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Study of Humic Substances and Dissolved Organic Matter Isolated from the Groundwater of ONKALO, Olkiluoto, 2014

Jani Mäkelä, Mervi Mäkelä, Katariina Koikkalainen, Pentti Manninen

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Ramboll Finland Oy

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Working Reports contain information on work in progress or pending completion.
ABSTRACT

The dissolved organic matter (DOM) at groundwater from ONKALO, Olkiluoto was studied. DOM can be divided into several fractions: Humic substances (HS) i.e. humic and fulvic acids, hydrophobic neutrals (HON), hydrophilic acids (HIA), hydrophilic bases (HIB) and hydrophilic neutrals (HIN). The purpose of this study was to determine the apparent molecular size distribution and the amount of humic substances (HS) and also to investigate the amount and distribution of DOM other than humic substances. These other groups of compounds can be divided into four fractions: hydrophobic neutrals (HON), hydrophilic acids (HIA), hydrophilic bases (HIB) and hydrophilic neutrals (HIN). In addition metal concentrations from the groundwater samples were determined. Groundwater samples were taken from the groundwater stations ONK-PVA1, ONK-PVA5 and ONK-PVA10. Different fractions of DOM were isolated using DAX-8, XAD-4 and ion exchange resins. The molecular size distribution of humic substances was determined with high performance size exclusion chromatography (HPSEC) and mass spectrometric (MS) analyses were performed in order to evaluate the structure. The amount of humic substances and other fractions of DOM were determined using total organic carbon (TOC) measurements. Metal concentrations were determined using ICP-MS/MS method. The amount of DOM in general was found to decrease in the groundwater stations situating lower beneath the ground. Sulfur concentration was also found to decrease when going further beneath the ground. Differences were found also in the molecular size distribution of HS and results from MS measurements between the groundwater stations. The results of both molecular size distribution and amount of humic substances were similar with previous years' studies.

Keywords: DAX-8, fulvic acids, groundwater, humic acids, HPSEC, mass spectrometry, DOM.
ONKALON POHJAVESINÄYTTEIDEN HUMUSAINET JA LIUENNUT ORGAANINEN AINES 2014

TIIVISTELMÄ

Tässä tutkimuksessa tutkiin Olkiluodon ONKALON pohjavesinsäiden liuennetun organismin aineksen (DOM) koostumusta. Yleisesti ottaen liuennut organisminaines voidaan jakaa karkeasti eri fraktioihin: humusyhdisteet (HS, joihin luetaan humushapot ja fulvohapot), hydrofobiset neutraalit yhdisteet (HON), hydrofiiliset hapot (HIA), hydrofiiliset emäksiset yhdisteet (HIB) sekä hydrofiiliset neutraalit yhdisteet (HIN). Tämän tutkimuksen tarkoitukseena oli tutkia humusyhdisteiden määrää ja molekyylilikokojaamaa. Tarkoituksen oli myös tarkastella liuennetun organismin aineksen muiden fraktioiden kuin humusyhdisteiden määrää ONKALON pohjavesinsäteissä. Tarkastelussa huomioitavat fraktiot olivat hydrofobiset neutraalit yhdisteet (HON), hydrofiiliset hapot (HIA), hydrofiiliset emäksiset yhdisteet (HIB) ja hydrofiiliset neutraalit yhdisteet (HIN). Lisäksi tutkiin pohjavesinsäiden metallipitoisuuksia ja pitoisuksien muuttumista eri pohjavesiasemien välillä.

Pohjavesinsäteet otettiin pohjavesiasemilta ONK-PVA1, ONK-PVA5 ja ONK-PVA10. Eri DOM fraktiot erotettiin toisistaan käyttämällä DAX-8, XAD-4 ja ioninvaihtohartseja. Humusyhdisteiden molekyylilikokojaamasta määriteltiin käyttämällä korkean erotuskyvyn kokoeksluusikromatografian (HPSEC) ja yhdisteiden rakennetta tutkettiin massaspektrometrin (MS) avulla. Humusyhdisteiden ja muiden DOM fraktioiden määrä selvitettiin kokonaisorganisen hiilen (TOC) määritystehoja käyttäen. Metallipitoisuuksia tutkittiin ICP-MS/MS menetelmällä.

Liuennetun organismin aineksen määrän havaittiin pienenevän pohjavedessä pohjavesiaseman syvyyden kasvaessa. Myös humusaineissa olevan rikkipitoisuuden havaittiin pienenevän syvyyden kasvaessa. Lisäksi humusyhdisteiden molekyylilikokojaamasta ja rakene vaihteli pohjavesiasemien välillä. Sekä molekyylilikokojauma että humusyhdisteiden määrä olivat yhtenevät aiempien vuosien tulosten kanssa.

Avainsanat: DAX-8, fulvohapot, pohjavesi, humushapot, HPSEC, massaspektrometri, DOM.
LIST OF SYMBOLS AND ABBREVIATIONS

ρ Polydispersivity
ACN Acetonitrile
DOC Dissolved organic carbon
DOM Dissolved organic matter
FA Fulvic acids
HA Humic acids
HIA Hydrophilic acid
HIB Hydrophilic base
HIN Hydrophilic neutral
HON Hydrophobic neutral
HPSEC High performance size exclusion chromatography
HRMS High resolution mass spectrometer
HS Humic substances
IHSS International Humic Substances Society
LOQ Limit of quantification
mDa Millidalton
$M_n$ Number-average molecular weight
$M_w$ Weight-average molecular weight
MS Mass spectrometry
MW Molecular weight
NOM Natural organic matter
ppm Parts per million
PSS Poly(styrene sulfonate) sodium salt
QC Quality control
RI Refractive index
RT Retention time
SEC Size exclusion chromatography
TOC Total organic carbon
TOF Time of flight
UPLC Ultra performance liquid chromatography
UV Ultraviolet
UV$_{254}$ Ultraviolet detection at 254 nm
ICP-MS/MS Inductively coupled plasma tandem mass spectrometer
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1. INTRODUCTION

Aquatic humic substances (HS) are major components of the natural organic matter (NOM) in soil and water. They generally comprise one third to over half of the dissolved organic carbon (DOC) in natural surface waters. HS are complex organic compounds, which are mainly derived from soil humus and terrestrial and aquatic plants. The colour of the dissolved and precipitated HS varies from light to dark brown. The precise composition and properties of the HS varies depending on the source. HS are highly chemically reactive but recalcitrant to biodegradation.

