Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Aquatic Science and Assessment

# Association of hydrophobic organic contaminants to size fraction of natural humic substances

– Using ultrafiltration and high-performance liquid chromatography

*Kristina Tattersdill*

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## Abstract

Ultrafiltration and high-performance size exclusion chromatography (HPSEC) were used to determine interactions between different size-fractions of natural humic substance and carbofuran, lindane, chlorpyrifos and PCB-105. Ultrafiltration, using filter MWCO size of 1 kDa and 100 kDa, showed an adsorption of pesticide to filter. Chlorpyrifos (1 kDa) had the highest bioconcentration factor on filter (BCF) of 208±4.0. There was an overall trend of loss of pesticide recovery. Lindane had the lowest recovery 62±2.3% at 1 kDa. Fractionation using HPSEC gave different size-fractions of humic substance with recovered pesticide. However, HPLC/MS/MS showed that both carbofuran and chlorpyrifos fastened in the column. This was most likely the case for lindane also, although this could not be tested due to lindane's volatility. PCB-105 was lost during incubation previous to HPSEC fractionation.

When adding humic substance, there was a decreased adsorption of pesticide to filter (ultrafiltration) and column (HPSEC). Even though no pattern between pesticides and specific size fractions of humic substance could be detected, it is obvious that humic substance alters the behavior of HOC and making them more mobile.

# Content

# Abbreviations

| | |
|---|---|
| $^{14}C$ | Radioactive Isotope of Carbon (6 Protons and 8 Neutrons) |
| DOM | Dissolved Organic Matter |
| DEAE | Diethyl-aminoethyl cellulose |
| HOC | Hydrophobic Organic Contaminants |
| HPLC | High-Performance Liquid Chromatography Coupled with Tandem Mass Spectroscopy |
| HPSEC | High-Performance Size Exclusion Chromatography |
| $K_{OW}$ | Octanol-Water Partitioning Coefficient |
| MilliQ | Highly Purified and Desalted Water (a Millipore$^{TM}$-system) |
| MWCO | Molecular Weight Cut-off |
| PCB | Polychlorinated Biphenyls |
| PSS | Polystyrene Sylphonate |
| SR FA | Suwannee River Fulvic Acid |
| SR HA | Suwannee River Humic Acid |
| UF | Ultrafiltration |
| VS DOA | Vikasjön Dissolved Organic Acid |
| VS HA+FA | Vikasjön Humic Acid + Fulcic Acid |

# 1 Introduction

Hydrophobic organic contaminants (HOC) are compounds that are not found natural in the environment. Pollution of aquatic ecosystems is of vast concern due to the vulnerability of these systems. HOC may reach aquatic systems during precipitation, through surface runoff and groundwater or by direct exposure. Once in the system organic contaminants may be activated or detoxified by hydrolysis, redox reactions and/or photolysis (Scrudato et al., 1999; Katagi, 2006). Due to the hydrophobic nature of these contaminants, dissolved and particulate matter determines the bioavailability and mobility of HOC (Chiou et al., 1986; Kukkonen and Oikari, 1991; Nikkilä and Kukkonen, 2000, Katagi, 2006). Studies have shown that contaminants adsorb to sediment particles (Ying and Williams, 2000; Kukkonen and Landrum, 1996; Gao et al., 1998), where stirred sediments are more susceptible for HOC adsorption (Ying and Williams, 2000). Gao et al. (1998) showed that high adsorption in specific particle size fraction of sediment was due to the presence of organic matter. Aquatic organic matter is derived from addition and degradation of terrestrial primary production (allochthonous) or degradation of aquatic primary production (autochthonous). Composition of allochthonous and autochthonous matter determines the microbial degradation, where autochthonous is degraded more easily. (Tranvik et al., 2009)

The most important properties determining the environment in aquatic ecosystems are temperature, pH and concentration of dissolved organic matter (DOM) (Khan, 1972; Kukkonen and Oikari, 1991; Katagi, 2006). DOM regulates light penetration and associate to metals and hydrophobic organic contaminants. Chemical properties for both molecules, determines the association between HOC and DOM, where humus from diverse sources show different binding properties (Uhle et al., 1999; Albert at al., 2002). The aromatic parts, determine the association to HOC (Gauthler et al., 1987; Uhle et al., 1999). Interactions between DOM and organic

contaminants may be through hydrogen bonding, ion exchange, van der Waals, hydrophobic bonding, and charge transfer (Khan, 1972; Katagi, 2006). These interactions and the concentration of DOM make the contaminants less bioavailable and hence decrease uptake by pelagic filter feeders (Kukkonon and Oikari, 1991; Nikkilä and Kukkonen, 2000).

The majority of DOM is composed of humic substances, which are yellow organic acids, giving humic lakes its coloured water. Humic substances consist of fulvic acid (40%), humic acid (10%) and humin (Thurman, 1985). Due to their large impact on the aquatic ecosystem, knowledge of molecular weight and molecular size distribution is of importance. Humic substances have large variation in size and structure due to origin and properties, making it difficult to determine a molecular structure. However, researchers have tried, and Schulten and Schnitzer (1993) presented one model (figure 1). Conte and Piccolo (1999a) showed that humic substances are small molecules with hydrophobic interactions between them building larger molecular arrangements.

Establishing molecular weight and size distribution of humic substance can identify the molecular structure and consequently determine the interaction with HOC. This descriptive information can be achieved using field-flow fractionation, viscosimetry, ultrafiltration, size-exclusion chromatography and methods based on physical/chemical properties (vapour-pressure osmometry, variation of pH-value and freezing-point depression). At present high performance size exclusion chromatography (HPSEC) is most frequently applied. It is possible to quantify humic and fulvic acid using the system, together with fractionation of humic substances (Peuravuori and Pihlaja, 1997; Müller et al., 2000; Wu et al., 2007). It has long been believed that molecular weight of humic substance varies between 1-100 kDa. Perminova, et al. (2003) wished to define humic substances as a specific class of chemical compounds and isolated 77 samples from different aquatic origin with a composition of either humic acid, fulvic acid or both. The obtained range was within 4.7-30.4 kDa.

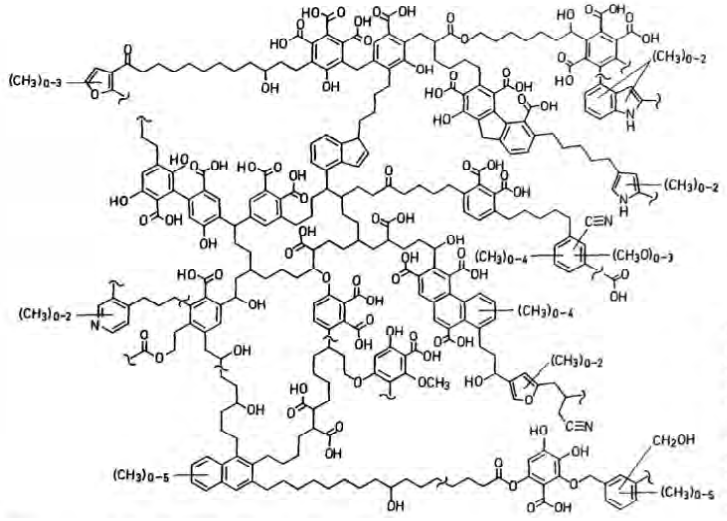

Figure 1. Schematic structure of humic acid developed by Schulten and Schnitzer (1993)

The objective of this study was to develop a method for fractionating humic substance in association with hydrophobic organic contaminants. Where the adsorption to size fractions will be examined and also, if the hydrophobic properties (i.e., log $K_{ow}$-values) of contaminant effects the association. Both ultrafiltration and HPSEC were used to find a suitable method for fractionation of HOC associated to humic substance. Our findings can contribute to the knowledge of the interaction between HOC and DOM.

