0037)	3	12.2 (5.54)	3	0.3360 (0.120)	10	1.67 (0.144)	9	0.526 (0.074)
Middle soil	**S**	10	2.44 (0.080)	7	0.160 (0.008)	10	0.0047 (0.0002)	10	0.0030 (0.0002)	7	22.9 (1.59)	7	0.1120 (0.007)	9	1.99 (0.091)	9	0.316 (0.019)
Deep soil	**F**	12	2.02 (0.476)	6	0.200 (0.034)	1	0.078 (na)	5	0.0196 (0.0033)	1	69.3 (na)	1	0.3056 (na)	7	2.59 (0.466)	7	0.197 (0.025)
Deep soil	**S**	9	3.42 (0.002)	3	0.122 (0.025)	9	0.0045 (0.0074)	9	0.0027 (0.0002)	3	31.3 (5.25)	8	0.1180 (0.025)	8	2.24 (0.077)	8	0.622 (0.044)
Hillslope	**F**	107	2.02 (0.015)	53	0.088 (0.002)	10	0.0255 (.0041)	23	0.0130 (0.0004)	14	16.3 (0.96)	14	0.0950 (0.001)	106	2.79 (0.024)	102	0.444 (0.005)
Hillslope	**S**	18	1.26 (0.032)	14	0.095 (0.002)	6	0.0022 (0.0002)	17	0.0048 (0.0004)	14	56.3 (4.76)	14	0.0920 (0.002)	18	2.16 (0.073)	14	0.159 (0.007)
Lower stream	**F**	96	3.36 (0.027)	74	0.103 (0.001)	11	0.0237 (0.003)	26	0.0072 (0.0002)	24	17.7 (0.36)	24	0.1109 (0.003)	95	3.44 (0.033)	94	0.978 (0.009)
Lower stream	**S**	18	1.72 (0.041)	16	0.92 (0.003)	7	0.0011 (0.0002)	18	0.0052 (.0005)	16	43.4 (2.07)	18	0.0890 (0.003)	18	2.49 (0.052)	18	0.253 (0.008)
Left fork	**S**	8	1.35 (0.061)	9	0.093 (0.005)	5	0.0061 (0.0008)	9	0.0025 (0.0002)	9	34.5 (4.82)	9	0.0880 (0.005)	8	2.97 (0.180)	9	0.173 (0.017)
Right fork	**S**	9	2.80 (0.167)	9	0.105 (0.005)	9	0.0028 (0.0002)	10	0.0054 (0.009)	9	22.4 (1.64)	9	0.100 (0.005)	9	3.16 (0.142)	10	0.567 (0.038)
Upper stream	**S**	16	2.03 (0.094)	15	0.069 (0.003)	8	0.0018 (0.0002_	13	0.0026 (.0001)	11	48.6 (5.43)	12	0.0810 (0.003)	16	2.80 (0.071)	16	0.289 (0.012)

Table 2.2: Linear **regression results of phenols on DOC and UV_{254} by source and season. Number of samples (n).**

Fall 2007										
Source	**Phenols ~ DOC**					**Phenols ~UV_{254}**				
	n	**p**	**r2**	**intercept**	**slope**	**n**	**p**	**r2**	**intercept**	**slope**
Organic horizon	13	0.00000	0.94	0.288	0.283	13	0.00000	0.98	-0.057	0.126
Shallow soil	11	0.18759	0.18	0.296	0.137	11	0.19134	0.18	0.138	0.042
Middle soil	9	0.69283	0.02	0.283	0.091	9	0.90237	0.00	0.067	-0.003
Deep soil	7	0.19027	0.31	-0.005	0.089	7	0.99024	0.00	0.036	0.001
Hillslope	104	0.00000	0.39	0.002	0.228	104	0.00000	0.39	0.026	0.043
Lower stream	96	0.00000	0.67	-0.093	0.331	96	0.00000	0.75	0.020	0.081
Spring 2008										
Source	**Phenols ~ DOC**					**Phenols ~UV_{254}**				
	n	**p**	**r2**	**intercept**	**slope**	**n**	**p**	**r2**	**intercept**	**slope**
Organic horizon	5	0.04631	0.78	0.070	0.169	5	0.04365	0.79	0.050	0.134
Shallow soil	10	0.00309	0.69	-0.235	0.250	10	0.00099	0.76	0.032	0.080
Middle soil	9	0.37315	0.11	0.133	0.069	9	0.00057	0.83	0.010	0.120
Deep soil	8	0.00030	0.90	-0.241	0.245	8	0.00005	0.95	0.015	0.111
Hillslope	14	0.99862	0.00	0.117	0.000	14	0.00008	0.63	0.013	0.086
Lower stream	18	0.06975	0.19	0.028	0.132	18	0.00003	0.67	0.017	0.099
Left fork	8	0.84200	0.01	0.174	-0.019	9	0.02697	0.53	0.028	0.065
Right fork	9	0.01120	0.57	0.086	0.193	10	0.00000	0.95	0.019	0.116
Upper stream	16	0.05622	0.24	0.148	0.061	16	0.00002	0.75	0.026	0.085