Dissolved organic matter (DOM) has a significant role in the physical, chemical and microbiological processes in water ecosystems. For instance they have an effect on the transportation, toxicity and bioavailability of metals. Altogether DOM can be divided into six fractions: HA, FA, hydrophobic neutrals (HON), hydrophilic acids (HIA), hydrophilic bases (HIB) and hydrophilic neutrals (HIN) (Wang et al., 2009; Leenheer, 1981; Leenheer and Croué, 2003). HON consist of few groups of compounds with relatively low molecular sizes and a degree of condensed aromatic moieties (Świetlik and Sikorska, 2005). The characteristics are similar with HIA and HIB. HIN, however, consist of non-humic, aliphatic and low molecular weight components. For instance, amino acids can be either hydrophobic or hydrophilic and acidic or alkaline. Examples of the compound groups of the fractions HON, HIA, HIB and HIN are presented below:

- **HON**: hydrocarbons, tannins, fats/oils
- **HIA**: carboxylic acids, polyuronic acids (i.e. citric acid)
- **HIB**: peptides, many amino acids
- **HIN**: alcohols, amines, sugars

Because of the low concentration of aquatic HS, the material must first be isolated and concentrated for further studies. The fraction of aquatic HS, that is not soluble in water at a pH lower than 2, are defined as humic acids (HA). The fraction of HS that is soluble under all pH conditions are referred to as fulvic acids (FA) (Malcolm 1990). The method used in concentrating and isolating the dissolved organic matter should be effective in isolation of the aquatic humic matter at the same time keeping the chemical and structural composition unaffected as it occurs in its original state in an aquatic environment (Aiken et al. 1985). The most frequently used isolation procedure for HS is the column chromatographic method by non-ionic sorbents such as XAD resins (polymethyl methacrylate) or analogous materials (Leenheer 1981, Thurman et al. 1981, Peuravuori et al. 1997).

Molecular weight (MW) is one of the fundamental properties that need to be known in order to understand the physical and chemical characteristics and chemical reactivity of HS. The MW of HS has an effect especially on their adsorption and metal complexing behaviour. Determination of the MWs is a difficult task because HS are a complex mixture of natural, heterogeneous organic materials with different structures and a broad molecular size distribution. Size exclusion chromatography (SEC) has been frequently used especially for characterization of aquatic and soil HS (Janoš and Zatřepálková 2007).
This study continues previous work among HS characterization and initiates a study among other groups of compounds in DOM. Detailed information of the geo-chemical behaviour of radioactive and thus possibly hazardous metal ions under environmental conditions is necessary for the long-term storage of radioactive waste. In the immobilization or mobilization of the metal ions due to the complexation and colloid formation, DOM might play an important role. Therefore, the goal of the current study was to determine the amount and distribution of different DOM fractions and the molecular size distribution of HS in the groundwater in the bedrock at Olkiluoto. During previous years the method for HS has been optimized and these studies were utilised in the current study. Newly developed method for other DOM fractions were utilized for real samples at different sampling depths. Since the molecular weights are strongly operational, the values calculated from the SEC measurements are "apparent molecular weights". In addition, mass spectrometric (MS) studies were performed in order to evaluate the structure and differences of HS in different sampling depths more precisely.
2. EXPERIMENTAL

2.1 Sampling

Groundwater samples were collected on the 11th – 14th of November from groundwater stations ONK-PVA1 (depth -15 m.a.s.l.), ONK-PVA5 (depth -229 m.a.s.l.) and ONK-PVA10 (depth -366 m.a.s.l.) in ONKALO, Olkiluoto, Finland. Figure 1 illustrates the locations of the groundwater stations ONK-PVA1, ONK-PVA5 and ONK-PVA11 in ONKALO.

![Figure 1. The groundwater monitoring stations in ONKALO, Olkiluoto (Pöyry Finland Oy).](image)

The samples were on-line pressure filtered in situ, using Whatman mixed cellulose ester, 0.45 μm filters. The tubing material used in the sampling was nylon. The PE-HD containers (30 l) used to collect and store the samples were washed with methanol, 1M NaOH, 1M HCl and high-purity MQ water. Two parallel 30 l samples were taken from every sampling point. The samples were stored at 6 ºC and, since the photosensitivity of HS (Vidali et al. 2010), protected from the light by dark plastic coating around the containers.

2.2 Chemicals and standards

All the solutions used were made using high-purity MQ water.

Methanol (J. T. Baker) was of the “Baker Analyzed” quality.

n-Hexan e was of the “Baker resi-analyzed” quality (J. T. Baker).

Analysis grade 1.0 M HCl and NaOH (Reagecon).

Acetonitrile (Prolabo) was of LC-MS grade.
Phosphate buffer was made of K$_2$HPO$_4$ and KH$_2$PO$_4$, both "EMSURE®" (99%) quality (Merck).

DAX-8 resin (Supelite DAX-8, Supelco) was used for the isolation of HS from groundwater.

XAD-4 resin (Amberlite® XAD-4, Sigma-aldrich)

DOWEX 50WX8 hydrogen form ion-exchange resin (Sigma-aldrich).

DOWEX®Marathon™MSC hydrogen form ion-exchange resin (Sigma-Aldrich).

Diaion WA10 free base ion-exchange resin (Supelco)

Reference solution containing humic and fulvic acids were made by dissolving International Humic Substances Society (IHSS) Nordic Aquatic Humic Acid Reference (1R105H) and IHSS Nordic Aquatic Fulvic Acid Reference (1R105F) in phosphate buffer. The reference humic and fulvic acids were dissolved by shaking.

Poly(styrene sulfonate) sodium salt standards (PSS) with MWs 210, 3 610 and 6 520 g/mol were used as a calibration standards for the molecular size distribution investigations (Figure 1). PSS standards were received from PSS Polymer Standards Service GmbH, except for the 210 g/mol standard, which was ordered from Sigma-Aldrich.

QCWW4 reference solution (Eurofins A/S) was used as a quality control sample for the total organic carbon (TOC) determinations.

2.3 Sample isolation

Prior to isolation, DAX-8 and XAD-4 were first cleaned according to Thurman et al. (1981) with slight modifications. The DAX-8 and XAD-4 materials were cleaned by rinsing the resins in a beaker with 0.1 M NaOH and thereafter with methanol until pH were neutral. Next, the resins were Soxhlet-extracted sequentially for 6 h with n-hexane and methanol. Both DAX-8 and XAD-4 were stored in methanol at 6 ºC until used. Both DOWEX cation-exchange resins were soxhlet-extracted overnight with methanol. Diaion anion-exchange resin was soxhlet-extracted overnight with acetone.

Isolation of the organic fractions were made similarly to previous studies with humic and fulvic acids (Luste et al. 2012, Luste et al. 2013). DAX-8 and XAD-4 resins as well as ion exchange resins were used in isolation of fractions (Wang et al., 2009). The multistep method is presented in the following diagram (Figure 1).
2.3.1 Isolation of solid humic substances

Because the HRMS sample introduction requires a solid sample, larger sample amount was taken (30 l). This was necessary because of the low concentration of level of humic substances in samples, especially in ONK-PVA10. Approximately 23 l of sample was acidified to pH 2 with 6M HCl directly in PE-HD container. A HPLC column was slurry packed with DAX-8 resin resulting in a 13.3 ml sorbent bed. MQ waters was pumped through the packed column until it was free of methanol. Next, the sample was circulated through the column with an LC pump in 3 ml/min during about 5 days resulting in a saturation of DAX-8 polymer with humic substances. After sampling, the humic substances were eluted from the column with 100 ml of 0.1 M NaOH. The eluted samples were neutralized with 6 M HCl and freeze dried. The resulting solid humic substances (Figure 2) were stored in vials at -20 °C.
Figure 22. Isolated solid humic substances and direct sample introduction cups for HRMS. Left cup is filled with sample and right cup is empty.

