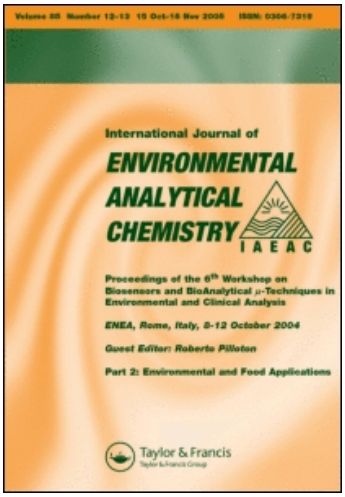

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Artefacts in XAD-8 NOM fractionation

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The XAD-8 resin has been widely used during the last decades to characterize and isolate natural organic matter (NOM) in water. The present work focuses on the performance and limitations of the XAD-8 method. A number of different NOM samples (mostly RO-isolates) have been XAD-8 fractionated with the purpose to study (1) the impact of DOC concentration of the samples on the quality of the resulting fractions and (2) the stability of NOM in the different fractions during the XAD-8 fractionation procedure and storage. Focus is placed on the method's independence of NOM concentration, the stability (quantity and quality) of the hydrophilic fraction during the fractionation, the relationship between hydrophilic and hydrophobic fractions, and the stability of the obtained fractions after the fractionation is completed.

The main conclusions are that the division into hydrophobic and hydrophilic fractions are not independent of the NOM concentration and not constant during the procedure, furthermore that the XAD-8 fractions may undergo irreversible alteration of structure due to the procedure or storage that will influence on the interpretation of the data. The possible consequences for the interpretation of results and further analysis or use of the XAD-8 based fractions are discussed.

Keywords: NOM; XAD-8; Hydrophobic; Hydrophilic; Analytical artefacts

1. Introduction

Natural organic material (NOM) is an important component in the terrestrial and aquatic environment because of its involvement in a large number of geochemical and biological processes such as weathering, pH buffering and metal complexation, as well as transport of pollutants.

In 1976 Leenheer and Huffman published an operational defined chromatographic method for the fractionation of NOM into fractions based on their hydrophilic/hydrophobic and acid/basic character using a sequence of three different column materials [1, 2]. There are now a large variety of modifications and clones from the original method of 1976, adapted to suit very different analytical purposes. The first column in the sequence contained the Amberlite XAD-8 resin from Rohm and Haas: A methylmethacrylate copolymer with weak polar properties and relative high surface area. This material has over the last few decades also become a popular tool

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for isolation and subsequent investigation of the more hydrophobic fraction of NOM from an aqueous sample. This method was introduced by Thurman and Malcolm in 1981 and resulted in what they defined as a fulvic acid- and a humic acid fraction [3]. The method was adopted by International Humic Substances Society (IHSS http://www.ihss.gatech.edu/) and the international standards and reference material of fulvic- and humic acids is based on isolation using XAD-8.

XAD-8 is widely used to fractionate NOM into hydrophilic and hydrophobic fraction by simply pumping a given volume of water sample at a fixed pH through the resin: The material retained on the resin is defined as *hydrophobic*, while the NOM that passes though the column is defined *hydrophilic*. The concentration ratio between the fractions is used to express the hydrophobic character of the NOM. As stressed in the original method development [1, 2], the XAD-8 technology provides an operationally defined chromatographic method of separation and investigation of the NOM material. The most important implication of this is that small differences in methodology may make comparison between data from various methods and operators difficult or even impossible. Several publications address this problem in critical reviews of the fractionation and isolation techniques [4–6]. The aim of the work presented in this article is to search for methodical artefacts that may have influence on the interpretation of XAD-8 generated data and demonstrate the order of their influence. This is conducted by testing a set of hypotheses regarding the behaviour of NOM material and the XAD-8 materials performance and ability to separate hydrophobic from more hydrophilic NOM material:

(1) Concentration effect: The ratio of the hydrophobic acid fraction (HPOA) to hydrophilic fraction (HPI) is independent of the original DOC concentration.
(2) Chromatographic effect: The composition of the eluded HPI fraction will remain constant during the XAD-8 fractionation.