Table 2.3: Mean % phenolic DOC, % aromatic DOC, and % phenolic aromatic DOC by season and source. Number of samples (n), standard error (se).

Fall 2007									
Source	**% Phenolic DOC**			**% Aromatic DOC**			**% Phenolic Aromatic DOC**		
	n	**mean**	**se**	**n**	**mean**	**se**	**n**	**mean**	**se**
Organic horizon	12	16.6	1.0	12	26.1	0.9	12	63.6	3.4
Shallow soil	11	10.1	1.2	11	23.7	1.3	11	43.4	5.3
Middle soil	9	10.8	2.9	10	16.8	1.8	9	55.7	9.8
Deep soil	7	4.8	1.5	7	15.7	2.9	7	35.6	12.0
Hillslope	102	11.9	0.8	106	20.9	0.7	102	55.9	3.3
Lower stream	95	15.8	0.6	96	24.2	0.7	95	65.8	1.7
Spring 2008									
	% Phenolic DOC			**% Aromatic DOC**			**% Phenolic Aromatic DOC**		
	n	**mean**	**se**	**n**	**mean**	**se**	**n**	**mean**	**se**
Organic horizon	5	9.8	0.4	5	19.7	0.5	5	50.1	3.0
Shallow soil	10	10.2	0.6	10	19.3	0.7	10	52.7	2.7
Middle soil	9	7.4	0.9	9	16.2	1.2	9	44.5	2.7
Deep soil	8	9.3	1.4	8	18.4	1.1	8	48.2	5.8
Hillslope	14	7.8	1.0	18	16.8	1.5	14	41.0	4.4
Lower stream	18	8.9	0.9	18	20.2	1.3	18	43.1	3.3
Left fork stream	8	6.3	1.6	8	22.1	1.5	8	27.5	6.0
Right fork stream	9	12.1	1.7	9	24.2	1.7	9	48.3	4.2
Upper stream	16	8.6	1.0	16	21.5	1.2	16	39.5	3.5

Table 2.4: Linear regression results of DON:DIN and DON on total phenols by source and season. Number of samples (n), not applicable (na).

Fall 2007										
Source	**DON:DIN~Phenols**					**DON~Phenols**				
	n	**p**	**r2**	**intercept**	**slope**	**n**	**p**	**r2**	**intercept**	**slope**
Organic horizon	9	0.2536	0.18	2	2	12	0.0001	0.87	-0.101	0.079
Shallow soil	7	0.8429	0.01	17	2	11	0.1975	0.18	0.111	0.045
Middle soil	3	0.9199	0.02	52	-17	7	0.9259	0.00	0.214	0.013
Deep soil	1	na				4	0.0426	0.92	0.056	0.392
Hillslope	14	0.9127	0.002	20	3	50	0.4618	0.01	0.089	-0.011
Lower stream	26	0.0081	0.26	-24	51	62	0.0261	0.07	0.067	0.046
Spring 2008										
Source	**DON:DIN~Phenols**					**DON~Phenols**				
	n	**p**	**r2**	**intercept**	**slope**	**n**	**p**	**r2**	**intercept**	**slope**
Organic horizon	4	0.6118	0.15	35	-3	4	0.7477	0.06	0.159	0.023
Shallow soil	1	na				1	na			
Middle soil	6	0.3120	0.25	51	-80	6	0.2887	0.27	0.301	-0.417
Deep soil	2	na		168	-150	2	na		0.702	-0.624
Hillslope	15	0.1676	0.14	26	191	15	0.8402	0.00	0.085	0.019
Lower stream	16	0.1694	0.13	19	84	16	0.8018	0.00	0.096	-0.022
Left fork	9	0.6438	0.03	24	62	9	0.5637	0.05	0.102	-0.080
Right fork	9	0.0016	0.78	3	30	9	0.0651	0.41	0.068	0.051
Upper stream	12	0.5665	0.03	40	121	13	0.8600	0.00	0.079	-0.013