2.3.2 Isolation of humic substances

For the isolation of different fractions of DOM, the cleaned DAX-8 polymer in methanol was slurry-packed into a 160-mm I.D. class column, resulting in an 8-cm sorbent bed. The packed column was rinsed with MQ water until it was free of methanol. The groundwater sample, acidified to a pH 2.5 with 1.0 M HCl, was placed in a separatory funnel and slowly drained through the resin. The humic substances (HS) adsorbed on the resin were eluted with 60 ml of 0.1 M NaOH. The final solution was neutralized with HCl prior to analysis. For the TOF-MS analyses the samples were also passed through the cation exchanger (DOWEX 50WX8 hydrogen form). The eluate from the DAX-8 column was collected for the isolation of hydrophilic fractions of DOM.

2.3.3 Extraction of hydrophobic neutrals

The eluted DAX-8 resin, containing the hydrophobic neutrals (HON), was first air dried and placed into a soxhlet extraction thimble and extracted overnight with methanol. The resulting extract was evaporated to dryness with rotary evaporator at 50°C and 50 mbar. 30 ml of MQ water was added into the residue and the sample was evaporated into dryness again. This was repeated four times in order to get methanol out of the sample.

2.3.4 Isolation of hydrophilic fractions

For the isolation of hydrophilic fractions of DOM, XAD-4 column was packed similarly to the DAX-8 column. The packed column was rinsed with MQ water until it was free of methanol. The collected eluate from the DAX-8 column was placed in a separatory funnel and slowly drained through the XAD-4 resin. Next the resin was eluted with 60
ml 0.1 M NaOH. The resulting fraction was acidified to pH 2 with 1 M HCl. The acidified sample was then treated on strong cation-exchange resin (DOWEX MARATHON). The resin was eluted with 60 ml of 1 M NH₄OH. This fraction was named hydrophilic bases (HIB).

The eluate from the cation exchange resin was treated on a weak anion-exchange resin (Diaion WA10). This resin was eluted with 1 M NH₄OH and this fraction was named hydrophilic acids (HIA). The eluate from the anion-exchange resin was named hydrophilic neutrals (HIN).

### 2.4 TOC analysis

All the TOC measurements of samples in this study were performed with the TOC-V CPH (Shimadzu Corporation) analyzer connected to an ASI-V autosampler (Shimadzu Corporation). The TOC analyzer was calibrated to the range of 0.5 to 20 mg/l. Quality control samples (QC WW4, 2.0 and 20.0 mg/l) were analyzed at the beginning and at the end of each sample sequence. Prior to measurement, the samples were acidified with concentrated phosphoric acid.

### 2.5 Molecular size distribution

#### 2.5.1 Analytical method

Size exclusion chromatography (SEC) is a widely used method for determining the molecular size distribution. It can provide simultaneously the number-average molecular weight ($M_n$) and the weight-average molecular weight ($M_w$), which are parameters used to describe the $MW$s of mixtures of molecules like HS. Since the molecular weights are strongly operational, the values calculated from the SEC measurements are "apparent molecular weights". In SEC the elution time depends of the molecular size and the elution of large molecules is faster than the elution of small molecules.

High performance SEC (HPSEC; Waters 2695 Separations Module) with ultraviolet (UV) detection (Hewlett Packard, Series 1050) at 254 nm (UV254) was used to investigate the molecular size distribution of isolated humic substances.

Macroporous silica-based TSKgel G3000SW column (7.5 i.d. × 600mm) were used for the HS separation. The selected column has found to provide effective separation of the humic substances in HPSEC (Laborda et al. 2008, Peuravuori et al. 2005).

PSS standards with MWs 210, 3 610 and 6 520 g/mol were used as calibration standards for the molecular size distribution investigations. PSS standards have been extensively employed in HS detection, even though they are less branched and cross-linked than humic substances (Asakawa et al. 2011, Asakawa et al. 2008, Guéguen and Cuss 2011, Laborda et al. 2008, Peuravuori and Pihlaja 1997). Nonetheless, the PSS standards have been considered to be suitable standards in studying the molecular weights of humic substances, since they behave similarly during chromatographic elution on HPSEC (Peuravuori and Pihlaja 1997, Chin et al. 1994). In our earlier studies changing the SEC conditions changed the elution time of HS and PSS rather equally (Luste et al. 2011).
Background, i.e. ionic strength, pH (≈7) and amount of phosphate buffer of standards, reference samples and groundwater samples were kept the same. The pH was not altered since the column suits best for the pH neutral or slightly acidic conditions (Janoš and Zatřepálková 2007). The used eluent was 25% ACN in 10 mM phosphate buffer solution. Studies concerning the effect of the used eluent can be found in Luste et al. (2011). The flow rate of 0.6 ml/min was used and the sample volume was 40 µl. All the samples were filtered with 0.45 µm teflon filters prior to analysis.

2.5.2 Result calculations

Molecular size distribution was determined with HPSEC. The calibration curve (log \( MW \) vs. peak retention time) was used to define the \( MW \) of the samples and \( M_n \) and \( M_w \) were further determined using Equations 1 and 2. In the equations, \( h_i \) is the height of the measured peak eluted at retention time \( R_i \) and \( M_i \) is the molecular weight of the peak at retention time \( R_i \), determined from the calibration curve. When the peak response exceeds 10 times the standard deviation of the average values of the baseline, it is considered as a peak (limit of quantification, LOQ). Peaks with significant intensity for HS were not found from the blank samples (MQ samples).

\[
M_n = \frac{\sum_i^n h_i}{\sum_i^n M_i} \tag{1}
\]

\[
M_w = \frac{\sum_i^n h_i M_i}{\sum_i^n h_i} \tag{2}
\]

Polydispersity, \( \rho \), measures the sample heterogeneity and can be calculated using Equation 3.

\[
\rho = \frac{M_w}{M_n} \tag{3}
\]

2.6 MS analyses

MS analyses were performed in order to evaluate the structure and differences of HS in different sampling depths. Two analytical MS instruments were used: Time of Flight – Mass Spectrometer (TOF-MS) and High Resolution Mass Spectrometer (HRMS).

2.6.1 TOF-MS

The used equipment was LCT Premier XE time of flight mass spectrometer (Waters Micromass, Manchester, UK), equipped with an electrospray source (z-spray) operating in negative ion mode. Capillary voltage was set to -3 000 V. Nitrogen was used as a source cone gas (10 l/h) and for desolvation gas (900 l/h). Cone voltage was set in all experiments to 20 V. Desolvation temperature was 350 °C and source temperature 120 °C. Spectra were recorded over a range of 100–1 000 m/z with a scan time of 1 s. All data were recorded in continuum mode. The instrument was operating in W-mode and was adjusted to a mass resolution of 10 500 (leucine encephalin, m/z 556). System
calibration was done over the mass range of 100–1 000 m/z using sodium formate solution. Samples were directly infused into a mass spectrometer using a 500 µl syringe (Hamilton).

MS data were analyzed using MassLynx 4.0 software. Spectra were generated by summing as many spectra as possible in a given chromatogram. No smoothing or background subtraction was used in the data treatment process. The signal intensity of molecules in the range m/z 275–375 was determined from the summed spectrum for the Van Krevelen plots. H/C and O/C values for each molecular formula were calculated and the signal intensity was normalized to the largest peak in the mass range.