Secondly the questions of chemical change due to fractionation or storage of the fractions are addressed:

(3) Stability and artefact essay: HPI and HPOA represent stable chemical defined fractions. Neither chemical alteration during the fractionation procedure nor artefacts due to chromatographic methodology will establish a new HPI/HPO equilibrium after a removal of either of the fractions.

Laboratory experiments using reverse osmosis isolates of NOM material were conducted to test these hypotheses. Method reproducibility, especially in regards to different scaling of the methodical procedure and DOC concentration, as well as stability of the generated fractions is addressed.

2. Experimental

2.1. *Samples and sample preparation*

The NOM material used in this study are from selected RO-isolates produced from Nordic surface water locations [7]. These RO-isolates are produced on site by processing 500–1100 L of surface water through a mobile reverse osmosis (RO) unit (PROS2/2S) resulting in a 25 L concentrated DOM sample. To prevent precipitation

of insoluble salts, such as $CaCO_3$ and $CaSO_4$, the water is first pumped through an inline cation exchanger replacing other cations with Na^+. The 25 L samples were then rota-evaporated and freeze dried [8]. This RO-isolation technique provides a stable NOM material representing practically all the organic material in the original sample and is believed to become the standard method of isolation of DOM in the future [9].

RO-isolates were dissolved in carbon-free water (MilliQ) to the desired concentration at least 24 h prior to fractionation. An approximately 250 mL sample was required per fractionation, since part of the sample was spent during filtration and some was used for DOC measurements. The samples were filtered using a 0.45 μm membrane filter. Samples were stored in pre-baked brown glass bottles in a dark cooling-room until they were fractionated and analyzed.

DOC was measured by high temperature catalytic combustion. Optical properties are measured as absorption at 254, 400 and 600 nm in samples that were pH adjusted to pH 2.0 (±0.1).

2.2. *Methods*

2.2.1. XAD-8 fractionation. Unless otherwise noted the XAD-8 fractionations were performed by fractionating 180 mL sample adjusted to pH 2 (±0.1) through a XAD-8 column (length 8 cm and inner diameter 5 mm; 3 mL resin volume) at a flow speed of 1 mL min^{-1}. The sample was acidified to pH 2.0 (±0.1) by addition of 200–400 μL 37% HCl (p.a.). The whole sample was then without delay pumped through the XAD-8 column. The hydrophobic fraction, (HPO), is adsorbed by the XAD-8 material and the hydrophilic fractions (HPI) pass through the column. The first 30 mL passing through the column were discarded as the dead-volume of the column-system. The rest of the eluate was collected and analyzed as the HPI fraction. The system was then flushed by passing 30 mL of carbon-free water at pH 2 through the column.

The hydrophobic acid fraction (HPOA) was eluated by back-flushing the column with 1 N NaOH solution at a flow speed of 1 mL min^{-1}. 30 mL of eluated HPOA was collected and then diluted back to its original concentration by adding 150 mL carbon-free water. The XAD-8 fractions (HPI, HPOA) were stored in pre-baked brown glass bottles in the dark at 4°C until analyzed or further fractionated. The hydrophobic neutrals (HPON) are defined as the fraction that remains on the XAD-8 column after the NaOH extraction. The size of this fraction is obtained by subtracting the amount of carbon in the HPI and HPOA fractions from the total sample DOC. HPON compounds are removed from the column by a washing procedure using NaOH, HCl and carbon free water between the samples. Blind samples of carbon-free water were treated and run as an ordinary sample frequently in between the ordinary samples. These blind samples revealed a background DOC leakage from the column ranging from 0.1–0.3 mgC L^{-1} for the HPI fractions and 0.2–0.5 mgC L^{-1} for the HPOA fractions (depending on the column and number of runs performed on it). These background values are subtracted from the sample fraction DOC.

2.2.2. Optical properties. Optical properties were measured as absorption at 254, 400 and 600 nm on a Hitachi U2000 Spectrophotometer using a 1 cm quartz cell.

Unless otherwise specified, all samples were acidified to pH 2.0 (±0.1) with 37% HCl just prior to measurements, as the absorbtivity is known to be pH dependent [10]. pH 2 was used since this is the key reference pH of the XAD-8 procedure. Re-measurements of a random subset of fractions revealed no significant or systematic change in absorptivity over a 48 h period. Normalized 254 nm absorption, defined as 254 nm absorption of a fraction aliquot divided by initial 254 nm absorbance of the total sample, was used to monitor the consistency of the fractions at different NOM concentrations. Specific Absorption Ratio (SAR), defined as absorption of 254 nm divided by absorption at 400 nm, and the specific UV absorption ($sUVa = A_{254\,nm}/DOC$) were used as indicators of structural differences between compared fractions.