# 2 Material and methods

## 2.1 Sampling and extraction of humic substances.

Water from the pelagic zone of lake Vikasjön, Sweden (N 60° 17'; E 17° 52'; total organic carbon, 30.1±1.1 mg/l (Reference lake, SLU, Sweden)) was collected at 20 cm depth using two 25-L containers. Fifty gram pre-treated DEAE-cellulose (fibrous diethyl-aminoethyl cellulose; Sigma Aldrich) (1 g/l) was added to the water samples. (For pre-treatment of DEAE see Miles et al. (1983). The suspension (water and DEAE) was mixed for 1 hour to allow adsorption of humic substances to the DEAE. The containers were placed cold over night to let the DEAE and associated humic compounds settle. The overlying water was discarded and the suspended DEAE in water was filtered through 0.45-µm membrane filters (sterilized membrane, PALL). The DEAE was then washed with 200 ml 0.3M NaOH; this desorbed the organic acids from DEAE. The solution was then acidified with 2 M and 0.5 M HCl until neutral pH was reached, according to Miles et al. (1983). The extracted dissolved organic acid (DOA) was frozen over night, followed by freeze-drying for four days.

To desalt dissolved organic acid from Vikasjön a 30 cm long desalting- coil (Spectra/por 6, 18mm; Fisher scientific) (1cm/ml and an additional 10%) was rinsed in distilled-deionised water for 30 min. An amount of 0.2g DOA was weighed up and mixed with 20 ml of milliQ water and added to the coil, using a knot as a stopper at one end and a clamp closing the coil. The coil was then added to a beaker containing 2.5 litres (over 100x the volume in the coil), and stirred using a magnetic stirrer. Water in the beaker was changed frequently and the conductivity of the solution was measured until steady state was reached at 1.2 mS/m. The solution was frozen over night and freeze-dried to a crystallised solution.

Together with dissolved organic acid (DOA), humic- and fulvic acids (HA+FA) from lake Vikasjön, Sweden (previously extracted on a XAD-resin done by Anna Lundqvist (Thurman and Malcom, 1981)) was also used in the experiments. In addition commercially produced standards where acquired; Suwannee River (International Humic Substance Society), extracted as humic acid (SRHA) and fulvic acid (SRFA).

## 2.2 Organic test chemicals

Three widely used insecticides and one PCB congener, covering log-$K_{OW}$-gradient from 1.5 to 6.65 were used to get a realistic picture of the behaviour of these contaminants in the environment (figure 2). Radioactive compounds were used to allow for accurate and fast quantification of experimental concentrations and participation in different size fractions of organic acids.

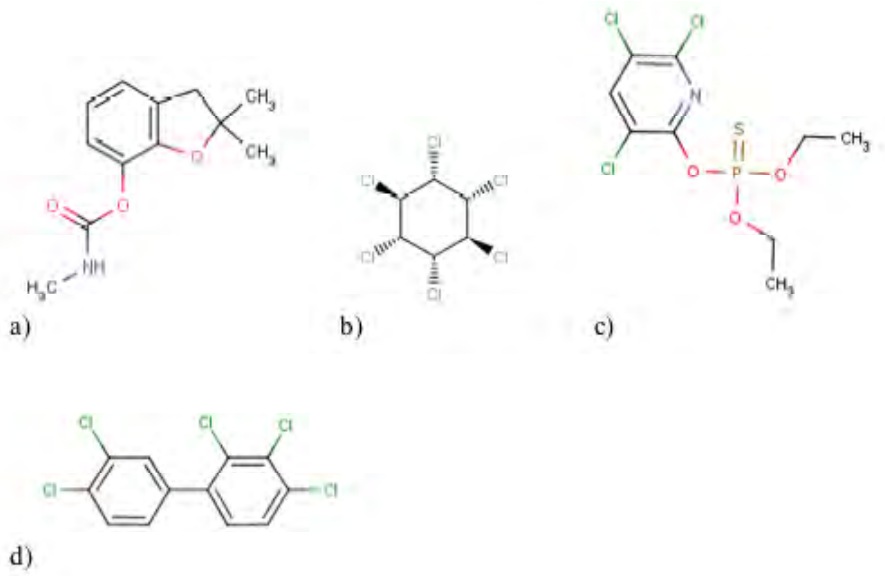


Figure 2. Chemical structure of carbofuran, CAS no.1563-66-2 (a), lindane, CAS no. 58-89-9 (b), chlorpyrifos, CAS no. 2921-88-2 (c) and PCB-105, CAS 32598-14-4 (d) (Homepage of Toxicology Data Network (Toxnet))

14C-labeled carbofuran (log $K_{OW}$ of 1.5 specific activity 29.3 mCi/mmol, purity > 95%, Izotop, Institute of Isotopes Co, Budapest, Hungary), lindane (log $K_{OW}$ 3.5, 29.7 mCi/mmol, > 99%, International Isotope, Munich, Germany), chlorpyrifos (log $K_{OW}$ of 4.7, 32 mCi/mmol, > 99%, American Radiolabeled Chemicals, St.

Louis, MO, USA) and PCB-105 (log $K_{OW}$ 6,65, 26.5 mCi/mmol, >99%, Stockholm University, Sweden) were applied. Compounds stock solutions were diluted with acetone to appropriate concentrations (see section; 1.3.1 and 1.4.2)

## 2.3 Ultrafiltration, pilot study

Ultrafiltration is a simple way to achieve different molecular size fractions. It is mostly used when up concentrating DNA-fragments and proteins (Information leaflet on centrifugal devices, 2008), but has also been used to filter organic matter (Pedersen et al, 1999; Kerc et al, 2004; Alberts et al, 2002; Revchuk and Suffet, 2009). There are various types of membranes on the market; the most commonly used are cellulose or polyethersulfone membranes, where the latter has low molecular binding (Information leaflet on centrifugal devices, 2008). Molecular weight is of interest when filtering a solution, but also ionic state of molecule, conformation and interactions between molecules may affect the retention (Product manual for Microsep, 2001; Revchuk and Suffet, 2009).

14C-labeled pesticides carbofuran, lindane, and chlorpyrifos were tested in this experiment.

### 2.3.1 Filtration of pesticide

Fifty µl pesticide (0.4 µg/ml) was added to 20 ml milliQ-water (n=3) to achieve nominal concentrations of 1 µg/l. From each pesticide solution, 0.5 ml was transferred to a scintillation vial containing 10 ml of scintillation cocktail (Optiphase Hisafe 2; PerkinElmer) for later liquid scintillation counting analysis (see below). Also, 50 µl of initial pesticide solution (n=3) was transferred to scintillation vials for later scintillation quantification. For each pesticide three millilitres of pesticide solution, were added to ultrafiltration tubes (n=3, Mircosep, PALL Life Science) with filter size (molecular weight cut-off, MWCO) of 1 kDa and 100 kDa. 1 kDa-tubes were centrifuged (Biofug primo R, Heraeus) at 7000xg for 100 min (20°C), and 100 kDa-tubes at 1000xg for 20 min (20°C) (according to manufacturer's recommendations). After centrifugation, 0.5 ml filtrate sample was taken, and 10 ml scintillation cocktail was added. The filters were removed from tubes, weighed to the nearest mg and transferred to scintillation vials with addition of 10 ml scintillation cocktail. After 24 h, concentrations of pesticides were quantified by scintillation counting for 10 min (Tri-Carb 2100TR, Liquid Scintillation Analyser, Packard). The results for samples were adjusted with background radiation values obtained from the blanks (only MilliQ-water).