The unsaturation of a molecule can be expressed by the so-called double bond equivalent (DBE), which basically means the number of double bonds and ring systems in the molecule. For C, H, O and N containing molecules, the following simplified equation for DBE calculation from molecular formula can be used, where C is number of carbon atoms, H is number of hydrogen atoms and N number of nitrogen atoms:

\[
DBE = C - \frac{H}{2} + \frac{N}{2} + 1
\]

2.6.2 HRMS

Humic substances were investigated with HRMS method in order to find more detailed information of the molecular structures. The used instrument was Autospec Premier double focusing magnetic sector mass spectrometer (Waters Micromass, Manchester, UK). The measurements were done for all the solid samples; ONK-PVA1, ONK-PVA5 and ONK-PVA10.

In the HRMS method the sample is placed in a small glass cup (Figure 2) and inserted directly inside the ion source where it desorbs and degrades. Finally the sample will be ionized in the ion source (chemical ionisation with methane). The analysis was done with magnet-scan method by scanning 5.85 min the mass range 50–500 m/z with scan time of 5 s and inter scan delay time of 0.5 s. The instrument was tuned to 10 000 resolution with perfluorokerosene (PFK) using a mass 330.97922 m/z. The direct sample insertion probe was water-cooled and temperature programmed by starting with 115 °C with 1 minute hold and then increasing the temperature 100 °C/min until 500 °C. The ion-source temperature was set at 140 °C and the data was recorded at continuum mode.

2.7 ICP-MS/MS analysis

ICP-MS/MS analysis was performed in order to examine the nature of metal complexation by humic substances and to detect a possible change in the concentration between different groundwater stations. The measurements were performed using 8800 Triple Quadrupole ICP-MS (Agilent). The following metals were determined 31→47 m/z P [O2], 32→48 m/z S, [O2], 52 m/z Cr [He], 55 m/z Mn [He], 56 m/z Fe [He], 59 m/z Co [He], 60 m/z Ni [He], 63 m/z Cu [He] and 95 m/z Mo [He] since they were thought to form complexes with HS or to be found in the HS structure based on
literature. The measurements were done from both the original untreated samples and isolated (DAX-8) samples in ONK-PVA1, ONK-PVA5 and ONK-PVA10.

The isolated samples were digested with HNO$_3$ using Milestone Ethos One microwave since acidification of samples without digestion would lead to precipitation of HS in the sample. Blank samples were also digested and measured to record possible contamination.
3. RESULTS AND DISCUSSION

The estimated amount of different organic fractions in DOM in the groundwater was determined with TOC measurements. The amount of each fraction was determined as a percentage of the initial TOC in the sample. Molecular weights of the humic substances were determined with SEC. Molecular size distribution determinations with SEC describe the MWs of the mixtures of humic substances. Analyses were performed for three parallel sample and results are given for each sample. Thus, the variation (or error) of the method can be seen between the differences in the results of parallel samples.

3.1 The amount of different fractions of DOM

3.1.1 Results for humic substances

The results of the TOC measurements for the fraction of humic substances are presented at Table 1. The other result was measured in HS isolation process and the other in the whole DOM isolation process. The TOC content in isolated sample was expected to be HS. In this study humic substances covered around 20–30 % on average of the TOC content in groundwater samples. TOC recoveries are slightly lower than in previous studies by Luste et al. (2013) as shown in the Table 2. However the difference to previous studies is not significant considering the numerous steps in the isolation process. The results of isolated samples were calculated to equate the volume of the original sample. The results of QC samples prior and after each sample sequence at the TOC instrument, were always at the limits of laboratory’s accredited method.

Table 1. The results of the TOC measurements for the fraction of humic substances measured from HS isolation process and *DOM isolation process. **Calculated for the original volume.

<table>
<thead>
<tr>
<th></th>
<th>TOC at the original sample (mg/l)</th>
<th>TOC (HS) at isolated sample (mg/l) **</th>
<th>TOC recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONK-PVA1</td>
<td>13.68*</td>
<td>3.4*</td>
<td>25**</td>
</tr>
<tr>
<td></td>
<td>16.92</td>
<td>2.9</td>
<td>17</td>
</tr>
<tr>
<td>ONK-PVA5</td>
<td>2.68*</td>
<td>0.51*</td>
<td>19*</td>
</tr>
<tr>
<td></td>
<td>2.06</td>
<td>0.65</td>
<td>32</td>
</tr>
<tr>
<td>ONK-PVA10</td>
<td>1.41*</td>
<td>0.23*</td>
<td>16*</td>
</tr>
<tr>
<td></td>
<td>1.32</td>
<td>0.33</td>
<td>25</td>
</tr>
</tbody>
</table>
Table 2. The results of TOC measurements (Luste et al. 2013). *Calculated for the original volume.

<table>
<thead>
<tr>
<th></th>
<th>TOC at original sample (mg/l)</th>
<th>TOC (HS) at isolated sample (mg/l)*</th>
<th>TOC recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONK-PVA1</td>
<td>9.6</td>
<td>3.4</td>
<td>34</td>
</tr>
<tr>
<td>ONK-PVA5</td>
<td>2.1</td>
<td>0.7</td>
<td>31</td>
</tr>
<tr>
<td>ONK-PVA10</td>
<td>1.4</td>
<td>0.3</td>
<td>27</td>
</tr>
<tr>
<td>Reference</td>
<td>10.6</td>
<td>6.5</td>
<td>58</td>
</tr>
</tbody>
</table>

3.1.2 HS amount - follow-up

The amount of humic substances at ONKALO, Olkiluoto has been studied for many years. The current results have been compared with the previous ones at Table 3. Between the samplings, the amount of HS has remained at equal level in each groundwater station. According to the results, the amount of HS reduced when the sample was taken from the groundwater station situating further beneath the ground. This is reasonable since the amount of HS in the surface water is usually much greater compared to groundwater. Also, in the groundwater stations situating closer to the ground, humus originating from the surface water/soil is most probably leached into groundwater.

Table 3. The HS (mg/l) results at ONKALO, Olkiluoto in the previous and current study.

<table>
<thead>
<tr>
<th>Groundwater station</th>
<th>Reference</th>
<th>Sampling date</th>
<th>Amount of HS (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONK-PVA1</td>
<td>Current study</td>
<td>11.–14.11.2014</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Luste et al. 2013</td>
<td>28.8.2012</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Luste et al. 2011</td>
<td>14.10.2011</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Vilhunen and Manninen 2010</td>
<td>3.6.2010</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>Manninen and Mäkelä 2006</td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td>ONK-PVA5</td>
<td>Current study</td>
<td>11.–14.11.2014</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>Luste et al. 2013</td>
<td>28.8.2012</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Vilhunen and Manninen 2010</td>
<td>3.6.2010</td>
<td>0.75</td>
</tr>
<tr>
<td>ONK-PVA10</td>
<td>Current study</td>
<td>11.–14.11.2014</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Luste et al. 2013</td>
<td>28.8.2012</td>
<td>0.35</td>
</tr>
</tbody>
</table>
3.1.3 Results for other fractions of DOM

The results for multistep isolation process (Figure 1) are shown in the Table 4.