2.2.3. DOC/TOC analysis. DOC concentrations were determined on a Shimadzu TOC 5000A where organic carbon (OC) is combusted to CO_2 by means of high temperature and catalysis. The CO_2 is subsequently measured using an IR detector. Combustion temperature was 680°C and platinum is used as catalyst.

2.2.4. Filtration at 0.45 µm. All filtrations were performed with 0.45 µm Sartorius cellulose nitrate filters (Diameter 5 cm). Prior to each filtration the filters were rinsed with 50–100 mL carbon-free water. Filtration is the performed using Millipore equipment under mild suction.

2.3. *Experimental designs*

2.3.1. Concentration effect experiment. This experiment was conducted in order to test Hypothesis 1. Samples with approximate DOC concentration of 40 mgC L^{-1} were prepared using RO isolates from the sites Birkenes (fall) and Svartberget (spring) (table 1). These two materials were chosen to represent NOM originating from spruce forests in sites of different physicochemical and geological character [11]. Birkenes is a boreal coastal site with thin acid brown soils and a high annual precipitation and sulphur deposition. Svartberget is a boreal inland site, mainly covered by thick podzol and histosol. This catchment receives significantly less precipitation and sulphur deposition. Both catchments are of approximately the same size [7]. The samples were then filtrated and diluted into estimated TOC concentrations of 0–40 mgC L^{-1} (see table 1 for the measured DOC concentrations). Four replicates of each sample and concentration were then XAD-8 fractionated. The relative proportions of the HPI, HPOA and HPON fractions are compared for the various concentrations.

The DOC concentrations of the samples in this experiment were designed to deliberately exceed the recommendations found in the original XAD methodology [1–3] in order to test the capacity of the XAD-8 material.

2.3.2. Consistency of HPI during fractionation. This experiment was conducted to test Hypothesis 2. In this experiment the stock solution prepared from the Svartberget (Spring) RO-isolate, described in the previous experiment, was used. The samples were fractionated on the same XAD-8 columns, but instead of collecting the HPI fraction as a whole, seven 20 mL aliquots were collected

Table 1. The materials and samples of experiment to test concentration and scaling effects.

DOC material	DOC (mgC L)$^{-1}$	ID of subsample	Comment
Site: Birkenes	0	A-0	Concentration experiment
Time: Fall 1999	1.1	A-1	
Type: RO-isolate	2.1	A-2	
	4.3	A-3	
	10.7	A-4	
	21.3	A-5	
	42.6	A-6	
Site: Svartberget	0	B-0	Concentration and scaling
Time: Spring 2000	0.9	B-1	experiment
Type: RO-isolate	3.5	B-2	
	8.7	B-3	
	17.5	B-4	
	34.9	B-5	

throughout the fractionation after discarding the dead volume (30 mL) and the last 10 mL of the 180 mL sample. Normalized 254 nm absorption and specific absorption ratio (SAR) of these aliquots were then studied in order to detect changes in the HPI-fraction during the fractionation as well effect of different DOC concentrations in the fractionated sample.

2.3.3. Stability and artefact essay. This experiment was conducted in order to make a stability and artefact essay of the fractionated material and thereby test Hypothesis 3. In this experiment stored HPI and HPOA fractions from five previous XAD-8 fractionations were re-fractionated on XAD-8 columns. The stored fractions used in this experiment were from four natural surface water samples (samples from Svartberget and Skjervatjern, table 2) and a corresponding RO-isolate to one of them. The original XAD-8 fractions had been pH adjusted back to the natural pH of the original samples (pH 4.5–5.5), and had been in storage for a period of 12 to 24 months (dark and at 4°C). Furthermore, in order to investigate any continued reproduction of HPI and HPOA in the fractions on a shorter time frame, the 2nd HPI and HPOA fractions from the original XAD-8 fractionation were subjected to a third XAD-8 fractionation only 24–48 h after the second XAD-8 fractionation. These two re-fractionations of XAD-8 fractionated material are considered to represent the shortest and longest possible timeframe for the occurrence of any chemically or biologically induced changes to the fractions. Although the original fractionation was performed on a larger scale XAD-8 method (20 mL XAD-8 column and a sample volume 2 L), the second and third re-fractionations were performed in 3 to 4 parallel sub-sample series on the original size 3 mL XAD-8 columns to obtain standard deviation on the results.