Table 2.5: Baseflow and peak DOC, phenols, N, and SUVA254 during storms. Not applicable (na), not detected (nd)

	Fall Storm 2007								
Parameter	**Organic Horizon**			**Hillslope**			**Lower Stream**		
	Baseflow	**Peak**	**Change (factor)**	**Baseflow**	**Peak**	**Change (factor)**	**Baseflow**	**Peak**	**Change (factor)**
DOC (mg/L)	27.6	36.43	1.3	0.839	4.424	5.3	1.071	5.625	4.3
Phenols (mg/L)	11.7	9.85	0.8	0.046	1.278	27.8	0.232	1.766	6.6
SUVA254	3.841	3.895	1.0	1.410	5.55	3.9	2.533	5.574	1.2
Total N (mg/L)	0.715	2.52	3.5	0.068	0.117	1.7	0.069	0.2141	2.1
NO3- (mg/L)	0.004	0.0164	4.1	nd	0.0317	na	nd	0.0141	na
NH4+ (mg/L)	0.157	1.17	7.5	nd	0.0847	na	nd	0.0306	na
	Spring Storm 2008								
Parameter	**Organic Horizon**			**Hillslope**			**Lower Stream**		
	Baseflow	**Peak**	**Change (factor)**	**Baseflow**	**Peak**	**Change (factor)**	**Baseflow**	**Peak**	**Change (factor)**
DOC (mg/L)	30.53	32.16	1.1	1.713	1.713	1.0	1.536	2.398	1.6
Phenols(mg/L)	5.55	5.55	1.0	0.046	0.302	5.6	0.046	0.488	9.6
$SUVA_{254}$	2.594	2.612	1.0	1.226	4.044	2.3	2.083	3.516	1.7
Total N (mg/L)	0.4246	0.4246	1.0	0.1451	0.1451	1.0	0.1007	0.1168	1.2
NO3- (mg/L)	0.0034	0.0050	1.5	0.0035	0.0056	1.6	0.0035	0.0084	2.4
NH4+ (mg/L)	0.0158	0.0158	1.0	0.0032	0.0032	1.0	0.0059	0.0059	1.0

Table 2.6: %RPP determination using casein precipitation method and solid phase extraction method (SPE). Standard error in parentheses.

	Casein method	SPE method
Total number of assays performed	111	228
Number of invalid results (≤0)	72	22
Number of valid results (n)	39	206
Mean value (%RPP)	67.8 (3.12)	74.1 (1.43)
Number of duplicate assays performed	5	45
Mean difference between duplicate results	31.0 (9.13)	17.9 (2.57)

Chapter 3:

General Conclusion

Janet K. Rasmussen

Why is N-limitation of pristine forested ecosystems associated with significant loss of DON? Aggrading forests require N inputs in excess of outputs as well as conservation of existing N stores. Productivity is limited when N cycling rates are slowed and when N is lost from the system. It has long been suspected that polyphenols derived from tannins and other plant products may play an important role in the sequestration of recalcitrant DON. Tannins are also thought to inhibit decomposition of DON by interfering with microbial processes.

In our study, we hoped to discover whether the positive correlation between phenols and DON:DIN or DON found by others in litter extracts would be evident in water leached from the organic horizon. We hoped to find a positive correlation between phenols and DON in stream water to support the hypothesis that reactive polyphenols are a mechanism of DON transport from N-limited catchments. We also wanted to know how the character of phenols, DOC, and N changed with source and time over changes in the hydrograph. We separated the reactive portion of polyphenols using two methods to determine whether %RPP varied with source or time. Our study site was a well-characterized N-limited watershed in an unpolluted headwater catchment in the H. J. Andrews Experimental Forest, Western Cascades, Oregon. We sampled from the lysimeters, an isolated hillslope, and the stream over two storms: one in October 2007, and one in April 2008. Our key findings were:

The DOC and phenol concentrations in the hillslope rose and fell synchronously with the stream concentrations over a fall storm, which suggested that nutrient-rich water from preferential flow, near-stream sources, and a small tributary may supplement the nutrient-depleted groundwater delivered to the stream.