**Table 4. Results from the DOM isolation process.**

<table>
<thead>
<tr>
<th></th>
<th>ONK-PVA1 TOC recovery (%)</th>
<th>ONK-PVAS TOC recovery (%)</th>
<th>ONK-PVA10 TOC recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOA (humic substances)</td>
<td>25</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>HIB</td>
<td>0.36</td>
<td>0.77</td>
<td>1.5</td>
</tr>
<tr>
<td>HIA</td>
<td>0.85</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>HIN</td>
<td>1.3</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>HON</td>
<td>12</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>sum (%)</td>
<td>39</td>
<td>52</td>
<td>43</td>
</tr>
</tbody>
</table>

In comparison with the previous study major improvements in the isolation process were achieved. From the results it can be seen that hydrophobic acids (HOA) i.e. humic substances, hydrophobic neutrals (HON) and hydrophilic neutrals are the major components of DOM. The amount of hydrophilic bases (HIB) and hydrophilic acids (HIA) in the ONK-PVA samples can be considered negligible. The amount of hydrophilic fractions seems to increase with increasing sampling depth, but with this amount of measured data no definite conclusions can be drawn.

When the amount of TOC that was not retained by XAD-4 resin was included in the calculations, the sum of all fractions was approximately 100%. From the theoretical point of view this fraction may consists of hydrophobic basic compounds. However this conclusion needs further investigations. The approximate recovery of 100% indicated that the multistep isolation process (Figure 1) is feasible for the treatment and no residual TOC from the used resins was present in significant amounts.

**Table 5. Results from the DOM isolation process. Unknown fraction included.**

<table>
<thead>
<tr>
<th></th>
<th>ONK-PVA1 TOC recovery (%)</th>
<th>ONK-PVAS TOC recovery (%)</th>
<th>ONK-PVA10 TOC recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOA</td>
<td>25</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>unknown fraction</td>
<td>42</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>HIB</td>
<td>0.36</td>
<td>0.77</td>
<td>1.5</td>
</tr>
<tr>
<td>HIA</td>
<td>0.85</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>HIN</td>
<td>1.3</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>HON</td>
<td>12</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>sum (%)</td>
<td>81</td>
<td>107</td>
<td>98</td>
</tr>
</tbody>
</table>
3.2 Molecular size distribution

3.2.1 Calibration

The calibration was done using PSS standards 210, 3 610 and 6 520 g/mol. The calibration curve is presented in the Figure 3.

![HPSEC calibration curve of PSS standards (MWs 210, 3 610 and 6 520 g/mol).](image)

\[ y = -40.37 \log M + 6.146 \]
\[ R^2 = 0.9999 \]

**Figure 3.** The HPSEC calibration curve of PSS standards (MWs 210, 3 610 and 6 520 g/mol).

3.2.2 Results

The chromatograms of blank, ONK-PVA1, ONK-PVA5 and ONK-PVA10 samples are presented in Figures 4–7. In the Figures 4, 5 and 6 there are two chromatograms of the same sample other being from the HS isolation process (blue) and the other from the DOM isolation process (red). As can be seen, both isolation processes resulted in similar kind of profiles. The intensity of the DOM isolated sample was higher due to larger sample volumes and hence more concentrated sample. In the Figure 7 all the samples are shown in the same chromatogram.
Figure 4. HPSEC Chromatograms of ONK-PVA1 with two different isolation processes. Red chromatogram is from the DOM isolation process and blue from the HS isolation.

Figure 5. HPSEC Chromatograms of ONK-PVA5 with two different isolation processes. Red chromatogram is from the DOM isolation process and blue from the HS isolation.
The profile of the chromatogram of ONK-PVA1 sample differed significantly from the other chromatograms. Also the amount of humic substances seemed to be much lower in ONK-PVA10 sample compared to samples taken from ONK-PVA5 and especially from ONK-PVA1, according to the responses in chromatograms. The chromatograms were divided into four to five fractions, and the latter fractions (RT's around 30-35 min) were similar to each other. However, the similarity concerned only the location of the peaks since the ratio of the peak heights varied. The main differences were found from the earlier parts of the chromatogram, representing the molecules with the highest molecular weights. According to the chromatograms (Figure 7) the molecular size distribution appears to get lower as the sampling point goes further beneath the ground.

**Figure 6.** HPSEC Chromatograms of ONK-PVA10 with two different isolation processes. Red chromatogram is from the DOM isolation process and blue from the HS isolation.

**Figure 7.** HPSEC chromatograms of blank, ONK-PVA1, ONK-PVA5 and ONK-PVA10.
The calculated $M_n$ and $M_w$ values for each groundwater sample are presented in Table 6 (calculations using Equations 1 and 2). The values were lower when the sample was from the groundwater station situating further beneath the ground. When exploring the results, it should be noted that the HS in the groundwater sample does not represent the original situation but the isolated HS in completely different environment. Results of the reference samples (Luste et al. 2013) have shown that the treatments have some effect on the results by slightly reducing the values. Thus, also in case of the groundwater samples the expected values are somewhat higher than the measured ones.

Table 6. Molecular size distribution results for two samples and results from previous study. (*samples from DOM isolation process.)

<table>
<thead>
<tr>
<th></th>
<th>ONK-PVA1</th>
<th></th>
<th>ONK-PVA5</th>
<th></th>
<th>ONK-PVA10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M_n$</td>
<td>$M_w$</td>
<td>$M_n$</td>
<td>$M_w$</td>
<td>$M_n$</td>
</tr>
<tr>
<td>Current study</td>
<td>418</td>
<td>879</td>
<td>386</td>
<td>557</td>
<td>529</td>
</tr>
<tr>
<td>Current study*</td>
<td>518</td>
<td>1089</td>
<td>440</td>
<td>816</td>
<td>577</td>
</tr>
<tr>
<td>Luste et al 2013</td>
<td>790</td>
<td>1020</td>
<td>523</td>
<td>633</td>
<td>443</td>
</tr>
</tbody>
</table>

The values for polydispersivity, i.e. sample heterogeneity, are presented in Table 7 (calculated using Equation 3). According to the results, polydispersivity was the highest at the ONK-PVA1 groundwater sample and there was a slight difference compared to previous study (Luste et al. 2013). There seemed to be more dispersion in ONK-PVA1 and ONK-PVA5 samples than in the previous study whereas in ONK-PVA10 samples there were less. Polydispersivity is a measure of variability in molecular size distribution of a sample. The result indicated that in the groundwater sample taken from closest to groundsurface, there was a greater variation in molecular size of HS compared to groundwater samples taken deeper from the groundsurface. HS in samples taken deeper from the groundsurface had a more uniform molecular size distribution.

Table 7. Polydispersivity ($\rho$) values for two samples and results from previous study. (*samples from whole DOM isolation process.)

<table>
<thead>
<tr>
<th></th>
<th>ONK-PVA1</th>
<th>ONK-PVA5</th>
<th>ONK-PVA10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current study</td>
<td>2.1</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Current study*</td>
<td>2.1</td>
<td>1.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Luste et al 2013</td>
<td>1.3</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>
3.3 Structural determination by MS

3.3.1 TOF-MS analyses for the selected samples

The mass spectra by direct infusion for blank, ONK-PVA1, ONK-PVA5 and ONK-PVA10 sample are shown in Figures 8–10.

**Figure 8.** Mass spectrum of ONK-PVA1 sample (TOF-MS). Circled peaks below 250 m/z in the spectrum are most likely due to impurities from cation exchange step in the sample treatment.