The fractions were not filtrated through a 0.45 μm filter prior to further XAD-8 fractionation, as required by the procedure. This step was dropped in order to prevent disturbance of larger NOM entities caused by aggregation during the storage. The individual HPI and HPOA fractions from each parallel from the second fractionation were pooled together to provide sufficient material for the third XAD-8 fractionation. TOC and the absorbance at 254, 400 and 600 nm were measured for all individual fractions.

Table 2. The materials and samples of the experiment to test stability and method artefacts.

DOC material	Sample for XAD8 fractionation	DOC (mgC L^{-1})	ID of subsample	Comment
Site: Svartberget	TOTAL	16.0	C1	Original sample
Time: Spring 2000	HPI*	3.1	C2	HPI of C1
Type: RO-isolate	HPOA*	11.4	C3	HPOA of C1
	HPI_i**	2.2	C4	HPI of C2
	$HPOA_o$**	6.2	C5	HPOA of C3
Site: Svartberget	TOTAL	11.0	D1	Original sample
Time: Fall 1999	HPI*	2.9	D2	HPI of D1
Type: Surface water	HPOA*	7.3	D3	HPOA of D1
	HPI_i**	2.0	D4	HPI of C2
	$HPOA_o$**	4.8	D5	HPOA of D3
Site: Svartberget	TOTAL	16.5	E1	Original sample
Time: Spring 2000	HPI*	5.1	E2	HPI of E1
Type: Surface water	HPOA*	10.6	E3	HPOA of E1
	HPI_i**	4.1	E4	HPI of E2
	$HPOA_o$**	7.0	E5	HPOA of E3
Site: Skjervatjern	TOTAL	10.5	F1	Original sample
Time: Fall 1999	HPI*	3.7	F2	HPI of F1
Type: Surface water	HPOA*	6.7	F3	HPOA of F1
	HPI_i**	3.1	F4	HPI of F2
	$HPOA_o$**	4.4	F5	HPOA of F3
Site: Skjervatjern	TOTAL	4.8	G1	Original sample
Time: Spring 2000	HPI*	1.7	G2	HPI of G1
Type: Surface water	HPOA*	1.7	G3	HPOA of G1
	HPI_i**	1.1	G4	HPI of G2
	$HPOA_o$**	1.1	G5	HPOA of G3

*2nd fractionation 12–24 months after the 1st, **3rd fractionation 24–48 h after the 2nd. Suffix i = derived from re-fraction of HPI, o = derived from re-fraction of HPOA.

3. Results and discussion

3.1. *Comparison of the HPI to HPOA ratio at various DOC concentrations*

When the relative proportions of the XAD-8 fractions were compared at different concentrations, both samples revealed a tendency of increase in the relative proportion of the HPI fraction and thereby an apparent higher "hydrophilic" character at higher concentrations. Figure 1 display the results of the concentration series based on the Svartberget RO-isolate. The most concentrated samples in both series are outside the analytical range of the method, but the increase in hydrophilic character appears to occur over the whole concentration range. Even though the standard deviation of the HPON fraction is much larger that the two other (HPON is obtained by subtraction), the average values follows the general trend that the hydrophobic character decreases with increasing concentration.

The specific UV absorption ($sUVa = A_{254\,nm}/DOC$) and specific absorption ratio ($SAR = A_{254\,nm}/A_{400\,nm}$) were compared for all concentrations and fractions. For the total sample and the HPOA fractions no significant differences were found between the different DOC concentration levels. For the HPI fractions, however, sUVa was found to increase significantly with the DOC concentration for both tested materials, and SAR from one of the materials (Svartberget) decreased significantly with concentration. Both these results indicate a clear change in the *quality* of the HPI fraction