### 2.3.2 Filtration of pesticide-humus solution

A humic- and fulvic acid mix previously extracted from Vikasjön (HA+FA) (4.5 mg) was added to 45 ml of milliQ water (50 mgTOC/l). This volume was then divided into 3 glass jars where one 14C-labeled pesticide was added to each jar, á 15 ml. A volume of 38 µl of carbofuran and lindane (initial concentration 0.4 µg/ml) and 63 µl of chlorpyrifos (0.24 µg/ml) was added, reaching a final concentration of 1 µg/l in the solution. The jars were plugged and placed in the dark on a shaking table (100 rpm) for 48h at room temperature. 0.5 ml of the solution was mixed with 10 ml scintillation cocktail (n=2) for further scintillation counting. The filters in the ultra filtration tubes were then pre-washed twice by filtering through 3ml of milliQ water by centrifugation, to reduce non-specific adsorption (1kDa; 7000xg, 100kDa; 1000xg). Three ml pesticide-humus solution were added to an ultrafiltration tube (1kDa) and centrifuged for 140 min at 7000xg. After centrifugation, samples were taken from the filtrate (0.5 ml), the filter (weighed), the concentrate on top of filter (20-200 µl depending on the residual) and 10 ml scintillation cocktail was added. The 100 kDa tubes were treated similarly with centrifugation time of 30 min at 1000xg. Scintillation counting was conducted as described above (2.3.1. Filtration of pesticide)

## 2.4 High-performance size exclusion chromatography (HPSEC)

As always with chromatography there is a stationary phase and liquid phase in high-performance size exclusion chromatography (HPSEC). The stationary phase is situated inside a column containing defined pore sizes of porous molecules. It takes around 30 min for the sample to go through the column, depending on flow rate and column length. SEC separates on the basis of molecular size. The largest molecules come out first while the pores may adsorb the smaller molecules and these molecules have therefore a longer retention time through the column. The column may be calibrated by using standards with known molecular weight and from that achieve a standard curve to use against other unknown fractions. HPSEC do not give absolute measurements, but fraction sizes may be obtained, and may therefore be used to fraction out humic substances among others.

For experiment explained in this section, 14C-labeled pesticides carbofuran, lindane, and chlorpyrifos, together with PCB-105 were applied.

### 2.4.1 Apparatus and column

A Tsk-gel column (G2000SW; 7.5x300mm, particle size, 10 µm, Tosoh Bioscience) was coupled to a high-pressure liquid chromatography machine (Waters 2695, separations module, Alliace), together with a UV-detector (Spectromonitor III-Loc) and a writer (Linear). The detector was set at 254nm, and flow rate at 0.7ml/min. The injection volume was 100µl. The effluent was 500ml 0.05M NaH2PO4 buffer with a pH adjusted to 6.8 with 0.1M NaOH (Ionic strength = 0.1, conductivity = 4.699±0.019 mS), vacuum filtrated before use (0.45 µm, Millipore). Prior to use, the column was rinsed with phosphate buffer, and after completed work, washed with 20% ethanol (1-2 column volumes). At flow rate 0.7 ml the columns void volume (V0 = 5.23 ml) was determined by blue dextran (20 mg/l, 2000 kDa, >99%, Farmacia), and permeation volume probe by 0.1% acetone (Vp = 10.15 ml). Based on previous reports polystyrene sulfonate (PSS) showed good correlation with the properties of humic substances (Wu et al. 2007; Albert et al. 2002; Müller at al.2000) and therefore chosen as standard. The standard molecular size 32, 17, 4.3 kDa (Sigma-Aldrich) and 8 and 1 kDa (Dalco Chromtech) of PSS were prepared with milliQ water and phosphate buffer (1:1) reaching a final concentration of 20 mg/L and added each to a 1.5 ml glass vial (Crimp vials, nornal opening, KTK Kemi). The standard's retention times at UV-absorbance (254 nm) set the base for the standard curve.

### 2.4.2 Mixing and fractionation of humus.

Size-fractionation of humic substances was achieved using HPLC, based on retention time for different molecular weights. The sample solution of phosphate buffer, 0.05 M, pH 6.8 was prepared as explained above, and mixed with milliQ water in the ratio 1:1. The four different humic substances, SRFA, SRHA, HA+FA and DOA, was added according to their total organic carbon (TOC) content to achieve 30 mgTOC/l in 15 ml solution (milliQ and 0.05M phosphate buffer; 1:1) (see table 1). This was done to mimic a natural humic lake (like lake Vikasjön; TOC 30.1±1.1 mg/l (Reference lake, SLU, Sweden))

*Table 1. Total organic carbon (TOC) content in the humic substances Suwannee River fulvic acid (SR FA), Suwannee River humic acid ( SR HA), Vikasjön humic and fulvic acid (VS HA+FA) and Vikasjön dissolved organic acid ( VS DOA) and the amount added to the experimental volume of 15 ml to achieve a TOC content of 30mg/L*

| Humic substance | TOC (mg/g) | Volume (L) | Weight (mg) |
|---|---|---|---|
| SR FA | 523,4 | 0.015 | 0.9 |
| SR HA | 526,3 | 0.015 | 0.9 |
| VS HA+FA | 501,7 | 0.015 | 0.9 |
| VS DOA | 221,3 | 0.015 | 2.0 |

The solution was then filtered (0.2 µm, regenerated cellulose, Scantec lab). Fifty microlitres of 14C-marked pesticide (initial concentration ~9.0 µg/ml) was added to the solution in a tin foiled Teflon tube (30 µg/l) (figure 3), with the top loosely screwed on (for normalized gas exchange) (n=4, SRFA, SRHA, HA+FA and DOA). The samples were placed in the dark on a shaking table (100rpm) for 48h at room temperature.

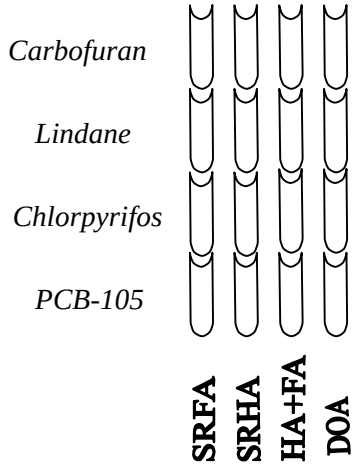


Figure 3. Experimental setup during 48h of shaking, where each tube in addition to solvent (milliQ water : phosphate buffer; 1:1) contained one organic chemical and one humic substance.

A volume of 1 ml was added to a 1.5 ml glass vials (Crimp vials, nornal opening, KTK Kemi) and placed in the HPSEC for fractionation (n=4). The fractions were collected in scintillation bottles according to retention time for desired fraction

size based on calibration curve, figure 4. Scintillation liquid was added to each fraction collected (see section 2.3.1). The results where modified with values obtained from the blanks (milliQ-water and humus substance,) and use of internal standard (Internal standard kit for liquid scintillation counting, 14C, PerkinElmer) on the different fractions from the four different humus (n=2).