**Figure 9.** Mass spectrum of ONK-PVA5 sample (TOF-MS). Circled peaks below 250 m/z in the spectrum are most likely due to impurities from cation exchange step in the sample treatment.
Figure 10. Mass spectrum of ONK-PVA10 sample (TOF-MS). Circled peaks below 250 m/z in the spectrum are most likely due to impurities from cation exchange step in the sample treatment.

The mass spectra showed typical patterns for humic substances. With the groundwater samples the maximum of mass distributions shifted towards lower m/z values as the sampling depth increased indicating lower molecular masses. For the groundwater samples the maxima of mass distributions were 425 m/z, 400 m/z and 375 m/z for ONK-PVA1, ONK-PVA5 and ONK-PVA10 respectively. The peaks below 250 m/z in the spectrum were most likely due to cation exchange step in the sample treatment. These impurities stayed in the resin despite the soxhlet-extraction with acetone.

Because of the large number of molecules detected by MS, it is necessary to find ways to visualize the data. When calculating the molecular formulas, the following chemical constrains were applied: the maximum number of elements C(4−100), H(unlimited), N(0−5), O(2−80), P(0−20) and S(0−2). Also nitrogen rule was applied and thresholds for molecular elemental ratios (0<H/C<2.5, 0<O/C<1). An error of 5 ppm was allowed in molecular formula calculations by the Masslynx software. An example of calculated molecular formulas in millidaltons (mDa) and in parts per million (ppm) for sample ONK-PVA10 are shown in Table 8.
Table 8. Example for calculated molecular formulas for sample ONK-PVA10.

<table>
<thead>
<tr>
<th>Measured mass m/z</th>
<th>Theoretical mass (m/z)</th>
<th>mDa</th>
<th>ppm</th>
<th>Formula</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>O</th>
<th>P</th>
<th>S</th>
<th>O/C</th>
<th>H/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>291,0930</td>
<td>291,0929</td>
<td>0.1</td>
<td>0.3</td>
<td>C15 H17 N O3 S</td>
<td>15</td>
<td>17</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0.20</td>
<td>1.1</td>
</tr>
<tr>
<td>292,1169</td>
<td>292,1167</td>
<td>0.2</td>
<td>0.7</td>
<td>C13 H24 O3 S2</td>
<td>13</td>
<td>24</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>0.23</td>
<td>1.8</td>
</tr>
<tr>
<td>293,1013</td>
<td>293,1012</td>
<td>0.1</td>
<td>0.3</td>
<td>C13 H15 N3 O5</td>
<td>13</td>
<td>15</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0.38</td>
<td>1.2</td>
</tr>
<tr>
<td>294,1039</td>
<td>294,1038</td>
<td>0.1</td>
<td>0.3</td>
<td>C14 H18 N2 O3 S</td>
<td>14</td>
<td>18</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0.21</td>
<td>1.3</td>
</tr>
<tr>
<td>295,1830</td>
<td>295,1827</td>
<td>0.3</td>
<td>1</td>
<td>C17 H28 O2 P</td>
<td>17</td>
<td>28</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0.12</td>
<td>1.6</td>
</tr>
<tr>
<td>296,0061</td>
<td>296,0061</td>
<td>0</td>
<td>0</td>
<td>C16 H9 O2 P S</td>
<td>16</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0.13</td>
<td>0.56</td>
</tr>
<tr>
<td>297,0887</td>
<td>297,0892</td>
<td>-0.5</td>
<td>-1.7</td>
<td>C14 H18 O5 P</td>
<td>14</td>
<td>18</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0.36</td>
<td>1.3</td>
</tr>
<tr>
<td>298,0981</td>
<td>298,0987</td>
<td>-0.6</td>
<td>-2</td>
<td>C13 H18 N2 O4 S</td>
<td>13</td>
<td>18</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0.31</td>
<td>1.4</td>
</tr>
<tr>
<td>299,0924</td>
<td>299,0923</td>
<td>0.1</td>
<td>0.3</td>
<td>C13 H18 N O5 P</td>
<td>13</td>
<td>18</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0.38</td>
<td>1.4</td>
</tr>
<tr>
<td>300,0634</td>
<td>300,0634</td>
<td>0</td>
<td>0</td>
<td>C16 H12 O6</td>
<td>16</td>
<td>12</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0.38</td>
<td>0.75</td>
</tr>
<tr>
<td>301,1198</td>
<td>301,1191</td>
<td>0.7</td>
<td>2.3</td>
<td>C12 H20 N3 O4 P</td>
<td>12</td>
<td>20</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0.33</td>
<td>1.7</td>
</tr>
<tr>
<td>302,0940</td>
<td>302,0943</td>
<td>-0.3</td>
<td>-1</td>
<td>C20 H14 O3</td>
<td>20</td>
<td>14</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0.15</td>
<td>0.70</td>
</tr>
<tr>
<td>303,0866</td>
<td>303,0869</td>
<td>-0.3</td>
<td>-1</td>
<td>C16 H15 O6</td>
<td>16</td>
<td>15</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0.38</td>
<td>0.94</td>
</tr>
<tr>
<td>304,0977</td>
<td>304,0977</td>
<td>0</td>
<td>0</td>
<td>C15 H17 N2 O3 P</td>
<td>15</td>
<td>17</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0.20</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The molecular formulas derived from the mass spectral data can be graphically arranged in a Van Krevelen diagram that displays the C/H ratio versus the O/C ratio of each molecule (Kim et al. 2003). The molar ratio of hydrogen to carbon (H/C) was set as the x-axis and the molar ratio of oxygen to carbon (O/C) as the y-axis. As a result, classes of compounds were able to identify in a certain place in the diagram. Elemental compositions for each peak was calculated from the mass spectra and then converted to H/C and O/C ratios. An example of a 2D Van Krevelen plot obtained from the sample from ONK-PVA10 is shown in the Figure 11. Also Van Krevelen plots from formulas that contain N, P or S are shown in the Figures 12–14. There did not seem to be any apparent distribution of formulas containing these elements.
Figure 11. Van Krevelen plot for ONK-PVA10 sample.

Figure 12. P containing formulas of ONK-PVA10 on Van Krevelen plot.

Figure 13. S containing formulas of ONK-PVA10 on Van Krevelen plot.
Figure 14. N containing formulas of ONK-PVA10 on Van Krevelen plot.

In the three dimensional Van Krevelen plot the ion intensity is plotted in the 3rd dimension. In figures 15–20 contour plots generated from the intensity data of all detected signals in the mass range $m/z$ 275–375 are illustrated.

Figure 15. Contour plot for ONK-PVA1 TOF sample.
Figure 16. Contour plot for ONK-PVA5 TOF sample.

Figure 17. Contour plot for ONK-PVA10 TOF sample.
Figure 18. Contour plot for ONK-PVA10 TOC-N sample.

Figure 19. Contour plot for ONK-PVA10 TOC-S sample.
Compared to the previous study (Luste et al. 2013), the plots appear slightly different due to different kind of data smoothing. However, the same characteristics can be seen between different sampling depths. When sample was taken closer to the ground the intensity maximum of H/C was lower and of O/C higher. This indicated higher aromaticity and higher carboxylate/oxygen content. According to this, humic substances sampled further from the ground surface contained less oxygen in their molecular structure. However, it has to be noticed, that visible differences in contour plots between these isolated samples were due to different intensity distributions and not due to occurrence of completely different set of molecules.