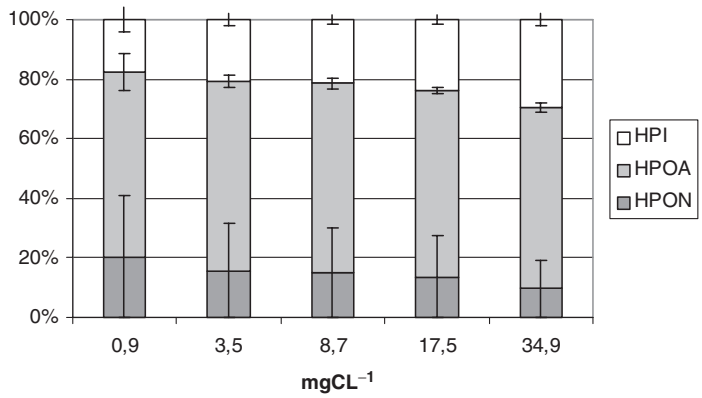

Figure 1. The relative proportion of HPI fractions obtained from XAD-8 fractionation increase significantly with increasing DOC concentrations (Svartberget Spring RO-isolate, three replicate fractionations at each concentration).

in addition to the shift in *distribution* between the fractions found at various DOC concentration levels. A decrease in SAR is a relative shift towards longer wavelength (red-shift). An increase in sUVa is an increase in the relative UV absorption for a given mass unit of DOC. Both these changes are associated with increased length/size of conjunct double bounds structures of the humic material, in NOM literature commonly associated with more hydrophobic and larger molecular structures like quinoide- and ketophenol systems [12–14]. This indicates that when XAD-8 fractionation is performed at high DOC concentration, larger and more aromatic molecular structures are found to be present in the HPI fraction than when the same material is XAD-8 fractionated at a lower concentration.

Since the experiment is performed on the same NOM material, only two possible explanations can be the cause of these results: (1) That the higher concentration of sample truly has a more hydrophilic character, or (2) that the results are caused by some methodical bias related to the concentration of the sample. It is difficult to explain any mechanisms leading to a higher level of hydrophilic matter in a sample of higher concentration. On contrary, due to aggregation as the concentration approaches saturation the conceptual assumption would be to expect a more hydrophobic character in a sample of high concentration. The other explanation, a methodical bias seems sounder. If the amount of hydrophobic substance locally exceeds the capacity of the stationary phase then the NOM will leek faster through and out of the column at higher concentration. This would lead to a relatively larger HPI fraction, and this HPI fractions will on average consists of larger and more aromatic NOM. This explanation is well supported by the sUVa and SAR observations. If this is the cause then this will have implications for studies of the relative proportions of hydrophobic to hydrophilic material fractionated on XAD-8 when the samples were of different DOC concentration.

3.2. *Consistency of the HPI fraction during fractionation*

As discussed above a concentration dependency could be due to a HPO breakthrough of the column (saturation point) or be explained by an increased bleeding of

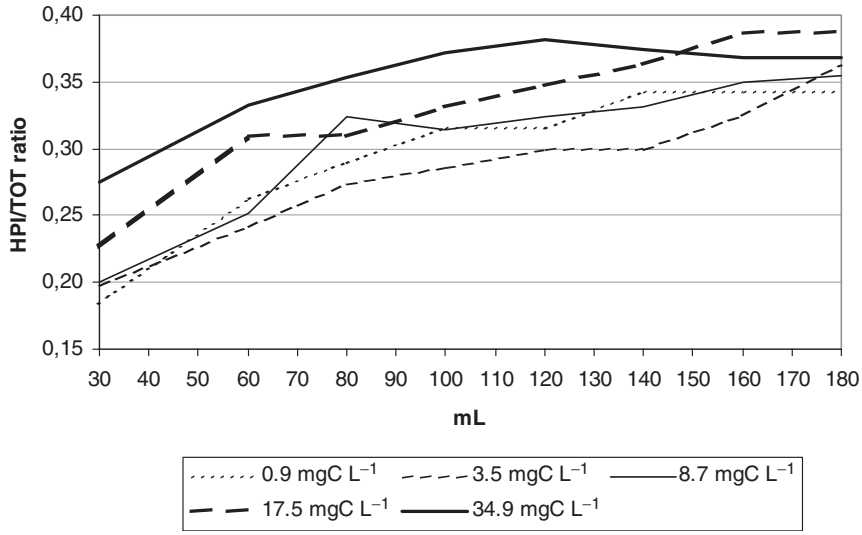

Figure 2. The proportion of the HPI fraction relative to the total NOM of the original un-fractionated sample (measured as UV_{254} absorbance), given as a function of HPI fractionation volume (in mL) over the whole 180 mL fractionation. Svartberget RO-isolate samples at various concentrations.

hydrophobic matter. Figure 2 shows the results from the scaling experiment where smaller aliquots of the 180 mL (HPI fraction) are collected during the whole fractionation time.