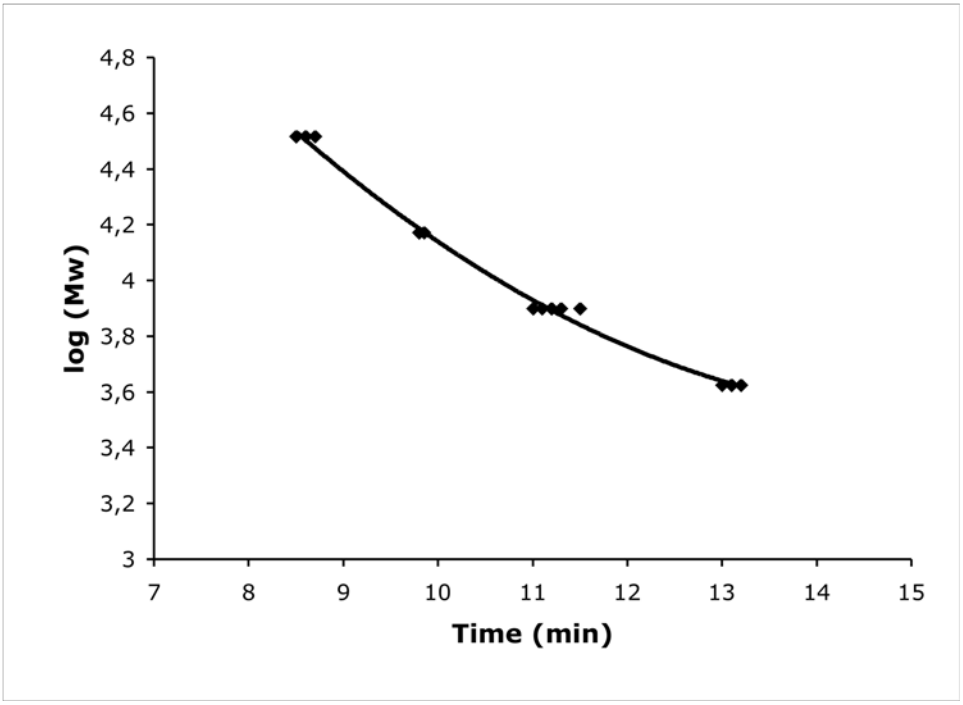


Figure 4. Standard curve based on retention time for polystyren sulfonate (PSS), Log10 (molecular weight (Mw)) 32, 14.7, 7.9, 4.3 kDa. Mw = $0.0215x^2$-0.6603x+8.5947; $R^2$=0.9963

### 2.4.3 Possible absorption of pesticide to Tsk-Gel® column

Recovery (%) of pesticides, before and after the gel permeation chromatography, were analysed by high-performance liquid chromatography coupled with a tandem mass spectrometry (HPLC/MS/MS). Pesticide solutions (carbofuran and chlorpyrifos), internal standard solution, HPCL-equipment and -parameters were as described in Jansson and Kreuger (2010). 75 µL stock solution of carbofuran and chlorpyrifos (2µg/mL) were dissolved in 5 ml 1:1 MilliQ:phosphate buffer (for buffer see section 2.4.1) or the DOM-treatment SR FA at a concentration of 30

µg/L. The SR FA sample was covered with tinfoil and allowed for pesticide adsorption on shaking table (100 rpm) over night, at room temperature. Pesticide + eluent and Pesticide + SR FA were fractionated by the Tsk-Gel® column and fractions collected of the void volume (0 – 7.5 min), the permeation volume (7.5 – 19.0 min) (divided in two equal samples), and two fractions after the sample had passed through the column (19.0 – 29.0 and 29.0 – 39.0 min, respectively). Samples of fractions from two injections in succession were collected. In between the fractionation of pesticide and of pesticide + SR FA, the column was rinsed with ethanol (10%), followed by rinsing with 0.05M phosphate buffer. 1.5 ml of each fraction was transferred to vials, then added 30 µL internal standard (Jansson and Kreuger 2010), and samples were analysed by HPLC/MS/MS. Also, samples (n=2) of pesticide and of pesticide + SR FA were diluted 50 times and analysed by HPLC/MS/MS, this to compensate for dilution by the mobile phase during fractionation.

## 2.5 Statistics

Prior to statistical analysis data was log10-transformed, except for bioconcentration factor for filter which was arcsine-transformed ($x'=\text{arc-sin}\sqrt{x}$). Analysis of variance (ANOVA, two-way) was used to analyse effects of types of pesticide and MWCO filter-size. Tukey-Kramer's HSD tests were used for pair wise comparisons. In all statistical analyses p-value was set to 0.05

# 3 Results

## 3.1. Ultrafiltration

### 3.1.1 Filtration of pesticide

Pesticide concentrations in filtrate decreased markedly (ranging from 8 to 100%) as a result of filtration (figure 5). Carbofuran and chlorpyrifos filtered through MWCO of 100 kDa, showed the lowest loss of pesticide in filtrate (8 and 31% decreased concentration in filtrate, respectively). The binding of pesticide to filters decreased with increasing pore size for all three pesticides, where carbofuran had the highest difference of adsorbed pesticide to filters. The filtration of lindane through 1 kDa-filter, gave no measurable concentration in the filtrate, but over 60% had adsorbed onto the filter.

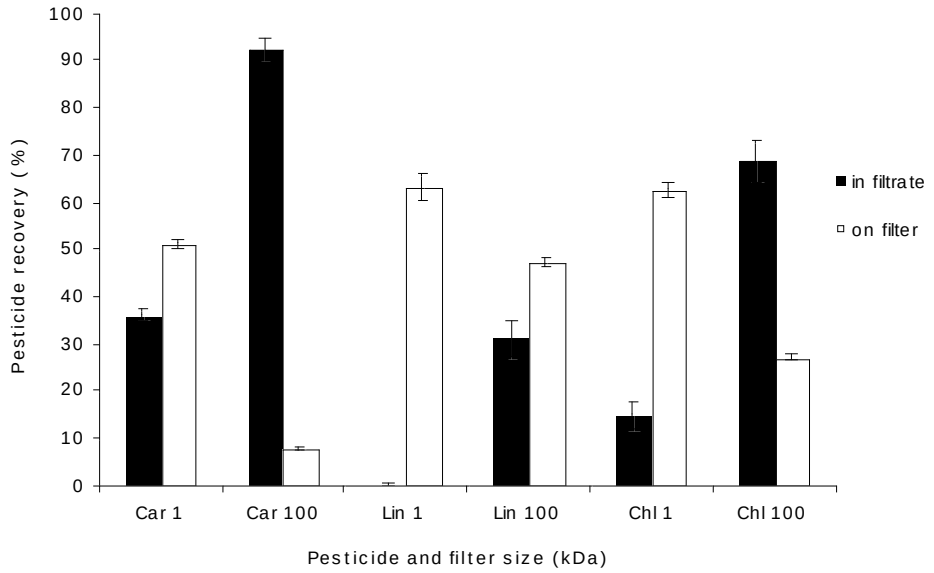


Figure 5. Recovery of pesticides (%, mean±1 SE) after filtration; in filtrate (black bars), and adsorbed onto filters (white bars), for carbofuran (Car), lindane (Lin), and chlorpyrifos (Chl), using ultrafiltration tubes with MWCO 1 kDa and 100 kDa.

Since concentrations in water phase differed prior to filtration for all pesticides, a bioconcentration factor (BCF) for filters were calculated (figure 6), i.e., a quota of filter concentration (ng/g) and water concentration (µg/L). Filter BCFs were significantly lower for carbofuran treatments for both MWCO-filters (One-way ANOVA $F=8.81$, $p=0.0164$, and $F=81.1$, $p=<0.0001$; for 1 kDa, and 100 kDa, respectively). Only for 100 kDa-filters, lindane showed a higher BCF than chlorpyrifos. For all three pesticides, 1 kDa-filters had a higher BCF, i.e., adsorbed a higher amount of pesticide, than 100 kDa-filters. Carbofuran had the highest difference between the MWCO-filters BCF, with an 87% higher adsorption to 1 kDa-filter than to100 kDa-filter.