When plotting the N, S and P containing formulas in a similar way no apparent differences can be seen. Molecules containing above-mentioned elements seemed to spread rather evenly among all molecules.

**Defining the saturation by DBE**

The unsaturation of a molecule can be expressed by DBE (Equation 4), i.e. the number of double bonds and ring systems in the molecule. Normalizing DBE to the number of carbon atoms within a molecule (DBE/C) provide a measure of the aromaticity. Frequency distribution of carbon normalized double bond equivalents are shown in Figures 21–23.

DBE/C-plots support the interpretation of the contour plots. It can be seen, that molecules found from the ONK-PVA10 groundwater station settle on the lower values (not so much datapoints in the circled area) than molecules found in the ONK-PVA1 indicating less aromatic compounds being present in the samples taken deeper from the ground.
Figure 21. Frequency distribution of carbon normalized DBE of ONK-PVA1.

Figure 22. Frequency distribution of carbon normalized DBE of ONK-PVA5.
3.3.2 HRMS analysis for ONK-PVA1 sample

The total ion chromatograms from HRMS analyses of ONK-PVA1, ONK-PVA5, ONK-PVA10, reference FA and reference HA samples are shown in the Figures 24−28.

Figure 23. Frequency distribution of carbon normalized DBE of ONK-PVA10.

Figure 24. Total ion chromatogram of reference FA (HRMS analysis).
**Figure 25.** Total ion chromatogram of HA reference (HRMS analysis).

**Figure 26.** Total ion chromatogram of ONK-PVA1 sample (HRMS analysis).
As shown in the Figures 24–28, the sample started to desorb from the sample cup around 1.5 minutes (the probe temperature program is around 215 °C). However the humic-like spectrum can be seen at around 3.25 min when the sample cup temperature was around 340 °C. The spectra were summed from 3.25 min until the end of the program resulting in 29 scans for all the samples. The resulting mass spectra are shown in the figures 29–34.
**Figure 29.** Mass spectrum of calibrant PFK (HRMS analysis).

**Figure 30.** Mass spectrum of ONK-PVA1 sample (HRMS analysis).
**Figure 31.** Mass spectrum of ONK-PVA5 sample (HRMS analysis).

**Figure 32.** Mass spectrum of ONK-PVA10 sample (HRMS analysis).
The mass spectra show similar patterns as in the spectra obtained with ESI-TOF method. The shift in the maximum of mass distribution as in the ESI-TOF spectrum is not so clearly seen. This is most likely due to ionization process in the direct sample insertion where the temperature program of the probe determines the molecules to desorb and ionized whereas in the ESI-TOF the whole sample is ionized when it is infused into the ion source.
The Van Krevelen plots were made similarly to ESI-TOF experiments. Only C, H and O were allowed in the molecular formula construction. An example of the Van Krevelen diagram constructed from the HRMS data is shown in the Figure 35.

![Van Krevelen plot of ONK-PVA1 analyzed with HRMS.](image)

**Figure 35.** Van Krevelen plot of ONK-PVA1 analyzed with HRMS.

The Kendrick mass defect analysis (KDM) can be very useful when studying DOM and it has been used extensively (Hatcher et al. 2007, Freitas et al. 2002). With KMD analysis, the homologous series of compounds can be identified within a sample. A homologous series is a series of \( m/z \) values that differ only by the exact mass of a certain functional group, such as CH\(_2\), COOH or H\(_2\). All the observed masses are normalized to the nominal mass of a functional group, for example CH\(_2\), as in the equations 5 and 6.

\[
\text{Kendrick mass} = \frac{\text{observed mass} \times \text{nominal mass of CH}_2}{\text{exact mass of CH}_2} \quad (5) \\
\text{KDM} = (\text{Kendrick nominal mass} – \text{Kendrick mass}) \quad (6)
\]

The Kendrick mass defect (KDM) was then calculated and all data were sorted according to KDM. Compounds with identical KMD values represent members of a CH\(_2\) homologous series and are easily visualized from the plot as a straight line parallel to x-axis (Figures 36–38).
**Figure 36.** Kendrick mass defect plot of ONK-PVA1 analyzed by CH$_2$ KMD analysis.

**Figure 37.** Kendrick mass defect plot of ONK-PVA5 analyzed by CH$_2$ KMD analysis.
Figure 38. Kendrick mass defect plot of ONK-PVA10 analyzed by CH$_2$ KMD analysis.

Figure 39. Kendrick mass defect plot of ONK-PVA1, ONK-PVA5 and ONK-PVA10 analyzed by CH$_2$ KMD analysis.
When plotting all the Kendrick mass defect plots together differences between ONK-PVA samples can be seen (Figure 39). Most of the compounds overlap completely suggesting similar if not identical chemical composition. However, with PVA10 a larger amount of compounds has a lower Kendrick mass defect (KMD) than within PVA1, an observation which is consistent with larger aliphatic contribution in PVA10. Saturation and reduction increases with decreasing KMD while the degree of oxidation and unsaturation increases with increasing KMD. The HRSM analysis support the ESI-TOF analyses: The humic substances taken closer to the ground had higher aromaticity and higher carboxylate/oxygen content, whereas the humic substances in the deeper ONK-PVAs were less aromatic and contain less oxygen.

3.4 Analysis of metal content in groundwater samples by ICP-MS/MS

Elemental composition of HS typically consists of carbon, hydrogen, oxygen, nitrogen, sulfur and trace elements (Frimmel 2005). Hetero elements like oxygen, nitrogen and sulfur are found in several functional groups such as carboxyl groups, phenolic and carbohydrate hydroxyl groups, amino groups, quinonic groups and reactive positions in aromatic structures (Frimmel 2005, Klavins et al. 2012). Due to the different functionalities HS have an ability to interact with metal ions and have an impact on their mobility, behavior and speciation forms in the environment.

After the isolation of humic substances with DAX-8 metal concentrations were measured from the samples taken from three different groundwater stations ONK-PVA1, ONK-PVA5 and ONK-PVA10 using Agilent 8800 ICP-MS triple quad. The isolated samples were digested with HNO3 using Milestone Ethos One microwave since acidification of samples without digestion would lead to precipitation of HS in the sample. For comparison metals were also measured from the original untreated samples. Since the metal binding capacity of humic substances is strongly pH-dependent (Perdue et al. 1987) the metal concentrations in the isolated groundwater samples may not be transferred directly to the original samples. Therefore the metal recovery in HS is an estimate since the metal binding capacity of HS may have changed during the isolation procedure.