For all samples the normalized 254 nm absorption of the HPI fraction (relative to the 254 nm absorption of the total sample) increases significantly during the fractionation. Only in the highest concentration sample, the normalized 254 nm absorption approaches a stabile level after about two thirds of the fractionation time. It should be noticed that the DOC level in this sample is clearly higher than recommended in the original Leenheer and Huffman procedure of 1976. The extinction coefficient (i.e. sUVa) differ between different NOM species and the DOC concentration can therefore not be deduced directly from 254 nm absorption data. Thus an increase in the 254 nm absorption could be caused by two factors separately or in combination; (a) an increase in the overall NOM concentration of the aliquot, and/or (b) an increase in the average sUVa of the NOM species in the aliquot. Regardless of cause, an increase in relative 254 nm absorption is a clear indication of instability in the composition of the HPI fraction during the fractionation.

The specific absorption ratio ($A_{254\,nm}/A_{400\,nm}$; SAR) of the HPI fraction in the aliquots decreases throughout the fractionation for all concentrations. This decrease was most profound in the samples with low NOM concentrations. In the 3.5 and 8.7 mgC L^{-1} samples, SAR dropped from an initial value of 16 in both samples to 8 and 11, respectively. Even in the 17.5 mgC L^{-1} sample the decrease in SAR was significant from 12 initially to 10 at the end of the fractionation. This red-shift in UV-VIS absorbance indicates a shift toward larger and more aromatic structures with the progress of the fractionation. The only sample where SAR could be characterized as reasonably stabile was the 34.9 mgC L^{-1} sample where the SAR value of the HPI fraction only dropped slowly from an original value of 11 to an end value of 10 over the period

of the fractionation. Again it should be noted that the concentration of this sample is higher that the recommended limit for the method.

These results are consistent with a constant increase in the leakage of more hydrophobic matter through the column due to the chromatographic nature of the method and the increasing saturation of the column with higher DOC concentrations. When different concentrations are compared it becomes apparent that samples with low DOC concentrations display a much larger change in the HPI fraction during the fractionation time, than samples of higher concentration. In the first aliquot of HPI (after discarding a dead volume of 30 mL) there is a close to 50% difference in the relative proportion of the 254 nm absorption of the HPI fraction between the lowest and the highest DOC concentrations. Furthermore, the increase in the relative 254 nm absorption with DOC concentration in this initial aliquot forms a near linear response to the DOC concentration of the sample.

This artefact has implications in studies using XAD-8, especially where the resin is used in tandem with other columns. In a sequential fractionation procedure [1, 2] the aliquots used to describe various DOC fractions are collected manually as smaller sub-samples during the procedure after the columns in the sequence. It will then be of major importance exactly when the sub-samples are collected. The results of these methods will therefore be highly operationally defined, and great care should be taken when performing the fractionation.

3.3. *Consistency of properties in the HPI and HPOA fractions during storage*

When the stored XAD-8 fractions were subjected to a second XAD-8 fractionation in the stability experiment, all samples revealed a pattern of "re-generation" of HPI and HPOA in their respectively opposite fractions. Apparently new HPI is formed in the HPOA fraction and HPOA is formed in the HPI fraction (figure 3). The boundaries between the two fractions seem therefore weak or altered through storage. These patterns are repeated in the third fractionation only 24–48 h after the second one. One could argue that the method and the matrix of the original sample in the first fractionation are different from the second fractionation (12–24 months later), since the column system is smaller and the ionic strength of the sample is greater in the second than the first fractionation. However these differences do not concern the step from the second to the third fractionation 24–48 h later as they are performed according to the exact same method and on samples with a high but very similar ionic strength. Only minor differences in the fractionation/re-fractionation pattern could be found when comparing the two natural samples with the samples prepared from the RO isolates from the Svartberget site (C 1–5 (RO-isolate, spring), D 1–5 (surface water, fall) and E 1–5 (surface water, spring), table 2). This leads to the conclusion that the regeneration of HPI, HPOA and HPON is formed by mechanisms independent of whether the NOM material origins from a natural sample or an RO-isolate.