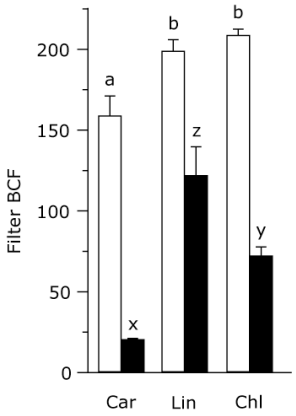


Figure 6. Bioconcentration factor (BCF, mean±1 SE, L/kg) in filters for treatments with carbofuran (Car), lindane (Lin) and chlorpyrifos (Chl) for ultrafiltration filters with molecular weight cut-off (MWCO) 1 kDa (white bars) and 100 kDa (black bars). Bars with different letters, within each MWCO-treatment, are significantly different.

The proportion of recovered pesticide, i.e., the amount of pesticide present in filtrate and on filter after filtration, was affected by, both the type of pesticide and filter MWCO-size (Two-way ANOVA $F=22.3$, $p<0.0001$). Lindane had the lowest recovered amount of all three pesticides, where treatments with 1 kDa-filter showed the lowest recovery between the two filter MWCO-sizes (table 2)

*Table 2. Initial amount (ng) of pesticide filtered through ultrafiltration tubes with molecular cut-off (MWCO) of 1 and 100 (kDa) and recovered amount (ng) and quota (%).*

| Pesticide | MWCO (kDa) | Initial amount (ng) | Recovered amount (ng) | Recovery (%) |
|---|---|---|---|---|
| Carbofuran | 1 | 3.26±0.07 | 2.85±0.04 | 87±0.6 |
| | 100 | 3.26±0.07 | 3.26±0.05 | 100±3.0 |
| Lindane | 1 | 2.91±0.06 | 1.81±0.03 | 62±2.3 |
| | 100 | 2.91±0.06 | 2.27±0.04 | 78±2.9 |
| Chlorpyrifos | 1 | 1.77±0.02 | 1.38±0.05 | 78±2.4 |
| | 100 | 1.77±0.02 | 1.70±0.05 | 96±3.9 |

### 3.1.2 Filtration of pesticide-humic substance solution

All three pesticides had a recovery lower than 60 % in filtrate after ultrafiltration (figure 7). For lindane and chlorpyrifos the lowest values were seen in MWCO of 1 kDa (5 % and 7 % respectively). The contrary was observed for carbofuran (see figure 3) where pesticide recovery was 42 % for MWCO-size 1 kDa and only 8 %

for 100 kDa. Carbofuran-humus solution filtered through MWCO of 100 kDa, showed low values for all variables, where the highest was seen in the concentrate. However, the recovery of carbofuran in the concentrate on top of filter after filtration was highest for MWCO 100 kDa compared to the other pesticides/filter size (One-way ANOVA $F=41.9$, $p<0.0001$). Concentration on filter was highest on 1 kDa, compared to 100 kDa, across pesticide treatments (Two-way ANOVA $F=87.9$, $p<0.0001$). Chlorpyrifos-humus had the highest adsorbed concentration on filter, where MWCO 1 kDa retained 64 % of the pesticide concentration after filtration (Figure 7).

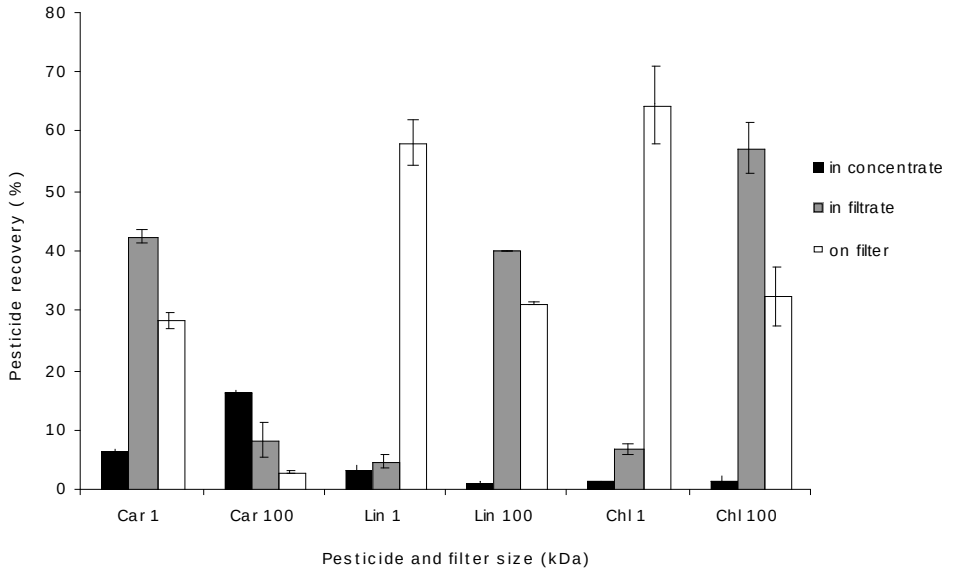


Figure 7. Recovery of pesticides when mixed with humic substance (%, mean±1 SE) after filtration; in concentrate on top of filter (black bars), in filtrate (grey bars), and adsorbed onto filters (white bars), for carbofuran (Car), lindane (Lin), and chlorpyrifos (Chl), using ultrafiltration tubes with MWCO 1 kDa and 100 kDa.

Both for lindane and chlorpyrifos MWCO 1 kDa filter showed bioconcentration factors close to 180 (figure 8). On the contrary carbofuran 1 kDa showed same BCF as lindane 100 kDa and chlorpyrifos 100 kDa. Filter BCF decreased from 1 kDa to 100 kDa across pesticide treatment (Two-way ANOVA $F=352.8$, $p<0.0001$, Figure 6)

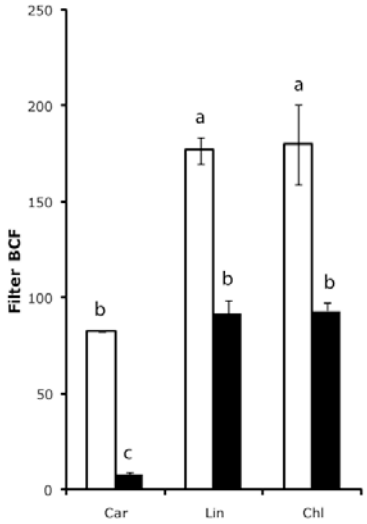


Figure 8 Bioconcentration factor (BCF, mean±1 SE, L/kg) in filters for humus treatments with carbofuran (Car), lindane (Lin) and chlorpyrifos (Chl) for ultrafiltration filters with molecular weight cut-off (MWCO) 1 kDa (white bars) and 100 kDa (black bars). Bars with different letters, are significantly different.

When comparing figure 6 and 8, there is a small decrease in filters BCF. However, only carbofuran adsorption decreased significantly with 52% for MWCO 1 kDa and 41% for 100 kDa (One-way ANOVA F=42.8, P=0.007 and F=61.1, P=0.004 respectively).

The proportion of recovered pesticide, i.e., the amount of pesticide present in filtrate, on filter and in concentrate on top of filter after filtration never reached 100 % (Table 3). Chlorpyrifos showed the highest recovery of 90 % after filtration through MWCO 100 kDa (Table 3). Contradictory, carbofuran showed the lowest recovery for this particular MWCO. For lindane and chlorpyrifos, the recovery was higher for MWCO 100 kDa than that for 1 kDa.