Aquatic HS is known to contain sulfur ranging from 0.5 to 1.43 % (Xia et al. 1998) in the structure and therefore sulfur concentration could be used as a tool to estimate the change in the amount of HS between different groundwater stations. Tables 9–11 present the results for metal determinations of both the original (i.e. the untreated samples) and isolated HS samples. Recovery column indicates the percentage of metals complexed to the humic substances.
Table 9. The results of the metal measurements of HS in the ONK-PVA1 sample.
*Calculated for the original volume

<table>
<thead>
<tr>
<th></th>
<th>Original sample (µg/l)</th>
<th>RSD-% (n=7)</th>
<th>Isolated sample (µg/l)*</th>
<th>RSD-% (n=7)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>148</td>
<td>5.2</td>
<td>1.16</td>
<td>8.8</td>
<td>0.78 %</td>
</tr>
<tr>
<td>S</td>
<td>64959</td>
<td>10</td>
<td>384</td>
<td>16</td>
<td>0.59 %</td>
</tr>
<tr>
<td>Cr</td>
<td>81.2</td>
<td>6.8</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mn</td>
<td>112</td>
<td>5.4</td>
<td>0.175</td>
<td>12</td>
<td>0.16 %</td>
</tr>
<tr>
<td>Fe</td>
<td>331</td>
<td>5.4</td>
<td>1.70</td>
<td>6.6</td>
<td>0.51 %</td>
</tr>
<tr>
<td>Co</td>
<td>0.64</td>
<td>7.3</td>
<td>0.0210</td>
<td>13</td>
<td>3.3 %</td>
</tr>
<tr>
<td>Ni</td>
<td>212</td>
<td>2.7</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cu</td>
<td>2.62</td>
<td>6.8</td>
<td>0.162</td>
<td>20</td>
<td>6.2 %</td>
</tr>
<tr>
<td>Mo</td>
<td>6.86</td>
<td>2.3</td>
<td>0.958</td>
<td>10</td>
<td>14 %</td>
</tr>
</tbody>
</table>

Nd = not detected

Table 10. The results of the metal measurements of HS in ONK-PVA5 sample.
*Calculated for the original volume

<table>
<thead>
<tr>
<th></th>
<th>Original sample (µg/l)</th>
<th>RSD-% (n=8)</th>
<th>Isolated sample (µg/l)*</th>
<th>RSD-% (n=7)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>Nd</td>
<td>-</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S</td>
<td>42589</td>
<td>12</td>
<td>154</td>
<td>11</td>
<td>0.36 %</td>
</tr>
<tr>
<td>Cr</td>
<td>48.5</td>
<td>5.9</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mn</td>
<td>90.7</td>
<td>4.8</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fe</td>
<td>148</td>
<td>4.9</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Co</td>
<td>0.147</td>
<td>4.7</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ni</td>
<td>40</td>
<td>2.2</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cu</td>
<td>2.10</td>
<td>5.3</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mo</td>
<td>1.94</td>
<td>4.6</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Nd = not detected
Table 11. The results of the metal measurements of the humic substances in ONK-PVA10 sample. *Calculated for the original volume

<table>
<thead>
<tr>
<th></th>
<th>Original sample (µg/l)</th>
<th>RSD-% (n=8)</th>
<th>Isolated sample (µg/l)*</th>
<th>RSD-% (n=7)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>Nd</td>
<td>-</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S</td>
<td>220</td>
<td>12</td>
<td>8,0</td>
<td>8.1</td>
<td>3.6 %</td>
</tr>
<tr>
<td>Cr</td>
<td>Nd</td>
<td>-</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mn</td>
<td>141</td>
<td>6,1</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fe</td>
<td>Nd</td>
<td>-</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Co</td>
<td>Nd</td>
<td>-</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ni</td>
<td>0.141</td>
<td>14</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cu</td>
<td>Nd</td>
<td>-</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mo</td>
<td>Nd</td>
<td>-</td>
<td>Nd</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Nd = not detected

Original and extracted blank samples were also measured. Metals from the original untreated blank sample (0.1 M NaOH) were not detected. After DAX 8-isolation the blank sample contained 3.1 µg/l sulfur and the amount was subtracted from the results of the isolated samples. According to Tables 9–11 only the HS isolated from ONK-PVA1 sample has formed metal complexes with the metals that were determined. Overall, the metal concentrations in the samples decreased when going further beneath the ground both in the original and isolated samples. When comparing the different groundwater samples sulfur concentration also decreased but sulfur was still found in the isolated HS structures.

Sulfur concentration of the isolated samples (HS) decreased by 98% when comparing ONK-PVA1 and ONK-PVA10 samples. This supports the results obtained from TOC analysis and indicates that the HS content decreased when going further beneath the ground. It is to be noted that some changes in the sulfur content might also be due to changes in HS structure between different groundwater stations.
4. FURTHER SUGGESTIONS

In next study, groundwater samples will be taken most probably from ONK-PVA1, ONK-PVA5, ONK-PVA10 and ONK-PVA11. In SEM analysis, the micrograph magnifications need to be selected so, that there is no significant gaps in the range of noticeable particles. In the future, SEM samples will be analysed at more regular magnification intervals (e.g. 2000x) to help bridge the gap between the 1000 and 4000 magnifications.

In 2016 or 2017, groundwater samples from the same sampling points will be collected for the analyses of humic substances and dissolved organic matter. In addition to ONK-PVA1, ONK-PVA5, ONK-PVA10 and ONK-PVA11, samples may be collected also from ONK-PVA9 and ONK-KR16. Since for next years, the emphasis is on the colloid studies, less detailed analyses on HS and DOM will be carried out. These analyses will concentrate on DOM fractionation and determination of HS concentration and molecular size distribution.
5. CONCLUSIONS

The annual follow-up of HS in the ONKALO groundwater was continued including determinations of other DOM fractions in the study. As a result of the isolation method the average amount of humic substances were 3.2 mg/l in ONK-PVA1, 0.58 mg/l in ONK-PVA5 and 0.28 mg/l in ONK-PVA10. These results are similar to the previous studies. The amount of HS covered around 20 – 30 % of the TOC content in groundwater samples. The amount of HS in the groundwater appears to be lower at the water from the groundwater stations situating further beneath the ground. Within the other fractions of DOM, the hydrophopic fractions seemed to be the major fractions in groundwater samples. However significant unknown fraction of DOM was still present. Even though the isolation method was proven to be suitable since the mass balance of all the fractions, including the unknown was close to 100%.

The molecular size distribution measurements in order to find out the $MW$, $M_n$ and $M_w$ of the humic matter were performed using HPSEC with UV$_{254}$ detection. The eluent was 25% ACN in 10 mM phosphate buffer. The apparent $M_n$ values of ONK-PVA1, ONK-PVA5 and ONK-PVA10 water were around 468, 413 and 553, respectivley, whereas the $M_w$ values were 984, 687 and 593 (averages from two samples). According to the results, the size of HS molecules varied as a function of groundwater sampling depth, i.e. the molecular size distribution showed the highest values near the ground surface (ONK-PVA1) and the value became smaller in the samples taken further beneath the ground (ONK-PVA5 and ONK-PVA10). Altogether, according to the TOC measurements and the responses in SEC-analyses the amount HS was considerably lower in ONK-PVA5 and ONK-PVA10 samples compared to the ONK-PVA1 sample.

The structure of the studied humic substances was evaluated by two different MS methods. Similar kinds of results were achieved with two different MS methods and two different ways of data analysing. According to the results, the aromaticity of the HS content in the groundwater seemed to get lower when going further from the ground. At the same time the oxygen content in the molecular structures of organic material decreased.

Metal concentrations were also measured from the three groundwater samples in order to evaluate the change between different groundwater stations. The decrease in sulfur concentration in isolated groundwater samples when going further beneath the ground was found to support the results of TOC analysis.
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