The results from the fractionations of the Svartberget RO-isolate (given as an example in figure 4), show as expected that for each time the sample is fractionated the HPOA fraction becomes generally more "hydrophobic" and the HPI fraction more "hydrophilic" of nature based on the total division into HPON, HPOA and HPI. Comparing the SAR values of the HPI fractions it can also be noticed that the

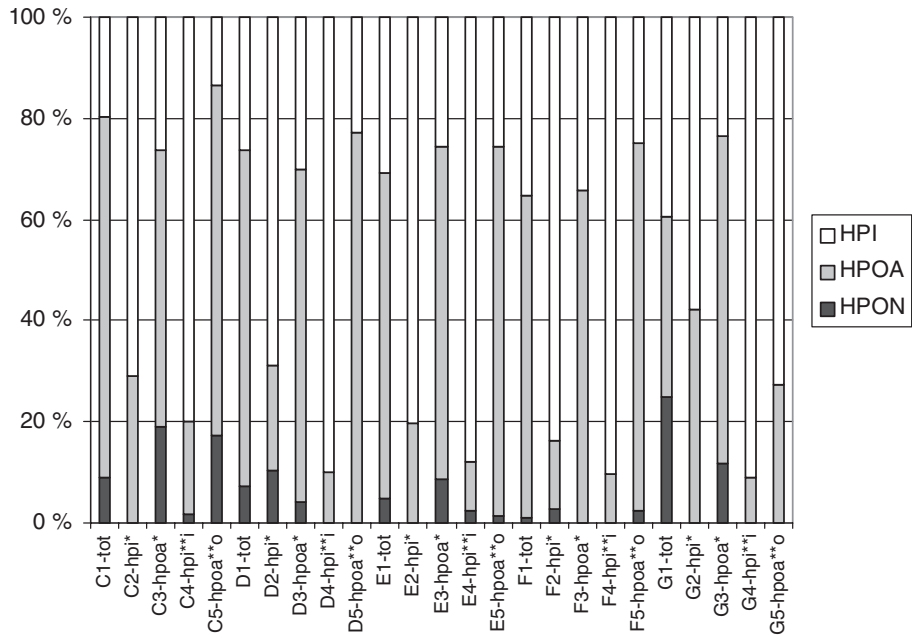

Figure 3. Relative proportions of HPI, HPOA and HPON in a RO-isolate and four fresh samples from Svartberget and Skjervatjern. Fractionated and re-fractionation in two steps of the HPI and HPOA fractions. * = 2nd fractionation after 12–24 months, ** = 3rd fractionation 24–48 h after the 2nd. Suffix i = derived from re-fraction of HPI, o = derived from re-fraction of HPOA.

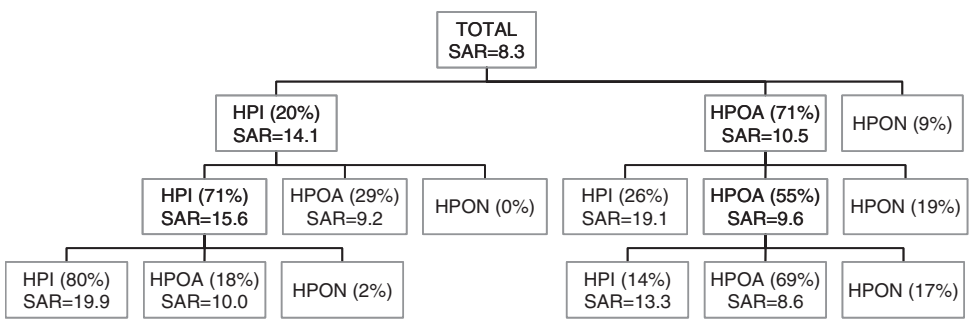

Figure 4. Fractionation scheme, relative distribution and SAR values of fractions from repeated XAD-8 fractionation of HPI and HPOA fractions (e.g. the Svartberget RO-isolate).

values increase with each re-fractionation (figure 4). This indicates that the average molecular size of this fraction (HPI) decreases for each repeated fractionation while the average proportion of the fraction is increasing. If the ''HPOA-line'' of the diagram in figure 4 is observed, it can be noticed that SAR of the HPOA-fractions are decreasing. This indicates larger and more aromatic compounds in the HPOA fraction for each repeated fractionation.