Phenol concentration was strongly correlated to DOC in the fall, except in the soils. Phenols were positively correlated to aromatic DOC in all sources during the spring, and all but the soils in the fall. The phenolic proportion of aromatic DOC varied considerably in the hillslope over a fall storm, coincident with the hydrograph, while in the stream, the decline was roughly linear. Variation in phenolic content of DOC and aromatic DOC was greater in the fall than in the spring between soil and surface water. Therefore, we cannot support the use of $SUVA_{254}$ as a proxy for phenol concentration.

DOC was estimated to be between 4.8% and 16.6% phenolic in fall deep soil and organic horizon leachate, respectively.

Phenol concentrations were positively correlated to DON:DIN only in two stream sampling sites, but was correlated to DON in organic horizon and stream in the fall, with

suggestive evidence of a positive correlation in the right fork of the stream in the spring. This was an observational study and only water-soluble phenols were examined, still the results, while inconclusive, lend support to our hypothesis that reactive polyphenols are a mechanism of DON transport in N-limited catchments. Controlled conditions and more sensitive N analysis may return more conclusive results.

We estimated the proportion of reactive polyphenols to be 74% in all sources, with the highest proportion being found in the organic horizon and right fork of the stream. The Solid Phase Extraction procedure was more sensitive and reproducible in separating reactive polyphenols from total phenols than the casein precipitation method for dilute samples.

Future research

Many of our samples were below detection limits for one or all fractions of N. Therefore, a study similar to that which we performed should be repeated in an unpolluted, but somewhat less N-limited headwater catchment with ample supply of polyphenol-producing vegetation. Another alternative would be to use a more sensitive method for N analysis. Extraction of organic horizon using solvents would be helpful to confirm that solvent-extractable phenols are positively correlated with DON:DIN in the study site.

More specific characterization of the phenols and reactive fraction of polyphenols in the samples would be useful. Reactive polyphenols from larger volumes of sample could be eluted from the polyamide resin and analyzed further for N content and characterization of the polyphenol species present.

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Appendix: Lab Protocols

Janet K. Rasmussen

Standard Curve for Purified Salal Tannins:

Materials:

Purified salal (*Gaultheria shallon*) tannins provided by Caroline Preston, Pacific Forestry Centre, Vancouver, BC.

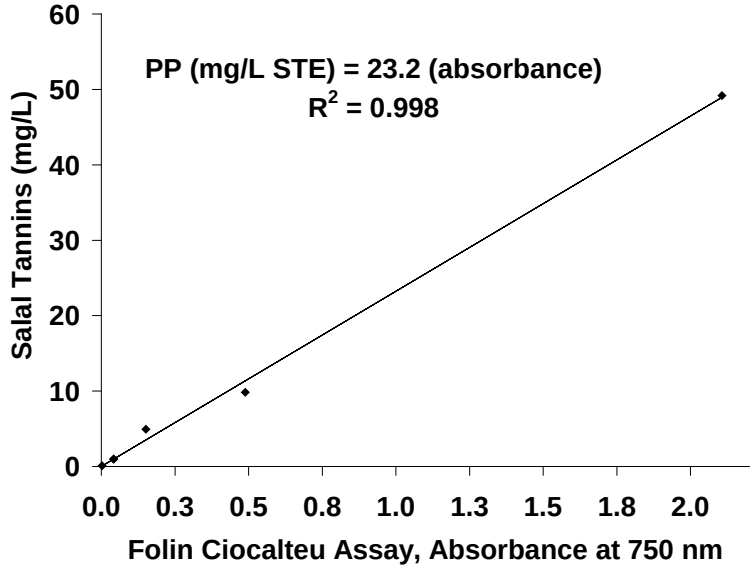
Whatman GFF filter

Procedure:

Whatman GFF filter was dried at 60°C for one hour, and weighed.