*Table 3. Initial amount (ng) of pesticide associated with humic substance from Lake Vikasjön (humic and fuvic acid) filtered through ultrafiltration tubes with molecular cut-off (MWCO) of 1 and 100 (kDa) and recovered amount (ng) and quota (%).*

| Pesticide associated with humic substance | MWCO (kDa) | Initial amount (ng) | Recovered amount (ng) | Recovery (%) |
|---|---|---|---|---|
| Carbofuran | 1 | 4.11±0.10 | 3.18±0.05 | 77±0.5 |
| | 100 | 4.11±0.10 | 1.14±1.01 | 28±2.8 |
| Lindane | 1 | 2.06±0.08 | 1.37±0.09 | 66±4.1 |
| | 100 | 2.06±0.08 | 1.50±0.10 | 73±0.1 |
| Chlorpyrifos | 1 | 3.30±0.19 | 2.39±0.13 | 73±7.6 |
| | 100 | 3.30±0.19 | 2.99±0.03 | 90±9.8 |

## 3.2 High-performance size exclusion chromatography

High performance liquid chromatography separates humic particles depending on the molecular size. Humic substances had an impact on carbofuran and lindane concentrations (Table 4) with significantly lower concentrations in Suwannee River fulvic acid (SR FA) treatment than the other three humic substances treatments. When looking at the recovery of pesticides in the different particle size fractions collected, carbofuran and lindane were similar, while chlorpyrifos differed from the other two.

*Table 4. Two-way ANOVA statistics of the effect of humic substance and size fraction of amount of carbofuran, lindane and chlorpyrifos (ng) in each size fraction for each humic type*

| | Carbofuran | | | Lindane | | | Chlorpyrifos | | |
|---|---|---|---|---|---|---|---|---|---|
| | **df** | **F** | **p** | **df** | **F** | **p** | **df** | **F** | **p** |
| Humic substance | 3 | 13.18 | <0.0001 | 3 | 57.31 | <0.0001 | 3 | 2.428 | ns |
| Size fraction (kDa) | 8 | 11.03 | <0.0001 | 8 | 33.34 | <0.0001 | 8 | 37.77 | <0.0001 |

*df: degrees of freedom, ns=not significant

Carbofuran concentrations were highest in the DOA treatments and lowest in the SRFA treatment (table 4, figure 9). All four humic substance treatments showed similar patterns, where there was a high association at particle fraction size of >30

kDa, followed by at drop in recovery in fraction size 25-30 kDa. A steady increase in recovery of carbofuran followed until fraction 5-10 kDa (figure 9). However this trend was only significantly for DOA with fraction size >30 and "fastened in the column" compared to carbofuran measured in fraction sizes 20-25 and 25-30 (One-way ANOVA $F=233.9$, $P<0.0001$).

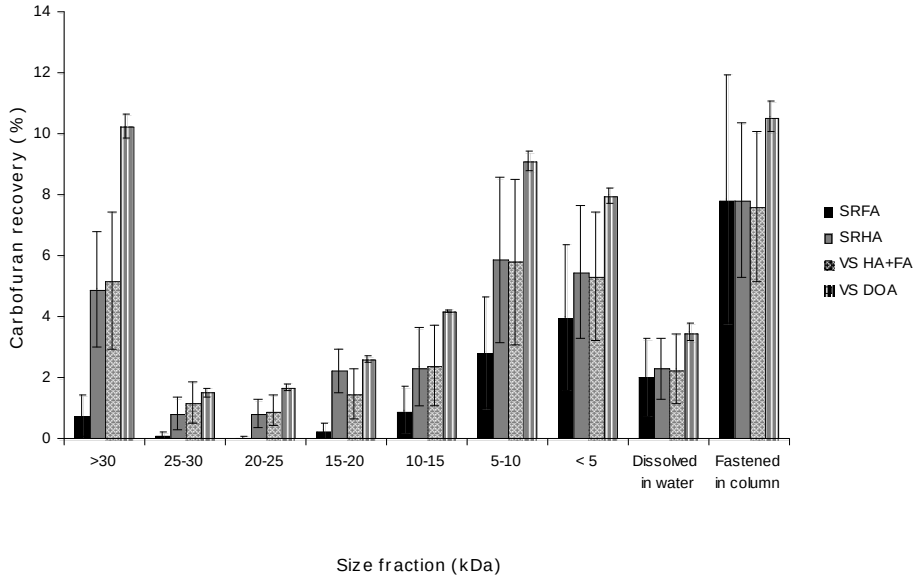


Figure 9. Percent (mean±1 SE) of carbofuran recovery of addition in each size fraction, dissolved in water and fastened in the column, for humic types Suwannee River fulvic acid (SR FA, black bars), Suwannee River humic acid (SR HA, dark grey bars), Lake Vikasjön fulvic and humic acids (VS FA+HA, white bars) and Lake Vikasjön dissolved organic acids (VS DOA, light grey bars).

Lindane showed similar trend as carbofuran, with significantly lower pesticide association with particle size fraction 20-25 and 25-30 (Table 4, figure 10). While the highest recovery was in fraction "fastened in column". SR FA showed the significant lowest association with lindane while SR HA had the highest association, This difference was largest in fraction "fastened in column" where the recovered pesticide was 1 % for SR FA and 15 % for SR HA.

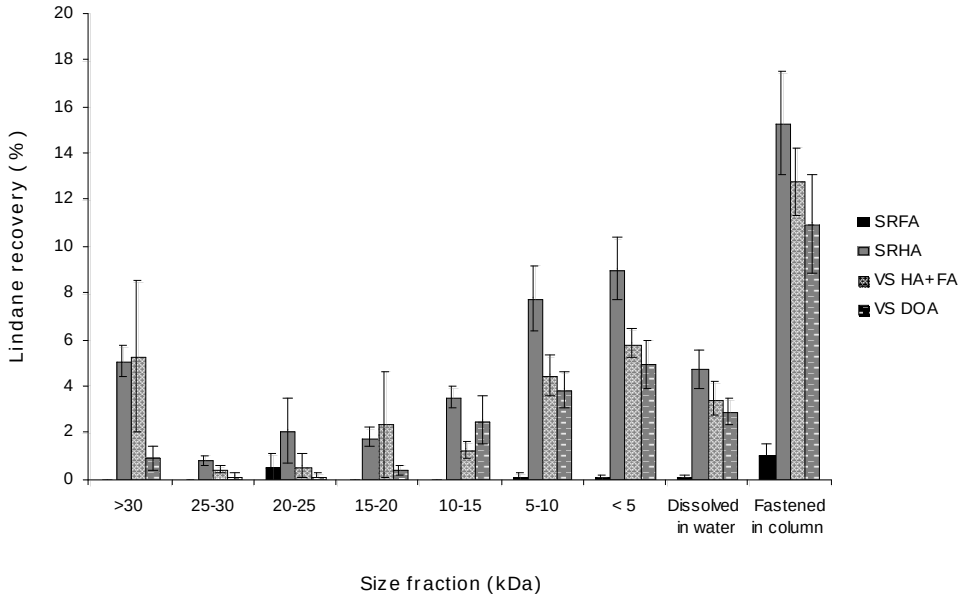


Figure 10. Percent (mean± 1SE) of lidane recovery of addition in each size fraction, carbofuran dissolved in water and fastened in the column, for humic types Suwannee River fulvic acid (SR FA, black bars), Suwannee River humic acid (SR HA, white bars), Lake Vikasjön fulvic and humic acids (VS FA+HA, grey bars) and Lake Vikasjön dissolved organic acids (VS DOA, dotted bars)

Chlorpyrifos, on the other had differed compared to the other two pesticides, with concentrations in SR FA treatment more similar to the other humic substance´treatments, only being significantly different to SR HA (table 4). Size fraction (kDa) <5 showed the highest recovery in all humic substances, and fraction 20-25 kDa the lowest recovery (figure 11, table 4) In addition, SR FA fraction 10-15, 15-20, 25-30 and >30 kDa were low, and for SR HA 25-30 kDa chlorpyrifos concentration was also low.