Three main mechanisms could be proposed to explain these results alone or in combination. One could be that the reproduction of HPI in the HPOA fraction and

vice versa could be explained by irreversible changes in the chemical structures of the NOM material due to the tough chemical environment and changes the sample is subjected to as a result of the XAD-8 method. After all the material is first acidified to pH 2, then run mechanically through an adsorbing column and the adsorbed material is then desorbed at a high pH. The conditions during this procedure differ radically from the natural conditions of NOM and are very likely to cause permanent chemical alteration to the material. Extremely high pH is found to cause a significant amount of breakdown due to hydrolysis in NOM material [15]. It is therefore very likely that the regenerated HPI found in the re-fractionation of HPOA could be created as a result of the original desorption from the XAD-8 column with 1 N NaOH. The relatively high SAR values of the HPI fraction in the re-fractionated HPOA fractions support this theory.

Another explanation could be that the cut-off boundary between the HPI and HPOA fractions is not so rigid. Due to the chromatographic nature of the XAD-8 procedure, the more hydrophobic compounds among the HPI fraction no longer have to "compete" with the more hydrophobic HPOA compounds of the affiliation toward the XAD-8 material. These compounds are therefore more delayed than they would be if more hydrophobic matter would be present and will therefore appear to be part of a HPOA fraction. This corresponds quite well with the theory of capacity, saturation and chromatographic nature of the XAD-8 column discussed in the two previous experiments.

The third mechanism is that the apparent generation of the HPOA in the HPI fractions and vice versa could be explained by mechanical, chemical or biological induced change during storage, i.e. new equilibrium processes that aggregates or breaks down the NOM material.

The alteration in the chemical structure of the individual species of NOM present in XAD-8 fractions during storage will not have any practical importance as long as the purpose of the fractionation is only to investigate the mass ratio between the various fractions, and compare different samples. However, modern use of the XAD-8 method goes beyond that, and intentions are made to specify these fractions further and to interpret their physicochemical properties with various mean of instrumentation and analytical methods. XAD-8 is also used as a method of isolation of NOM intended as standard material. The user of the XAD-8 methodology for the fractionation and/or isolation of NOM should therefore be aware of this lack of stability of the adsorbed or eluated NOM from an XAD-8 column.

4. Conclusions

The ratio HPOA to HPI is not independent of the original DOC concentration, as higher concentrations have a tendency to give a relatively higher level of HPI than found at lower concentrations. This is believed to be an effect of the chromatographic nature of the XAD-8 procedure in combination with the capacity and saturation of NOM on the XAD-8 column. The DOC level increases and the quality shifts towards larger and more aromatic NOM in the HPI fraction through the fractionation of a 180 mL sample on an ordinary 3 mL XAD-8 column. This rise is highest on the lower concentrations, but is significant in the whole concentration range from 1 to 35 mgC L^{-1}. This is also believed to be the result of the chromatographic nature of

the XAD-8 procedure and due to the relatively small structural and chemical difference between species of NOM in the operationally defined HPI and HPOA fractions.

Re-fractionation of isolated XAD-8 fractions (HPI, HPOA) can produce a new HPI/HPOA cut-off, due to a combination of three possible mechanisms: (1) Irreversible chemical changes in the NOM due to the fractionation procedure, (2) Chromatographic aspect of the XAD-8 fractionation (XAD-8 capacity and competition between NOM species), and (3) Establishment of a new equilibrium between "hydrophobic" and "hydrophilic" NOM, due to mechanical aggregation or chemical and biological processes during treatment and storage.

The main conclusion from this study is not that studies based on XAD-8 fractionation are not useful. XAD-8 is considered an important tool in the study of NOM properties and function in the environment. This study illustrates instead that the user of these XAD-8 fractionation or isolation techniques should keep in mind the highly operationally defined nature of these methods and be aware of the discussed analytical artefacts and their influence on the interpretation of results.

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