100 mg salal tannins were added to 1 L water, and filtered using precombusted filter. The filter was again dried for one hour at 60°C, and reweighed. 2.4% of the tannins were retained on the filter, thus 97.6% of the dry tannins were soluble in water at 25°C. Serial dilutions were prepared ranging from 0.0984 mg/L to 98.4 mg/L. A standard curve was prepared so that phenols in environmental samples could be measured in salal tannin equivalents (STE). The formula used for this is

Phenol concentration (mg/L STE) = 23.2 (absorbance (cm) at 750 nm following Folin Ciocalteu assay)



$SUVA_{254}$:

Materials:

Filtered samples at 25°C

1 cm quartz cuvette

Shimadzu UV 1201 spectrophotometer

Procedure:

The spectrophotometer was set at UV 254 nm, and zeroed to air. Three blanks were prepared of NANOpure water. 2 mL of water was placed into the cuvette, and absorbance at UV 254 nm was recorded. The average values of the blanks were subtracted from the absorbance value of each subsequent sample, while the instrument could be zeroed to air at any time during the laboratory session.

The environmental samples, at 25°C, were measured in a similar fashion. Duplicate samples were tested and the average difference between sample results was 0.002, n = 21.

Folin Ciocalteu Assay for Total Phenols:

Materials:

Filtered samples at 25°C

Folin Ciocalteu reagent (Sigma F9252)

20% Na_2CO_3 (J. T. Baker 3604-05) (buffer solution)

1 cm quartz cuvette

Shimadzu UV 1201 spectrophotometer

Procedure:

The spectrophotometer was set at 750 nm, and zeroed to air. Three blanks were prepared of NANOpure water.

2 mL of sample or blank were measured into a 5mL test tube using a volumetric pipette. Folin Ciocalteu reagent, 0.1 mL, was added while vortexing. After 1-8 minutes, 0.3 mL buffer solution of 20% Na_2CO_3 was added while vortexing. Tubes were incubated at 25°C for 1 hour, and absorbance read at 750 nm. The average values of the blanks were subtracted from the absorbance value of each subsequent sample, while the instrument could be zeroed to air at any time during the laboratory session.

Duplicate samples were tested, and the difference between results averaged 0.002 absorbance units (n = 25).

Casein Precipitation Assay for Reactive Polyphenols:

Materials:

Filtered samples at 25°C

Casein powder (Sigma C5890)

Mechanical shaker

Centrifuge

5µ filter needle (Becton-Dickinson 305200)

Folin Ciocalteu reagent (Sigma F9252)

20% Na_2CO_3 (J. T. Baker 3604-05) (buffer solution)

1 cm quartz cuvette

Shimadzu UV 1201 spectrophotometer

Procedure:

Undiluted water samples at 25°C, 6mL, were added to 200 mg casein powder, in 20 mL glass bottles with rubber stoppers, and shaken 3 hours in mechanical shaker. Three blanks were prepared in the same manner using NANOpure water in place of sample. After centrifugation at 2700 rpm for 15 minutes, the supernatant was aspirated through a 5µ filter needle, and re-assayed for phenols by the Folin Ciocalteu method previously described. The difference between the pre-casein and post-casein absorbance values was designated as the reactive polyphenol fraction.

Duplicate samples were tested, and the difference between final results (in %RPP), after excluding those test for which results were less than or equal to zero (38 of 45), averaged 31.0 (SE = 9.13, n =5).

Solid Phase Extraction (SPE) Procedure with Polyamide Resin (Supelco DPA6S) for Reactive Polyphenols:

Materials:

Filtered samples at 25°C

Polyamide filter tubes (SPE) (Supelco DPA 6-S 6 mL capacity)

Methanol (Fisher Scientific A412-1)

70:30 acetone (Supelco 1998)

Folin Ciocalteu reagent (Sigma F9252)

20% Na_2CO_3 (J. T. Baker 3604-05) (buffer solution)

1 cm quartz cuvette

Shimadzu UV 1201 spectrophotometer

Procedure:

SPE tubes were conditioned according to manufacturer's recommendation by passing 2 mL of methanol through the tube at a rate of 2mL/min., repeating 3 times. This was followed by passing 2 mL NANOpure water through the tube at the same rate, 3 times. Undiluted water samples at 25°C, 5-10mL, were passed through the SPE tubes at an approximate rate of 2mL/min., repeating 3 times; and the depleted sample was kept for repeat Folin Ciocalteu analysis. The difference between the pre-SPE and post-SPE absorbance values was designated as the reactive polyphenol fraction.

Duplicate samples were tested, and the difference between final results (in %RPP), after excluding those tests for which results were less than or equal to zero (10 of 55), averaged 17.9 (SE = 2.52, n = 45).

Reactants were eluted from the tube by rinsing three times at the same rate with 70:30 acetone. Eluted samples were archived for future analysis.