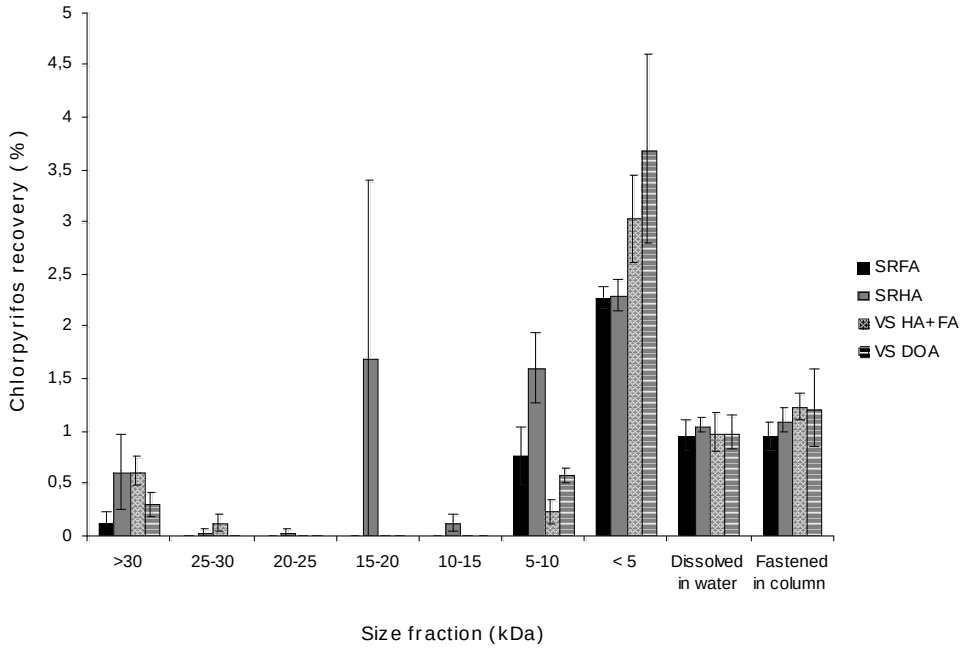


Figure 11. Percent (mean ±1SE) of chlorpyrifos recovery of addition in each size fraction, carbofuran dissolved in water and fastened in the column, for humic types Suwannee River fulvic acid (SR FA) (black bars), Suwannee River humic acid (SR HA) (white bars), Lake Vikasjön fulvic and humic acids (VS FA+HA) (grey bars) and Lake Vikasjön dissolved organic acids (VS DOA) (dotted bars)

Pesticide recovery ranged from 1.9 to 51 % for all humic substance treatments, with the highest recovery for carbofuran in association with Lake Vikasjön DOA (table 5, figure 8). The highest recovery of lindane and chlorpyrifos was seen in humic substance treatment SR HA (50% and 8.5%, figure 10 and 11, respectively). Lindane had the lowest recovery in SR FA with only 1.9%. SR FA had the lowest recovery within all pesticides (figure 8, 10, 11). Due to this low recovery, pesticide injection over time (data not shown) was plotted. The injection sequence was the same for all pesticides (n=4); SR FA (first), SR HA, VS HA+FA, VS DOA (last). It gave low $R^2$-values between 0.01 (chlorpyrifos) and 0.27 (carbofuran).

*Table 5. Initial amount injected (ng) in high-performance liquid chromatography (HPLC), recovered amount from all size fractions (ng) and quota (%) of pesticides, carbofuran, lindane and chlorpyrifos when associated with humic substance, Suwannee River fulvic acid (SR FA) Suwannee River humic acid (SR HA), Lake Vikasjön fulvic and humic acids (VS FA+HA).*

| Pesticide | Humic substance | Initial amount (ng) | Recovered amount (ng) | Recovery (%) |
|---|---|---|---|---|
| Carbofuran | SR FA | 2.76±0.07 | 0.52±0.31 | 19±11 |
| | SR HA | 2.83±0.07 | 0.92±0.37 | 33±13 |
| | VS HA+FA | 2.87±0.07 | 0.93±0.40 | 32±13 |
| | VS DOA | 2.85±0.07 | 1.45±0.04 | 51±1.5 |
| Lindane | SR FA | 2.71±0.07 | 0.05±0.02 | 1.9±0.8 |
| | SR HA | 2.26±0.06 | 1.13±0.19 | 50±8.3 |
| | VS HA+FA | 2.99±0.11 | 1.09±0.23 | 36±7.7 |
| | VS DOA | 2.28±0.13 | 0.8±0.14 | 26±5.0 |
| Chlorpyrifos | SR FA | 2.50±0.05 | 0.13±0.01 | 5.1±0.2 |
| | SR HA | 2.40±0.05 | 0.21±0.06 | 8.5±2.5 |
| | VS HA+FA | 3.01±0.09 | 0.16±0.02 | 6.2±0.7 |
| | VS DOA | 2.53±0.10 | 0.17±0.03 | 6.7±1.1 |

PCB-105 showed deviations from the initial concentration of 30 µg/L for the humic substances SRFA, SRHA, HA+FA in samples taken 30 min after addition of PCB. However after 48h of mixing the recovery of PCB was around 10 % for all humic substances, with an eleven-folded decrease for DOA (figure 12). Results of fractionations of PCB + humic substance were similar to background levels (data not shown).

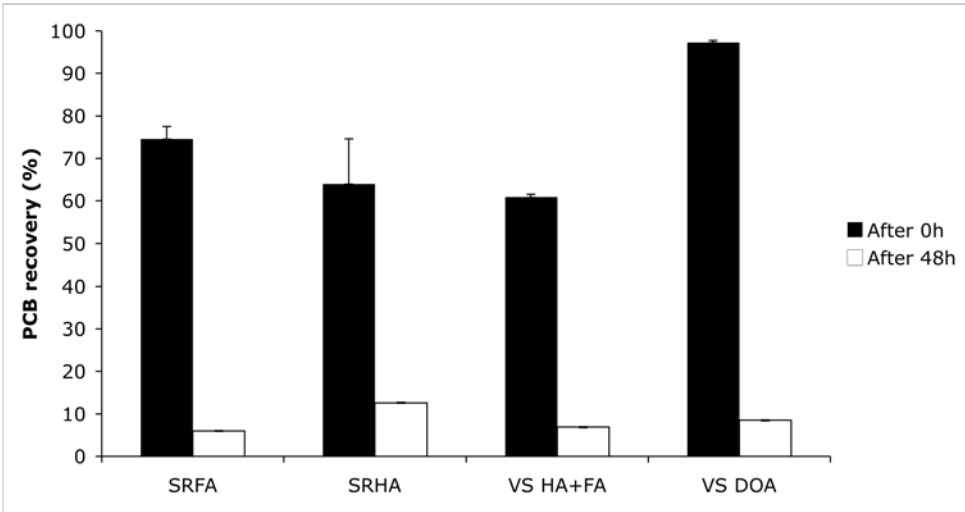


Figure 12. Recovery of PCB-105 (% mean±1 SE) when associating with different humus solutions; SRFA, SRHA, VS HA+FA, and VS DOA,; after 0h (black bars) and after 48h (white bars) of mixing. Bars with different letters are significantly different.

### 3.2.1 Possible absorption of pesticide to Tsk-Gel® column

Carbofuran was not detectable in fractions from the first injection. Only in the second injection at fraction 19-29 min, there was a carbofuran recovery (figure 13). In the following fraction, 29-39 min, the recovery increased 3-folded. When mixed with humic substance (SR FA), however, the recovery increased for the last two fractions of the second injection.

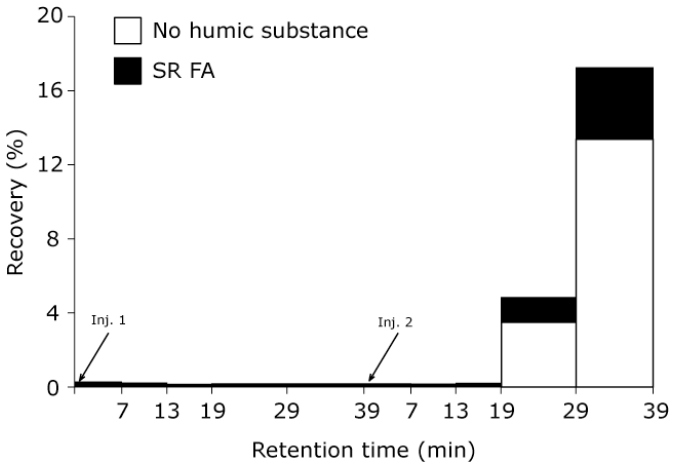


Figure 13. Recovery (%) of carbofuran from an HPLC/MS/MS-analysis (high-performance liquid chromatography/mass spectrometry /tamdem mass spectometry in treatments with only eluent (phosphate buffer solution, white bars) and in presence of humic substances (Suwanne River fulvic acid, black bars).

When chlorpyrifos was fractioned without humic substance present, no pesticide was evident after 78 min and two injections. However, in presence of humic substance (SR FA) chlorpyrifos was slightly detectable in the fraction 29-39 min after injection, followed by a constant increase in recovery trough out the following fractions. The largest recovery was observed in injection no.2 in fraction 29-39 min, having 8% recovery (figure 14).

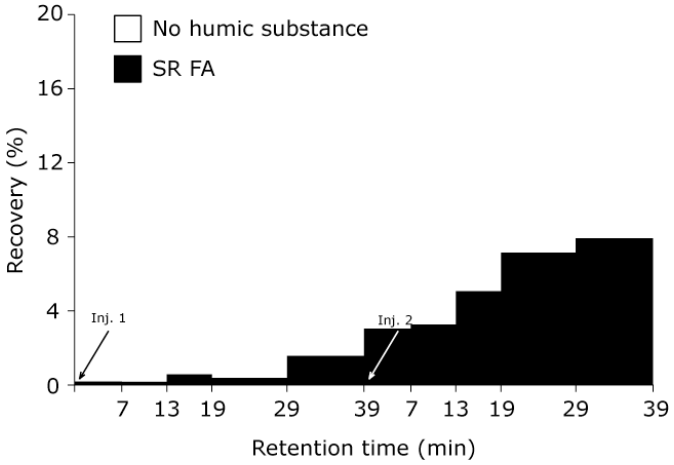


Figure 14. Recovery (%) of chlorpyrifos from an HPLC/MS/MS-analysis in treatments with only eluent (phosphate buffer solution, white bars) and in presence of humic substances (Suwannee River fulvic acid, black bars).

# 4 Discussion

The pesticides lindane, carbofuran and chlorpyrifos adsorbed to filter during ultrafiltration and to the Tsk-gel column during HPSEC. Humic substance decreased the association to filter/column, indicating that humic substance is an important molecule for HOC mobility. It has been reported that the hydrophobic properties and origin of humic substance determines this association (Gauthler et al., 1987, Uhle et al., 1999). Dissolved organic matter is significant to aquatic ecosystems, by affecting both chemical and physical properties. DOM is part of the carbon-cycle, where degradation by UV-radiation or by microbes or deposition in sediment is of importance (Tranvik et al., 2009). Organic contaminants bound to DOM are evidentially of considerable concern and hence their destiny in aquatic systems.

Difference in pesticide absorption between the filter sizes after ultrafiltration was likely due to centrifugation time (100 min for 1 kDa, compared to 20 min for 100 kDa). However, there was also a possible adsorption of pesticides to the tube walls and potential evaporation, reducing the pesticide recovery rate. Ultrafiltration was disregarded as a good method based on the results obtained.

Adsorption to the Teflon tube wall may have affected PCB-105 results. It's low water solubility ($K_{ow}$= 6.65), favoured tube wall association compared to humic substance in solution (Scrudato et al. 1999).

Due to the adsorption of pesticides to column the recovered pesticide in each fraction was most likely related to the injection sequence of the humic substances in to HPSEC. Pesticide recovery was not due to specific molecular size as first predicted, but rather the pesticides retention time in the column, which showed to be dissimilar from the retention time of the specific size fraction of humic substance.

The fraction "dissolved in water" also recovered some pesticide, among others, chlorpyrifos. However, it is unlikely that this might have happened with chlorpyri-

fos, as it is hydrophobic (log $K_{OW}$ of 4.7) and dissolves poorly in water (Katagi, 2006). Hence, we can yet again relate this to the adsorption of pesticide in column.

Due to these results it is not possible to draw any good conclusions from the relationship between different log $K_{OW}$ of applied chemicals as mentioned in the aim for the experiment. However, as seen between carbofuran and chlorpyrifos, humic substance has a larger impact on the recovery rate for chlorpyrifos compared to carbofuran.

Despite the negative results on our experiment a similar study have been done with pyrene (Chin et al., 1997) where association of pyrene increased with increasing molecular weight of the humic substance. Pyrene's planar configuration had low steric hindrance and henceforth would show increased association with humic substance, compared to larger/bulkier molecules. However, Chin et al. (1997) did not attempt to find differences in association within a humic substance, as we were unable to achieve. Nevertheless, HPSEC analysis is a respectable method at present time for fractionation of humic substance. A future study may involve adding HOC to pre-fractioned humic substance, and measure the amount of adsorbed HOC, and furthermore perform bioavailability studies of HOC on aquatic test organisms. Studies on availability of HOC depending on dissolved organic matter (DOM) concentration have shown that bioavailability decreases with increasing DOM-concentrations (Kukkonon and Oikari, 1991; Nikkilä and Kukkonen, 2000)

There is no denying the influence DOM has on the aquatic system. The information that can be obtained from size fractionation may give a better estimate of organic contaminants distribution in the aquatic system. Wu et al. (2007) showed that molecular weight defined dispersal in stream when measuring the occurrence of humic and fulvic acids along the length of a stream. They concluded that humic acid decreased downstream, while fulvic acid increased. This study indicates that humic substance composition may vary depending on physical conditions. By knowing the size distribution of humic substance and the corresponding HOC association, dispersal of HOC can be estimated depending on molecular size of humic substance.

# 5 Conclusion

The importance of humic substances is not to be undermined, and so the knowledge of its interaction with hydrophobic contaminants (HOC) is of interest. The molecular size of a humic substance is known, however, the interaction between HOC and different size fractions within a humic substance is not. All though this was not achieved in this experiment, a deeper understanding has been reached. The pesticides, carbofuran, lindane and chlorpyrifos, adsorbed to both the filter during ultrafiltration and also to the Tsk-gel® column during HPSEC. However, when humic substance was present, the adsorption to filter/column decreased, indicating that there is an important association formed between the pesticide and the humic substance.

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